ON THE ROLE OF THE ENZYME PEPTIDE METHIONINE SULFOXIDE REDUCTASE IN THE RESPONSE OF ARABIDOPSIS PLANTS TO OXIDATIVE STRESS

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ABSTRACT

Reactive oxygen species (ROS) are produced during cellular metabolism. Low steady state levels of ROS are maintained by enzymatic and non-enzymatic mechanisms. Under extreme environmental conditions, ROS production can overwhelm the detoxification systems and oxidative stress results. ROS can oxidize methionine (Met) residues to methionine sulfoxide (MetSO) thereby inactivating enzymes or tagging proteins for hydrolysis. The enzyme peptide methionine sulfoxide reductase (PMSR) reduces MetSO to Met, repairing damaged proteins. The MetSO-PMSR-Met system has been referred to as the “last chance” defense mechanism against ROS. The role of PMSR in protection against oxidative stress is well recognized in bacteria, yeast and animal cells; however the function of PMSR in plants is yet to be fully established. The objective of this research was to determine the role of PMSR in the response of plants to oxidative stress. One family of PMSR proteins, PMSRA of Arabidopsis was used as a model system. Arabidopsis has five *PMSRA* genes being expressed in different organs. PMSRA1 to PMSRA3 are cytosolic proteins, PMSRA4 is plastidic and PMSR5 is secreted. *PMSRA4* transcript is the most abundant; *PMSRA2, PMSRA3* and *PMSRA5* are highly expressed in roots and stems; *PMSRA1* is abundant in flower buds; and *PMSRA4* is up-regulated by ozone, methyl viologen, cercosporin and high light. Kinetic analysis of recombinant enzyme showed that PMSRA4 had twice the $k_{\text{cat}}$ and was four times more efficient ($k_{\text{cat}}/K_{\text{m}}$) than PMSR3. Transgenic plants over-expressing PMSRA3 and PMSRA4 were more resistant to methyl viologen and ozone. PMSRA3 over-expressing plants were more resistant to cercosporin and PMSRA4 over-expressing plants more resistant to high light. Transgenic plants with low levels of PMSRA4 were susceptible to oxidative stress in the chloroplast. Treatments that lower PMSRA4 levels in wild-type plants, such as Mn$^{2+}$ excess, increased the susceptibility of plants to oxidative stress damage (e.g. a modest 2-day water deprivation in plants grown under Mn$^{2+}$ excess induces toxicity). PMSRA activity is essential for plant survival to oxidative stress. It appears that specific isozymes protect discrete cellular compartments. PMSRA4 is the most abundant and most efficient PMSRA in Arabidopsis and protects against oxidative stress derived from different cell compartments.
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Chapter 1

LITERATURE REVIEW

REACTIVE OXYGEN SPECIES

Aerobic organisms depend on the passage of electrons from a variety of donors to oxygen in order to generate the metabolic energy necessary for life. This is a tightly regulated process where the reduction of molecular oxygen occurs at cytochrome oxidase. Despite great care to prevent the leakage of electrons to oxygen outside of cytochrome oxidase, under certain metabolic stresses, oxygen may be reduced to generate several reactive oxygen species (ROS) (Fig. 1.1). ROS can also be generated photochemically. The rather un-reactive dioxygen can be activated by transfer of excitation energy which results in spin inversion. The main source of the activation energy comes from photo-activated compounds (photosensitizers). The absorption of a photon by a photosensitizer produces singlet states which transfer energy to dioxygen resulting in the formation of singlet oxygen ($^1$O$_2$) (Smirnoff, 1993). Singlet oxygen reacts with molecules with paired electrons and diffuses easily. It is a prevalent reactive species that can cause damage to biological membranes especially in the chloroplast (Perl-Treves and Perl, 2002). There are many naturally-occurring photosensitizers in plants such as chlorophyll, protoporphyrin IX, quionones, furanocoumarines, polyacetylenes and thiphenes (Smirnoff, 1993). Most of the singlet oxygen in the cell is formed in the chloroplasts when photo-excited chlorophyll in the triplet state reacts with dioxygen. This formation of singlet oxygen may play a role in the dissipation of excitation energy when the normal transfer of electrons by photosynthesis is
inhibited, as may occur under different stress conditions that limit the utilization of ATP and NADPH by the Calvin cycle reactions (Perl-Treves and Perl, 2002).

Figure 1.1: Formation of major reactive oxygen species. The free energy for each species is given in italics. (1) Dioxygen biradical. (2) Singlet oxygen. (3) Superoxide radical. (4) Perhydroxyl radical. (5) Hydrogen peroxide. (6) Hydroxyl radical. Based on Perl-Treves and Perl (2002)

Dioxygen can also be chemically activated by addition of electrons. Each molecule of dioxygen can accept up to four electrons (and four H\(^+\)), two for each atom, resulting in two molecules of water. However, due to the spin restrictions the additions of electrons is one by one (univalent reduction) and if the transfer of electrons is not completed (only one, two or three electrons are received by dioxygen), ROS are formed (Levine, 1999).

The first reduction product of electron transfer to ground state oxygen is superoxide (O\(_2^-\)). Superoxide is a source of other ROS. It reacts with itself to yield H\(_2\)O\(_2\) in a reaction referred to as dismuation. Dismutation can also be enzyme catalyzed. However, when the reaction is not catalyzed by an enzyme, the number of molecules superoxide can react with is very limited (Levine, 1999). O\(_2^-\) is produced during electron transport when the electrons do
not follow the normal course in the chain. As such, $O_2^{-}$ can be produced in the electron transport chain in the mitochondria and in the reducing side of photosystem I (PSI) in the chloroplast. At low pH the rate of dismutation of $O_2^{-}$ to $H_2O_2$ is the highest; otherwise it may interact with plastocyanin or cytochrome $f$ and reduce them, resulting in superoxide-mediated cyclic electron flow around PSI (Perl-Treves and Perl, 2002). There is also evidence of NADPH-mediated superoxide production by microsomal membranes as a result from autoxidation of reduced cytochrome P-450 or cytochrome P-450 reductase (Smirnoff, 1993).

The dismutation product of superoxide, hydrogen peroxide, is not a free radical but takes part in reactions with several cellular components. It is highly diffusible and directly inactivate enzymes even at very low concentrations (Perl-Treves and Perl, 2002). Due to its stability, the toxicity of $H_2O_2$ itself is not very high but has the potential to generate the highly reactive hydroxyl radical ($OH^\bullet$). There are many sources of $H_2O_2$ in the cell; but it is produced mostly in chloroplasts during the superoxide dismutase-catalyzed disproportionation of superoxide to $H_2O_2$. Another source of $H_2O_2$ generation occurs during photorespiration at the step of glyoxalate formation from glycolate (Levine, 1999).

The one-electron reduction of hydrogen peroxide yields the hydroxyl radical. The hydroxyl radical is one of the most reactive molecules known in biology. It can react with almost any cellular molecule, which limits its diffusion to twice its molecular diameter. Oxidation of organic molecules by hydroxyl radical is indiscriminate and it rapidly attacks virtually all macromolecules, leading to serious damage in cellular components. DNA lesions and the resultant mutations can occur or the damage can also results in irreparable metabolic dysfunction and cell death. In living cells, $OH^\bullet$ production occurs by the metal-catalyzed Haber-Weiss reaction:

\[
H_2O_2 + O_2^{-} \longrightarrow O_2 + OH^\bullet + OH^\bullet
\]

However, superoxide cannot directly reduce hydrogen peroxide. This reaction requires a metal catalyst such as copper or iron. Superoxide reduces the metal:

\[
O_2^{-} + Fe^{+3} \longrightarrow O_2 + Fe^{+2}
\]
and the reduced metal then reduces hydrogen peroxide.

\[
H_2O_2 + Fe^{+2} \rightarrow OH^- + OH^- + Fe^{+3}
\]

**OXIDATIVE STRESS**

ROS are produced constantly in the cell as part of metabolism. To avoid the deleterious effects of ROS, the plant has evolved several mechanisms (see below) to remove oxygen radicals. However, when the production of ROS exceeds its removal, the cells are exposed to oxidative stress.

The effect of ROS on the cell depends upon its steady state concentration. Thus, ROS can activate antioxidant responses, programmed cell death or necrosis. These three responses can occur at different times during the life cycle of the plant or they can act simultaneously although at different cellular locations. When the plants are under stress conditions, the production of ROS increases and the plants are more susceptible to photo-inhibition which results in chlorosis and death (Arora et al., 2002). Photo-oxidative damage is magnified by atmospheric pollutants (like ozone), herbicides (like methyl viologen), heavy metals, photosensitizers (like the fungal toxin cercosporin) and natural occurring conditions such as high light, cold, heat, flooding and drought (Levine, 1999). Also, the accumulation of ROS after pathogen attacks (known as oxidative burst) is well known (Bolwell et al., 2002). Here, the plant actually utilizes ROS to defend itself from pathogens.

The link between abiotic stress and oxidative stress has been well established for different types of stress conditions. For example, under drought conditions, stomata are closed, reducing CO₂ availability. As a result, NADPH and ATP formed in the light reactions of photosynthesis are not consumed and they accumulate. This results in inhibition of electron transport in the photosystems. The excess of energy that is not transformed in the photosystems increases the transit of energized electrons to oxygen, creating ROS (Smirnoff, 1993).

Other stress conditions result in a more direct production of ROS. For example, tropospheric ozone is absorbed through the stomata. Ozone is highly reactive and readily
oxidizes a large number of organic molecules. It also reacts in aqueous solutions generating O$_2^{•−}$ and H$_2$O$_2$. As has been shown before, both O$_2^{•−}$ and H$_2$O$_2$ react with transition metals generating highly reactive OH• via the Haber-Weiss reaction (Rao et al., 2000).

Herbicides like methyl viologen (MV), also known as paraquat, generate ROS directly in the chloroplast in a light-catalyzed reaction or in any other organelles where electron transport systems may be uncoupled by methyl viologen. Upon exposure to the herbicide it enters the cell and the divalent methyl viologen cation (MV$^{2+}$) can remove electrons from electron systems such as PSI in the chloroplast. The reduction product is a blue, monocationic methyl viologen radical (MV$^{+•}$), which in turn is rapidly oxidized by molecular oxygen to regenerate MV$^{2+}$ and O$_2^{•−}$. The paraquat molecule is then ready for another cycle of oxidation reduction (Dodge, 1994).

Photosensitizers like cercosporin are produced by pathogens (e.g. fungi). When the photosensitizer absorbs energy (from light), the molecule is excited to a triplet state which may react with molecules in the cell such as lipids. However, the toxicity comes mostly from the reaction of the excited triplet state with oxygen to produce ROS, by electron transfer (O$_2^{•−}$, H$_2$O$_2$, OH•) or by direct energy transfer to generate $^{1}$O$_2$ (Daub and Ehrenshaft, 2000).

**CELLULAR PROTECTION AGAINST OXIDATIVE STRESS**

ROS can cause damage to an array of biomolecules which include membrane lipids, DNA and proteins (Apel and Hirt, 2004). Extensive damage can lead to cell death. The cell has developed several protection mechanisms against oxidative stress which include the scavenging of ROS and also the repair of oxidative damage caused by ROS. ROS-scavenging systems have been well studied and several reviews are available (Perl-Treves and Perl, 2002; Apel and Hirt, 2004; Laloi et al., 2004). These scavenging systems include non-enzymatic antioxidants such as glutathione, vitamin E (tocopherol) and vitamin C (ascorbate) (Figure 1.2), and enzymes such as superoxide dismutase (Scandalios, 1993), catalase and glutathione peroxidase (De Felipe et al., 1988).
Figure 1.2: Chemical structure of some important cellular antioxidants (Perl-Treves and Perl, 2002)
**Superoxide Dismutase**

Superoxide dismutases (SOD) are ubiquitous metalloenzymes that convert O$_2$•$^-$ to H$_2$O$_2$ in the following reaction:

\[
2 \text{O}_2\cdot^- + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{O}_2 + \text{H}_2\text{O}_2
\]

SOD activity maintains a low steady state concentration of O$_2$•$^-$ to reduce the probability of formation of hydroxyl radicals (Smirnoff, 1993). Unlike most other organisms, plants have multiple enzymatic forms of SOD localized in the chloroplast (Cu/ZnSOD), mitochondria (MnSOD) and the cytosol (Cu/ZnSOD) (Scandalios, 1997). SOD is also present in peroxisomes, glyoxisomes and in the extracellular space (Perl-Treves and Perl, 2002). SOD is the fastest enzyme known, reacting with superoxide as soon as it collides with it. It is considered the “primary defense” against oxygen radicals (Bannister et al., 1987).

**Ascorbate Peroxidase**

The activity of SOD produces H$_2$O$_2$ which is still toxic and must be further transformed. Ascorbate peroxidase (APX) reduces H$_2$O$_2$ to water using ascorbate (Asc) as a reductant. In the reaction Asc is oxidized to monodehydroascorbate (MDA) as follows:

\[
2 \text{ascorbate} + \text{H}_2\text{O}_2 \xrightarrow{\text{APX}} 2 \text{MDA} + 2\text{H}_2\text{O}
\]

In the chloroplast, MDA can be transformed into dehydroxyascorbate (DHA). In order to regenerate the Asc pool, both MDA and DHA must be reduced. This reduction is produced by different reactions.

- Ferredoxin-mediated non-enzymatic reduction:

\[
\text{MDA} + \text{Fd}_{\text{red}} \rightarrow \text{Asc} + \text{Fd}_{\text{ox}}
\]
• NADPH-mediated reduction of MDA by MDA reductase (MDAR) in the chloroplast stroma:

\[
2 \text{MDA} + \text{NADPH} \xrightarrow{\text{MDAR}} \text{Asc} + \text{Fe}_{\text{ox}}
\]

• Reduction of DHA to Asc by DHA reductase (DHAR) with reduced glutathione (GSH) as the reducing substrate:

\[
\text{DHA} + 2 \text{GSH} \xrightarrow{\text{DHAR}} \text{Asc} + \text{GSSG}
\]

The APX enzyme system is localized throughout the plant cell. There is one APX system operating at the thylakoid surface near PSI to reduce the escape of ROS. A second system is in the stroma to protect enzymes of the Calvin cycle and a third one operates in the cytoplasm (Dalton et al., 1987).

**Glutathione Reductase**

The activity of DHAR depletes the GSH pool and accumulates oxidized glutathione (GSSG). GSH is regenerated through the NADPH-mediated reduction of GSSG by the enzyme glutathione reductase (GR) in the following reaction:

\[
\text{GSSG} + \text{NADPH} \xrightarrow{\text{GR}} 2 \text{GSH} + \text{NADP}^+
\]

GR is a flavoprotein, mostly found in the chloroplasts, although there are mitochondrial and cytosolic isozymes.

**Catalase**

Another enzyme involved in the scavenging of ROS is catalase (CAT). Catalase efficiently scavenges H\(_2\)O\(_2\) and does not require a reducing substrate for the reaction. Here, H\(_2\)O\(_2\) is oxidized to molecular oxygen and the two electrons are transferred to a second H\(_2\)O\(_2\) yielding water:
Catalase is localized in the peroxisomes, to scavenge the H$_2$O$_2$ produced during photorespiration by the enzyme glycolate oxidase. This metabolic process seems to be important in the tolerance to oxidative stress (Scandalios et al., 1997). There are several CAT isozymes in plants (three in Arabidopsis) with different expression patterns depending on the organ and the type of stress that generates the H$_2$O$_2$ (Scandalios et al., 1997). Catalase is photo-inactivated and requires continuous de novo synthesis. Stress factors that affect protein synthesis (e.g. heat, chilling) may lead to catalase inactivation (Perl-Treves and Perl, 2002).

**PEPTIDE METHIONINE SULFOXIDE REDUCTASE**

If ROS are able to escape cellular scavenging systems, the damage they cause, depending on the biomolecule, may be repaired. Repair mechanisms are an active area of research, although these systems have not been studied as intensely as that of scavenging systems (Klatt and Lamas, 2000). One of the more obscure repair mechanisms involves protein repair. The work of Levine et al. (1996) showed that Met residues, even more than Cys or Tyr, are susceptible to damage by ROS. Proteins with exposed Met residues are highly susceptible to oxidative damage from free radicals which escape the scavenging systems (Davies, 2005). The two-electron oxidation of the Met residues to MetSO is often of little consequence to protein structure or function (Levine et al., 1996). However, continued oxidation of Met groups by ROS can lead to inactivation or ultimately proteolysis. Met residues have been proposed to protect the active site of selected enzymes from oxidative damage from ROS (Levine et al., 1996). In that way, key structural or functional residues are not accessible to free radicals and the protein/enzyme can continue to be fully functional. The oxidized Met residues in the protein damaged by oxidative stress can be reduced back to Met by the enzyme peptide methionine sulfoxide reductase (PMSR). The affected protein returns (is repaired) to its original state (Moskovitz et al., 1999) (Fig. 1.3). This repair mechanism has been proposed to be the “last chance” defense of the cells against the oxidative stress (Levine et al., 1999).
How PMSR provides protection against ROS is not clear, but its function as an endogenous antioxidant is very well supported (Levine et al., 1996; Weissbach et al., 2005). The observation that Met oxidation in some enzymes does not have any effect on the protein function indicates that the cyclic oxidation-reduction of surface-exposed Met residues is an

**Figure 1.3:** Mode of action of the enzyme peptide methionine sulfoxide reductase (PMSRA). Mild oxidation results in the formation of MetSO, which is repaired by PMSR activity with the reductive thioredoxin-thioredoxin system. NADPH is the ultimate electron donor. Strong oxidation produces MetSO₂ which cannot be repaired (Adapted from Hoshi and Heinemann (2001))
effective scavenger of free radicals (Stadtman et al., 2002). However, extensive oxidation of Met residues can lead to loss of biological activity, loss of regulatory capacity or change in activity (Stadtman et al., 2002). For example, studies with glutamine synthase have shown that the surface-exposed Met residues act as free radical scavengers. Oxidation of 8 of the 10 surface-exposed Met residues has no effect on the susceptibility of the enzyme to proteolytic degradation and the enzyme is fully functional. However with further oxidation of the last two surface-exposed Met residues, the protective (scavenging) function of Met oxidation is surpassed and enzyme activity is lost resulting in a dramatic increase in the susceptibility of the enzyme to proteolytic hydrolysis (Levine et al., 1996).

**Discovery of PMSR Activity**

The enzymatic reduction of MetSO was observed for the first time in yeast in 1960 (Black et al., 1960). In 1966, PMSR activity was observed in plants by Doney and Thompson, working with leaf extracts of turnip and beans (Doney and Thompson, 1966). In 1970, based on the findings of Black et al. (1960) that the enzymatic activity responsible for MetSO reduction required three different protein components, Gonzalez Porque et al. (1970) found that the yeast enzymatic system was composed of thioredoxin, thioredoxin reductase and NADPH, which was the electron donor for PMSR. In 1980, it was shown that the thioredoxin system could be replaced by dithiothreitol as an electron donor, making the assay of the enzyme “in vitro” much easier (Ejiri et al., 1980). In the following year, PMSR activity was found in *E. coli* by Brot et al. (1981) while working on the biological activity of ribosomal protein L12. From the beginning of this initial work, the investigators recognized the role of PMSR in preserving protein activity. The authors showed that oxidation of Met residues of L12 resulted in the lost of the biological function, and that the reduction of the resulting MetSO back to Met restored the function. This same kind of inactivation/reactivation or oxidation/reduction was found for the α-1-proteinase inhibitor (Abrams et al., 1981), which is inactivated after oxidation of Met residues, and recovers activity when the MetSO residues are reduced back to Met by PMSR. These initial activities
were associated with Met residues of proteins. Later a new enzyme was discovered, capable of reducing free MetSO (the amino acid) to Met (Ejiri et al., 1980) which later on was found to be the same PMSR previously described.

During the 1980’s, studies on PMSR mainly involved survey studies describing the distribution of PMSR activity in different organisms. The studies also described proteins affected by Met oxidation. Sanchez et al. (1983) found PMSR activity to be ubiquitous in plants. The authors reported that 85% of the PMSR activity was confined to the chloroplast. Despite these initial findings, very little research effort was expended on PMSR in plants during that decade. This was due in part, to the lack of recognition of the importance of ROS and protein oxidation. The protein was subsequently renamed as MsrA\(^1\) (except in plants in which the name remains PMSR) and the MsrA gene was cloned in the early 1990’s. First the \textit{E. coli} \textit{MsrA} gene was cloned (Rahman et al., 1992) and then the bovine \textit{MsrA} (Moskovitz et al., 1996). The first plant \textit{PMSR} gene was cloned in 1996 from \textit{Brassica napus} (Sadanandom et al., 1996). Sequence alignment showed a conserved sequence of GCFWG among the different proteins, which was subsequently found to be the active site of the enzyme (Lowther et al., 2000).

**PMSR and Oxidative Stress**

Physiological studies on the role of PMSR in the response to oxidative stress were first performed using a PMSRA null mutant of \textit{E. coli}. Under normal growth conditions, the mutant did not have any phenotype. However, it was extremely sensitive to hydrogen peroxide (Moskovitz et al., 1995) and other ROS (St John et al., 2001). The same susceptibility to ROS was observed in yeast PMSRA null mutants (Moskovitz et al., 1997). Studies with pathogens such as \textit{Streptococcus pneumoniae} and \textit{Erwinia chrysanthemi} also revealed the importance of PMSR in response to oxidative stress (Wizemann et al., 1996; Hassouni et al., 1999). Although these two pathogens have different host organisms (humans

\(^1\) For simplification in the present review PMSR is used indistinctively of the organism.
and plants respectively), mutations in the \textit{PMSRA} gene reduced the pathogenicity in both microbes. In both cases, the host cells respond to the pathogen invasion with an oxidative burst. Mutations in the \textit{PMSRA} gene may compromise the pathogen’s ability to repair the oxidized proteins (Weissbach et al., 2005). In the case of \textit{S. pneumoniae}, the reduced pathogenicity is attributed to the reduced adherence of the microorganisms to the lung cells (Wizemann et al., 1996). With the mutant \textit{Erwinia}, it has a defective interaction with the plant cell host (Hassouni et al., 1999).

Other evidence supporting the importance of PMSR in response to oxidative stress comes from studies with organisms over-expressing PMSR. Yeast and human lymphocytes over-expressing PMSRA are more resistant to oxidative stress generated by hydrogen peroxide or methyl viologen (Moskovitz et al., 1998a; Moskovitz et al., 1998b). Also, transgenic \textit{Drosophila} over-expressing PMSRA are more resistant to methyl viologen, with a death rate of 10\% in the transgenic flies and 80\% death rate in the untransformed flies (Ruan et al., 2002).

\textbf{Possible Role of PMSR in Protein Regulation}

Bigelow and Squier (2005) showed that the oxidation of just one key Met residue can change the activity of calcium regulator proteins. Others have shown that oxidation of one Met residue can lead to protein hydrolysis (Ferrington et al., 2001). Thus, the cyclic oxidation-reduction of these key Met residues are important regulators of activity (Bigelow and Squier, 2005). Based on these observations, Sharov et al. (1999) proposed that PMSR might be involved in covalent regulation of enzyme activity. The oxidation-reduction cycle would regulate protein activity in a manner similar to phosphorylation-dephosphorylation cycle. For example, selective Met oxidation could be responsible for activation-deactivation of potassium channels. Here, the selective reduction by PMSR of the oxidized Met residues could be part of that regulatory system (Ciorba et al., 1997; Chen et al., 2000). The same kind of activation-inactivation has been observed in \textit{\alpha}1-protease inhibitor (Abrams et al., 1981), the \textit{\alpha/\beta}-type small, acid soluble protein of \textit{Bacillus} sp. (Hayes et al., 1998) and other proteins.
The regulatory function of PMSR is even more critical in cases where the protein with the redox active Met residues is involved in a signal transduction cascade. In this case, protein regulation by Met oxidation would not be limited only to the protein itself, but would be expanded to all the proteins downstream of the susceptible one. An example of regulation of a signal cascade by oxidation of Met residues of one individual protein in the cascade is calmodulin (Sharov et al., 1999; Sun et al., 1999). Calmodulin is involved in many cascades involving calcium signaling in the cells. When the Met residues of calmodulin are oxidized, the Ca\(^{2+}\) binding capacity is reduced and calmodulin conformational changes necessary to regulate protein function are lost (Gao et al., 1998). However, PMSR is able to repair oxidized calmodulin restoring the regulatory capacity (Sun et al., 1999).

The regulation of protein function by the Met oxidation-reduction cycle has been shown by studies in vitro with the plastid small heat shock protein HSP21 (Gustavsson et al., 2002). HSP21 functions as a chaperonin. Chaperonins assist in refolding proteins denatured by exposure to stress conditions (Sundby et al., 2005). HSP21 has a distinctive Met-rich domain (Chen and Vierling, 1991). The oxidation of the Met residues in the domain results in changes in the structure of HSP21 protein (Gustavsson et al., 1999; Harndahl et al., 1999) and the protein loses its chaperonin activity (Harndahl et al., 2001). However, PMSR, in reducing the MetSO residues in the oxidized HSP21, recovers the chaperonin activity of the protein (Gustavsson et al., 2002). Since there are many potential substrate proteins for the HSP21 chaperonin activity in the chloroplast, the number of proteins whose activity could be regulated by HSP21 oxidation-reduction upon exposure to stress conditions is very high (Sundby et al., 2005).

**PMSR and Aging**

Late in the 1990’s, research on PMSR exploded, driven, in part, by the finding that older cells contained (accumulated) higher MetSO content (Seppi et al., 1991). Subsequent research linked reduced PMSR activity with MetSO accumulation. Stadtman et al. (2002) found that PMSR activity declines with age in rat tissues. The same decrease in activity was
observed in age-related diseases such as Alzheimer’s disease (Gabbita et al., 1999), Parkinson’s disease (Krishnan et al., 2003) and emphysema (Vogt, 1995). Transgenic mice with lower PMSR activity exhibited a 40% decrease in the maximum life span (Moskovitz et al., 2001). Furthermore, PMSR over-expression in flies nearly doubled their life span (Ruan et al., 2002). Other age-related processes involving decreased PMSR activity and/or increased MetSO content include age-related damage of the eye protein crystalline (Fujii et al., 2002); increase in the level of MetSO in human skin collagen over the range of 10 to 80 years (Wells-Knecht et al., 1997) and the loss of calmodulin’s ability to regulate ATP hydrolysis and ATP-dependant Ca\textsuperscript{2+} transport (Gao et al., 1998; Gao et al., 2001).

**Biochemical Properties of PMSR**

When Met residues are oxidized by ROS, two different MetSO diastereoisomers which are referred to as Met-(R)-SO and Met-(S)-SO, are produced (Fig. 1.4). Most organisms have two different methionine sulfoxide reductases that reduce both the free and protein-bound MetSO residues. Within the family that reduces the protein-bound MetSO, one PMSR is specific for the reduction of the Met-(S)-SO and is referred to as PMSRA (Sharov et al., 1999). The second form of the enzyme is specific for the reduction of Met(R)-SO and is referred to as PMSRB (Etienne et al., 2003). Both enzymes are monomeric and do not have any sequence identity and are structurally unrelated (Kauffmann et al., 2005). However, the catalytic mechanism of the two enzymes is similar. The physiological reductant for both PMSRs is thioredoxin. This can be replaced *in vitro* by dithiothreitol. The mechanism (Fig. 1.5) involves two but can also involve three Cys residues, with the formation of a sulfenic acid as intermediate (Boschi-Muller et al., 2000). In the first step, the “catalytic” Cys attacks the sulfoxide group of MetSO (I) with the formation of a trigonal-bipyramidal intermediate (II). The intermediate is protonated resulting in sulfenic acid formation and the release of reduced Met as product (III). Then, an intramolecular disulfide bond is formed between the catalytic Cys and the “recycling” Cys with the release of water (IV). If the
Figure 1.4. Two diastereomers (R and S) of L-methionine sulfoxide with the sulfur as the chiral center are produced during the oxidation of L-methionine by ROS. Each diastereomer is the substrate of a different family of PMSR proteins (PMSRA and PMSRB). The reaction involves a third “final” Cys there is a series of exchange reactions via the “recycling” Cys and the “final” Cys (V). Finally, the disulfide bond is reduced by DTT or the thioredoxin/thioredoxin reductase/NADPH system (TR/TRR/HADPH) and the active site returns to the fully reduced state (Boschi-Muller et al., 2000; Weissbach et al., 2002).
The biochemical properties (Km, kcat) of PMSRA and PMSRB have been determined for the enzymes of different organisms (Moskovitz et al., 2000b; Boschi-Muller et al., 2005) and are summarized in Table 1.1. The kinetics of PMSRs involved a ping-pong mechanism in the presence of thioredoxin or DTT as a reductant (Boschi-Muller et al., 2001). However, the kcat with thioredoxin is two to three orders of magnitude higher than with DTT (Boschi-Muller et al., 2005).

**Figure 1.5:** Reaction mechanism for PMSRA catalysis. See text for details. (Based on Boschi-Muller et al. (2000) and Weissbach et al. (2002)).
PMSR in Plants

PMSR activity has been detected in a number of different plant species (Sanchez et al., 1983; Ferguson and Burke, 1994). ESTs have been identified in tomato, strawberry, soybean, sunflower, canola, cotton and other species. In Arabidopsis, five PMSRA genes (Sadanandom et al., 2000) and three PMSRB genes (Rodrigo et al., 2002) have been reported; which is in contrast to other studied organisms that have only one PMSRA and up to two PMSRBs. In the PMSRA family, one of the gene products is targeted to the chloroplast (PMSRA4). Three are localized to the cytosol (PMSRA1 to PMSRA3) and the fifth gene (PMSRA5) is part of a secretion pathway. In the PMSRB family, the three genes have been identified by in silico analysis based on their homology to the human SelX protein (selenoproteins), a PMSRB protein (Jung et al., 2002). The 3 genes found in Arabidopsis have been assigned to the AtSXL family (Arabidopsis thaliana selenoproteinX-like) (Rodrigo et al., 2002). However a more detailed sequence analysis (from the gene annotation The Arabidopsis Information Resource) shows that there are 9 genes with high homology to PMSRB, two are targeted to the chloroplast (PMSRB1 and PMSRB2) and the others are in the cytosol (PMSRB3 to PMSRB8) (Dos Santos et al., 2005).

Table 1.1. Kinetic parameters for the PMSR activity of different organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reductant</th>
<th>Enzyme</th>
<th>Km (µM)</th>
<th>kcat (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em>¹</td>
<td>DTT</td>
<td>PMSRA</td>
<td>170</td>
<td>20</td>
</tr>
<tr>
<td><em>S. cervisiae</em>¹</td>
<td>DTT</td>
<td>PMSRA</td>
<td>44</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. taurus</em>¹</td>
<td>DTT</td>
<td>PMSRA</td>
<td>33</td>
<td>ND</td>
</tr>
<tr>
<td><em>E. coli</em>²</td>
<td>Trx</td>
<td>PMSRA</td>
<td>1900</td>
<td>222</td>
</tr>
<tr>
<td><em>M. pneumoniae</em>²</td>
<td>Trx</td>
<td>PMSRB</td>
<td>1200</td>
<td>66</td>
</tr>
</tbody>
</table>

¹ Substrate Dabsyl-MetSO (Moskovitz et al., 2000a)
According to Sanchez et al. (1983) 85% of the PMSR activity in plants is due to the plastid forms and the remaining 15% to the cytosolic forms. This observation is consistent with the role of PMSR in repair of proteins damaged by ROS since the production of ROS has been shown to be the greatest in chloroplast (Allen, 1993). The role of PMSRA have also been documented by Ferguson and Burke (Ferguson and Burke, 1994) who found increased activity in plant PMSR in response to water deficit and high temperature. The function of PMSRA2 in the protection of Arabidopsis against oxidative stress induced by long nights has also been shown (Bechtold et al., 2004).

In the case of PMSRB, null mutants for one of the cytosolic isozymes are more susceptible to oxidative stress induced by hydrogen peroxide (Rodrigo et al., 2002) and it has been shown that the chloroplast PMSRB is one of the targets of CDSP32, a thioredoxin whose exclusive function is for response against oxidative stress (Rey et al., 2005).

The PSMR genes are not equally expressed in Arabidopsis. Their responses to light induction or stress conditions are different among the genes. Moreover, PMSR expression is apparently related to the age of the plants. Northern and Western analysis of PMSRA4 and PMSRA3 have shown that the plastid form (PMSRA4) is expressed only after short light exposure (20 minutes), while the cytosolic form (PMSRA3) is not responsive to light induction (Sadanadom et al., 2000). The plastid form is expressed primarily in photosynthetic organs, while the cytosolic form is expressed in all parts of the plant. The expression of the plastid form declines with age of the plant, whereas the cytosolic form is equally expressed (Sadanandom et al., 2000). Finally, PMSRA2 activity increases when ROS content peaks after long nights (Bechtold et al., 2004).

Understanding the function of PMSR in the cell is growing, due mainly to the extensive research on bacteria, yeast and animal systems. The recognition of specific proteins acted upon by PMSR in those organisms are growing in number. The PMSR proteins are also actively characterized in terms of biochemical properties, structural analysis, and mechanism of action. However, the same is not true in the case of PMSR in plants. Forty five years after the initial discovery of PMSR activity, only 11 papers dealing with PMSR activity in plants have been published, 6 within the past 5 years (Sadanandom et al., 2000; Gustavsson et al.,
Only few of those papers have found a relationship between PMSR and the response of plants to stress (Rodrigo et al., 2002; Bechtold et al., 2004; Dos Santos et al., 2005). So the need for more research on PMSR on plants is evident.

The goal of this dissertation is to determine the function of PMSRA in Arabidopsis. The research attempts to clarify the role of the enzyme in the protection of Arabidopsis plants against oxidative stress; and to determine if there are differences in the function of PMSRA isozymes depending on the sub-cellular organelle where they are localized. Chapter two describes the gene expression of the five Arabidopsis PMSRA genes, in the different organs of the plant and under different stress conditions. This chapter also compares some biochemical properties of two recombinant PMSRA proteins expressed in E. coli. In Chapter three, the effect of the overexpression of a cytosolic PMSR (PMSRA3) is analyzed in transgenic Arabidopsis plants exposed to different stress conditions. Chapter four summarizes the results of exposing transgenic Arabidopsis plants under or overexpressing PMSRA4 to different stress conditions. Chapter 5 describes the effects of exposing transgenic plants overexpressing PMSRA4 to Mn$^{2+}$ toxicity and mild water deprivation. Finally, Chapter six summarizes the results of the thesis, which are used to construct a descriptive model of the function of PMSRA in Arabidopsis.

REFERENCES


Boschi-Muller, S., Azza, S., and Branlant, G. (2001). E. coli methionine sulfoxide reductase with a truncated N terminus or C terminus, or both, retains the ability to reduce methionine sulfoxide. Protein Sci 10, 2272-2279.


for peptide methionine sulfoxide reductase (MsrA) and its substrate stereospecificity. J Biol Chem 275, 14167-14172.


Chapter 2

EXPRESSION PROFILE ANALYSIS AND BIOCHEMICAL PROPERTIES OF THE PEPTIDE METHIONINE SULFOXIDE REDUCTASE A (PMSRA) GENE FAMILY IN ARABIDOPSIS

ABSTRACT

The peptide methionine sulfoxide reductase A (PMSRA) gene family in Arabidopsis consists of five members, three in chromosome V (encoding cytosolic proteins), one in chromosome IV (encoding a plastid proteins) and one in chromosome II (encoding a protein targeted for secretion). Analysis of the sequence showed that four of the proteins (PMSRA1 to PMSRA4) have a conserved catalytic domain GCFWG and a domain exclusive for plants PIRCYG (PMSRA 5 does not have any of them). To establish detailed expression data of this gene family, comprehensive in silico and RT-PCR analyses for the five PMSRAs were conducted. Specificity was determined for organs, stage of development and expression in response to oxidative stress induced by high light, methyl viologen (paraquat), cercosporin and ozone. All genes are expressed in the different plant organs. PMSRA4 is predominant in all tissues except roots. PMSRA1 to PMSRA3 have higher expression in roots and stems. PMSRA1 is very important in flower buds and PMSRA 5 in stems. Promoter analysis showed the presence of cis responsive elements to different stress conditions such as metals, extreme temperatures, pathogens, water stress, high light salinity and others. RT-PCR analysis showed that PMSRA4 is up-regulated by the specific stress conditions, especially by high light that induced an 8-fold increase in mRNA. The other genes were not as responsive. To further
characterize the PMSRA family, recombinant plastid PMSRA4 and one cytosolic PMSRA (PMSRA3) were over-expressed in *E. coli* and purified. Kinetic parameters (\(K_M\), \(k_{cat}\), \(k_{cat}/K_M\)) were measured for both proteins. PMSRA4 is twice as fast and more than 4 times more efficient than PMSRA3, and has a lower \(K_M\) for 9-fluorenylmethylchloroformate-methionine sulfoxide (FMOC-MetSO)

**INTRODUCTION**

Reactive oxygen species (ROS) can be generated photochemically or by non-enzymatic reduction of molecular oxygen. ROS oxidize all biomolecules including the amino acids of proteins. Methionine residues are easily oxidized by ROS generating methionine sulfoxide (MetSO). The presence of MetSO affects the structure and function of the proteins (Moskovitz, 2005). To repair damaged proteins, the enzyme peptide methionine sulfoxide reductase (PMSRA) catalyzes the reduction of free and peptide-bound MetSO back to Met.

Most organisms with genome sequence contain only one PMSRA or two at most (Weissbach et al., 2005), while in plants several different PMSRA isoforms are found (Sadanandom et al., 2000). The Arabidopsis PMSRA was first sequenced in 1996 (Sadanandom et al., 1996). Sequencing the Arabidopsis genome revealed five genes encoding PMSRA. Three genes encode cytosolic proteins, one gene for a plastid-localized protein and one for a protein targeted for a secretory pathway. All members of this PMSRA gene family share a highly conserved structure and thus, by inference, mechanism.

Accumulating evidence suggests that the various members of the PMSRA family in Arabidopsis play distinct roles in response to stress conditions. The plastid PMSRA protein protects the chloroplast against ROS produced after photooxidative stress induced by high light or methyl viologen (see Chapter 4). A cytosolic PMSRA confers plants with some tolerance to oxidative stress generated under long night conditions (Bechtold et al., 2004). The plastid PMSRA has also been proposed to be involved in repair of a chloroplast-localized small heat shock protein (Gustavsson et al., 2002) and possibly of components of the photosystem II (see Chapter 4).
Expression patterns for Arabidopsis *PMSRA* genes have been analyzed in the plastid and one cytosolic form (Sadanandom et al., 2000). The effect of light in regulation of the plastid PMSRA has been examined (Sadanandom et al., 2000). The accumulation of the plastid PMSRA in response to photooxidative stress in the chloroplast has also been reported (see chapter 4). Gene expression analysis of a cytosolic PMSRA showed its universal presence in all plant organs and its induction by the cauliflower mosaic virus (CaMV) (Sadanandom et al., 2000). Although these studies have given insight into the function of PMSRA in the cells, more comprehensive gene expression analyses of all the PMSRA isoforms in Arabidopsis are needed to provide information on the diverse roles of PMSRAs. Toward the elucidation of the different roles of PMSRAs in Arabidopsis, the expression profiles of the five *PMSRA* genes were analyzed in the present study. To this end, preliminary kinetic studies on the plastid PMSRA and one of the cytosolic isozymes were also performed. The distribution of the PMSRAs in the different organs of the plant was examined in response to different oxidative stress conditions. These experiments provide further information on the role of PMSRA in Arabidopsis.

**MATERIALS AND METHODS**

*In silico* Analysis of *PMSRA* Genes

Sequence analysis of Arabidopsis *PMSRA* genes was performed using The Arabidopsis Information Resource (TAIR) database. *PMSRA* open reading frames in other plants were found using the NCBI Gene Bank database. Gene structure and promoter identity were predicted using GeneScan. Promoter *cis*-acting elements were predicted using PlantCare database (Lescot et al., 2002). Protein prediction and sequence alignments (using Clustal W) were performed using SDCS Biology Workbench tools. Phylogenetic trees were constructed using TreeTop software (http://www.genebee.msu.su/services/phtree_full.htm). Tissue
specific expression analysis was performed using the Arabidopsis Massively Parallel Signature Sequencing (MPSS) database (Brenner et al., 2000).

**Plant Material**

*Arabidopsis thaliana* ecotype Columbia seeds were grown in petri dishes on solid media (0.5X Murashige and Skoog, 2% sucrose, 0.7 bactoagar) for experiments with 10-day-old plants. For other experiments, plants were grown in a commercial soil mix (Redi-earth Plug and Seedling mix, Scotts-Sierra, Marysvill, OH), watered every third day by sub-irrigation and fertilized once a week with commercial fertilizer (Peter’s Professional; Scotts-Sierra 20-20-20). Plants were grown in lighted chambers at 23°C day / 21°C night with a 12-h light / 12-h dark photoperiod. The irradiance was approximately 150 µmol m⁻² s⁻¹ at canopy high, provided by incandescent bulbs and cool-white fluorescent tubes. Samples were harvested and flash-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

**RNA Isolation and RT-PCR**

Organ-specific expression of PMSRAs was analyzed for roots, stems, leaves, flower buds, and open flowers from 5 week-old plants. PMSRA expression was examined in 4 week-old plants that were exposed to 10 µM methyl viologen, 100 µM cercosporin, photooxidative stress (600 µmol m⁻² s⁻¹ at 8°C / 16 hrs) and ozone (0.35 µL L⁻¹ for 6 hrs per day 3 consecutive days). Control plants were kept under the initial conditions without treatment.

Samples were ground in liquid nitrogen, and total RNA isolated according to manufacturer’s protocol using RNeasy plant kit (Qiagen, Valencia CA). RNA content was determined by 260 nm absorbance after treatment with RNase-free DNase (DNA-free Ambion, Austin TX) for 30 min at 37°C. Using the Retroscript kit (Ambion, Austin TX), 2.5 µg of total RNA were reverse transcribed into cDNA with random primer d(N)₁₀, then
amplified with gene specific primers (Table 2.2) using antibody-mediated hot start with a mixture of *Taq* polymerase (GoTaq, Promega, Madison WI) and *Taq* antibody (BD Biosciences, Mountain View CA). In a total volume of 25 µL, the RT-PCR reaction solution contained 2.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 8.5), 60 mM KCl, 2.4 mM MgCl₂ and 300 µM of each dNTP. For each RT-PCR reaction, a plant 18S universal internal standard (Ambion, Austin TX) was included as a loading control. This standard was amplified using a pair of 18S rRNA specific primers and a pair of competitive primers mixed at a ratio 2:8 (18s rRNA primers: competitive primers). PCR reactions for all genes were subjected to 27 cycles at 94°C (45 s), 55°C (45 s) and 72°C (1 min). For each analysis three rounds of RT-PCR were conducted with three independently-isolated total RNA samples. Twenty µL from each PCR reaction were fractionated by 1.5 % (w/v) agarose gel in Tris-borate EDTA buffer and stained with 0.5% (w/v) ethidium bromide. The ethidium-bromide-stained gels were digitally photographed with an Eagle Eye II System (Stratagene, La Jolla CA). ImageJ for windows program was used to quantify the intensity of the DNA bands.
PMSRA Expression in \textit{E. coli}

cDNAs were synthesized from 10 day-old plants grown in solid medium (0.5x M&S, 1% Suc, 0.7% bacto-agar). The plastid (\textit{PMSRA4}), without the sequence encoding the chloroplast signal peptide, and a cytosolic (\textit{PMSRA3}) full length cDNAs were obtained using the following gene specific primers:

\begin{itemize}
  \item PMSRA4-Forward, 5’-ATGAACAACCTTTTCAACAGACTC-3’
  \item PMSRA4-Reverse, 5’-GTCGTTGCAGCCTTTTTC-3’
  \item PMSRA3-Forward, 5’-ATG AACACTGCGATTGTTCC-3’
  \item PMSRA3-Reverse, 5’-GCCACACCTTTTGACAGATAC-3’
\end{itemize}

\begin{table}
\centering
\begin{tabular}{lll}
\hline
Gene & Primer & Sequence \\
\hline
\textit{PMSRA1} & PMSRA1 EXP-F & 5’-CAATCGCACAAGTAATCGAC-3’ \\
& PMSRA1 EXP-R & 5’-GTCGTTGCAGCCTTTTTC-3’ \\
\textit{PMSRA2} & PMSRA2 EXP-F & 5’-CAAACCTGCGATTGTTCC-3’ \\
& PMSRA2 EXP-R & 5’-TGACAAGGTACTGCTGATGG-3’ \\
\textit{PMSRA3} & PMSRA3 EXP-F & 5’-GATTGGGTGTTGGATCAAG-3’ \\
& PMSRA3 EXP-R & 5’-GCCACACCTTTTGACAGATAC-3’ \\
\textit{PMSRA4} & PMSRA4 EXP-F & 5’-TTGTCCTTTCCCTCAAACC-3’ \\
& PMSRA4 EXP-R & 5’-CAACAGCTTCACGAGCTATG-3’ \\
\textit{PMSRA5} & PMSRA5 EXP-F & 5’-CGGATACTGTGGATTCTC-3’ \\
& PMSRA5 EXP-R & 5’-GCTCTTCCTTCTACCATGTGTTCC-3’ \\
\hline
\end{tabular}
\caption{Oligonucleotide primers used in RT-PCR}
\end{table}
PMSRA3-Reverse, 5’-TTAGCCGTAACAGCGGATTGGGTCGTTG

Restriction sites for directional cloning into the NdeI and XhoI sites of the pET-15b expression vector (Novagen, La Jolla, CA) were added to the primers. PCR products were cloned into pGEM-T easy (Promega, Madison, WI) and the identity and integrity confirmed by sequence analysis. The cDNA was digested with NdeI and XhoI and then ligated into the expression vector. PMSRA3 and PMSRA4 expression was induced in BL21-(DE3)pLysS cells (Novagen, LA Jolla, CA) with 1 mM IPTG. After 3 hours of induction, cells were lysed and the soluble PMSRA purified by Ni-affinity column. After elution, β-mercaptoethanol was added to 5 mM and the proteins were concentrated to 1.5 ml using an Amicon with a 10 K cut membrane. The His-tag was removed with 1.5 units/mg biotinylated thrombin (Novagen, La Jolla, CA). Thrombin was removed with streptavidin agarose and PMSRA3 and PMSRA4 recovered by spin filtration.

PMSR activity was measured according to the method of Ferguson and Burke (1994) with some modifications. MetSO (Sigma) was derivatized with 9-fluorenylmethyl chloroformate (FMOC) according to the procedure of Ferguson and Burke (1992). The resultant FMOC-MetSO was used as substrate with DTT as the electron donor. The 120 µl reaction mixture consisted of various FMOC-MetSO concentrations as specified in the figure legends, 150 mM DTT, 50 mM Tris-HCl, pH 8.0 (PMSRA4) or pH 7.5 (PMSRA3). Pure PMSRA3 or PMSRA4 was as specified in the figure and table legends. The reaction was at room temperature and quenched with 1 volume of cold acetone and kept at -20°C for 30 minutes followed by centrifugation at 16,000 × g. The supernatant was passed through a 0.2 µm filter. FMOC-Met was separated and quantified by HPLC using a Supelcosil LC-18 column (Supelco) and a fluorescence detector. The mobile phase consisted of 50 mM acetate buffer pH 3.9 (Solvent A) and acetonitrile (Solvent B). Solvent B was increased from 38% to 90% in 20 minutes, and then further increased to 96% for 3 minutes and then return back to initial conditions for column equilibrium. Met and MetSO content was determined by peak area (excitation 260 nm, emission 313 nm) as compared to known standards.
RESULTS

The Plant PMSRA Family

Analysis of the completed sequence of the Arabidopsis genome revealed the presence of five genes encoding PMSRA (At5g61640, At5g07460, At5g07470, At4g25130, At2g18030), which were named from ATPMSRA1 to ATPMSRA5 respectively (Table 2.1). In addition, four PMSRA-like proteins were found in rice, two in poplar, two in cabbage, two in Arabidopsis arenosa, two in Olimarabidopsis plumila, one in Capsella rubella, one in strawberry, one in cotton, one in lettuce, one in tomato, one in rye, one in canola and one in the red algae Gracilaria gracilis. The accession numbers of all the sequences are in Table 2.2. ATPMSRA4, BNPMSRA1, LSPMSRA1, PBPMRSA2 and GBPMSRA1 contain a putative chloroplast target sequence (not shown).

Amino acid sequences of the above PMSRA proteins (excluding ATPMSRA5 and OSPMSRA4) were aligned using the ClustalW program (Fig. 2.1). All sequences contained the conserved catalytic site GCFW followed by either Gly (as in other organisms) or by Ser. There are also two additional Cys residues at the C-terminus which have been shown to be involved in catalysis (Boschi-Muller et al., 2000; Lowther et al., 2000a). One of the Cys residues is part of the conserved region DPIRCYG which is only found in plant PMSRAs (BLAST search using this sequence resulted in hits only in plant PMSRAs and the red algae G. gracilis). In an alignment including all the protein sequences (not shown), ATPMSRA5 and OSPMSRA4 showed high similarity to PMSRA proteins (>50%) but they lack the key Cys residue in the active site GCFWG (Boschi-Muller et al., 2000; Lowther et al., 2000a). These two proteins also did not have the conserved region DPIRCYG. However, the alignment of the complete amino acid sequences (Fig. 2.2) showed high similarity between the two proteins. In addition, the substitution of the active-site Cys by Ser was conserved. Moreover, both proteins have a conserved region GWP(V/I)LRDI at the C-terminus, which could be a variation of the DPIRCYG region of the other plant proteins. The high similarity of these two proteins with PMSRA, but the lack of the key residues for catalysis (Boschi-
Muller et al., 2000; Lowther et al., 2000b; Moskovitz et al., 2000; Tete-Favier et al., 2000), suggests that these genes either do not encode a PMSRA protein, or might have a novel mechanism for carrying out the reduction of MetSO.

An alignment of the complete amino acid sequences of all the proteins was used to derive a phylogenetic unrooted tree using the “neighbor joining” method of the Philip package (Fig. 2.3). Bootstrap analysis with 1,000 replicates was performed to assess the statistical reliability of the tree topology. As expected, the most distant PMSRA is that of *G. gracilis*, due to the position of the organisms in the evolutionary scale (red algae). ATPMSRA5 and OSPMSRA4 form a separated PMSRA class, in concordance to the changes at the active site and at the C-terminus. BNPMSRA1, ATPMSRA4, PBPMMSRA2, GBPMSRA1 and LSPMSRA1 are grouped together. The similarity amongst the proteins shows the common cellular localization within the chloroplast, and might be related to their respective functions inside the organelle. Most of the monocotyledon-PMSRAs are in a separated group,

**Table 2.2.** Arabidopsis PMSRA Family. Data were obtained from The Arabidopsis Information Resouce (TAIR) database

<table>
<thead>
<tr>
<th>Proposed Name</th>
<th>Locus Name</th>
<th>GenBank Accession #</th>
<th>Subcellular Localization</th>
<th>No. amino acids</th>
<th>Introns</th>
<th>No. ESTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSRA1</td>
<td>At5g61640</td>
<td>NP_194243</td>
<td>Cytosol</td>
<td>202</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>PMSRA2</td>
<td>At5g07460</td>
<td>NP_196363</td>
<td>Cytosol</td>
<td>218</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>PMSRA3</td>
<td>At5g07470</td>
<td>NP_196364</td>
<td>Cytosol</td>
<td>202</td>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td>PMSRA4</td>
<td>At4g25130</td>
<td>NP_568937</td>
<td>Plastid</td>
<td>205</td>
<td>1</td>
<td>35</td>
</tr>
<tr>
<td>PMSRA5</td>
<td>At2g18030</td>
<td>NP_179394</td>
<td>Secretory Pathway</td>
<td>254</td>
<td>3</td>
<td>12</td>
</tr>
</tbody>
</table>
including those of rice and rye. Another group consisted of the cytosolic Arabidopsis PMSRAs ATPMSRA1 and ATPMSRA3 together with one from rice (OSPMSRA1), and one from each wild relative of Arabidopsis (OPPMSRA2, CRPMSRA2, AAPMSRA2, BOPMSRA2). Another group is conformed by the cytosolic ATPMSRA2, AAPMSRA1, OPPMSRA1 and BOPMSRA1. FAPMSRA1, LEPMSRA1 and PBPMSRA1 are grouped together and are distinctive from the other PMSRAs. The annotation of these enzymes in the GeneBank shows that they are particularly important during fruit ripening in strawberry and in tomato (E4 ripening enzyme in both species). Thus, the grouping in the phylogenetic tree may suggest a functional specialization of these PMSRAs.

**Table 2.3.** PMSRA proteins from plant species other than Arabidopsis. Sequences were obtained by BLAST search in the NCBI GeneBank using Arabidopsis PMSRA1, PMSRA3 and PMSRA5 sequences as query.

<table>
<thead>
<tr>
<th>Proposed Name</th>
<th>Species</th>
<th>Gene Bank Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNPMSRA1</td>
<td><em>Brassica napus</em></td>
<td>CAA62760</td>
</tr>
<tr>
<td>GBPMSRA1</td>
<td><em>Gossypium barbadense</em></td>
<td>AAO43182</td>
</tr>
<tr>
<td>LSPMSRA1</td>
<td><em>Lactuca sativa</em></td>
<td>AAF19789</td>
</tr>
<tr>
<td>OSPMSRA1</td>
<td><em>Oryza sativa</em></td>
<td>AAP55037</td>
</tr>
<tr>
<td>OSPMSRA2</td>
<td><em>Oryza sativa</em></td>
<td>XP472920</td>
</tr>
<tr>
<td>OSPMSRA3</td>
<td><em>Oryza sativa</em></td>
<td>XP472921</td>
</tr>
<tr>
<td>OSPMSRA4</td>
<td><em>Oryza sativa</em></td>
<td>BAD68657</td>
</tr>
<tr>
<td>LEPMSRA1</td>
<td><em>Tomato</em></td>
<td>AAB23481</td>
</tr>
<tr>
<td>FAPMSRA1</td>
<td><em>Fragaria x ananassa</em></td>
<td>CAA93442</td>
</tr>
<tr>
<td>SCPMSRA1</td>
<td><em>Secale cereale</em></td>
<td>CAE92372</td>
</tr>
<tr>
<td>GGPMSRA1</td>
<td><em>Gracilaria gracilis</em></td>
<td>AAD43253</td>
</tr>
<tr>
<td>PBPMSRA1</td>
<td>Hybrid Poplar</td>
<td>AAS46231</td>
</tr>
<tr>
<td>PBPMSRA2</td>
<td>Hybrid poplar</td>
<td>AAS46232</td>
</tr>
<tr>
<td>BOPMSRA1</td>
<td><em>Brassica oleracea</em></td>
<td>AAR13689</td>
</tr>
<tr>
<td>BOPMSRA2</td>
<td><em>Brassica oleracea</em></td>
<td>AAR13690</td>
</tr>
<tr>
<td>AAPMSRA1</td>
<td><em>Arabidopsis arenosa</em></td>
<td>AAR15485</td>
</tr>
<tr>
<td>AAPMSRA2</td>
<td><em>Arabidopsis arenosa</em></td>
<td>AAR15486</td>
</tr>
<tr>
<td>OPPMSRA1</td>
<td><em>Olimarabidopsis pumila</em></td>
<td>AAR15471</td>
</tr>
<tr>
<td>OPPMSRA2</td>
<td><em>Olimarabidopsis pumila</em></td>
<td>AAR15472</td>
</tr>
<tr>
<td>CRPMSRA1</td>
<td><em>Capsella rubella</em></td>
<td>AAR15455</td>
</tr>
</tbody>
</table>
Figure 2.1. Sequence alignment of plant and algae PMSRs. The amino acid sequences from the beginning of the PMSRA motif to the end of the proteins were used for the alignment. Light grey shade residues are the conserved active site for PMSRA activity (GCFWG). Dark grey shade residues are the conserved domain unique for plants (PIRCYG). AT, Arabidopsis thaliana. GG, Gracilaria gracilis. GB, Gossypium barbadensis. FA, Fragaria x Ananassa. LS, Lactuca sativa. LE, Lycopersicum esculentum. BN, Brassica napus. OS, Oryza sativa. SC, Secale cereale. PB, Hybrid poplar. OP, Olimarabidopsis pumila. AA, Arabidopsis arenosa. CR, Capsella rubella. BO, Brassica oleracea.
Figure 2.2. Sequence alignment of Arabidopsis (ATPMSRA5) and rice (OSPMSRA4) PMSRA-like proteins. The complete sequences were used for the alignment. The PMSRA active site localization (GCFWG) is shown on the upper part of the sequences at position 63. The two proteins share a high degree of similarity (> 50%) with PMSRA proteins but do not have the key Cys residue in the active site (position 65 in the alignment). Also the two proteins do not have the PMSR-Plant domain PIRCYG at the end of the sequence. Shade residues are identical.
Figure 2.3. Unrooted, bootstrapped tree of PMSRA proteins. An alignment of the entire sequence of amino acids was bootstrapped (n=1000) to create the final tree. Values indicate the number of times (in percent) that each branch topology was found during bootstrap analysis. AT, Arabidopsis thaliana. GG, Gracilaria gracilis. GB, Gossypium barbadensis. FA, Fragaria x Ananasa. LS, Lactuca sativa. LE, Lycopersicum esculentum. BN, Brassica napus. OS, Oryza sativa. SC, Secale cereale. PB, Hybrid poplar. OP, Olimarabidopsis pumila. AA, Arabidopsis arenosa. CR, Capsella rubella. BO, Brassica oleracea.
Expression of *PMSRAs* in the Organs of Arabidopsis

The expression of Arabidopsis *PMSRA* genes was analyzed to determine whether individual members of the family were expressed in particular organs. The analysis by RT-PCR (Fig. 2.4) showed that all the *PMSRA* genes are transcribed. *PMSRA4* transcripts were abundant in all the tissues except in roots. As expected, *PMSRA4* is highly expressed in photosynthetic tissues (leaves) and germinating seedlings. *PMSRA1* and *PMSRA5* have a very low expression in all the organs. *PMSRA2* and *PMSRA3* have the highest expression in roots and stems. The RT-PCR results are consistent with the *in silico* expression analysis using the MPSS database (Table 2.4). According to this analysis, *PMSRA4* is the most highly expressed *PMSRA* gene in Arabidopsis, followed by *PMSRA3* and *PMSRA2*. The expression levels of *PMSRA1* and *PMSRA5* are very low in the different organs. *PMSRA4* transcript is especially abundant in siliques, inflorescences in stages 3 to 6, and leaves. The highest expression level of *PMSRA1* is in leaves and immature flower buds. *PMSRA2* expression is the highest in roots and leaves. Roots are the organ with the highest *PMSRA5* expression.

Response of Arabidopsis *PMSRA* Genes to Stress Conditions

Analysis of the *PMSRA* promoters revealed many *cis*-regulatory elements that are responsive to stress (Table 2.5). In the *PMSRA* promoter there are *cis*-elements responsive to metals, water stress, heat shock, low temperatures, wound, elicitor induced, hormone, growth regulator responsive elements (related to stress conditions such as salicylic acid, methyl jasmonate, ABA) and. Each gene has its own unique set of *cis*-regulatory elements. Thus, all promoters have a heat stress element (HSE) and a wound responsive element (WUN); however, the metal responsive element MRE1 is present only in the promoters of *PMSRA1*, *PMSRA3* and *PMSRA4*. There are no drought stress or dehydration responsive elements in the promoters of *PMSRA1* and *PMSRA2*. *PMSRA1* and *PMSRA5* do not have *cis* elements responsive to methyl jasmonate or abscisic acid.
Figure 2.4. Expression of PMSRA genes in different organs. GS, germinated seedling; YL, young leaf; EL, mature fully extended leaf; CL, cauline leaf; ST, stem; FB, flower bud; MF, mature flower after anthesis; RT, roots. Signal values obtained from each gene were normalized with the 18S rRNA signal value and the resulting mean values were presented as relative units. Error bar represents SD (n=3).
RT-PCR analysis (Fig. 2.5) showed that \textit{PMSRA4} is the most responsive to the stresses tested (high light, methyl viologen, ozone, cercosporin). The most dramatic induction was observed with high light. The gene showed more than two-fold induction when the plants were exposed to cercosporin, ozone or methyl viologen (Table 2.6) and more than eight fold induction in the treatment with high light. \textit{PMSRA1} and \textit{PMSRA5} are repressed by cercosporin, ozone and methyl viologen, but slightly induced by high light. \textit{PMSRA2} expression is repressed by cercosporin but induced by the other treatments. \textit{PMSRA3} expression is only responsive to ozone showing only a slight induction.

\begin{table}[h]
\centering
\begin{tabular}{lcccccccc}
\hline
 & AP1 & AP3 & INS & ROS & LES & GSE & CAS & SIS \\
\hline
PMSRA1 & 20 & 5 & 30 & 24 & 39 & 0 & 0 & 41 \\
PMSRA2 & 0 & 11 & 0 & 185 & 62 & 0 & 20 & 22 \\
PMSRA3 & 12 & 22 & 24 & 20 & 39 & 26 & 88 & 8 \\
PMSRA4 & 21 & 109 & 144 & 23 & 95 & 37 & 142 & 207 \\
PMSRA5 & 7 & 10 & 9 & 23 & 3 & 17 & 8 & 23 \\
\hline
\end{tabular}
\caption{Quantitative estimate of expression of PMSRA genes using Massively Parallel Signature Sequencing (MPSS). The values are in transcripts per million (TPM) and correspond to the relative expression of 17 bp long sequences in the corresponding libraries. AP1, flower buds stages 1 to 10; AP3, flower buds stages 3 to 6; INS, inflorescence with immature buds; ROS, roots 21 days-old plants; LES, leaves 21 days-old plants; GSE, three days-old germinating seedlings; CAS, actively growing callus; SIS, siliques 24-48 hrs after fertilization.}
\end{table}
Table 2.5. Predicted cis elements in the promoters of Arabidopsis PMSRA genes.

Promoter sequences were analyzed in silico for the presence of cis-regulatory elements (+) using the PLANTCARE database. Promoter regions were determined using GENESCAN.

<table>
<thead>
<tr>
<th>ELEMENT</th>
<th>PMSRA PROMOTER</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>ABRE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ASF1-Motif</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AT-RICH_SEQUEN</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BOX-W1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CGTCA-MOTIF</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DRE</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EIRE</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ELI-BOX3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EMBP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ERE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GC-MOTIF</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HSE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LTR</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MBS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MRE1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MYB-CORE</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MYC</td>
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<td>-</td>
</tr>
<tr>
<td>SARE</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TCA-ELEMENT</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>WB-BOX</td>
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<td>-</td>
</tr>
<tr>
<td>WUN-MOTIF</td>
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<td>+</td>
</tr>
</tbody>
</table>
Figure 2.5. Response of PMSRA genes to oxidative stress conditions. UC, untreated control; CC, 100 µM cercosporin; O₃, 0.350 µL L⁻¹ ozone, 6 hrs per day for 3 days; MV, 10 µM methyl viologen; HL, High light, 600 µmol m⁻² s⁻¹ 16 hrs. Signal values obtained from each gene were normalized with the 18S rRNA signal value and the resulting mean values were presented as relative units. Error bar represents SD (n=3).
Recombinant PMSRA3 and PMSRA4 were expressed in *E. coli* and purified by affinity chromatography (Fig. 2.6A and 2.7A). Kinetic analyses of the two proteins were performed with FMOC-MetSO as a substrate. FMOC-MetSO was used to mimic a peptide bond. The time course of product formation is shown in Fig. 2.6B and 2.7B for PMSRA3 and PMSRA4, respectively. The activity is linear up to 60 minutes. For subsequent kinetic analysis where the initial velocity was measured, product formation was measured at 10 minutes.

Prior to steady state kinetic analysis, the pH optimum for activity was determined. For the cytosolic PMSRA3, the optimum is pH 7.5 (Fig. 2.6C). The pH profile shows an inflection at approximately 6.5 for an ionization which needs to be in the basic form and an inflection near 9.5 for an ionization which needs to be in the acid form. For the chloroplast

Table 2.6. Effect of oxidative stress treatments in PMSRA gene expression in Arabidopsis. The numbers correspond to the fold induction ± SD (n=3) in relation to the untreated controls. Numbers are derived from Fig. 2.5.

<table>
<thead>
<tr>
<th></th>
<th><em>PMSRA1</em></th>
<th><em>PMSRA2</em></th>
<th><em>PMSRA3</em></th>
<th><em>PMSRA4</em></th>
<th><em>PMSRA5</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cercosporin</td>
<td>0.58±0.23</td>
<td>0.49±0.01</td>
<td>0.91±0.03</td>
<td>2.18±0.30</td>
<td>0.60±0.06</td>
</tr>
<tr>
<td>Ozone</td>
<td>0.73±0.23</td>
<td>1.76±0.05</td>
<td>1.34±0.09</td>
<td>2.58±0.09</td>
<td>0.29±0.03</td>
</tr>
<tr>
<td>Methyl viologen</td>
<td>0.45±0.04</td>
<td>1.49±0.08</td>
<td>0.90±0.03</td>
<td>2.28±0.35</td>
<td>0.62±0.04</td>
</tr>
<tr>
<td>High Light</td>
<td>1.54±0.62</td>
<td>1.90±0.09</td>
<td>0.78±0.04</td>
<td>8.64±0.81</td>
<td>1.15±0.03</td>
</tr>
</tbody>
</table>

Biochemical Properties of PMSRA3 and PMSRA4

Recombinant PMSRA3 and PMSRA4 were expressed in *E. coli* and purified by affinity chromatography (Fig. 2.6A and 2.7A). Kinetic analyses of the two proteins were performed with FMOC-MetSO as a substrate. FMOC-MetSO was used to mimic a peptide bond. The time course of product formation is shown in Fig. 2.6B and 2.7B for PMSRA3 and PMSRA4, respectively. The activity is linear up to 60 minutes. For subsequent kinetic analysis where the initial velocity was measured, product formation was measured at 10 minutes.

Prior to steady state kinetic analysis, the pH optimum for activity was determined. For the cytosolic PMSRA3, the optimum is pH 7.5 (Fig. 2.6C). The pH profile shows an inflection at approximately 6.5 for an ionization which needs to be in the basic form and an inflection near 9.5 for an ionization which needs to be in the acid form. For the chloroplast
PMSRA4, the optimum is pH 8.0 (Fig. 2.7C), in agreement with previous work (Sadanandom et al., 2000). Inflections are at pH 6.5 and at 9.5 for the chloroplast enzyme.

Figure 2.6. Biochemical characterization of PMSRA3. A, Purification of recombinant PMSRA-3 from E. coli. PMSRA-3 containing fractions were subjected to SDS-PAGE and stained with Coomasie blue R-250 Lane 1: Non-induced control; Lane 2: after induction with 1 mM IPTG; Lane 3: after affinity purification using a Ni++ column; Lane 4: after thrombin digestion of the His-Tag. B, Time dependence of PMSRA3 activity. C, pH optima. D, Michaelis-Menton for FMOC-MetSO. The reaction mixture consisted of 100 mM Tris-HCl, pH 8.0; 150 mM DTT in a total volume of 120 µL. For B and C, 3 mM FMOC-MetSO was used as a substrate. In all the reactions PMSRA3 concentration was 1.34 µM. The reaction time for C and D was 10 minutes.
Figure 2.7. Biochemical characterization of PMSRA4. A, Purification of recombinant PMSRA-3 from *E. coli*. PMSRA-4 containing fractions were subjected to SDS-PAGE and stained with Coomasie blue R-250 Lane 1: Non-induced control; Lane 2: after induction with 1 mM IPTG; Lane 3: after affinity purification using a Ni\(^{2+}\) column; Lane 4: after thrombin digestion of the His-Tag. B, Time dependence of PMSRA4 activity. C, pH optima. D, Michaelis-Menten for FMOC-MetSO. The reaction mixture consisted of 100 mM Tris-HCl, pH 8.0; 150 mM DTT in a total volume of 120 µL. For B and C, 3 mM FMOC-MetSO was used as a substrate. In all the reactions PMSRA4 concentration was 0.60 µM. The reaction time for C and D was 10 minutes.
Steady-state kinetic parameters using FMOC-MetSO were determined. $K_M$ of both enzymes was in the millimolar range, $2.12 \pm 0.36 \text{ mM}$ for PMSRA3 and $1.09 \pm 0.12 \text{ mM}$ for PMSRA4 (Table 2.7). The $k_{\text{cat}}$ was measured at $0.91 \pm 0.14 \text{ s}^{-1}$ for PMSRA3 and $1.59 \pm 0.12 \text{ s}^{-1}$ for PMSRA4. The catalytic efficiency, $k_{\text{cat}}/K_M$ was calculated at $432 \text{ M}^{-1}\text{s}^{-1}$ and $1,450 \text{ M}^{-1}\text{s}^{-1}$ for PMSRA3 and PMSRA4 respectively.

<table>
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<tr>
<th>Enzyme</th>
<th>$K_M$ (mM)</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$k_{\text{cat}}/K_M$ (M$^{-1}\text{min}^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>PMSRA3</td>
<td>2.12±0.36</td>
<td>54.8±8.2</td>
<td>25906±4277</td>
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<tr>
<td>PMSRA4</td>
<td>1.10±0.12</td>
<td>95.2±7.3</td>
<td>86940±6127</td>
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</tbody>
</table>

**DISCUSSION**

Most organisms have one or two genes encoding PMSRA (Hoshi and Heinemann, 2001) whereas plants have multiple genes for PMSRA. The presence of several PMSRA isoforms might be related to the function of the enzyme in the response to ROS produced in different sub-cellular compartments by the metabolism of the cell (Perl-Treves and Perl, 2002).

Since plants are sessile and unable to avoid detrimental conditions that may yield ROS, evolution of multiple PMSRA enzymes to repair damaged proteins may be protective.
PMSRA is ubiquitous in the plant. The induction of each *PMSRA* is not only responsive to different stress conditions, but also is organ specific. *PMSRA4* is abundant in photosynthesizing tissues while *PMSRA3* is abundant in stems and roots, and *PMSRA1* is highly expressed in flower buds. The metabolic processes responsible of ROS production are characteristic of each organ and it is expected that the resultant oxidized proteins are going to be different. For example, proteins oxidized by ROS during the auxin-induced gravitropic response (Joo et al., 2001) are likely to be different from proteins oxidized by ROS after long nights (Bechtold et al., 2004); thus, the PMSRA necessary for repairing the oxidized proteins should be different in each case (PMSRA3 and PMSR2 respectively).

MPSS analysis shows that *PMSRA4* is the most highly expressed *PMSRA* gene in the different organs of Arabidopsis (Table 2.4). Furthermore, when the cell senses increased oxidative stress, *PMSRA4* transcript increases several times (Table 2.6). This pattern of gene induction suggests the production of unique chloroplast-derived signaling molecules when the level of ROS in this organelle increases. Photosynthesis and photorespiration are responsible for the production of large amounts of ROS (Foyer, 2002), making the chloroplast the major source of ROS in the cell. In fact, it has been shown that as a result of the highly oxidative environment proteins in the chloroplast have a higher percentage of MetSO than proteins from other compartments (see Chapter 4). Studies with yeast (Moskovitz et al., 1997) showed that under normal growth conditions, both WT and a knockout line contained approximately 3% MetSO content. Exposure to H$_2$O$_2$ results in an increase to 7% MetSO in WT and 15% in the knockout line. In Arabidopsis chloroplasts, under normal growth conditions, MetSO content is over 25% in WT and transgenic lines (see Chapter 4).

PMSRAs in plants apparently can have a more specialized role than a general repair of ROS-damaged proteins. For example, the sequence of an E4 ripening enzyme revealed features attributable to PMSRA in strawberry and tomato. The homology of these enzymes with PMSRA like proteins is more than 50% and showed the characteristic signatures GCFWG of active PMSRAs and PIRCYG of plant PMSRAs. This finding is very intriguing, since the E4 protein in tomato seems to be regulated by the ethylene burst typical of climatic fruits (Brady and Speirs, 1991), however, strawberry is not a climatic fruit and yet the E4 ripening enzyme is also present. This finding suggests that the induction of
PMSRA like proteins might be a general response of both climateric and non-climateric fruits to the production of free radicals during ripening. Fruit ripening is an organized process linked to oxidative phenomena (Masia, 1998), in which the coordinated activity of several hydrolytic enzymes is necessary. Thus, PMSRA activity would be crucial in maintaining the functionality of those enzymes involved in the maturation process.

Other PMSRA genes were not as responsive to the applied oxidative stress treatments as PMSRA4. This lack of induction of the genes of cytosolic localized PMSRAs (PMSRA1 to PMSRA3) is unexpected because some of the stressors used are known for inducing ROS in the cytosol, such as ozone (Langebartels et al., 2002) and cercosporin (Steinkamp et al., 1981) and one would expect repair mechanisms to be equally responsive in this cellular compartment. However, the differences among the promoter regions of each PMSRA gene (Table 2.5) are such that it is plausible that the responsiveness of the other genes (different from PMSRA4) to oxidative stress conditions was not achieved with the treatments used in this study.

In addition to investigating expression of the various PMSRA isozymes, two of the enzymes were kinetically characterized. This is part of an overall effort to understand the physiological role of these enzymes in different organelles of the plants. PMSRA3 (cytosolic) and PMSRA4 (chloroplastic) were characterized. Both have uncharacteristically high Km values in the millimolar region. However, FMOC-MetSO is not a physiologically relevant substrate. PMSRA4 has a $k_{cat}$ two times the value for PMSRA3 and the catalytic efficiency $k_{cat}/K_M$ is four times that of PMSRA3. PMSRA4 is localized in the chloroplast, where the majority of ROS are produced in the plant cell (Apel and Hirt, 2004) with the resulting accumulation of MetSO (see Chapter 4). To cope with the excess of oxidation, PMSRA4 must be abundant, fast and efficient and with the ability to react with MetSO from any protein. On the other hand, not only are there three PMSRAs in the cytoplasm, but the ROS production in the cytoplasm most likely is limited to electron leaking from other organelles such as chloroplasts, reticulum endoplasmic, mitochondria and peroxisomes (Dodge, 1994; Apel and Hirt, 2004). As a result, protein oxidation and MetSO accumulation should be lower than in the chloroplast and the individual PMSRAs do not have to be as efficient and fast as the plastid PMSRA.
The values of the kinetic properties determined for Arabidopsis PMSRA3 and PMSRA4 were comparable to the values measured in other species. $K_M$ values were similar to the $K_M$ measured for MsrA from *E. coli* (1.9 ± 0.2 mM) by Boschi-Muller et al. (2001) and the $K_M$ of a MsrB from *Neisseria meningitides* (1.2 ± 0.2 mM) reported by Boschi-Muller et al. (2005). However, the $K_M$ value for FMOC-MetSO was almost 10 times higher than that reported for *E. coli* (Moskovitz et al., 2000), but was very similar to the values reported for PMSRB in mammals (Kim and Gladyshev, 2004) and *Drosophila* (Kumar et al., 2002). The $k_{cat}$ values were lower than those reported for PMSRA *E. coli* (222 ± 30 min$^{-1}$) (Boschi-Muller et al., 2005), but more than 6 times (PMSRA4) and 4 times (PMSRA3) higher than the $k_{cat}$ recorded for a mammal PMSRB (13.8 min$^{-1}$) (Kim and Gladyshev, 2004) and more than 20 times that of Arabidopsis PMSRBs (4.2 and 1.2 min$^{-1}$) (Dos Santos et al., 2005). In terms of efficiency, $k_{cat}/K_M$ was slightly lower in PMSRA4 than in *E. coli* MsrA (121,200 ± 30,000 M$^{-1}$min$^{-1}$) (Boschi-Muller et al., 2005), similar to mammal MsrB (81,180 M$^{-1}$min$^{-1}$) (Kim and Gladyshev, 2004) and higher than Arabidopsis PMSRBs (53,460 and 22,200 M$^{-1}$min$^{-1}$) (Dos Santos et al., 2005).

In summary, the experiments have shown that the expression patterns of PMSRA genes are distinct and the differential expression patterns could be linked to responses to specific oxidative stress conditions in determined sub-cellular compartments. Based on the expression patterns a role for PMSRA4 can be ascribed to ROS accumulation in the chloroplast regardless of the initial origin of the oxidative stress condition. With the oxidative stress treatments employed, it is not possible to infer the function of the other PMSRAs. However, the organ-specific induction of *PMSRA* genes is an indicator that the different PMSRAs play different roles in the plant with unique natural substrates in each case. Furthermore, the specific induction of *PMSRA1* in flower buds and the presence of *PMSRA* genes during fruit production of tomato and strawberry suggest a role of PMSRA in plant development.
REFERENCES


Boschi-Muller, S., Azza, S., and Branlant, G. (2001). E. coli methionine sulfoxide reductase with a truncated N terminus or C terminus, or both, retains the ability to reduce methionine sulfoxide. Protein Sci 10, 2272-2279.


Dos Santos, C.V., Cuine, S., Rouhier, N., and Rey, P. (2005). The Arabidopsis plastidic methionine sulfoxide reductase B proteins. Sequence and activity characteristics,


Chapter 3

OVER-EXPRESSION OF A CYTOSOLIC PEPTIDE METHIONINE SULFOXIDE REDUCTASE (PMSRA3) CONFFERS INCREASED TOLERANCE TO OXIDATIVE STRESS IN ARABIDOPSIS

ABSTRACT

Methionine (Met) is the most easily oxidized amino acid. Exposure of proteins to reactive oxygen species (ROS) results in methionine sulfoxide (MetSO) formation. The enzyme peptide methionine sulfoxide reductase (PMSR) repairs oxidized proteins by reducing the MetSO back to Met. Transgenic Arabidopsis plants over-expressing PMSRA3 (cytosol localized PMSRA) under the control of the CaMV 35S promoter were generated to determine the role of the enzyme, in protection from ROS. Under optimal growth conditions, five independent transgenic lines with increased PMSRA3 expression exhibited no phenotypic difference from wild-type (WT) plants. However, plants over-expressing PMSRA3 were more resistant to methyl viologen, cercosporin and ozone, agents that caused oxidative stress. WT and transgenic plants exhibited different levels of photosynthesis, electrolyte leakage and MetSO in response to each treatment. Differences among the WT and transgenic lines in chlorophyll fluorescence and stomatal conductance were observed only in response to methyl viologen. The resistance of PMSRA3 over-expressing plants to methyl viologen appears to be mediated, in part, by Cu/Zn superoxide dismutase activity.
INTRODUCTION

The fidelity of passage of electrons from donor to acceptor, the energetic basis for all life, is central for metabolic efficiency. Utilization of oxygen as the terminal electron acceptor provides aerobes with an increased thermodynamic yield in catabolism. However the price tag for this yield is the potential for generation of reactive oxygen species (ROS) that can be toxic or even lethal to all aerobes. Oxygen is capable of “stealing” electrons from a variety of biochemical sources. The production of partially reduced oxygen species can lead to hydroxyl radical formation which can result in the oxidation of all biological macromolecules. Aerobes have evolved a variety of enzymatic and non-enzymatic mechanisms to scavenge ROS (Alscher et al., 2002; Apel and Hirt, 2004). Superoxide dismutase, catalase, peroxidase and other non-enzymatic antioxidants provide the first line of defense against ROS. However, under conditions of oxidative stress, the defense mechanism can be overwhelmed by ROS production resulting in oxidative damage to cellular components including proteins, membrane lipids and DNA.

Proteins, due to their abundance in the cell, are especially susceptible to oxidation by ROS. Met, even more than Cys or Tyr is the most susceptible amino acid to oxidation (Levine et al., 1996). The oxidation of Met to methionine sulfoxide (MetSO) can alter the structure of the protein and leads to improper folding (Schenck et al., 1996; Davies, 2005). Conformational changes in proteins can lead to loss of function and tagging for degradation (Vogt, 1995; Davies, 2005). The oxidized Met can be reduced back to Met by the enzyme peptide methionine sulfoxide reductase (PMSR). The enzyme uses thioredoxin as the biological reductant. The oxidation of surface-exposed Met and the subsequent reduction back to Met by PMSR has been proposed as a last-chance defense against ROS (Levine et al., 1996); (Levine et al., 2000). The oxidation of Met by ROS produces two stereoisomers of the sulfoxide (Sharov et al., 1999; Sharov and Schoneich, 2000). There are two families of PMSR enzymes; one family for each stereoisomer of MetSO. PMSRA reduces specifically the S stereoisomer and PMSRB, reduces the R stereoisomer (Sharov and Schoneich, 2000). There are nine open reading frames encoding proteins with high similarities to PMSRB proteins (Dos Santos et al., 2005) and five genes encoding PMSRA like proteins.
(Bechtold et al., 2004). By far, most work has been performed on the PMSRA family of enzymes in Arabidopsis. One is targeted to the chloroplast (PMSRA4) and three to the cytosol (PMSRA1 to PMSRA3) (Sadanandom et al., 2000). A fifth gene (PMSRA5) has a high degree of similarity to the PMSRA family and contains a leader sequence for secretion (see Chapter 4). However, conserved active site amino acids, proposed to be essential for activity (Boschi-Muller et al., 2000), are not found in the protein sequence. Studies thus far all indicate that the PMSR enzymes play a protective role against ROS. Studies have shown that PMSRA2 is involved in response to oxidative stress conditions arising from long nights (Bechtold et al., 2004). Chapter 4 shows that PMSRA4 plays a major role in the protection of chloroplast from oxidative stress. Under expression of PMSRB results in increased susceptibility of Arabidopsis to H₂O₂ (Rodrigo et al., 2002).

The present paper investigates the function of cytosolic PMSRA3 in protection of Arabidopsis from oxidative stress. Transgenic plants that over express the enzyme have been generated and compared to wild type (WT) under conditions of oxidative stress imposed by three different agents.

**MATERIALS AND METHODS**

**Construction of Transgenic Plants**

The cDNA encoding PMSRA3 (At5g07470) was obtained by reverse transcription of 2.5 µg of mRNA from *Arabidopsis thaliana* ecotype Columbia. A commercial kit (Retroscript System. Ambion, Austin, Texas) with oligo(dt) primer was used to obtain the cDNAs. Gene-specific primers PMSRA3-IntL (5'-ATGAACATACTCAACAGATTTGTTTGGG-3') and PMSRA3-intR (5'-TTAGCCGTAACACGGATTGTCGTTG-3') were used to obtain the coding region of PMSRA3 cDNA. The cDNA was cloned into pGem-T easy and then completely sequenced. The cDNA was cloned in the sense direction into PBI121. Fifteen plants were transformed by
Agrobacterium-mediated transformation using the floral dip method (Clough and Bent, 1998). To assure the independence of the insertions, seeds from each plant were always harvested separately. Screening for the transformed plants and selection of the homozygous T3 lines was performed in 0.5X M&S medium supplemented with 50 µM kanamycin. Homozygous T3 seeds were used for all the experiments. The independence of the lines was assured by keeping the seeds from each line isolated from the others after transformation.

**Analysis of Gene Expression**

RNA was extracted from 10 day-old plants grown in solid medium (0.5× M&S, 1% Suc, 0.7% bacto-agar). Leaf tissue was ground in liquid nitrogen and total RNA was extracted from 100 mg of tissue (RNeasy, Qiagen, Chatsworth, CA). RNA (1 µg) was treated with DNase I and reverse transcribed to synthesize the first strand cDNA using the Retroscript System (Ambion, Austin, TX) in a two-step reaction with previous denaturing of the RNA at 80°C and cDNA synthesis at 44°C using oligo d(T). The synthesized cDNA (2 µl) was used for PCR amplification of the genes of interest using gene specific primers. The expression of the endogenous *PMSRA3* was determined by RT-PCR using the PMSRA3-intL primer and the PMSRA3-extR primer (5’-TCGGAGAGGGACAGAGGA AAGAAGCAG-3’ targeted the 3’ end UTR). The total *PMSRA3* mRNA content (endogenous PMSRA3 plus the *PMSRA3* under the 35S promoter) was determined by with the PMSRA3-intL and PMSRA3-intR primers. The amount of transcript produced by the *PMSRA3* transgene under the 35S promoter was determined using the PMSRA3-intL primer and the Nost-R primer (5’-TCGCAAGACCGGCAACAGGATTC-3’) derived from the sequence of the Nos terminator pBI121. The mRNA levels were normalized using PCR amplified cDNA of a constitutively active actin cDNA (Jambunatan et al., 2001).
Oxidative Stress Treatments

Methyl viologen was applied to mature rosettes of 4 week-old plants by spraying a 10 µM solution of the herbicide with 0.05% Tween 20. Approximately 3 ml of methyl viologen per plant were applied 3 hours before the beginning of the light period. The plants were then returned to the growth chamber.

Ozone was applied to 4 week-old plants at 0.35 µL L⁻¹ for 6 hours during 4 days. Ozone was generated by passing oxygen through an ozonator (OREC V1-0, Ozone Research and Equipment, Phoenix), and O₃ concentrations in the growth chamber were monitored continuously with a UV photometric O₃ analyzer (Model 49, Thermo Environmental Instruments, Franklin, MA).

Plants were sprayed with 100 µM cercosporin in 20% acetone according to Orendi et al. (2001). Photosynthetic rates, stomatal conductance and the maximal quantum yield of PSII photochemistry (Fv/Fm ratio) were measured 24 hours before and after treatments. Photosynthetic rates and stomatal conductance were measured with a Li-Cor 6400 gas exchange system fitted with an Arabidopsis chamber (Li-Cor, Lincoln, NE). The gas exchange cuvette was maintained at 23°C block temperature, 400 µl·L⁻¹ CO₂ and 600 ±30 µmol photon·m⁻²·s⁻¹ light intensity. Chlorophyll fluorescence was measured using a pulse amplitude modulated fluorometer PAM-2000 equipped with an Arabidopsis Chamber and Clip (Walz, Effeltrich, Germany). Photosynthesis, stomatal conductance and chlorophyll fluorescence were determined in leaves 4 and 5 from the apex, of at least 8 plants (photosynthesis) or 12 plants (fluorescence). Photosynthesis and stomatal conductance was measured 4 hours after the beginning of the light period. Fv/Fm ratio was determined in dark-adapted leaves before the beginning of the light period. The percentage of reduction in the photosynthesis and in Fv/Fm ratio was calculated as the reduction in the variable after the treatment in relation to the value measured before the treatment.
Electrolyte Leakage

Twenty-four hours after oxidative stress treatments, leaves 4 and 5 were used for electrolyte leakage analysis. Leaves were submerged in individual glass tubes for 24 hrs in 5 ml of distilled de-ionized water at 22°C. Conductivity was measured using a Model 35 conductance meter (YSI Scientific). The tubes were then autoclaved for 10 min and then cooled to room temperature before measuring the final conductivity. The ratio between the first and second readings represents the relative injury of the tissues.

Protein Analysis

Homogenous tissue from leaves 4 to 8 from the apex was harvested from at least 5 different plants and divided into sub-samples, frozen in liquid nitrogen and stored at -80°C. The extraction of total soluble protein was as previously described for potato (Eckardt and Pell, 1994) except that 0.25 g of tissue were used per sample, a ratio of 1:5 (w/v) of tissue:buffer was used and 5 mM β-mercaptoethanol was used instead of DTT. Soluble protein concentration was measured by the Bio-Rad protein assay (Hercules, CA) with BSA as a standard. Rubisco large subunit (RbcL) and PMSRA content were determined by Western Blot analysis. Catalase and superoxide dismutase activities were determined by activity gel assays (Orendi et al., 2001). The MetSO content was measured as per Levine et al. (1996). The protein extracts were incubated with cyanogen bromide (CNBr) along with non-treated controls. CNBr does not react with MetSO whereas it oxidizes Met to homoserine. Subsequent acid hydrolysis with DTT reduces MetSO back to Met. Thus, in samples treated with CNBr, the Met content is equal to the MetSO content. Analysis of the untreated samples (no CNBr) yields the total Met content. Thus, the percent MetSO content can be determined by comparison between CNBr-treated samples and non-treated samples. MetSO content is expressed as percent MetSO: \[ \frac{\text{MetSO}}{(\text{MetSO} + \text{Met})} \times 100. \]

PMSR activity was measured as per Ferguson and Burke (1994) with some modifications. MetSO (Sigma) was derivatized with 9-fluorenylmethyl chloroformate
(FMOC) according to the procedure of Ferguson and Burke (Ferguson and Burke, 1992) (1992). Reaction mixture contained 3 mM FMOC-MetSO, 150 mM DTT, 50 mM Tris-Cl, pH 8.0 in a total volume of 120 µl. Activity was measured in the soluble fraction of chloroplasts. The reaction was carried out at room temperature and quenched with 1 volume of cold acetone. Protein was precipitated at -20°C for 30 minutes followed by centrifugation at 16,000 × g. The supernatant was passed through a 0.2 µm filter. FMOC-MetSO and the FMOC-Met formed were quantified by HPLC using a Supelcosil LC-18 column (Supelco). The mobile phase consisted of 50 mM acetate buffer, pH 3.9 (solvent A) and acetonitrile (solvent B). Solvent B was increased from 38% to 90% in 20 minutes, and then further increased to 96% for 3 minutes and then return back to initial. The amount of Met and MetSO in each reaction was determined by peak area.

**Statistical Analysis**

Analysis of variance (ANOVA) for physiological variables was performed using the general linear model in SAS (SAS System for Windows version 8.2; SAS Institute, Cary, NC). Treatment means were compared using Bonferroni test at the p ≤ 0.05 level.

**RESULTS**

**Expression of PMSRA3 in Transgenic and WT Plants**

Arabidopsis plants were transformed with CMV-35S promoter: PMSRA3 cDNA (35S::PMSRA3) using Agrobacterium tumefaciens. Five independent transgenic lines were further characterized. The expression level of PMSRA3 was measured by semi-quantitative RT-PCR (Fig. 3.1A). The transgenic plants consistently expressed more PMSRA3 than the WT untransformed plants (Fig 3.1A, “Total”). In all of the transgenic lines, the amount of
total message encoding PMSRA3 (endogenous $PMSRA3$ plus 35S::$PMSRA3$) was higher than the amount of the endogenous $PMSRA3$ transcript (Fig. 3.1A, “$PMSRA3$”). The transgene was found only in the transformed plants (Fig. 3.1A “Transgene”). When measured by Western blot, PMSRA3 protein content was also higher in the transformed plants (Fig. 3.1B). Over-expressing line 802 contained more than 3 times the PMSRA3 content of WT plants. The higher amount of PMSRA3 protein was also reflected by a 3-fold increase in total PMSR activity (Fig. 3.1C).

Under optimal growth conditions, there were no phenotypic differences between the transgenic lines and the WT plants. Physiological parameters photosynthetic rate, stomatal conductance (g), maximum quantum yield (Fv/Fm), effective quantum yield and peptide bound MetSO were all similar (Table 3.1).
Figure 3.1. Characterization of PMSRA3 expression in wild-type and transgenic Arabidopsis plants overexpressing PMSRA3. (A) RNA was isolated from 10 days-old wild type (WT) and 5 overexpressing (35S::PMSRA3) plants and subjected to RT-PCR analysis. Actin was used as an internal control. Gene specific primers were used to determine the combined level of endogenous mRNA and the transgenic mRNA (“Total”), the level of the natural gene (PMSRA3) and the transgene. (B) PMSRA3 content was determined by Western blot. 20 µg of protein were separated by SDS-PAGE, blotted and reacted with anti-PMSR antibodies. (C) Mean PMSR ± SD (n=3) activity in the soluble fraction of the plants. Activity was measured using FMOC-MetSO as a substrate and DTT as the electron donor. Experiments in A and B were repeated three times with similar results.
Leaves of Transgenic and WT Plants After Oxidative Stress.

The response of the over-expressing plants to cercosporin, methyl viologen and ozone was characterized. Cercosporin, which localizes to the cell membranes (Daub and Ehrenshaft, 2000) is a photosensitizer which generates singlet oxygen. Methyl viologen undergoes cyclic reduction/oxidation in the chloroplast being reduced by photosystem I and then being

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**Table 3.1.** Physiological parameters of WT and PMSRA3 overexpressing lines under optimal growth conditions. The results represent the mean ± SD. Measurements were taken in five-week old plants. Twelve different plants were measured for photosynthesis, stomatal conductance (g) and chlorophyll fluorescence (Fv/Fm, $\Phi_{PSII}$). MetSO corresponds to the percentage of MetSO in relation to the total Met+MetSO pool form soluble proteins of leaf tissue. Leaves from 4 plants were used in each replicate and the result corresponds to three replicates.

<table>
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<th>Line</th>
<th>Photosynthesis $\mu$mol CO$_2$ m$^{-2}$s$^{-1}$</th>
<th>g mol m$^{-2}$s$^{-1}$</th>
<th>Fv/Fm</th>
<th>$\Phi_{PSII}$</th>
<th>MetSO %</th>
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Leaves of Transgenic and WT Plants After Oxidative Stress.

The response of the over-expressing plants to cercosporin, methyl viologen and ozone was characterized. Cercosporin, which localizes to the cell membranes (Daub and Ehrenshaft, 2000) is a photosensitizer which generates singlet oxygen. Methyl viologen undergoes cyclic reduction/oxidation in the chloroplast being reduced by photosystem I and then being
oxidized by oxygen to produce superoxide (Dodge, 1994). Ozone, due to its mechanism of entry into the plant and its reactivity, is localized in the apoplast and the cytosol of plant cells (Langebartels et al., 2002).

Transgenic plants over-expressing PMSRA3 were more resistant to the oxidizing agents (Fig. 3.2). WT plants collapsed 24 hrs after treatment with methyl viologen. Most leaves were wilted and wrinkled with a paper-like consistency. The transgenic lines were not as severely affected. Over-expressing line 1002 showed some symptoms from methyl viologen treatment. Some of the leaves developed white spots and some leaves were curling. However over-expressing line 802 did not show any symptom of methyl viologen toxicity. The leaves were turgid, without any spot or symptom of damage.

WT plants treated with cercosporin showed symptoms of toxicity 24 hrs after the treatment (Fig. 3.2). A white halo in the base of the leaves close to the middle rib and the presence of watery spots (indicated by red arrows) were indicative of the cercosporin effect. PMSRA3 overexpressing lines did not show any symptom of cercosporin induced toxicity. In the case of ozone, WT plants showed symptoms of damage such as downward leaf rolling and dry regions (Sharma and Davis, 1994) 24 hrs after the end of the treatment, while the over-expressing lines did not show any visible injury.

**Electrolyte Leakage After Oxidative Stress**

Electrolyte leakage was used as an indicator of the damage of leaf cell plasma membranes (Fig. 3.3). In response to oxidative stress, WT plants showed higher electrolyte leakage than PMSRA3 over-expressing plants. WT plants lost 25%, 20% and 14% more electrolytes than PMSRA3 over-expressing plants when treated with methyl viologen, cercosporin and ozone, respectively. These differences between WT and the overexpressing lines were statistically significant (p≤0.05).
Figure 3.2. Representative 5 week-old WT and PMSRA3 over-expressing plants (35S::PMSRA3) 24 hrs after the oxidative stress. Treatments are 10 µM methyl viologen, 100 µM cercosporin and 0,350 µL L⁻¹ ozone, 6 hours a day/3 days. Arrows indicate the damage in the leaves. WT plants treated with methyl viologen showed symptoms in all expanded leaves.
Photosynthetic rate and stomatal conductance were similar amongst genotypes before the treatments and under optimal growth conditions; however, both physiological parameters were severely impaired by the oxidative stress treatments (Table 3.2). After treatment with methyl viologen, in WT plants photosynthetic rate decreased by 88% and stomatal conductance decreased almost 50%. In contrast, treatment of the PMSRA3 over-expressing plants with methyl viologen resulted in 50% to 60% decrease in photosynthetic rates for all genotypes.

**Figure 3.3.** Analysis of cellular damage induced by methyl viologen, cercosporin or ozone in wild-type (WT) and PMSRA3-overexpressing (203 to 1002) plants. Damage is indicated by the percent solute leakage in treated tissues after the treatments, compared with the electrolyte leakage of the same tissues after autoclaved. Values are means±SD (n=5 plants of each genotype per treatment). Within each treatment, the bars with the same letter are not statistically different (p≤0.05).
lines. Stomatal conductance was not affected by methyl viologen in the PMSRA3 overexpressing lines. The differences in photosynthesis and stomatal conductance between WT and the transgenic lines were statistically significant (p≤0.05).

Table 3.2. Gas exchange analysis of Arabidopsis plants overexpressing PMSRA3 after treatment with methyl viologen, cercosporin and ozone. Leaves 4 and 5 of 8 different plants were measured 24 hrs before and 24 hrs after treatments. Stomatal conductance (mol m⁻² s⁻¹) and photosynthetic rate (µmol m⁻² s⁻¹) are presented. Values correspond to the mean ± SD. The percentage of inhibition for each parameter was calculated as \{[(before) – (after)] / [before]\} * 100. Significance: **P≤0.01. ND no difference.

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<td></td>
<td>Before</td>
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71
Cercosporin and ozone affected the photosynthetic rate but not the stomatal conductance of WT plants. Cercosporin induced 40% reduction of photosynthesis. In the plants exposed to ozone photosynthesis was reduced by 70% after the treatment with this oxidative stress. In PMSRA3 over-expressing plants, cercosporin resulted in 20% of photosynthesis inhibition. Ozone-induced inhibition of photosynthesis was between 20% and 35%. Differences were statistically significant for both treatments (p≤0.05) between the WT and transgenic plants.

**Chlorophyll Fluorescence After Oxidative Stress**

Chlorophyll fluorescence was affected by methyl viologen treatment but not by cercosporin (Table 3.3). The maximum quantum yield of dark-adapted plants (Fv/Fm) was inhibited in all the plants after methyl viologen treatment. However, WT plants were more affected than the transgenic plants and the differences were statistically significant. The reduction of Fv/Fm was 33% in the WT plants and approximately 10% in the PMSRA3 over-expressing plants. In light-adapted plants, the effective quantum yield (ΦPSII) decreased by 22% in WT plants and 5% to 7% in the transgenic plants. The differences between the WT and the transgenic plants were statistically significant.

Chlorophyll fluorescence of dark-adapted plants (Fv/Fm) was not affected by the treatment with ozone, whereas the light-adapted plants decreased by approximately 24% in the effective quantum yield. There were no differences between WT and transgenic plants. The treatment with cercosporin did not affect chlorophyll fluorescence in any of the lines.

**MetSO Content After Oxidative Stress**

Under optimal growth conditions, the MetSO content of cytosolic proteins is approximately 15% (Table 3.1). In WT plants, treatment with 10 µM methyl viologen (MV), cercosporin and ozone resulted in 38%, 29% and 21% MetSO formation, respectively (Fig.
In the plants over-expressing PMSRA3, the MetSO content was lower after all of the treatments. After methyl viologen treatment, the MetSO content varied from 20% and 27%, after cercosporin, between 18% and 22% and after ozone, between 15% and 18%. The differences were statistically significant between WT and the transgenic lines with methyl viologen and cercosporin treatment but not with ozone.

**Table 3.3.** Effect of different oxidative stress treatments on the chlorophyll fluorescence of dark adapted leaves (Fv/Fm) or light adapted leaves (ΦPSII). Measurements were performed in the same leaves of the gas exchange analysis of Table 3.2. Values correspond to the mean ± SD. The percentage of inhibition for each parameter was calculated as in Table 3.2. Stars (**) denote statistic differences (p≤0.01). ND, no difference.

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Rubisco and Antioxidant Enzymes levels After Oxidative Stress

The RbcL content was analyzed by Western blot analysis. The content of WT and the PMSRA3 over-expressing plants were similar after treatment with methyl viologen, cercosporin or ozone (Fig. 3.5A). Catalase was measured by activity gel assays (Fig. 3.5B). The gels showed one predominant band which corresponded to isozyme CAT3 based on the position in the gel (Orendi et al., 2001). The activities were similar between WT and

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**Figure 3.4.** MetSO content of the cell soluble proteins from Arabidopsis plants overexpressing PMSRA3, after exposure to oxidative stress. MetSO is expressed as the percent MetSO in relation to the total Met pool (Met + MetSO). Values are means±SD of 3 replicates. Within each treatment, means with the same letter above the bars are not statistically different (p≤0.05)
transgenic plants treated with methyl viologen or ozone. However, the transgenic plants showed higher CAT3 activity than the WT when treated with cercosporin.

Gel activity assay of superoxide dismutase showed a predominant band (Fig. 3.5C). identified as a Cu/Zn SOD based on H2O2 and NaCN inhibition (Samis et al., 2002). There were no differences in superoxide dismutase activity among the different genotypes after treatment with cercosporin or ozone. However, after the treatment with methyl viologen the PMSRA3 over-expressing plants exhibited higher Cu/Zn superoxide dismutase activity than WT plants.

**DISCUSSION**

*PMSRA3* is second only to plastid PMSR in expression level in Arabidopsis (22 ESTs are found in the Arabidopsis Genome Sequence Database). The gene is expressed in all the plant organs with increased expression in the photosynthetic tissues (Sadanandom et al., 2000). In the present study, the role of PMSRA3 in protection of plants from oxidative stress has been characterized. The hypothesis was that oxidative stress induction in the cytosol would benefit from the activity of PMSRA3 and by extension plants overexpressing PMSRA3 would exhibit greatest protection from such stresses. To test this hypothesis, plants overexpressing PMSRA3 were treated with methyl viologen, cercosporin and ozone. Under non-stress conditions, over-expression of PMSRA3 has little or no effect on the parameters measured. Under optimal growth conditions total MetSO content varies between 13% and 17% (Table 3.1). This is lower than the 30% found in chloroplast (see Chapter 4). Thus, under optimal growth conditions, the MetSO content of cytoplasmic proteins is very low and the bulk of oxidized proteins are localized in other cell compartments (chloroplast). Under conditions of oxidative stress, cytoplasmic proteins are oxidized and over-expression of PMSRA3 afforded some protection to the plants.
Figure 3.5. Effect of oxidative stress treatments in rubisco content, CAT3 activity and SOD activity. Protein was extracted from leaves 4 and 5 of 5-week old plants 24 hrs after the treatment. Rubisco content was determined by Western blot analysis after the fractionation of 5 µg of protein. CAT and SOD activities were determined by activity gel assays. The results were scanned using a densitometer and quantified using Scion Image for windows. Relative intensity was calculated as the ratio between the measured activity / content compare to the activity / content of WT plants. Values are means±SD (n = 3).
Ozone has been proposed to react in the apoplast and the cytosol of plant cells. Due to its reactivity, it is not likely to reach the chloroplast (Langebartels et al., 2002). Cercosporin is localized in the membrane. It is a photosensitizer, generating singlet oxygen resulting in lipid peroxidation (Daub and Ehrenshaft, 2000). However, the activity of cercosporin is not confined to the plasma membrane as the toxin can initiate peroxidation of chloroplasts and mitochondrial membranes (Steinkamp et al., 1981). Methyl viologen undergoes cyclic oxidation/reduction in the chloroplast (Stevens and Sumner, 1991) generating superoxide. Superoxide dismutate activity yields hydrogen peroxide and in the presence of iron or copper, can generate the hydroxyl radical via the metal-catalyzed Haber-Weiss reaction (Babbs et al., 1989).

Ozone-induced visual injury observed in WT plants was less evident in the transgenic plants overexpressing PMSRA3 (Fig. 3.2). Ozone enters the cell through the stomata and reacts at the cell wall, apoplast, and cytoplasm, yet, signs of oxidative stress are also observed in the mitochondria, peroxisomes (Pellinen et al., 1999) and chloroplasts (Eckardt and Pell, 1995). In fact, ozone, or oxidants, derived from ozone, caused an increase in MetSO content of the chloroplast (see chapter 4) and reduction of photosynthesis (Torsethaugen et al., 1997). In plants over-expressing cytosolic PMSRA3, one would predict that proteins oxidized in the cytosol by ozone could be repaired but proteins in the chloroplast such as rubisco or PSII proteins would not be repaired. Indeed, the effective quantum yield measurements indicate that components of the PSII were affected by ozone but over-expression of cytosolic PMSRA3 failed to protect PSII proteins (Table 3.3). Also, despite only a slight increase in total soluble MetSO content (Fig. 3.4), ozone did cause a large decrease in photosynthesis (Table 3.2) and resulted in higher percentages of electron leakage (Fig. 3.3). Stomatal conductance was not significantly impacted by ozone treatment and PMSRA3 over-expression did not have an effect in stomatal conductance compared to WT (Table 3.2).

Visual symptoms of cercosporin-induced injury were observed in the WT plants but not in the plants overexpressing PMSRA3 (Fig. 3.2). The action of cercosporin is not confined to just the plasma membrane. Treatment with this photosensitizer induces the peroxidation of membrane components of chloroplasts and mitochondria (Steinkamp et al., 1981). In order to reach these organelles, cercosporin can either be localized there or alternatively, ROS
generated by cercosporin may diffuse through the cytoplasm oxidizing proteins and other molecules on the way. PMSRA3 would function in the cytoplasm not only to repair oxidized proteins important for the metabolism of the cell, but also to keep Met residues in the reduced form to serve as scavengers of the free radicals. Although membrane lipids are the primary target for cercosporin (Daub and Ehrenshaft, 2000), the results indicate that proteins are also damaged by the toxin. Cercosporin caused an increase in MetSO (Fig. 3.4) and the cells had higher electrolyte leakage (Fig. 3.3). Moreover, the over-expression of PMSRA3 reduced both MetSO content and electrolyte leakage. Cercosporin-treated plants had lower photosynthetic rate, and PMSRA3 over-expression partially restored the photosynthetic rate (Table 3.2). Leaf conductance was not affected by cercosporin, and the ROS produced as a result of cercosporin treatment were unlikely to reach the interior of the chloroplast because neither rubisco (Fig. 3.5A) nor the PSII components (Table 3.3) were affected.

Methyl viologen-induced visual injury was observed in all the fully extended leaves of WT plants. PMSRA3 overexpressing plants showed milder symptoms in fewer leaves. In fact, plants of the line 802 did not show any visual symptom at all (Fig. 3.2). The induction of ROS in the chloroplast by methyl viologen is well known (Babbs et al., 1989; Dodge, 1994). Since PMSRA3 localizes to the cytoplasm and methyl viologen produces ROS mainly in the chloroplast, it was not expected that PMSRA3 over-expression would affect the response of the plants to the herbicide. However, all the measured parameters showed that PMSRA3 over-expression effectively increased the resistance of Arabidopsis plants to methyl viologen. Two possible mechanisms can explain the increased resistance. The first involves methyl-viologen-dependent production of ROS at sites away from the chloroplast. It is possible that methyl viologen is reduced elsewhere in the cell. The second mechanism involves PMSRA3 affecting the overall antioxidant potential of the entire cell. This increase would also impact the anti-oxidative system inside the chloroplast, thereby increasing the resistance of the plants to the herbicide. The two mechanisms are not mutually exclusive. There is strong evidence that supports the first mechanism. In fact, the participation of mitochondria in the metabolism of methyl viologen has been documented in animals and plants (Vicente et al., 2001; Peixoto et al., 2004). Also, methyl viologen has been shown to be reduced by NADPH-cytochrome c reductase, an enzyme located in the endoplasmic reticulum (Bus et al., 1976a; Bus et al.,
It is possible that ROS induced by methyl viologen leak out of the chloroplast and the other cell compartments causing protein oxidation in the cytoplasm (Stevens and Sumner, 1991; Dodge, 1994). The lower protein-bound MetSO percent in the transgenic plants over expressing PMSRA only in the cytoplasm (Fig. 3.4) supports the hypothesis that ROS concentration is increased in the cytosol by methyl viologen. Furthermore, PMSRA3 over-expressing plants had higher photosynthetic rates than the WT, and the stomatal conductance in the transgenic plants is similar to the stomatal conductance of untreated plants (Table 3.2). Since PMSRA3 is not imported into the chloroplast, the proteins protected by the PMSR3 overexpression should be oxidized in the cytosol, where PMSRA3 is located. This mechanism, however, cannot explain all of the results, especially with chlorophyll fluorescence (Table 3.3). Methyl viologen affects PSII proteins (Fv/Fm and the effective quantum yield are decreased) to a greater extent in WT plants than in the over-expressing lines. Since PMSRA3 does not enter in the chloroplast, it cannot reach the PSII proteins; the impact it has on chlorophyll fluorescence in the over-expressing lines cannot be the result of the direct activity of PMSRA3. In this case, the second mechanism is the only plausible explanation where the protective effect of PMSRA3 overexpression must be indirect.

Superoxide dismutase activity in plants treated with methyl viologen is higher in the PMSRA3 over-expressing lines than in the WT after treatment (Fig. 3.5C). This indicates that PMSRA3 activity is somehow impacting superoxide dismutase activity. It is plausible that PMSRA3 overexpression affects signaling that controls superoxide dismutase activity in response to methyl viologen. This may be accomplished by regulation of proteins involved in signal transduction such as calmodulin (Bigelow and Squier, 2005). Also, the higher PMSRA3 activity would increase the Met residues in the reduced state which act as ROS scavengers in the cytoplasm. Consequently, the need for antioxidants in the cytoplasm is lowered and the cell can divert more resources towards the chloroplast, resulting in the activation of more anti-ROS defenses such as superoxide dismutase.

From the results it can be concluded that PMSRA3 is important in the response of the cell to oxidative stress in the cytoplasm. From this study with three different agents of oxidative stress, one putatively localized in the chloroplast, another in the membranes and a third in the cytoplasm, it can be concluded that the oxidative stress is manifested distal to the
site of action and that over-expression of a protective enzyme in the cytoplasm can impact the other compartments of the cell.

REFERENCES


Chapter 4

INVESTIGATIONS INTO THE ROLE OF THE PLASTIDIAL PEPTIDE METHIONINE SULFOXIDE REDUCTASE IN RESPONSE TO OXIDATIVE STRESS IN ARABIDOPSIS

Hernán M. Romero, Barbara S. Berlett, Philip J. Jensen, Eva J. Pell and Ming Tien
Plant Physiology Vol 136, 3784-3794, November 2004
Investigations into the Role of the Plastidial Peptide Methionine Sulfoxide Reductase in Response to Oxidative Stress in Arabidopsis

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Peptidyl Met residues are readily oxidized by reactive oxygen species to form Met sulfoxide. The enzyme peptide Met sulfoxide reductase (PMSR) catalyzes the reduction of Met sulfoxides back to Met. In doing so, PMSR is proposed to act as a last-chance antioxidant, repairing proteins damaged from oxidative stress. To assess the role of this enzyme in plants, we generated multiple transgenic lines with altered expression levels of the plastid form of PMSR (PMSR4). In transgenic plants, PMSR4 expression ranged from 95% to 40% (antisense) and more than 600% (overexpressing lines) of wild-type plants. Under oxidative stress conditions—methyl viologen, ozone, and high light—differences were observed in the rate of photosynthesis, optimal growing conditions, there is no effect of the transgene on the phenotype of the plants. When exposed to different oxidative stress conditions—methyl viologen, ozone, and high light—differences were observed in the rate of photosynthesis, and the Met sulfoxide content of the isolated chloroplast. Plants that overexpressed PMSR4 were more resistant to oxidative damage localized in the chloroplast, and plants that underexpressed PMSR4 were more susceptible. The Met sulfoxide levels in proteins of the soluble fraction of chloroplasts were increased by methyl viologen and ozone, but not by high-light treatment. Under stress conditions, the overexpression of PMSR4 lowered the sulfoxide content and underexpression resulted in an overall increase in content.

The generation of reactive oxygen species (ROS), especially under conditions of metabolic stress, is an unavoidable side effect of life in an oxygen atmosphere. These species include singlet oxygen, hydrogen peroxide, superoxide, and hydroxyl radical. To protect against ROS, aerobic organisms have evolved both enzymatic and nonenzymatic scavenging systems (Scandalios, 1993; Smirnoff and Wheeler, 2000). The enzymatic systems include superoxide dismutases (Scandalios, 1993), catalases, and peroxidases (Defelipe et al., 1988). The nonenzymatic systems include antioxidants such as glutathione, vitamin E, carotene, and vitamin C. ROS that escape the scavenging system can cause oxidative damage to virtually all biomolecules (Klatt and Lamas, 2000). Once damaged, certain macromolecules, such as DNA, may be repaired by the cell. Research within the past decade has shown that proteins can also be repaired.

Amino acids vary in susceptibility to oxidative damage, with Met residues the most vulnerable followed by Cys and Tyr (Levine et al., 1996). ROS readily oxidize Met residues by two electrons to form the sulfoxide (MetSO), which can be in the R or S configuration. The sulfoxide, in turn, can be reduced back to Met by the peptide Met sulfoxide reductase (PMSR). This enzyme, named MsrA in Escherichia coli (Moskovitz et al., 1995), utilizes thioredoxin to reduce only the S stereoisomer back to Met. This enzyme is active with either the free amino acid sulfoxide or the peptidyl MetSO. For the R stereoisomer of the sulfoxide, a gene encoding a PMSR was only recently discovered in E. coli (Grimaud et al., 2001) and has since been found in other cells (Lowther et al., 2002; Olry et al., 2002). This enzyme, named MsrB in E. coli, is homologous to a protein from Neisseria gonorrhoeae that can catalyze the reduction of the R isomer of MetSO (Lowther et al., 2002; Olry et al., 2002).

Plant PMSR activity in turnip and bean leaves was first reported in 1966 by Doney and Thompson (1966). The first isolation of a plant gene for PMSR was from Brassica napus (Sadanandom et al., 1996). Subsequently, it has been shown that Arabidopsis (Arabidopsis thaliana) has several copies of PMSR (Sadanandom et al., 2000). One of the gene products is targeted to the chloroplast, whereas the other three are believed to be cytosolic. A fifth gene with a high degree of homology to PMSR does not contain the conserved residues believed to be essential for activity and, according to the gene annotation in The Arabidopsis Information Resource (TAIR, At2g18030), is targeted to a secretory pathway. Results thus far have not clarified the role of the various PMSRs in...
Arabidopsis. Sadanandom et al. (2000) examined the expression of the plastid form and one cytosolic form and found the plastid form to be expressed in green tissues, whereas the cytosolic form was expressed in all tissues examined. No change in expression of either form was observed when exposed to high or low temperature, wounding, jasmonic acid treatment, or the virulent pathogen *Pseudomonas syringae*. Induction of the cytosolic form was only observed 35 d post-inoculation with the cauliflower mosaic virus.

To examine the role of PMSR in plants, we have transformed Arabidopsis plants to alter expression levels of the plastid form of PMSR (PMSR4). Multiple lines were generated expressing different levels of PMSR4 ranging from 95% to 40% (antisense) and more than 600% (overexpressing lines) of wild-type plants. Our results show that PMSR4 plays a role in protection from ROS in the chloroplast.

**RESULTS**

**Oxidative Stress and Wild-Type PMSR4 Activity**

Wild-type plants were exposed to three agents that have been shown to cause oxidative stress: ozone, methyl viologen, and high-light intensity. The PMSR4 activity was measured in chloroplasts isolated from foliage after these treatments and compared to non-treated controls. Because the PMSR activity was measured in isolated chloroplasts, other nonchloroplastic isozymes do not contribute to the total measured enzyme activity. Figure 1 shows PMSR4 expression as measured by western blot (Fig. 1A) and by enzyme activity (Fig. 1B) after the oxidative stress treatments. The intensity of the immunoblots was measured and revealed that PMSR content was 3.5 times higher after high-light treatment and 4.5 times higher after methyl viologen treatment. In contrast, PMSR4 content was only 1.6 times higher in plants exposed to ozone. The pattern in PMSR4 enzyme activity was similar to that obtained from the western blots.

**Purity of Chloroplast Preparations**

Because our studies of transgenic plants involve characterization of the chloroplast, we needed to initially determine the integrity of our chloroplast preparations. The NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (G3PDH) activity is a marker enzyme for the chloroplast, whereas phosphoenolpyruvate carboxylase (PEPC) is a marker enzyme for the cytosol. Transgenic and wild-type plants were grown under optimal growth conditions and the activity of these enzymes in the crude (chloroplast-containing) extracts and chloroplast preparations of the different lines was determined (Table I). After chloroplast isolation, the activity of the marker enzyme G3PDH was 2 to 3 times higher in the crude extract, showing that the procedure enriched for the targeted organelle. When assaying the isolated chloroplast for the cytosolic marker PEPC, a maximum of 5% of the activity was recovered in the plastidial preparation. This shows the high purity of the isolated chloroplasts.

The purity of the chloroplast was confirmed by Asp aminotransferase (AAT) activity gels. This method separates the cytosolic and the chloroplastic isoforms of the enzyme (Schultz and Coruzzi, 1995) and detects their activity in the gels. Both isoforms were detected in the crude extract, but only one band corresponding to the chloroplastic form of AAT is present in the isolated chloroplasts (Figs. 1C, 3B, and 4B). Since the results obtained with the AAT activity gels were consistent with those of the marker enzymes, we decided to use AAT gel activity assay to routinely monitor the purity of the chloroplast preparations. Furthermore, PEPC activity was determined in several more chloroplast isolations and in no case was it
detected more than 5% of cytosolic contamination (data not shown).

Characterization of Transgenic Plants

To study the role of PMSR4 in the response of Arabidopsis plants to oxidative stress conditions, we obtained transgenic plants with altered expression of PMSR4. A full-length PMSR4 cDNA was placed in the sense (overexpression) and in the antisense (underexpression) direction, behind the cauliflower mosaic virus 35S promoter (35S::PMSR4 and 35S::anti-PMSR4, respectively) and transformed into Arabidopsis. The presence of the construct was confirmed by PCR analysis in several lines, and the independence of the lines was assessed by Southern-blot analysis. Three of the lines of each construct were further characterized.

The expression level of PMSR4 in the transgenic plants was analyzed by semiquantitative reverse transcription (RT)-PCR and by western blot. For overexpressing plants with the construct in the sense orientation (Fig. 2), RT-PCR showed a large increase in the PMSR message. Using specific primers (see "Materials and Methods") that amplified only the mRNA transcribed from the transgene, we confirmed that the increase in the PMSR4 mRNA levels was due exclusively to the effect of the transformation. Furthermore, analysis of the plants showed that the transformation did not induce gene silencing by cosuppression. All of the lines showed overexpression of PMSR4 mRNA (Fig. 2A). Similar analysis of the transgenic antisense lines showed a considerable reduction in the PMSR4 mRNA as seen by RT-PCR analysis (Fig. 3A). The reduction in the mRNA levels was in accord with the decrease observed in the protein levels (Fig. 3A).

The peptidyl PMSR activity in the chloroplast extracts from these transgenic lines was measured using 9-fluorenylmethyloxycarbonyl (Fmoc)-MetSO as the peptide mimic substrate (Fig. 4). The overexpressing lines (Fig. 4A) expressed higher PMSR4-specific activity than the untransformed wild-type plants. The PMSR4-specific activity in the overexpressing line 1001 was nearly 6 times higher than the activity of the untransformed wild-type plants. The enzyme activity of line 202 was almost 3 times that of the wild type. Consistent with the results of the western blot shown in Figure 2, the activity of line 802 was not much higher than that of the wild type, but the difference was statistically significant (P ≤ 0.05).

In contrast, the PMSR4-specific activities of the underexpressing lines (Fig. 4B) were significantly lower than that of the untransformed wild type, ranging from 0.15 nmol min⁻¹ mg⁻¹ protein in line 1003 to 0.27 nmol min⁻¹ mg⁻¹ protein in line 801.

MetSO content in the soluble fraction of the chloroplast preparations was also measured (Table II). Under optimal growing conditions, there were no statistical differences (P ≤ 0.05) in the percentage of MetSO among the different transgenic lines and the control (wild-type) plants. MetSO content of the chloroplast proteins was between 27% and 33% of the total Met pool.

PMSR4 Overexpression and Oxidative Stress

The wild-type and transgenic plants underexpressing and overexpressing PMSR4 were subjected to the

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<td>1.32</td>
<td>1.32</td>
<td>0.06</td>
<td>3.6 ± 2.3</td>
</tr>
</tbody>
</table>

aChloroplast marker, NADP-dependent G3PDH. bCytosolic marker, PEPC.

Table I. Activities of marker enzymes in cells and chloroplasts of wild-type and transgenic plants underexpressing (lines 801, 1002, and 1003) and overexpressing (lines 802, 202, and 1001) PMSR4

Data are presented as µmol min⁻¹ mg⁻¹ chlorophyll for a representative experiment. The percentage of activity in the chloroplast fraction corresponds to the mean ± sd for three independent chloroplast preparations of 32 plants grown under optimal growing conditions as described in "Materials and Methods."
oxidative stress treatments of high-light/low-temperature methyl viologen and ozone. Photosynthetic rates and chlorophyll $a$ fluorescence were measured and the percentage reduction was calculated (see "Materials and Methods"). Under optimal growing conditions, no phenotypic variations between the transgenic overexpressing lines and the untransformed wild-type plants were observed (data not shown). The photosynthetic activity ranged from 7.1 to 7.6 $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ and the maximum quantum yield ($F_v/F_m$ ratio) was around 0.83. However, oxidative stress treatments induced a reduction in the photosynthetic rate (Fig. 5A) and in the $F_v/F_m$ ratio (Fig. 5B) in wild-type and overexpressing plants. The overexpression of PMSR4 reduced the severity of the stress-induced impact on the photosynthetic rate and the $F_v/F_m$ ratio. The treatment that resulted in the largest difference in response between the wild-type and the overexpressing lines was methyl viologen (i.e. overexpression of the chloroplast PMSR4 afforded the most protection from methyl viologen stress). Whereas the photosynthetic rate and maximum quantum yield were inhibited 67% and 27%, respectively, in wild-type plants, these functions were inhibited only 38% and 19% in the overexpressing line 802; 16% and 8% in the overexpressing line 202, and 0.5% and 11% in the overexpressing line 1001.

PMSR4 overexpression also enhances the resistance of the plants against the oxidative stress imposed by the treatment of high-light intensity under low temperature. The inhibition caused by high-light treatment in wild-type plants was 59% in photosynthetic rate and 24% in $F_v/F_m$ ratio. In plants overexpressing PMSR4, lines 802, 202, and 1001, the photosynthetic rate was inhibited to a lesser extent at 38%, 34%, and 28%, respectively. The reduction of $F_v/F_m$ ratio was 18% (line 802), 15% (line 202), and 12% (line 1001).

Treatment with ozone resulted in statistically significant differences in the photosynthetic rate between the wild-type and the transgenic lines, but not in the $F_v/F_m$ ratio (Fig. 5B) or in the stomatal conductance (Fig. 6). The inhibition of photosynthesis in the wild type was 33%, and in the overexpressing plants, it was
16%, 14%, and 10% in lines 802, 202, and 1001, respectively.

PMSR4 Underexpression and Oxidative Stress

Underexpression of PMSR4 resulted in greater sensitivity to high light and to methyl viologen, but not to ozone (Fig. 7). In plants exposed to high light under low temperature, photosynthesis and $F_v/F_m$ ratio were reduced 55% and 23%, respectively, in the wild type, whereas the reduction in the transgenic lines was 70% and 24% (line 801), 68% and 23% (line 1002), and 66% and 23% (line 1003). The reduction in photosynthesis and $F_v/F_m$ ratio in the plants treated with methyl viologen was 59% and 46% (wild type), 74% and 54% (line 801), 74% and 55% (line 1002), 71% and 57% (line 1003).

MetSO Content of Chloroplasts

The MetSO content of the soluble fraction of chloroplasts was also determined in wild-type and transgenic lines after treatment with oxidative stress agents. The MetSO contents of the overexpressing lines 202 and 1001 after high-light treatment were slightly, but significantly, lower than that of wild type (Fig. 8A). The percent MetSO was 30% in wild-type plants and 25% in line 1001. No statistical differences were observed between the wild-type and the other overexpressing line 802. Treatment of the plants with methyl viologen resulted in a large increase in the MetSO content of all plants; however, in the overexpressing plants, the MetSO content was lower (50%) than in the wild-type plants (58%). A similar MetSO pattern was observed when plants were treated with ozone. Whereas the wild-type plants contained 56% MetSO, the overexpressing lines contained statistically significant lower amounts of MetSO (approximately 35% for all lines).

In plants underexpressing PMSR4 (Fig. 8B), the high-light treatment resulted in formation of approximately 30% MetSO in all the lines. There were no significant differences among them. In plants underexpressing PMSR4, methyl viologen treatment resulted in MetSO content significantly increasing to a much higher value (as much as 80% in line 1003) relative to wild type. A similar MetSO profile was also observed when plants were treated with ozone. Plants underexpressing PMSR4 contained as much as 85% MetSO content after ozone treatment, a value much higher than that observed in wild-type plants.

DISCUSSION

Plant Oxidative Stress and PMSR

Due to the ease of Met oxidation to yield MetSO and the ability of PMSR to repair the MetSO, Met has been proposed to act as a last-chance antioxidant for proteins (Levine et al., 1999). Whereas most organisms have one gene encoding the PMSR that reduces the S enantiomer of MetSO (Hoshi and Heinemann, 2001), plant genomes encode multiple copies possibly due to different subcellular localization (Perl-Treves and Perl, 2002). This study used transgenic plants with altered PMSR4 expression levels to assess its role in oxidative stress. The chloroplast should be highly sensitive to alterations in PMSR expression because photosynthesis and photorespiration are responsible for the production of large amounts of ROS (Foyer, 2002). While in vitro studies have documented the role of PMSR4 in

| Table II. Percent basal MetSO content of isolated chloroplasts from wild-type and transgenic plants underexpressing (35S::anti-PMSR4) or overexpressing (35S::PMSR4) PMSR4 |
|---|---|---|---|---|---|
|  | Wild Type | 802 | 202 | 1001 | 801 | 1002 | 1003 |
| Percent MetSO | 28 ± 5 | 31 ± 13 | 28 ± 11 | 27 ± 10 | 28 ± 5 | 33 ± 10 | 27 ± 12 |


Figure 4. PMSR activity of isolated chloroplasts from transgenic Arabidopsis plants overexpressing (A) or underexpressing (B) PMSR4. The control values are of plants transformed with the empty vector. Results shown are mean and SD of three independent chloroplast preparations. Chloroplasts were isolated from 32 4-week-old plants grown under optimal conditions (see “Materials and Methods”). Enzyme activity was measured as described in the legend of Figure 1. Means with the same letter above the values are not statistically different ($P \leq 0.05$).
repair of peptidyl MetSO (Gustavsson et al., 1999, 2002), the results from in vivo studies have not been as clear (Ferguson and Burke, 1994; Sadanandom et al., 1996). Only recently, Bechtold et al. (2004) found a correlation between decreased PMSR2 levels and increased sensitivity to oxidative stress in Arabidopsis.

Expression of PMSR4

Our results show that wild-type plants increase PMSR4 expression in response to oxidative stress (Fig. 1). This expression is dependent upon the type of oxidative stress and where it is localized. Oxidative stress, localized in the chloroplast and induced by high light and methyl viologen, caused an increase in PMSR4 expression. In contrast, ozone, which probably reacts before reaching the chloroplast, had little, if any, effect on PMSR4 expression. Similar results were found by Sadanandom et al. (2000) working with other non-chloroplast-localized stress conditions. These results suggest that the signal-inducing PMSR4 expression originates in the chloroplast. Evidence of such compartment-specific signaling has also been observed for cytosol-expressed PMSR2 from Arabidopsis (Bechtold et al., 2004). The authors found that a null mutant of this enzyme did not exhibit increased sensitivity to a 10-fold excess of light, methyl viologen, drought, or infection with cauliflower mosaic virus or P. syringae.

The mutant was more susceptible only to oxidative stress in the dark phase of short-day conditions. These data are consistent with PMSR2 being responsive to oxidative stress localized only in the mitochondria, but not from other cell compartments.

High-Light Stress

High-light stress caused a decrease in both photosynthetic rate and in chlorophyll a fluorescence (Table III). However, no change was observed in the MetSO content of the soluble proteins. These results are actually consistent with the known mechanism of high-light damage. Upon high-light stress, there is a decrease in quantum yield and photosynthetic productivity referred to as photoinhibition. This has been suggested to be caused by ROS oxidation of PSI proteins (Aro et al., 1993). Although our measurement of MetSO content was only of the soluble proteins and Met oxidation of membrane proteins of PSII would not be detected, our results are consistent with PMSR4 being active with membrane-associated proteins. This would explain the large differences in $F_v/F_m$ ratio and photosynthetic rate between the wild-type and the overexpressing lines.

Consistent with the findings from the overexpressing lines, with plants underexpressing PMSR4, the reduction of the photosynthetic rate is not accompanied by an increase in MetSO content. This again
suggests that when plants are exposed to high light, PMSR4 plays a key role in repairing membrane proteins in the chloroplast. Aro et al. (1993) found that the degradation and replacement of the D1 protein (and at lower level D2 protein) in PSII play a protective role against photodamage under both stress and nonstress conditions. Both of these are membrane proteins that have high turnover rates (replaced quickly in the cell) and Met residues may play a key role in this turnover. Both proteins have several Met residues, which, upon oxidation, can induce structural changes in the protein triggering the signaling for proteolysis (Aro et al., 1993; Satoh, 1998). Such oxidation of D1 and D2 Met residues after high-light stress has been observed with purified proteins from pea plants (Sharma et al., 1997b). If oxidation of these Met residues facilitates their proteolysis, then PMSR4 may play a key role in regulating D1 and D2 turnover and, in turn, in the integrity of PSII.

Ozone Stress

Due to its reactivity, ozone is proposed not to reach the chloroplast, reacting in the apoplast and the cytosol of plant cells (Langebartels et al., 2002). However, Torsethaugen et al. (1997) demonstrated that ozone treatment results in a dose-dependent reduction of photosynthesis. Similarly, our results show that, in all the lines analyzed, ozone caused a reduction in photosynthesis but not in \( F_v/F_m \) ratio. In plants underexpressing PMSR4, despite higher levels of MetSO in the soluble proteins of chloroplasts, no significant difference was observed in photosynthesis when compared to wild-type plants. This indicates that the response of plants to ozone is not impacted by the basal level of PMSR4 expression. One plausible explanation is that Met oxidation in the chloroplast-soluble
proteins reached a level beyond which it is not possible to obtain further reduction in the photosynthetic rate. Yet, when PMSR4 is overexpressed, MetSO content is lowered in the soluble proteins, resulting in protection of photosynthesis. In the latter case, PMSR4-dependent repair lowered the MetSO content below the threshold value limiting photosynthesis.

The finding that ozone caused a decrease in photosynthesis but not in \( F_v/F_m \) ratio indicates that ozone has little impact on the proteins of PSI. In contrast, the changes in MetSO content indicate that ozone treatment results in oxidation of the soluble proteins of the chloroplast. Because Rubisco is the major protein of the chloroplast, it is a likely target for Met oxidation and repair in vivo. Previous research has attributed the decrease in photosynthesis after ozone treatment to be due to a decrease in Rubisco content (Pell et al., 1992; Landry and Pell, 1993). This decrease in Rubisco has been attributed to oxidation, inactivation (Roshchina and Roshchina, 2003), and proteolysis (Pell et al., 1992; Landry and Pell, 1993). Our results here are consistent with these findings (Table III). The repair of proteins by PMSR4 overexpression in the chloroplast would then lower the MetSO content and, in turn, result in higher photosynthetic rates.

Another possible mechanism for protection is the ability of PMSR4 overexpression to protect guard cells in the stomata. Ozone treatment has been shown to cause a guard cell-dependent decrease in CO₂ exchange and thus in photosynthetic rates mainly due to changes in stomatal conductance (Torsethaugen et al., 1999). However, our results (Fig. 6) show that stomatal conductance of plants overexpressing PMSR4 is not statistically different from wild-type plants. This shows that PMSR4 protection of photosynthesis after ozone treatment is not linked to changes in the aperture and closure of stomata.

**Methyl Viologen Stress**

Upon illumination, methyl viologen is reduced by PSI. Reduced methyl viologen, in turn, reduces \( O_2 \) yielding superoxide. The resulting superoxide, in the presence of iron or copper, can lead to generation of the hydroxyl radical. Thus, methyl viologen has the capacity to oxidize all soluble and membrane-associated proteins of the chloroplast. Indeed, among the different oxidative stress treatments, methyl viologen caused the strongest inhibition of photosynthesis and of maximum quantum yield. Methyl viologen also caused the largest increase in MetSO content of chloroplast-soluble proteins.

Our results indicate that PMSR4 plays a significant role in plant response to methyl viologen. Underexpression caused a greater decrease in the \( F_v/F_m \) ratio, a greater increase in MetSO formation in the chloroplast, and a greater decrease in assimilation.

![Figure 8. MetSO content of soluble proteins from isolated chloroplasts of plants after exposure to oxidative stress. A, Plants overexpressing PMSR4. B, Plants underexpressing PMSR4. MetSO content is expressed as the percent of MetSO in relation to the total Met pool (Met + MetSO). For details on the treatments, see “Materials and Methods.” SD is shown as an error bar and represents the average of three independent chloroplast preparations. In each section, means with the same letter above the bars are not statistically different (\( P \leq 0.05 \)).](image)

### Table III. Summary of plant response to oxidative stress

The number of arrows reflects the magnitude of the increase or decrease.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Measurement</th>
<th>Wild Type</th>
<th>Overexpressing</th>
<th>Underexpressing</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Light</td>
<td>Photosynthesis</td>
<td>↓↓</td>
<td>Protect</td>
<td>Inhibit</td>
</tr>
<tr>
<td>High Light</td>
<td>( F_v/F_m )</td>
<td>↓↓</td>
<td>Protect</td>
<td>No change</td>
</tr>
<tr>
<td>High Light</td>
<td>MetSO</td>
<td>No change</td>
<td>↓</td>
<td>No change</td>
</tr>
<tr>
<td>Paraquat</td>
<td>Photosynthesis</td>
<td>↓↓</td>
<td>Protect</td>
<td>Inhibit</td>
</tr>
<tr>
<td>Paraquat</td>
<td>( F_v/F_m )</td>
<td>↓</td>
<td>Protect</td>
<td>Inhibit</td>
</tr>
<tr>
<td>Paraquat</td>
<td>MetSO</td>
<td>↑</td>
<td>↓</td>
<td>No change</td>
</tr>
<tr>
<td>Ozone</td>
<td>Photosynthesis</td>
<td>↓</td>
<td>Protect</td>
<td>No change</td>
</tr>
<tr>
<td>Ozone</td>
<td>( F_v/F_m )</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Ozone</td>
<td>MetSO</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
</tr>
</tbody>
</table>
capacity. In contrast, plants overexpressing PMSR4 were more resistant to methyl viologen as demonstrated by lower MetSO formation, higher \( F_v/F_m \) ratio, and higher photosynthetic rates (line 1001, Fig. 5C).

Summary of PMSR Function

The apparently wide range of functions of PMSR4 is still not clearly resolved and the natural substrates for the enzyme are yet to be identified. For example, Gustavsson et al. (1999) have shown that the chloroplast chaperone-like protein HSP21 has a Met-rich domain. Upon oxidation, the chaperone-like activity is lost; however, when the oxidized HSP21 is treated with recombinant PMSR4, the activity of HSP21 is recovered (Gustavsson et al., 2002). PMSR4 may also have a role in the regulation of chloroplast proteins. For example, the \( \beta \)-subunit of cytochrome b\(_{599} \) and the psbI gene product of PSI have four and two oxidizable residues, respectively. However, upon light treatment, only one specific Met residue in each protein is oxidized (Sharma et al., 1997a). The presence of these specific residues that are highly susceptible to oxidation is not well understood, but given the presence of PMSR4 in the chloroplast and its induction after high-light stress, the oxidation of these Met residues and their subsequent reduction by PMSR4 might play a regulatory role of PSI.

The lack of phenotypic variation between underexpressing and wild-type plants under nonstress conditions suggests that PMSR4 does not play a crucial role in ROS metabolism under low-stress conditions. However, PMSR4 appears to play a key role in plant response under conditions of oxidative stress. Our findings also indicate that, under basal nonoxidative stress conditions, plant chloroplasts contain a high level of MetSO and appear to be tolerant of these high levels. The average value of 27% to 33% MetSO in the soluble fraction of the chloroplasts of different lines under optimal growing conditions is considerably higher than the protein MetSO content reported for yeast (Saccharomyces cerevisiae), bacteria (Moskovitz et al., 1997), and mammals (Fliss et al., 1983). Also, MetSO content in the chloroplast is higher than the MetSO content found in the whole-cell proteins of cotton, pea, wheat, and potato (Ferguson and Burke, 1994). This is consistent with the highly oxidative environment of the chloroplast and is consistent with the proposal that Met serves as a last-chance antioxidant.

Construction of Transgenic Arabidopsis Plants

The full-length cDNA encoding PMSR4 from Arabidopsis (expressed sequence tag 226P20T7; GenBank accession no. X97326) was obtained from the Arabidopsis Biological Resource Center (Columbus, OH). The cDNA was cloned in the sense (overexpressing) and the antisense (underexpressing) direction into the pBI121 vector after removal of the \( \beta \)-glucuronidase gene from the vector using BamHI and SalI. The vector utilizes the 35S promoter. The orientation of the cDNA in each case was confirmed by restriction analysis. Fifteen plants were vacuum transformed with either the sense or the antisense construct and also with an empty vector as a control. Seeds from each plant were harvested separately. Seeds from the transformed plants were screened for resistance on 0.5 \( \times \) Murashige and Skoog plates supplemented with 50 \( \mu \)g ml\(^{-1} \) kanamycin. Resistant seedlings were transplanted and allowed to self. Seeds were harvested and screened again for kanamycin resistance. Resistant seedlings were transplanted and seed collected from individual T2 plants was again screened for kanamycin resistance to identify plants homozygous for kanamycin resistance. Homozygous T3 kanamycin-resistant plants were used in all the experiments.

Gene Expression Analysis

RNA was extracted from 10-d-old plants grown in solid medium (0.5 \( \times \) Murashige and Skoog, 1% Suc, 0.7% bacto-agar). Leaf tissue was ground in liquid nitrogen and total RNA was extracted from 100 mg of tissue (RNAeasy; Qiagen, Chatsworth, CA). RNA (1 \( \mu \)g) was treated with DNaseI and reverse transcribed to synthesize the first-strand cDNA using the Retroscript System (Ambion, Austin, TX) in a two-step reaction with previous denaturing of the RNA at 80°C and cDNA synthesis at 44°C using oligo(dT). The synthesized cDNA (2 \( \mu \)l) was used for PCR amplification of the genes of interest using gene-specific primers as in Zhong et al. (2003). For the overexpressing lines, the total amount of PMSR4 mRNA (the product of the natural endogenous \( PMSR4 \) plus the product of the introduced \( PMSR4 \) under the 35S promoter) was determined with the PMSR4-intL primer (5'-TCGACGACCTTTCTTCCA-CA-GACTCCG-3') and the PMSR4-intR primer (5'-TTACCATACAGTGATTGATC-3'), which were specific for the coding region of PMSR4. The amount of transcript product of the \( PMSR4 \) transgene under the 35S promoter control was determined using the same left primer as before (PMSR4-intL) and the Nost-R primer (5'-TCCGACAGCCCGACACAGGATTC-3') derived from the sequence of the Nos terminator in pBI121. For the antisense lines, the \( PMSR4 \) mRNA level was determined using the PMSR4-intR and the PMSR4-intL primers. The mRNA levels where normalized using the PCR-amplified cDNA of a constitutively active actin cDNA (Jambunatan et al., 2001).

Oxidative Stress Treatments

Methyl viologen was applied to mature rosettes of 4-week-old plants by spraying with 10 \( \mu \)M methyl viologen with 0.05% Tween 20. Approximately 3 ml of methyl viologen per plant were applied 3 h before the beginning of the light period. The plants were then returned to the growth chamber.

Ozone was applied to 4-week-old plants at 0.46 \( \mu \)L L\(^{-1} \) for 6 h during 4 d. Ozone was generated by passing oxygen through an ozonator (OREC V1-0, Ozone Research and Equipment, Phoenix), and \( O_3 \) concentrations in the growth chamber were monitored continuously with a UV photometric \( O_3 \) analyzer (model 49; Thermo Environmental Instruments, Franklin, MA).

To determine the effect of high light, 4-week-old plants were exposed to 600 \( \mu \)mol photon m\(^{-2} \)s\(^{-1} \) light intensity at 8°C for 48 h.

Photosynthetic rates, stomatal conductance, and the maximal quantum yield of PSI photochemistry (\( F_v/F_m \) ratio) were measured 24 h before and after treatments. Photosynthetic rates and stomatal conductance were measured with a LI-COR 6400 gas exchange system fitted with an Arabidopsis chamber (LI-COR, Lincoln, NE). The gas exchange cuvette was maintained at 23°C block temperature, 400 \( \mu \)L L\(^{-1} \) \( CO_2 \) and 600 ± 30 \( \mu \)mol photon m\(^{-2} \)s\(^{-1} \) light intensity. Chlorophyll \( a \) fluorescence was measured using a pulse amplitude-modulated fluorometer PAM-2000 equipped with an Arabidopsis chamber and clip (Walz, Effeltrich, Germany). Photosynthesis, stomatal conductance, and chlorophyll \( a \) fluorescence were determined in leaves 4 and 5 from the apex of at least 8 (photosynthesis) or 12 (fluorescence) plants. Photosynthesis and stomatal conductance were measured 4 h after the beginning of the light period. \( F_v/F_m \) ratio was determined in dark-adapted

MATERIALS AND METHODS

Plant Material

Arabidopsis (Arabidopsis thaliana) ecotype Columbia seeds were planted in a commercial soil mix (Redi-earth Plug and Seedling mix; Scotts-Sierra, Marysville, OH) and grown in lighted growth chambers, with approximately 120 \( \mu \)mol photon m\(^{-2} \)s\(^{-1} \) on a 23°C/21°C, 12-h day/night cycle. Plants were fertilized weekly with 20:20:20 fertilizer (Peters Professional; Scotts-Sierra).
leaves before the beginning of the light period. The percentage of reduction in photosynthesis and in $F_v/F_m$ ratio was calculated as the reduction in the variable after the treatment in relation to the value measured before the treatment. After measuring photosynthesis, plants were placed in the dark for 6 h and then leaves were harvested for chloroplast isolation.

**Chloroplast Preparation**

Chloroplasts were isolated from fully expanded leaves of 4-week-old plants (32/preparation). The purity of the preparations was determined by Asp transaminase activity gels according to the organelle preparation protocol (Weigel and Glazebrook, 2002). The chloroplast marker enzyme G3PDH was also measured (Stitt et al., 1989). Cytosolic contamination of the chloroplast was determined by the cytosolic marker enzyme PEPC (Stitt et al., 1989).

**Western-Blot Analysis**

Isolated chloroplasts were resuspended in 50 mM Tris-Cl, pH 7.5, 0.1% Triton X-100, 10% glycerol, 5 mM β-mercaptoethanol, 10 mM MgCl2, 50 mM KCl, and sonicated on ice for 2 min. To remove the chloroplast membranes, the sample was centrifuged at 4°C for 15 min. Protein content was determined by the Bio-Rad protein assay (Hercules, CA) following the manufacturer’s instructions. SDS-PAGE was performed using the Bio-Rad Mini-Protein system and the separated proteins were transferred to nitrocellulose using a Bio-Rad Transfer Blot Cell. PMSR4 was visualized using antibodies raised against the recombinant PMSR4 at 1:1,000 concentration and detected using a Bio-Rad Transfer Blot Cell. PMSR4 was visualized using antibodies raised against the recombinant PMSR4 at 1:1,000 concentration and detected using the Bio-Rad protein assay (Hercules, CA) following the manufacturer’s instructions. Immunoblots were digitized and the intensity of the bands measured with Scion Image freeware (Scion; http://www.scioncorp.com).

**Measurement of MetSO Content**

Lysed chloroplasts (100 μL) were centrifuged at 16,000g at 4°C and the supernatant proteins were isolated according to the method of Ferguson and Burke (1994). The MetSO content was measured as per Levine et al. (1996). The protein extracts were incubated with cyanogen bromide (CNBr) along with nontreated controls. CNBr does not react with MetSO, whereas it oxidizes Met to homo-Ser. Subsequent acid hydrolysis with dithiothreitol reduces MetSO back to Met. Thus, in samples treated with CNBr, the Met content is equal to the MetSO content. Analysis of the untreated samples (no CNBr) yields the total Met content. Thus, the percent MetSO content can be determined by comparison between CNBr-treated samples and nontreated samples.

**PMSR Activity Assay**

PMSR activity was measured as per Ferguson and Burke (1994) with some modifications. MetSO (Sigma, St. Louis) was derivatized with Fmoc according to the procedure of Ferguson and Burke (1992). Reaction mixture contained 3 mM Fmoc-MetSO, 150 mM dithiothreitol, 50 mM TRIS-Cl, pH 8.0, in a total volume of 120 μL. Activity was measured in the soluble fraction of chloroplasts. The reaction was carried out at room temperature and quenched with 1 volume of cold acetonitrile. Protein was precipitated at −20°C for 30 min followed by centrifugation at 16,000g. The supernatant was passed through a 0.2-μm filter. Fmoc-MetSO and the Fmoc-Met formed were quantified by HPLC using a Supercosil LC-18 column (Supelco, Bellefonte, PA). The mobile phase consisted of 50 mM acetate buffer, pH 3.9 (solvent A), and acetonitrile (solvent B). Solvent B was increased from 38% to 90% in 20 min, and then further increased to 96% for 3 min and then returned to initial. The amount of Met and MetSO in each reaction was determined by peak area.

**Statistics**

Data were subjected to ANOVA using statistical software (SAS System for Windows version 8.2; SAS Institute, Cary, NC). Means were compared by Duncan’s test at $P \leq 5\%$.

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**LITERATURE CITED**


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All the experiments listed in the paper were done by me, under the supervision of M. Tien and E. Pell. B. S. Berlett contributed in the determination of the methionine sulfoxide levels in the leaf proteins. P. J. Jensen contributed the development of the antibodies anti-PMSR that I used in the expression analysis.
Chapter 5

INTEGRITY OF THE PLASTID PEPTIDE METHIONINE SULFOXIDE REDUCTASE IS A DETERMINING FACTOR IN THE TOLERANCE TO Mn EXCESS IN ARABIDOPSIS

ABSTRACT

Reactive oxygen species (ROS) are toxic byproducts of aerobic metabolism and under conditions of metabolic stress, their production can overwhelm the ROS scavenging system. ROS oxidize all biological macromolecules including proteins of which methionine (Met) residues are most easily oxidized to yield methionine sulfoxide (MetSO). The enzyme peptide methionine sulfoxide reductase (PMSR) repairs the oxidized proteins by reducing the MetSO back to Met. In this report, the role of chloroplast PMSRA4 in Mn$^{2+}$ toxicity is explored. Mn$^{2+}$ toxicity reduces PMSRA4 content and activity. As a result peptidyl MetSO content increases. Gas exchange, chlorophyll fluorescence, chlorophyll and protein content and rubisco activity are affected. PMSRA4 over-expression protects plants against Mn toxicity. In the transgenic plants, in contrast to wild-type plants, Mn-induced ROS do not destroy all the PMSRA4 protein. As such, the physiology of these plants is not affected and plants are able to accumulate high levels of Mn without any measurable toxicity symptoms. Furthermore, PMSRA4 over-expressing plants are more resistant to oxidative stress caused by the combined effect of excess Mn and water deprivation when compared with wild-type.
INTRODUCTION

Reactive oxygen species (ROS) can be generated photochemically (Aro et al., 1993) or by the reduction of molecular oxygen in bulk phase leading to formation of superoxide, \( \text{H}_2\text{O}_2 \) and hydroxyl radical. Despite the meticulous manner in which aerobes reduce oxygen, leakage of electrons to oxygen from metabolic processes is known to cause oxidative damage in the chloroplast, mitochondria and peroxisomes (Alscher et al., 1997). ROS can oxidize all biomolecules including proteins (Antoine et al., 2003). Well known is the repair system for DNA damage, however, proteins are also repaired. The most easily oxidized amino acid is Met (Davies, 2005). Its two electron oxidation yields methionine sulfoxide MetSO. Conformational changes associated with MetSO formation can result in altered function (Chao et al., 1997) and proteolysis (Davies, 2005).

Peptide-bound MetSO can be repaired by reduction to Met by peptide methionine sulfoxide reductase (PMSR) (Weissbach et al., 2005). There are two stereoisomers of MetSO generated by ROS and there are two enzyme families to repair each stereoisomer. PMSRA isozymes repair the S isomer and PMSRB repairs the R isomer (Sharov and Schoneich, 2000; Shenkin, 2001). Both enzyme families protect against oxidative stress (Weissbach et al., 2005).

Arabidopsis has five genes encoding PMSRA, At5g61640, At5g07460, At5g07470, At4g25130, and At2g18030 (protein products PMSRA1 to PMSRA5 respectively) (Bechtold et al., 2004). PMSRAs 1 to 3 are cytosolic proteins, PMSRA4 is chloroplast targeted and PMSRA5 is apparently fated for a secretory pathway (see chapter 2). Bechtold et al. (Bechtold et al., 2004) found PMSRA2 to be involved in response of plants to oxidative stress after long nights (Bechtold et al., 2004). The results in Chapter 4 demonstrated that PMSRA4 plays a major role in the response to oxidative stress in the chloroplast.

At high levels in the soil, Mn has a deleterious effect on plant growth and development causing inhibition of photosynthesis and stomatal conductance, and reduction of chlorophyll content (Gonzalez and Lynch, 1997). Some studies suggest that Mn toxicity is mediated by ROS causing oxidative stress (Gonzalez and Lynch, 1999; Santandrea et al., 2000). In plants
Mn has been shown to accumulate in thylakoids destroying membranes and impairing electron transfer through the photosystems (Horst et al., 1999; Lidon et al., 2004).

The relative tolerance of plants to excess Mn is affected by climatic factors such as temperature, light and drought (El-Jaoual and Cox, 1998). Indeed, plants growing on metal rich soils must respond not only to metal excess but frequently to water deprivation. Besides inducing oxidative stress, heavy metals decrease water use efficiency in many species (Poschenrieder and Barcelo, 1999). Water deprivation causes similar effects as those of heavy metals, including induction of ROS; thus the combined effect of both stress conditions magnifies the individual effect of each one (Poschenrieder and Barcelo, 1999).

In the present chapter, the role of plastid PMSRA4 over-expression in Mn$^{2+}$ toxicity is investigated. Mn$^{2+}$ was chosen due to its role in chloroplast function and its proposed ability to initiate oxidative damage. Furthermore, Mn excess was combined with mild water deprivation to enhance ROS production in order to gain a better understanding of the function of PMSRA4 against oxidative stress. The results reveal that PMSRA4 integrity is fundamental for the plants to tolerate Mn$^{2+}$ toxicity and that the over-expression of PMSRA4 confers plants with resistance to oxidative stress imposed by Mn$^{2+}$ toxicity and its combination with water deprivation.

**MATERIALS AND METHODS**

**Mn$^{2+}$ Treatment**

Seeds of *Arabidopsis thaliana* ecotype Columbia and transgenic Arabidopsis over-expressing PMSRA4 (for a detailed description of the plants see Chapter 4) were planted in a mixture (1:1, v/v) of 20 mesh silica sand (U.S. Silica Co., Ottawa, IL, USA) and a commercial soil mix (Redi-earth Plug and Seedling Mix, Scotts-Sierra, Marysville, OH)). Pots were watered with distilled water every other day by sub-irrigation. One week after germination, the distilled water was replaced by a nutrient solution of Peters Professional
General Purpose fertilizer (Scotts Co.) which is a good alternative to MS salts (Pollock and Oppenheimer, 1999). The amount of the fertilizer was adjusted to a final Mn$^{2+}$ concentration of 5.0 µM, pH 5.0. Three weeks after germination, Mn$^{2+}$ content in the nutrient solution was adjusted to a final concentration of 0.5, 0.75, 1.0 and 1.5 mM using MnSO$_4$. Control plants were kept at 5.0 µM Mn$^{2+}$. After 14 days of treatment, photosynthesis, stomatal conductance and chlorophyll fluorescence were measured as in Chapter 4, and leaves were harvested and immediately placed in liquid nitrogen. Samples were stored at -80°C until analysis. Plants were grown in lighted growth chambers, at approximately 250 µmol photon m$^{-2}$s$^{-1}$ on a 23°C/21°C, 12-h day/night cycle.

In experiments involving water deprivation, irrigation ceased 14 days after initiation of Mn$^{2+}$ treatment. Four days after irrigation terminated (2 days of water deprivation) gas exchange and chlorophyll fluorescence were measured as before and tissue samples collected and stored at -80°C. Growing conditions were as before.

**Gene Expression Analysis**

Total RNA (2.5 µg), isolated from leaves, was used for cDNA synthesis with random decamers using the Retroscript System (Ambion, Austin, TX). Synthesis was in a two-step reaction with previous denaturing of the RNA at 80°C and cDNA synthesis at 44°C. The cDNAs produced by reverse transcription were amplified with gene-specific primers (10 pmol each). For each RT-PCR reaction, the 18S rRNA was amplified as an internal control using 18S Universal primers (Ambion, Austin, TX). To generate unsaturated RT-PCR signals of the internal control over the concentration range of total RNA used in this experiment, the 18S universal primers were mixed with a pair of competitive primers. Different ratios of 18S rRNA primers: competitive primers were used. A 4: 6 ratio and 25 cycles were used to amplify *PMSRA1*. A 3:7 ratio and 30 cycles were used for *PMSRA3*, *PMSRA4* and *PMSRA5*; and a 1:9 ratio and 35 cycles were used for *PMSRA2*. Experiments were conducted at least three times with three independent RNA preparations. The ethidium bromide stained gels were digitally photographed with an EagleEye II Digital Imaging System (Stratagene). Scion
Image for Windows (Scion, http://www.scioncorp.com) program was used to quantify the intensity of the ethidium bromide stained DNA bands.

**Protein Analysis**

Extraction of proteins was as described by Coba de la Pena et al. (2001) except that the extraction buffer contained 1 mM PMSF, 10 µM leupeptin and 20 µM pepstatin and 5 mM β-mercaptoethanol instead of DTT. After centrifugation, the soluble fraction was assayed for rubisco activity (Coba de la Pena et al., 2001). Ascorbate peroxidase, catalase and superoxide dismutase were measured by gel assays (Orendi et al., 2001). Protein extracts were also subjected to SDS-PAGE and Western blot analysis for HSP21, PMSRA and rubisco as in Chapter 4. Protein content, chlorophyll content and PMSRA activity were measured as in Chapter 4. MetSO content was measured in the soluble proteins following the procedure of Khor et al. (Khor et al., 2004).

**Mn²⁺ Analysis**

Leaf tissue was dried for 72 h at 65°C, ashed at 500°C for 14 h, dissolved in 0.1 N HCl and analyzed for Mn²⁺ by atomic absorption spectrophotometry (AAAnalyst 100, Perkin Elmer Co., Shelton, CT, USA)

**Statistics**

Data were subjected to ANOVA in a completely randomized split plot designed with the Line as the main plot and the Mn²⁺ concentrations as the subplot. The Bonferroni test was used to compare the means at P≤0.05. SAS System for Windows version 9.1 (SAS Institute, Cary, NC) was used in all the analyses.
RESULTS

Effect of Mn on PMSRA-Specific mRNAs and Protein Level

Symptoms of toxicity developed in Arabidopsis after six days of exposure to high Mn$^{2+}$. The first symptoms revealed chlorosis at the leaf margin and the leaf tip. The older leaves developed extensive reddish-purple flecks, then rapidly became bronze-yellow and died within 14 days. The effect of Mn$^{2+}$ treatment on PMSRA expression was analyzed by RT-PCR. All five PMSRAs of Arabidopsis were analyzed. Fig. 5.1 shows that excess Mn$^{2+}$ has little if any effect on expression of the PMSRA genes of Arabidopsis. Only PMSRA5, the gene targeted to a secretion pathway, shows repression with increasing concentrations of Mn$^{2+}$. However, when examined at the protein level, Mn$^{2+}$ treatment caused dramatic decreases in expression (Fig. 5.2A). Plastidial PMSRA4 is most abundant in leaves and is induced by high light (see chapter 2). Accordingly, Fig. 5.2A shows that it is the most predominant band in the blots and its protein levels are dramatically reduced by the Mn$^{2+}$ treatments.
Figure 5.1. Effect of Mn$^{2+}$ on PMSRA transcript levels. Relative RT-PCR reactions were performed with total RNA (2.5 µg) isolated from 5 week-old rosette leaves of Arabidopsis plants after 14 days of the indicated Mn$^{2+}$ treatments. Specific primers for each *PMSRA* gene were used for the cDNA amplification. A mixture of universal primers and competimers for the 18S rRNA were co-amplified in each reaction and used to obtain the normalized signal presented as bar graph (Relative Intensity). Bar graph shows the mean quantitative data (n=3). Error bars correspond to SD. C, control plants grown with 5 µM Mn$^{2+}$. Numbers in X-axis of the graphs correspond to the Mn$^{2+}$ concentration (mM).
Figure 5.2. Effect of Mn$^{2+}$ on protein expression and activity. (A) and (B) Immunoblot analysis of PMSRA (A) and Rubisco (RbcL) (B) after 14 days of Mn$^{2+}$ treatment. (C) to (D) activity gel assays of (C) APX, (D) CAT and (E) SOD. Assays were performed using total cell soluble proteins of leaf extracts of WT Arabidopsis plants after 14 days grown with different concentrations of Mn$^{2+}$. 20 µg of protein were loaded per lane except in Rubisco analysis in which 5 µg protein were loaded. Bar graph shows the mean percentage of change of content (A and B) or activity (C, D and E) in relation to the control plants grown at 0.5 µM Mn$^{2+}$ whose value correspond to 100%. Error bars correspond to SD (n=3). C, control plants grown with 5 µM Mn$^{2+}$. Numbers in X-axis of the graphs correspond to the Mn$^{2+}$ concentration (mM).
Effect of Mn on Expression of Selected Plastidial Proteins

To determine whether the decrease in PMSRA4 protein levels were due to general proteolysis, the effect of Mn$^{2+}$ on RbcL content was also examined. Western Blot analyses were performed with the RbcL-specific antibody. This not only allowed us to measure the rubisco levels but also detect proteolysis products. The results, (Fig. 5.2B) showed that excess Mn$^{2+}$ had no effect on the steady state levels of RbcL; no RbcL hydrolysis products were detected. The effect of Mn$^{2+}$ on ascorbate peroxidase, catalase and superoxide dismutase activity (Fig. 5.2C to 5.2E) was also determined. In contrast to results obtained with PMSRA, Mn$^{2+}$ treatment had no effect on the activity of ascorbate peroxidase and catalase activity but caused more than 5 fold increase in the activity of superoxide dismutase in the plants treated with 1.5 mM Mn$^{2+}$.

Effect of Mn on PMSRA4 levels

Transgenic Arabidopsis plants which over-express PMSRA4 exhibited more resistance to Mn$^{2+}$ toxicity. Fig. 5.3A shows representative untransformed plants (WT) and PMSRA4 over-expressing plants (line 1001). After 14 days of growth with 1.5 mM Mn$^{2+}$, 5 week-old WT plants showed advance symptoms of Mn$^{2+}$ toxicity. In contrast, the over-expressing plants showed only some chlorosis in the leaf tips. Similar results were obtained with the line 202.
Figure 5.3. Tolerance to Mn$^{2+}$ toxicity of PMSRA4 over-expressing plants.

(A) Representative 5 week-old WT and PMSRA4 over-expressing plants after 14 days of Mn$^{2+}$ treatment. (B) Immunoblot analysis of PMSRA4 expression of leaf extracts of WT and PMSRA4 overexpressing plants (-) plants grown with 0.005 mM Mn$^{2+}$; (+) plants grown with 1.5 mM Mn$^{2+}$. (C) PMSRA activity of leaf extracts of 5 week old WT and transgenic plants after 14 days of Mn$^{2+}$ treatment. Solid bar, WT; white bar line 202; hatch bar, line 1001.
After treatment with 1.5 mM Mn\(^{2+}\), Western blots showed little or no PMSRA4 protein in WT plants. Fig. 5.3B shows a representative Western showing reduced PMSRA. In over-expressing transgenic lines 202 and 1001, Mn\(^{2+}\) treatment also decreased the PMSRA4 protein levels. However, despite this decrease, line 202 still contained more than 50% of the PMSRA4 protein found in untreated WT plants. Similarly, line 1001 plants contained 1.3 times the PMSRA4 levels as found in the untreated WT plants.

Results obtained measuring PMSRA activity yielded a similar trend (Fig. 5.3C). In WT plants, treatment with 0.75 mM Mn\(^{2+}\) caused a 30% decrease in activity and 1.5 mM Mn\(^{2+}\) reduced activity by 80%. In line 202, the decrease in activity was 32% and 53% respectively. In line 1001, the reduction in activity was 19% and 36%. Irrespective of the percentage decrease, the PMSRA activity in the over-expressing plants was always higher than the activity of untreated WT plants. For example, after treatment with 1.5 mM Mn\(^{2+}\), the activity of line 202 was almost 120% of untreated WT and the activity of line 1001 was 220% of untreated WT.

**Effect of Mn Concentration on Mn Accumulation**

Over-expression of PMSRA4 had no effect on the Mn\(^{2+}\) content of the leaves under normal growth conditions (Fig. 5.4A). High Mn growth conditions resulted in an increase in Mn\(^{2+}\) accumulation in all the lines. The plants grown under control conditions contained approximately 80 µg Mn\(^{2+}\) per gram of dry weight. In plants treated with 0.75 mM Mn\(^{2+}\), the transgenic plants accumulated slightly more Mn\(^{2+}\) than WT. Line 1001 contained 550 ± 36 µg Mn\(^{2+}\) per gram of dry weight, line 202 contained 450 ± 37 µg Mn\(^{2+}\) per gram of dry weight and WT plants contained 420 ± 50 µg Mn\(^{2+}\) per gram of dry weight.
Effect of Mn on Chlorophyll and Rubisco

Mn$^{2+}$ treatment also caused a reduction in total chlorophyll (Fig 5.4B). This decrease was both dose dependent and line-specific where the PMSRA4 over-expressing plants contained significantly (p≤0.001) more chlorophyll than WT plants (Table 5.1). Mn$^{2+}$ treatment also affected the protein content (Fig. 5.4C). As with chlorophyll content, the decrease in protein was dependent on the dose of Mn$^{2+}$ (increasing Mn$^{2+}$ caused decreased
chlorophyll), however, no differences were observed between the different lines (Table 5.1). Rubisco activity of treated WT and transformed plants was also measured. Here, it was observed that the Mn\(^{2+}\) treatment affected rubisco activity only in the WT plants (Fig. 5.4D). The enzyme activity in the untransformed plants decreased as the Mn\(^{2+}\) concentration increased which was significantly different among the lines (Table 5.1). Detailed analysis using a multiple comparison test showed that over-expressing lines 202 and 1001 contained similar rubisco activity irrespective of the Mn\(^{2+}\) doses. However, when compared to WT plants, the rubisco activity in the transgenic lines was significantly higher in treatments of 0.75 mM Mn\(^{2+}\) and 1.5 mM Mn\(^{2+}\).

Table 5.1. F-values from ANOVA analysis of the different parameters measured during the response of WT and PMSRA4 over-expressing lines 202 and 1001 to Mn2+ (0.005, 0.75 and 1.5 mM). The table shows the statistic significance of the differences and is designated as *P ≤ 0.05, ** P ≤ 0.01 and *** P ≤ 0.001.

<table>
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<tr>
<th>Source of Variance</th>
<th>Mn(^{2+}) content</th>
<th>Soluble Protein</th>
<th>Chl. a+b</th>
<th>Rubisco activity</th>
<th>Photo</th>
<th>Cond</th>
<th>Rfd</th>
<th>Ft/Fv</th>
</tr>
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<tr>
<td>Line</td>
<td>6.91</td>
<td>2.6</td>
<td>21***</td>
<td>16.7*</td>
<td>117***</td>
<td>10.2*</td>
<td>37***</td>
<td>33***</td>
</tr>
<tr>
<td>Mn(^{2+})</td>
<td>569***</td>
<td>53***</td>
<td>74***</td>
<td>3.5</td>
<td>50***</td>
<td>9.7***</td>
<td>237***</td>
<td>115***</td>
</tr>
<tr>
<td>Mn(^{2+}) x line</td>
<td>2.89</td>
<td>1.1</td>
<td>8.7***</td>
<td>1.2</td>
<td>14***</td>
<td>3.5*</td>
<td>16***</td>
<td>16***</td>
</tr>
</tbody>
</table>

Effect of PMSRA4 over-expression on photosynthetic apparatus with Mn\(^{2+}\) excess

Photosynthetic rate, stomatal conductance and chlorophyll fluorescence were used to assess the effect of Mn\(^{2+}\) on the photosynthetic apparatus of PMSRA4 over-expressing and WT plants. In WT plants, treatment with 0.75 mM Mn\(^{2+}\) caused a 63% reduction in the photosynthetic rate (Fig. 5.5A). After 14 days plants treated with 1.5 mM Mn\(^{2+}\) had only 25% of the photosynthetic rate of the untreated controls. In contrast, plants over-expressing PMSRA4 were not as strongly affected by the treatments. In line 202, photosynthetic rates
decreased only 20% and 25% when grown with 0.75 mM and 1.5 mM Mn$^{2+}$ respectively. The photosynthesis of line 1001 was not affected by the Mn$^{2+}$ treatments.

**Figure 5.5.** Mn$^{2+}$ effect on Leaf Gas Exchange and Chlorophyll fluorescence of PMSRA4 over-expressing plants. (A) and (B) gas exchange was measured in leaves 4 and 5 of 5 week-old WT and PMSRA4 over-expressing plants after 14 days of treatment with different concentrations of Mn$^{2+}$ and photosynthetic rate (A) and stomatal conductance (B) were determined. Values represent the mean ± SD of the leaves of 8 different plants. (C) and (D) Chlorophyll fluorescence of dark adapted leaves of 5 week-old WT and PMSRA4 over-expressing plants after 14 days of treatment with different concentrations of Mn$^{2+}$ was measured and the vitality index (Fm-Ft)/Ft (C) and Fm/Fo index (D) were calculated. Leaves 4 and 5 of 8 plants were measured per treatment. Bars represent the mean ± SD. Solid bar, WT; white bar, line 202; hatch bar, line 1001.

Stomatal conductance (Fig. 5.5B) showed the same trend as photosynthesis. Mn$^{2+}$ treatment strongly reduced the stomatal conductance in the WT plants causing a 63%
reduction. The PMSRA4 over-expressing lines only exhibited a 30% inhibition of stomatal conductance in the line 202 and 12% in the line 1001.

Chlorophyll fluorescence (Fv/Fm ratio), the “vitality index” or Relative Fluorescence Difference (Rfd) of dark adapted leaves and the ratio Ft/Fv were also measured. Rfd calculated as (Fm-Ft)/Ft, was greatly decreased by Mn^{2+} treatment (Fig. 5.5C) in both WT and PMSRA4 over-expressing plants. WT plants were more affected with the index dropping 63% in WT plants exposed to 0.75 mM Mn^{2+}, and 82% in the plants exposed to 1.5 mM Mn^{2+}. In the PMSRA4 over-expressing plants, 0.75 mM Mn^{2+} caused 48% and 27% reduction in Rfd in lines 202 and 1001 respectively. The reduction in index was 62% in line 202 and 34% in the line 1001 when treated with 1.5 mM Mn^{2+}. The index Ft/Fv (Fig. 5.5D) increased 77% and 123% in WT plants exposed to 0.75 mM and 1.5 mM Mn^{2+} respectively. In the line 202 the increase was 48% and 74% and in line 1001 the index increased 14% and 28%. The differences observed were significant among the lines with the various Mn^{2+} doses (Table 5.1).

**Effect of Mn^{2+} treatment on protein-bound MetSO content**

The MetSO content of the soluble proteins was determined in wild-type and transgenic lines overexpressing PMSRA4 after 14 days of growth under Mn^{2+} excess. MetSO content increases with the Mn^{2+} treatments (Table 5.2). In wild-type plants MetSO content increases from 16.2% to 26% and 47% after treatment with 0.75 mM and 1.5 mM Mn^{2+}, respectively. PMSRA4 overexpression protected Met residues from Mn^{2+} oxidation. Thus, in the line 202, 0.75 mM Mn^{2+} causes oxidation of 11% more Met residues, and 1.5 mM Mn^{2+} treatment resulted in 21% more Met residues oxidized. In line 1001, the induced oxidation of Met residues was 5% and 14% more in plants grown under 0.75 mM Mn^{2+} and 1.5 mM Mn^{2+}, respectively, compare to the control plants under 5 µM Mn^{2+}. The basal MetSO content in the different lines and the wild-type plants grown under optimal Mn (5 µM) was around 16%.
Effect of Mn$^{2+}$ treatment and water deprivation

WT plants treated with Mn$^{2+}$ and subjected to water deprivation collapsed after 2 days of water stress (Fig. 5.6A). Plants grown with 1.5 mM Mn$^{2+}$ were especially susceptible and died from the water stress. Wild-type plants exposed to 0.75 mM had a better survival rate, but rubisco activity, Rfd, photosynthesis and stomatal conductance were affected (Fig 5.6B to 5.6E). Lines over-expressing PMSRA4 survived the treatment with a minimal change in the measured parameters (Fig. 5.6). Rfd was the only parameter affected in the transgenic plants (Fig. 5.6B). Photosynthesis was reduced in line 202 but the decrease was not as much as that observed in WT plants. The photosynthetic rate of line 1001 was not affected by the water deprivation (Fig. 5.6C). Water deprivation alone did not have any effect on the plants grown under optimal Mn$^{2+}$ concentration (0.005 mM). In these plants, visual symptoms of water stress were absent, and the parameters measured did not differ from those of the well-watered plants.

**Table 5.2.** Percent peptide-bound MetSO content of total cell extracts from wild-type and transgenic plants overexpressing PMSRA4 (lines 202 and 1001). Total soluble proteins were extracted from leaves four and five from the apex of five weeks-old plants (n=6) after 14 days of growth under the Mn$^{2+}$ treatment and MetSO content analyzed. The results represent the percentage of MetSO in the total Met pool and correspond to the mean ± SD of three independent protein extractions.

<table>
<thead>
<tr>
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<th>Mn 5 µM</th>
<th>Mn 0.75 mM</th>
<th>Mn 1.5 mM</th>
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<tbody>
<tr>
<td>WT</td>
<td>16.20±4.32</td>
<td>42.70±11.52</td>
<td>63.53±6.02</td>
</tr>
<tr>
<td>202</td>
<td>17.43±5.24</td>
<td>28.32±9.82</td>
<td>38.78±6.34</td>
</tr>
<tr>
<td>1001</td>
<td>14.58±5.96</td>
<td>20.87±4.49</td>
<td>29.82±7.40</td>
</tr>
</tbody>
</table>

**Effect of Mn$^{2+}$ treatment and water deprivation**

WT plants treated with Mn$^{2+}$ and subjected to water deprivation collapsed after 2 days of water stress (Fig. 5.6A). Plants grown with 1.5 mM Mn$^{2+}$ were especially susceptible and died from the water stress. Wild-type plants exposed to 0.75 mM had a better survival rate, but rubisco activity, Rfd, photosynthesis and stomatal conductance were affected (Fig 5.6B to 5.6E). Lines over-expressing PMSRA4 survived the treatment with a minimal change in the measured parameters (Fig. 5.6). Rfd was the only parameter affected in the transgenic plants (Fig. 5.6B). Photosynthesis was reduced in line 202 but the decrease was not as much as that observed in WT plants. The photosynthetic rate of line 1001 was not affected by the water deprivation (Fig. 5.6C). Water deprivation alone did not have any effect on the plants grown under optimal Mn$^{2+}$ concentration (0.005 mM). In these plants, visual symptoms of water stress were absent, and the parameters measured did not differ from those of the well-watered plants.
Figure 5.6. Tolerance to Mn$^{2+}$ toxicity of PMSRA4 over-expressing plants after 2 days of water deprivation. (A) Representative WT and PMSRA4 over-expressing plants after the combine Mn$^{2+}$-water deprivation treatment. (B) Chlorophyll fluorescence was measured in leaves 4 and 5 of 5 week-old WT and transgenic plants overexpressing PMSRA4 after the combine Mn$^{2+}$-water deprivation treatment and the Vitality Index ((Fm-Ft)/Ft) was calculated. Eight plants were measured and the results are mean ± SD. (C) Photosynthetic rate and (D) stomatal conductance after the Mn$^{2+}$-water deprivation treatment. Each value represents the average of 8 plants ± SD. Solid bar, WT; white bar line 202; hatch bar, line 1001. (E) Rubisco activity of leaf extracts of 5 week-old WT and transgenic plants overexpressing PMSRA4 was measured after the combine Mn$^{2+}$-water deprivation treatment. Results are mean ± SD (n=3).
DISCUSSION

In Chapter 4 it has been shown the importance of PMSRA4 in the oxidative stress response in the chloroplast. Since Mn accumulates in the chloroplast (Horst et al., 1999; Lidon et al., 2004) and induces oxidative stress (El-Jaoual and Cox, 1998) PMSRA4 is expected to play an important role in the response of Arabidopsis plants to Mn$^{2+}$ excess.

Growth with high Mn in the soil elicits a number of responses from Arabidopsis. With the exception of PMSRA5, Mn treatment caused no change in the PMSRA (PMSRA1, PMSRA2, PMSRA3, PMSRA4) message levels. However, PMSRA4 enzymatic activity and protein content were both decreased by Mn treatment. A similar effect was observed with Rubisco. Rubisco activity decreased in response to Mn$^{2+}$ treatment although the effect was not as strong as that observed in PMSRA4. Rubisco activity has been largely recognize as one of the targets of Mn$^{2+}$ treatment (McDaniel and Toman, 1994). The lower rubisco activity may have resulted from changes in protein structure due to Met oxidation (Chao et al., 1997). The reduction in PMSRA4 activity and content is not a general hydrolytic response of the cell to Mn treatment. When the activities of selected protective enzymes were measured by in-gel assays, Mn-superoxide dismutase, but not Cu/Zn superoxide dismutase activity increased upon Mn treatment. Ascorbate peroxidase and catalase activities were not affected.

Of the multiple effects of Mn toxicity, damage to the protective enzyme PMSRA4 may be the most far reaching, resulting in a series of effects on cellular events. The reduced PMSRA activity deprives the cell of an important line of defense against ROS and as such, would have a negative impact on the homeostasis of all proteins. Proteins with surface-exposed Met residues could scavenge ROS, yielding the sulfoxide which then would be repaired back to Met (Levine et al., 1996). The results of Chapter 4 showing the high content of chloroplast MetSO of plants under-expressing PMSRA4 demonstrate the importance of this enzyme in maintaining cellular redox balance (Petropoulos and Friguet, 2005). With this defense-repair mechanism compromised, protein bound MetSO content increases so that ROS can inactivate proteins that normally would be repaired by PMSRA and the accumulation of oxidized proteins would trigger the metabolic collapse that was observed in the plants.
exposed to Mn$^{2+}$. Decreased PMSRA activity would lead to the accumulation of oxidized proteins that are inactivated and targeted for proteolysis (Stadtman et al., 2005).

The data show that PMSRA4 is involved in the protection of proteins in both the light and dark reactions. Similar to PMSRA4, rubisco activity decreased upon Mn treatment. For the light reactions, it was previously demonstrated that plants under-expressing PMSRA4 experienced greater damage to PSII (see Chapter 4). This was found in the present study with Mn$^{2+}$. Rfd, the "vitality index", which reflects the vitality of a leaf and its potential photosynthetic activity (Lichtenthaler and Rinderle, 1988), was reduced by Mn$^{2+}$. The index Ft/Fv increased. These results are consistent with the reaction centers of PSII being damaged by Mn$^{2+}$ resulting in lowered capacity for light quanta conversion. Furthermore, higher Ft/Fm values reflect the incidence of stronger fluorescence associated with Mn$^{2+}$ toxicity. The damage of PSII by Mn$^{2+}$ treatment impairs electron flow and light harvesting, leading to increased steady state fluorescence (Ft) and reduced variable fluorescence (Fv). These results are consistent with the findings of Sharma et al. (1997) who demonstrated the susceptibility of Met residues of PSII components to oxidation. Damage to the PSII components compromised the ability of the plant to fix CO$_2$ due to lower NADPH and ATP synthesis, which together with lower rubisco activity caused the observed reduction in the photosynthetic rate. The reduction in CO$_2$ fixation raises the internal CO$_2$ concentration which would then lead to stomata closure and the observed reduction in the stomatal conductance.

The negative effect of impaired PMSRA activity has been documented in null mutants of PMSRA2 in Arabidopsis (Bechtold et al., 2004). The absence of PMSRA2 reduces the ability of these plants to repair damage caused by oxidative stress generated during long nights. Bechtold et al. (2004) observed an increase in protein oxidation and reduced growth rate. Similar results with plants that under-express PMSRA4 were obtained (see Chapter 4). These plants may also have reduced ability to repair damage caused by oxidative stress. Exposure to methyl viologen results in more than 80% of Met residues of the soluble proteins in the chloroplast being oxidized to the sulfoxide. Here again, it was observed that rubisco activity declined and proteins of the PSII were damaged.
Many of the symptoms observed with WT plants upon Mn treatment were alleviated by PMSRA4 over expression thereby demonstrating the importance of PMSRA4 in protecting plants from Mn\(^{2+}\) toxicity. Similar to WT, in the over-expressing plants Mn\(^{2+}\) treatment results in degradation of PMSRA4. However, not all of the PMSRA4 is degraded and apparently, the remaining lowered activity is still sufficient for protection of the cell. However, in the over-expressing lines, PMSRA4 activity is still higher than WT untreated plants after Mn\(^{2+}\) treatment.

Plants over-expressing PMSRA4 exhibited Mn\(^{2+}\) induced effects on chlorophyll content and the components of PSII, while Rubisco was unaffected. Expression of PMSRA4 in the transgenic plants after Mn\(^{2+}\) treatment only partially protected total chlorophyll and components of PSII (measured by Rfd and Ft/Fv). This is consistent with the schematic shown below. Different proteins would have varying Met content and also vary in their susceptibility to oxidative damage from ROS (k\(_1\)). Once oxidized, the MetSO can be repaired by PMSRA (k\(_3\)) or undergo proteolysis (k\(_2\)). The rate of k\(_1\), k\(_2\) and k\(_3\) would be unique for each protein. Thus, it is not surprising that steady state levels of rubisco or the proteins of PSII would vary under conditions of oxidative stress.

In the present experiments, Mn\(^{2+}\) decreased the drought tolerance in WT plants (Fig. 5.6). Over-expression of PMSRA4 conferred resistance to drought stress under conditions of Mn\(^{2+}\) treatment. Also, under normal Mn\(^{2+}\) conditions the plants over-expressing PMSRA4 had higher photosynthesis than WT when exposed to the water deficit. Thus, the reduction in PMSRA4 caused by Mn\(^{2+}\) toxicity contributed to the increased susceptibility to drought.

In addition to conferring some drought resistance under excess Mn\(^{2+}\), this work also shows the potential of PMSRA4 over-expressing plants in use of heavy metal
phytoremediation. Measurement of Mn$^{2+}$ content showed that when growing under Mn$^{2+}$ excess, Arabidopsis plants accumulate high levels of Mn in the leaves but only PMSRA4 over-expressing lines are able to accumulate Mn$^{2+}$ without showing toxicity symptoms. Mn$^{2+}$ levels measured are similar to that observed with metal-accumulator mutants under high Mn$^{2+}$ (Delhaize, 1996). However, in the transgenic plants photosynthesis and stomatal conductance were similar to those of the untreated plants.

In conclusion, the results demonstrate the importance of PMSRA4 in oxidative stress of the chloroplast. The decrease in PMSRA4 levels by Mn$^{2+}$ results in impaired ability to repair proteins oxidized by Mn$^{2+}$-dependent oxidation reactions of both the light and dark reactions. Finally, over-expression of PMSRA4 confers plant resistance to both Mn$^{2+}$ toxicity and drought stress (under high Mn$^{2+}$ conditions). The sensitivity of PMSRA-compromised plants to respond to oxidative stress may be useful in determining modes of action of a variety of stresses.

REFERENCES


Chapter 6
CONCLUSIONS

The primary objective of this thesis was to determine the role of the enzyme peptide methionine sulfoxide reductase in protection of plants from ROS. A number of experimental approaches were used: i) gene expression analysis, ii) biochemical and kinetic characterization of a cytosolic PMSRA and the plastidic PMSRA, iii) characterization of transgenic plants over-expressing a cytosolic PMSRA or the plastid PMSRA and iv) characterization of plants under-expressing plastid PMSR. Data derived from these experiments provide support for the hypothesis that PMSRA is an important component of the response of Arabidopsis plants to oxidative stress.

The physiological role of PMSRA in Arabidopsis had not been addressed when this research was started and several key findings have emerged:

1. The presence of multiple genes encoding PMSRA proteins supports cell survival under conditions of oxidative stress, especially inside the chloroplast. All of the PMSRA genes are expressed, each with different expression patterns and differential responsiveness to particular oxidative stress conditions. The specificity of the different isozymes is reflected by the sub-cellular compartment where the respective PMSRA proteins are localized and the variation in their biochemical properties. Thus, the plastid PMSRA4 is faster and more efficient than PMSR3, a protein localized in the cytosol where the conditions are milder and the oxidation lower than in the chloroplast (Chapter 1).

2. In response to the highly oxidative environment in the chloroplasts, plastid proteins are adapted to function with more than 30% of Met residues in the oxidized form;
more than seven times the normal percentage of Met oxidation in proteins of other organisms such as bacteria, yeast and mammals (Chapter 4).

3. Oxidative stress can cause a decrease in PMSRA content and activity. For example, Mn$^{2+}$ excess causes the reduction in content and activity of PMSRA4. As a result the plant undergoes extensive protein oxidation in the chloroplast and the physiology of the plant is compromised. Moreover, when PMSRA4 activity is low, plants are unable to survive mild drought stress conditions, which normally would not be harmful (Chapter 5).

4. Despite compartmentalization of each PMSRA isozyme and their apparent specificity in the response to particular stress conditions, there appears to be derived protection against oxidative stress throughout the cell as a result of over-expression of a single PMSRA isozyme. Thus, PMSRA4 over-expression, whose beneficial effects are expected to be limited to the chloroplast, increases the resistance of the plants to stress conditions imposed in other cell compartments, including the cytoplasm (Chapter 4). Conversely, over-expression of the cytosolic PMSRA3 increases the resistance of plants to chloroplast localized oxidative stress conditions (Chapter 3).

5. PMSRA genes are induced in response to oxidative stress conditions occurring during the development of the plant (flower buds, PMSRA1), normal photosynthesis within the chloroplast, (PMSRA4) or during oxidative stress events (PMSRA4 different stresses). The reduction of the content and activity of one PMSR is enough to severely affect the physiology of the plant under oxidative stress conditions (PMSR4, different stress conditions) and when the content of one PMSRA protein is increased, the plant is able to resist different stress conditions (PMSRA3 and PMSRA4, different stresses).

Figure 6.1 is a schematic that summarizes the main results of the thesis. Two subcellular compartments were analyzed, the chloroplast and the cytosol. In the chloroplast, metabolic processes of the cell continuously produce ROS. The oxidative consequences of the ROS are kept low by the activity of antioxidants and ROS scavengers. However, certain agents which cause oxidative stress disrupt the balance between ROS production and
scavenging. For example, high light (HL) affects the photosystem II (PSII) increasing the level of singlet oxygen (\(^{1}O_2\)); and methyl viologen (MV) treatment results in the generation of superoxide (\(O_2^{•-}\)) by the photosystem I (PSI). Consequently, ROS (HIGH ROS) react with proteins oxidizing Met residues to produce proteins rich in MetSO (MetSO-PROTEINS). 

*PMSRA4* is induced by the stress conditions and the result is increased PMSRA4 (big letters arrow pointing up). The increased activity of the enzyme reduces the MetSO residues back to Met, restoring protein function and the net effect is scavenging ROS (collaborates as antioxidant). However, there are conditions in which PMSRA4 is compromised (small letters, arrow pointing down). For example, Mn excess reduces the amount of the enzyme (or in transgenic plants under-expressing PMSRA4, Chapter 4). The lower amount of PMSRA4 implies lowered capacity to repair MetSO in the affected proteins. Oxidized proteins accumulate and important physiological functions such as photosynthesis are affected. With lower photosynthesis, and high production of ROS, proteins of the different photosystems are less active. Thus, there is an excess of energy that must be dissipated and the visual result is an increase in chlorophyll a fluorescence. With lowered repair and scavenging capacity due to PMSRA4 reduction, mild stress conditions such as water deprivation (drought) could be catastrophic for the plants.

Other stress conditions such as exposure to ozone or cercosporin result in the higher levels of ROS in the cytoplasm (some of the ROS can also reach the chloroplast). Also, elevated ROS levels in the chloroplast can result in leakage to the cytosol (dashed blue line) increasing ROS content in the cytoplasm. ROS oxidize cytosolic proteins which then lead to accumulation of MetSO-proteins. Such oxidation affects the physiology of the plant, lowering photosynthesis. However, PMSRA proteins present in the cytosol (PMSRA3 and PMSRA2) can repair the MetSO proteins. The schematic shows that in the cytosol, there is specialization of each PMSRA (represented by balck and purple colors); PMSRA3 responds to ROS accumulation induced by ozone and cercosporin (black), while PMSRA2 only functions in proteins oxidized by long night-induced ROS (purple) (Bechtold et al., 2004).
Figure 6.1: Schematic representation of the role of PMSRA in the response of plants to oxidative stress conditions. See text for details.

1 (Gustavsson et al., 2002)
2 (Bechtold et al., 2004)
FUTURE RESEARCH

The function of the other two PMSRAs (PMSRA1 AND PMSRA2) is yet to be determined. PMSRA1 is induced during flower formation (in flower buds), however, the role of the enzyme is not known. Also, the function of PMSRA5 needs to be investigated. First, the function of the PMSRA protein needs to be clarified. Kinetic studies are necessary to see if the enzyme is indeed a PMSRA. If the result is positive, the next step will be to analyze its role in the cell.

Another aspect that needs to be investigated is identification of the natural substrates of each enzyme. HSP21 seems to be a substrate of PMSRA4 (Gustavsson et al., 2002) and proteins of the PSII (D1, D2) are good candidates. In the case of the cytosolic enzymes, this is an area yet to be addressed. Also, the physiological electron donor for each isozyme has not been identified. It is clear that it is a thioredoxin; however there is high specificity in the thioredoxin targets in the cell and until individual thioredoxins are assigned to each PMSR, the true kinetics of the proteins will not be fully understood.

Finally, this thesis has focused on the subfamily PMSRA. The subfamily PMSRB also should be evaluated and then there should be a determination of the integrated role of PMSRA and PMSRB in protection from oxidative stress.

REFERENCES


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