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Glyoxalase System and Reactive Oxygen Species Detoxification System in Plant Abiotic Stress Response and Tolerance: An Intimate Relationship

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1. Introduction

Plants are sessile and sensitive organisms that inevitably encounter a variety of abiotic stresses in nature. Abiotic stresses such as salinity, drought, heavy metal toxicity and extreme temperatures are critical factors that reduce crop yields by more than 50% worldwide (Wang et al., 2003). The scenario is even more aggravated by the predicted forthcoming global changes in climate, foreseen extremization of environmental conditions, continuous increase of world population, ever-increasing deterioration of arable land, and scarcity of fresh water, all underscoring the importance of developing stress-resistant crops that are able to sustain growth and productivity in stressful environments. Plants tolerate abiotic stresses by modulating multiple genes and by coordinating the action of various genes from different pathways or systems (Sasaki-Sekimoto et al., 2005; Ahuja et al., 2010). During the past few years, the complex interrelationship of biochemical pathways that changes during stress has become appreciated, although we are far from understanding this complexity. A thorough understanding of biochemical and molecular responses of plants to various abiotic stresses and the interaction of different molecular pathways is, therefore, essential for a holistic perception of plant resistance mechanisms under stressful conditions. The regulatory roles of the glyoxalase system and reactive oxygen species (ROS) detoxification systems in plant abiotic stress tolerance have increasingly attracted much interest because excessive production of ROS and methylglyoxal (MG) is a common consequence of both abiotic and biotic stresses in plants (Veena et al., 1999; Chen et al., 2004; Yadav et al., 2005a, 2005b; Singla-Pareek et al., 2006; Hossain & Fujita, 2009, 2010; Banu et al., 2010; El-Shabrawi et al., 2010; Hossain et al., 2009, 2010, 2011). ROS and MG are highly toxic to plant cells, and in the absence of adequate protective mechanisms, they can react with proteins, lipids and nucleic acids and inactivate the vital defense system leading to irreparable metabolic dysfunction and death. Plants have a complex network of enzymatic

and non-enzymatic scavenging pathways or detoxification systems which function as an extremely efficient cooperative system to counter the deleterious effects of ROS and MG as well as to perform their signaling function. In plants, MG is detoxified mainly via the glyoxalase system. Besides detoxification of MG, the glyoxalase system could also play a role in oxidative stress tolerance by recycling reduced glutathione (GSH) that would be trapped nonenzymatically by MG to form hemithioacetal, thereby maintaining glutathione homeostasis. In addition, ROS levels are controlled via a versatile antioxidant network in plants. The specific interplay between ROS and components of the antioxidant and glyoxalase pathways could generate compartment-specific changes in both the absolute concentrations of ROS, MG and antioxidant compounds as well as in the ascorbate and glutathione redox ratios. Under stress conditions, these redox signals could interfere with the signaling networks complementary to the antioxidant system and regulate defense gene expression, thus coordinating the necessary readjustments in the redox-regulated plant defense to overcome oxidative stress (Foyer & Noctor, 2005a, 2011; Kuźniak, 2010; Mhamdi et al., 2010).

The results of numerous recent studies have shown that the alleviation of oxidative damage and increased resistance to abiotic stresses are often correlated with the more efficient antioxidative and glyoxalase systems. In this chapter, we will try to provide an overview of MG and ROS metabolism in plants and address a new metabolic relationship of AsA- and GSH-dependent antioxidative and glyoxalase systems in inducing abiotic stress tolerance. Further, we will discuss the progress made over the last few years in our understanding of the interaction between these two important pathways in improving abiotic oxidative stress tolerance either by exogenous chemical treatment (proline, betaine, selenium and nitric oxide) or by genetic engineering of different components of the glyoxalase system and ROS detoxification system in plants.

2. Methylglyoxal (MG) and its formation in biological system

MG is a highly reactive $\alpha\beta$ -dicarbonyl aldehyde compound. MG has a ketone group and an aldehyde moiety and the aldehyde group is more reactive than the ketone. Chemically MG is a yellow liquid with a characteristic pungent odor. Extensive studies have been carried out in mammalian and animal systems and different pathways have been proposed for endogenous MG formation from metabolic intermediates of carbohydrates, protein and lipid metabolism (Fig. 1). However, very little work has been done in plant systems regarding the endogenous production of MG. MG is formed spontaneously in plants by non-enzymatic mechanisms under physiological conditions from glycolysis and from photosynthesis intermediates, glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (Espartero et al., 1995; Yadav et al., 2005a). Under stress conditions, the rate of glycolysis increases, leading to an imbalance (in the initial and latter five reactions) in the pathway. Triosephosphates are very unstable metabolites, and removal of the phosphoryl group by β -elimination from 1, 2-enediolate of these trioses leads to the formation of MG (Richard, 1993; Yadav et al., 2005c). Therefore, spontaneous production of MG is an unavoidable consequence of the glycolysis pathway during stress. MG can also be formed enzymatically from G3P and DHAP. Triosephosphate isomerase hydrolyzes G3P and DHAP and removes phosphate to yield MG (Pompliano et al., 1990). Under physiological conditions, minor sources of MG formation include the metabolism of acetone (a metabolite of fatty acids). Acetone monooxygenase catalyzes acetone to acetol, and acetol

monooxygenase (AMO) converts acetol to MG (Casazza et al., 1984). Additionally, MG is also formed during the metabolism of aminoacetone (a metabolite of protein). Semicarbazide-sensitive amine oxidase (SSAO) is able to convert aminoacetone into MG (Lyles, 1996). In a bacterial system, MG is primarily produced from DHAP via MG synthase (Cooper, 1984). Degradation of lipid peroxidation products, in animal systems, generates products like 4-hydroxynon-2-enal and MG. Whether these mechanisms of MG formation are contributing to total MG in plants still needs to be established.

3. Detoxification of MG

MG is both a mutagen and a genotoxic agent. At high cellular concentration, it inhibits cell proliferation (Ray et al., 1994) and results in a number of adverse effects such as increasing the degradation of proteins through the formation of advanced glycation end products (AGEs) and inactivating the antioxidant defense system (Wu & Juurlink, 2002; Hoque et al., 2010). Additionally, MG causes increased sister chromatic exchange and endoreduplication (Chaplen, 1998). It also induces DNA strand breaks and increases point mutations (Chaplen, 1998). Therefore, efficient detoxification of MG overproduced during various abiotic or biotic stresses is one of the most important adaptive strategies of plant stress tolerance.

3.1 Glyoxalase system of MG detoxification

The glyoxalase system is an integral component and major pathway of cellular metabolism of MG in living systems present in the cytosol of cells and cellular organelles, particularly mitochondria. The function of the glyoxalase pathway has been studied extensively in animals, primarily because of its putative association with clinical disorders, such as cancer, diabetes and hypertension (reviewed in Chang & Wu, 2006; Desai et al., 2010). It consists of two enzymes: glyoxalase I (Gly I; lactoylglutathione lyase; EC 4.4.1.5) and glyoxalase II (Gly II; hydroxyacylglutathione hydrolase; EC 3.1.2.6). These enzymes act coordinately to convert MG and other 2-oxoaldehydes to their 2-hydroxyacids using GSH as a cofactor in a two-step reaction (Thornalley, 1990). The spontaneous reaction between GSH and MG forms hemithioacetal, which is then converted to S-D-lactoylglutathione (SLG) by Gly I. The second reaction is the hydrolysis of SLG to D-lactate catalyzed by Gly II and GSH is recycled back into the system (Fig. 1). MG detoxification is therefore strongly dependent on the availability of cellular GSH. Deficiency of GSH limits the production of hemithioacetal, leading to the accumulation of MG. The reactions catalyzed by the glyoxalase system are irreversible. The existence and widespread distribution of this shunt pathway documents its fundamental importance in biological systems. Recent investigations in plants have brought new developments in the involvement of the glyoxalase system in stress tolerance and its involvement with oxidative defense systems. Further insights into the biological function of the glyoxalase system came from the molecular cloning of their respective genes. The pioneering work of Dr. Sudhir Kumar Sopory and his associated co-workers (Veena et al., 1999; Singla-Pareek et al., 2003; Saxena et al., 2005; Yadav et al., 2005a, 2005b) provides a potential framework for interpreting the physiological roles of the glyoxalase system in higher plants against various abiotic stresses.

3.2 Non-glyoxalase metabolism of MG

There are other enzymatic systems through which MG could be detoxified in living systems, including plants. MG contains two functional groups that may be either oxidized or

The diagram illustrates the metabolic pathways leading to methylglyoxal (MG) and its downstream effects. Key components include:

- Glucose Metabolism:** Glucose is converted to G-6-P, then F-1,6-P, and finally G3P via Glycolysis. G3P can be converted to Pyruvate and then Acetyl CoA for further metabolism.
- Fructose Metabolism:** Fructose is converted to F-1-P, which can enter the Glycolysis pathway or be converted to DHAP.
- Amino Acid Metabolism:** Glycine and Threonine are converted to Aminoacetone, which then enters the MG pathway. Fatty acids are converted to Acetone, which also enters the MG pathway.
- Enzymes and Cofactors:** Aldolase, MG synthase, Triosephosphate isomerase, SSAO, and AMO are involved in the conversion of various substrates to MG. GSH and Glyoxalase system (Gly I, Gly II) are involved in the detoxification of MG.
- MG Pathways:** MG can be converted to Hemithioacetal, D-Lactic acid, or enter the Glyoxalase system. It can also be converted to DNA or RNA, leading to Mutagenesis, or to AGEs (Advanced Glycation End products), leading to Cellular injury.

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Juurlink, 2002) and more recently in plant systems (Yadav et al., 2005a; Singla-Pareek et al., 2006; Hossain et al., 2009; Banu et al., 2010). Yadav et al. (2005a) first showed that induction of the level of MG in response to various abiotic stresses is a common phenomenon in different crop species in which rice, *Pennisetum*, tobacco and *Brassica* seedlings showed a 2- to 6-fold increase in MG levels in response to salt, drought and cold stresses. To further check whether induction of MG in plants in response to various abiotic, hormonal and chemical stresses is a common phenomenon, we measured MG levels in pumpkin (*Cucurbita maxima* Duch.) seedlings subjected to drought, salinity, cold, high temperature, heavy metal, MG, 2,4-D, abscisic acid (ABA) and white light. MG levels were rapidly induced in response to different stresses and white light treatments within a short period of time (24 h) compared to the untreated control, and the levels ranged from 39.96 to 88.40 $\mu\text{mol g}^{-1}$ FW. Importantly, white light (60 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$) caused the highest induction (2.21-fold) in the level of MG followed by salinity (1.77-fold), MG (1.69-fold), drought (1.63-fold), heavy metal (1.55-fold) and ABA (1.37-fold) stresses (Hossain et al., 2009). MG was also reported to increase (2.5-fold) in a maize genotype (G4666) susceptible to fungus infected with *Aspergillus flavus* (Chen et al., 2004). Banu et al. (2010) showed that MG level increased significantly ($\approx$ 2-fold) in tobacco BY-2 cells in response to 200 mM NaCl stress. The rapid increase in the level of MG in plants due to different stresses clearly suggests that it is a general stress response. There is a possibility that MG could therefore act as a signal for plants to respond to stress.

5. Molecular characterization of Gly I protein and gene, induction of glyoxalase pathway enzymes (Gly I & Gly II) and Gly I protein expression in model plants in response to abiotic stresses

Since proteins are directly involved in plant stress tolerance, proteomics studies can significantly contribute to unravel the possible relationship between protein abundance and plant stress acclimation. Stress-induced Gly I protein and Gly I mRNA expression was first demonstrated by Espartero et al. (1995) in tomato (*Lycopersicon esculentum* cv. Rutgers) seedlings subjected to NaCl, mannitol and ABA treatment. In our study with pumpkin (*Cucurbita maxima* Duch.) seedlings, we found differential induction of Gly I activity and Gly I mRNA expression in response to various stresses and white light. A sharp increase in Gly I activity (1.82-fold) was observed in response to white light followed by salinity (1.42-fold), MG (1.34-fold), drought (1.27-fold) and heavy metal (1.19-fold) stresses. A sharp increase in Gly I activity due to white light suggested that Gly I expression might be under stringent regulation of photoreceptors in plants (Yadav et al., 2008). To know the genetic structure of the pumpkin Gly I gene, the pumpkin Gly I cDNA was also cloned and sequenced. The pumpkin Gly I cDNA (AB 303333) consists of a 975-bp nucleotide encoding a polypeptide of 185 amino acids and belongs to the short-type Gly I sequence of plants (Hossain et al., 2009). However, to clarify the mechanism by which stress-induced alteration of glyoxalase pathway enzymes and Gly I protein expression occurs, and with the hope of obtaining a new genetic sequence of the Gly I gene, we selected the bulbs of a model plant, onion (*Allium cepa* L.), which had very high Gly I activity compared to various other plant species and plant organs (Hossain et al., 2007). Gly I protein was purified from onion bulbs and the Gly I gene was characterized from onion cDNA libraries prepared from onion callus. The specific activity (356 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein) of the purified Gly I was the highest reported to date in plants (Hossain & Fujita, 2009). The relative molecular weight of purified onion

Gly I is approximately 25 kDa. We produced rabbit antiserum using purified protein and immunoscreened onion cDNA libraries with anti-Gly I antiserum. We isolated the onion Gly I cDNA, and purified, cloned and sequenced it. Onion Gly I cDNA (AB 630177) consisted of a 877-bp nucleotide encoding a polypeptide of 187 amino acids. Based on the number of amino acids, the onion Gly I gene was classified as a short-type Gly I, which showed significant homology with other known Gly I sequences of plants present in the database. Further, we studied the regulation of glyoxalase pathway enzymes (Gly I and Gly II) and Gly I protein expression in onion callus subjected to a range of abiotic stresses. Significant increases in Gly I activity and Gly I protein induction were observed following various stress treatments, except for heavy metal stress (CdCl_2). Low temperature stress showed the highest induction (2.57-fold) followed by salinity (2.52-fold) and drought stress (1.43-fold). A similar pattern of induction of Gly II activity was also observed in response to the various abiotic stresses: drought showed a 1.81-fold and chilling showed a 2.5-fold increase in activity. Moreover, the activity followed the same pattern as that of Gly I, except for salinity stress. In the case of heavy metal stress, the activity of both Gly I and Gly II decreased. The protein expression profile of Gly I also showed a true reflection of possible changes in activity levels due to various abiotic stresses. It is conceivable that an elevated level of Gly I activity is required to remove excessive MG produced in ample amounts under normal and various stressful conditions (Yadav et al., 2005a, 2005b; Hossain et al., 2009; Hossain & Fujita, 2009). The concomitant increase in Gly II activity convincingly indicated that both enzymes are critically important in MG detoxification and stress tolerance because, apart from MG, pathway intermediate SLG (substrate for Gly II) has also been found to be cytotoxic at high concentrations by inhibiting DNA synthesis (Thornalley, 1996). The presence and characterization of both Gly I and II has been reported in many plant species and the genes encoding these enzymes have been cloned and found to be regulated under various abiotic stress conditions (Espartero et al., 1995; Veena et al., 1999; Jain et al., 2002; Yadav et al., 2005a, 2005b; Saxena et al., 2005; Singla-Pareek et al., 2006; Hossain et al., 2009; Hossain & Fujita, 2009; Lin et al., 2010). Therefore, the multistress inducibility of Gly I and Gly II genes, and their overexpression, can lead to tolerance of multiple forms of stress in plants.

6. Similarity of Gly I activity, gene and protein expression between model plant (onion) and other plant species in response to environmental stresses

In agreement with our research results of stress-induced alteration of glyoxalase pathway enzymes and protein expression in a model plants species, onion (Hossain & Fujita, 2009), recent proteomic and transcriptomic studies also showed a similar pattern of Gly I activity, Gly I mRNA and protein expression in response to various stresses (biotic and abiotic). Salt-tolerant barley (*Hordeum vulgare* L.) genotype cv. Morex showed up-regulation of Gly I protein expression in response to salt stress but these proteins were down-regulated or remained unchanged in the susceptible line cv. Steptoe (Witzel et al., 2009). Durum wheat (*Triticum durum* Desf. cv. Svevo) subjected to heat stress showed up-regulation of Gly I protein expression (Laino et al., 2010) and rice (*Oryza sativa* L. cv. Dongjin) seedlings subjected to chilling stress (10°C) showed gradual up-regulation of Gly I protein expression (Lee et al., 2009). Consequently, rice seedlings treated with ABA (5 μM) showed up-regulation of Gly I activity (Li et al., 2010). Recent transcriptomic analysis of a salt-tolerant wild tomato species (*Solanum pimpinellifolium*) showed 2- to 3-fold

increases in Gly I gene transcript whereas the cultivated sensitive variety showed less than a 2-fold increase (Sun et al., 2010). Du et al. (2011) detected the up-regulation Gly I protein expression in rice leaves subjected to UV radiation. However, Tuomainen et al. (2011) did not observe an increase of the Gly I transcript in a metal hyperaccumulator plant *Thlaspi caerulescens*, similar to our finding in onion, in which we did not find an increase in protein expression in response to heavy metal (0.5 mM CdCl₂) stress (Hossain & Fujita, 2009). However, an increase in Gly I mRNA in response to ZnCl₂ (5 to 10 mM) was reported in *Brassica* and wheat (Singla-Pareek et al., 2006; Lin et al., 2010). This apparent discrepancy may be due to differences in genetic background or differential regulation of different Gly I isoforms (Tuomainen et al., 2011).

The potential for direct involvement of Gly I activity and protein expression in host resistance against aflatoxigenic fungi (*A. flavus*) in maize (*Zea mays* L.) was also investigated. Higher Gly I activity was observed in the kernels of resistant lines with or without *A. flavus* infection. After fungal infection, the level of MG did not increase in resistant genotypes. This lack of increase could be due to the relatively higher levels of Gly I activity and Gly I protein expression observed in resistant kernels (Chen et al., 2004). Later on, the involvement of Gly I protein in the *B. napus*/ *Sclerotinia sclerotiorum* pathosystem was investigated by qRT-PCR analysis. Gly I exhibited an increasing trend during a 36-48 h period, the timing of which paralleled the increase in abundance of a protein spot identified as Gly I, suggesting the potentially important role for this enzyme in biotic stress tolerance (Liang et al., 2008).

7. Influence of MG on oxidative stress and antioxidant defense system

There is a substantial evidence of MG-induced oxidative stress in various living cells, including those of plants. MG causes mitochondrial oxidative stress by increasing the generation of mitochondrial O₂^{•-}, NO and peroxynitrate. Additionally, MG significantly decreased the activities of MnSOD and complex III. MnSOD is the first-line enzyme in mitochondria to dismutate O₂^{•-} and complex III transfers electrons from ubiquinone to cytochrome c. The inhibition of complex III by MG may disrupt the electron transport chain, leading to electrons leaking out to form O₂^{•-} (Wang et al., 2009; Desai et al., 2010). MG was found to modify arginine, lysine and cysteine residues and inhibit a large number of enzymes (Thornalley, 1996). MG can also cause oxidative stress indirectly through the formation of advanced glycation end products (AGEs), the irreversible chemical modifications and cross-links in proteins (Desai et al., 2010). The activities of SOD, GST, CAT, Gly I and II, and GSH content decreased in a time- and dose-dependent manner following the administration of exogenous MG to rat liver cells whereas lipid peroxidation increased (Choudhary et al., 1997). Wu and Juurlink (2002) also found that MG (100 to 500 μM) induced oxidative stress by inactivating antioxidant enzymes such as GR and GPX and by a profound increase in oxidized glutathione (GSSG) content. They proposed that MG enhanced AGEs that in turn could activate receptors of AGEs (RAGE), thereby promoting O₂^{•-} production. MG-induced impairment of GR and GPX would also result in oxidative stress because GR plays an important role by reducing GSSG to GSH, whereas GPX scavenges peroxides by utilizing GSH, which can be converted to very reactive free radicals. The MG-induced impairment of GR and GPX activity was inactivated glycation because these proteins are known to be susceptible to glycation inactivation. However, very limited work has been done on the influence of exogenous MG in antioxidative defense systems in higher plants. Hoque et al. (2010) showed that exogenous application of MG (0.5 to 10 mM)

in tobacco (*Nicotiana tabacum* L. cv. BY-2) cells inhibits GST activity, whereas exogenous application of GSH can reverse the phenomenon. Thus, MG weakens the antioxidant enzymes and their modulation may contribute to oxidative stress.

8. Engineering glyoxalase pathway enzymes and abiotic stress tolerance of plants

Undoubtedly, the role of MG-scavenging systems in plant stress tolerance has increased through the use of gene transfer technology. A number of experiments clearly demonstrated that the enhancement of the MG-detoxification systems in plants provides partial protection from oxidative damage (Venna et al., 1999; Yadav et al., 2005a, 2005b; Singla-Pareek et al., 2003, 2006, 2008; Bhomkar et al., 2008). Overexpression of glyoxalase pathway genes in transgenic plants has been found to keep a check on the MG and ROS levels under stress conditions, regulate glutathione homeostasis, allowing the transgenic plants to survive and grow under various abiotic oxidative stresses.

Veena et al. (1999) first produced transgenic tobacco plants overexpressing the *B. juncea* short-type *Gly I* gene. The over-expressing transgenic plants showed tolerance to salt (400 and 800 mM NaCl) and toxic concentrations of MG (5 and 25 mM). The degree of tolerance was correlated with the degree of *Gly I* expression indicating the importance of the gene in plant stress tolerance. Similarly, Singla-Pareek et al. (2003) produced transgenic tobacco plants overexpressing glyoxalase pathway genes (*Gly I* and *Gly II*). Although *Gly II* transgenic lines showed improved tolerance to salinity stress and could set normal viable seeds, double transgenic lines showed a better response than either the single gene-transformed lines or non-transgenic line. Additionally, the double transgenic line showed a negligible (5%) yield reduction under salt stress (200 mM NaCl). The transgenic plants also showed lower chlorophyll destruction than wild-type (WT) plants. Later on, the suitability of the glyoxalase transgenic plants against heavy metal (ZnCl_2) stress was also tested by Singla-Pareek et al. (2006). Transgenic plants showed restricted MG accumulation and less lipid peroxidation at a high Zn concentration (5 mM ZnCl_2) and were able to survive, maintain growth, flower and set normal viable seeds without any reduction in yield. The most striking observation was that double transgenic plants performed better than either of the single-gene transformants as initially observed under salt stress. Maintenance of glutathione homeostasis in transgenic plants was the possible mechanism behind the tolerance against heavy metal toxicity. Subsequently, overexpression of the *Gly II* gene in transgenic rice plants showed higher tolerance to a toxic concentration of MG and salinity compared to non-transformed (NT) plants. The overproduction of *Osgly II* results in detoxification of MG and SLG and recycling of GSH that could be a possible mechanism behind stress tolerance and the *Osgly II*-overexpressing transgenic line. Importantly, the transgenic plants were able to maintain a high selectivity for K^+ over Na^+ uptake in the roots and reduced Na^+ sequestration in the young leaves compared with the WT plants leading to the maintenance of a high Na^+/K^+ ratio in both shoots and roots of transgenic plants. The maintenance of ideal Na^+/K^+ ratio in both the shoots and roots of transgenic plants was correlated with the normal growth of *Osgly II*-overexpressing transgenic tobacco plants that may be the basis of minimizing Na^+ toxicity under salt stress (Singla-Pareek et al., 2008). Additionally, overexpression of the *Gly I* gene using a novel *Cestrum yellow leaf curling virus* (CmYLCV) promoter in blackgram (*Vigna mungo* L.) showed tolerance to salinity stress. Exposure of transgenic plants to salinity stress (100 mM NaCl) revealed that the transgenic

plants survived under salt stress and set seed. In contrast, the untransformed control plants failed to survive. The higher level of Gly I activity in transgenic lines was directly correlated with their ability to withstand salt stress (Bhomker et al., 2008). The above findings clearly indicate that the glyoxalase pathway is one of the most important metabolic pathways that could have both a direct and indirect effect on plant stress tolerance by modulating multiple physiological responses and metabolic pathways.

9. Methylglyoxal as an initiator of activation of signal transduction pathways

Information regarding the signaling roles of MG in higher plants is scarce although MG was found to activate several signal transduction pathways in yeast. MG activates transcription factors such as Yap1 and Msn2, and triggers a Hog1 mitogen-activated protein (MAP) kinase cascade in *Saccharomyces cerevisiae*. Regarding the activation of Hog1 by MG, Sln1, an osmosensor possessing histidine kinase activity, functions as a sensor of MG (Maeta et al., 2005; Takatsume et al., 2006). Our understanding of the signaling role of MG in higher plants is at a rudimentary stage. However, critical analysis of glyoxalase transgenic plants reveals that they maintain MG at a certain level, which we predict to be an appropriate level that these transgenic plants maintain under stress, playing a secondary role in stress perception and signaling. In view of the finding in yeast, it would be worthwhile to investigate whether MG might be involved in signal transduction pathways in higher plants. Therefore, the signaling functions of MG in higher plants remain an open question.

10. Sites and sources of ROS production in plant cells

Abiotic stresses disrupt cellular homeostasis in plants leading to the onset of oxidative stress or the generation of ROS such as singlet oxygen ($^1\text{O}_2$), superoxide radical ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\bullet\text{OH}$). ROS are continuously produced during various metabolic processes. However, certain environmental stresses or genetic defects cause the production of ROS to exceed the management capacity. Organelles with a highly oxidizing metabolic activity or with an intense rate of electron flow, such as chloroplasts, mitochondria and peroxisomes (Fig. 2), are major sources of ROS production in plant cells (Mittler et al., 2004). In the chloroplast, during photosynthesis, energy from sunlight is captured and transferred to two light harvesting complexes, photosystem I (PS I) and photosystem II (PS II). $\text{O}_2^{\bullet-}$, which is produced mainly by electron leakage from Fe-S centers of PS I or reduced ferredoxin (Fd) to O_2 (Mehler reaction), is then converted to H_2O_2 by SOD (Gechev et al., 2006). $\text{O}_2^{\bullet-}$ can also be produced by the leaking of electrons to molecular oxygen from electron transport chains in PS I and II (Sgherri et al., 1996). Under excess light conditions PS II is able to generate $^1\text{O}_2$ by energy transfer from the triplet state chlorophyll (Asada, 2006). In peroxisomes, ROS is produced mainly during photorespiration and also during β -oxidation of fatty acids. The ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme, which catalyses the carboxylation of ribulose-1,5-bisphosphate (RuBP) during carbon assimilation, can also use O_2 to oxygenate ribulose-1,5-bisphosphate. Under abiotic stress conditions, which impair CO_2 fixation in the chloroplast, the oxygenase activity of RuBisCO increases and the glycolate that is produced moves from the chloroplast to peroxisomes, where it is oxidized by glycolate oxidase (GO) forming H_2O_2 (Takahashi & Murata, 2008). In peroxisomes, H_2O_2 can also be formed directly from O_2 by enzyme systems such as xanthine oxidase (XO) coupled to SOD (Mhamdi et al., 2010). The

mitochondrial electron transport chain consists of several dehydrogenase complexes which reduce a common pool of ubiquinone (Q). ROS production is likely to occur mainly in complex I and the Q zone (Blokhina & Fagerstedt, 2006). Additional sources of ROS in plant cells include the detoxifying reactions catalyzed by cytochromes in both the endoplasmic reticulum and the cytoplasm (Urban et al., 1989). In glyoxysomes, acyl-CoA oxidase is the primary enzyme responsible for H₂O₂ generation. Plasma membrane-bound NADPH oxidases as well as cell-wall associated peroxidases are the main sources of O₂^{•-} and H₂O₂ producing apoplastic enzymes (Mhamdhi et al., 2010). In the presence of redox-active metals, the extremely reactive •OH can be formed from H₂O₂ through the Fenton reaction or from H₂O₂ and O₂^{•-} through the Haber-Weiss reaction causing extensive oxidative damage of membranes and other macromolecules, including photosynthetic pigments, protein, DNA and lipids (Foyer & Noctor, 2005a; Gechev et al., 2006).

Fig. 2. Major sites and sources of ROS production of plant cells (modified from Blokhina & Fagerstedt, 2006; Mhamdi et al., 2010; Hossain & Fujita, 2011).

11. ROS scavenging and detoxification system in plants

Plants use an intrinsic mechanism known as the plant antioxidant system as a defense mechanism to regulate the ROS levels according the cellular needs at a particular time.

These antioxidants includes the enzymes superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), glutathione reductase (GR; EC 1.6.4.2), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPX; EC 1.11.1.9), glutathione S-transferase (GST; EC 2.5.1.18) and water-soluble compounds such as ascorbate (AsA) and GSH (Apel & Hirt, 2004; Hossain & Fujita, 2011). Although AsA and GSH function as cofactors of enzymes of the antioxidant and glyoxalase pathways, both can also directly quench ROS and regulate gene expression associated with biotic and abiotic stress responses to optimize defense and survival. Importantly, sustaining the ROS concentration (specially H_2O_2) at an appropriate level can promote plant development and reinforce resistant to environmental stressors by modulating the expression of genes and redox signaling pathways (Neill et al., 2002). Abiotic stress induced differential induction of different components of antioxidant defense systems, in particular those related to AsA and GSH briefly described below:

11.1 Ascorbate peroxidase (APX)

APX is one of the most important enzymes of the AsA-GSH cycle and plays a vital role in plant defense against oxidative stress by catalyzing the conversion of H_2O_2 to H_2O . APX activity and APX isoenzyme gene expression are variable even under the same stress conditions and some of them are constitutively expressed for the immediate and efficient detoxification of H_2O_2 under normal and oxidative stress conditions (Ishikawa & Shigeoka, 2008; Hossain & Fujita, 2011). Salt-tolerant plant species showed a significant increase in APX activity in response to salt stress but the activities decreased or remain unchanged in salt-sensitive genotypes (Mittova et al., 2003; Sekmen et al., 2007). A profound increase (≈ 5 -fold) in APX activity was observed in a drought-tolerant tomato variety (Zarina) in response to mild drought stress (Sánchez-Rodríguez et al., 2010). Sato et al. (2011) showed that transgenic plants overexpressing the OsAPX gene in rice showed cold tolerance at the booting stage as indicated by 2-fold lower H_2O_2 levels and MDA content. Spikelet fertility was significantly higher in transgenic lines than in WT plants. These results indicate that higher APX activity enhances H_2O_2 -scavenging capacity and protects spikelets from lipid peroxidation, thereby increasing spikelet fertility under cold stress.

11.2 Monodehydroascorbate reductase (MDHAR)

MDHAR, one of the major components of the AsA-GSH cycle, is a monomeric enzyme using NADPH as an electron donor to reduce monodehydroascorbate (MDHA) to AsA (Hossain et al., 1984). MDHAR activity increased in response to salt stress in wild salt-tolerant tomato species while the activity decreased in a salt-sensitive cultivar. Additionally, due to higher antioxidant capacity, salt-tolerant wild relatives maintained a lower level of H_2O_2 and MDA, whereas cultivated species showed greater oxidative damage (Shalata et al., 2001; Mittova et al., 2003). Mung bean and rapeseed seedlings subjected to salinity and heavy metal stresses showed a decrease in MDHAR activity (Hossain et al., 2010, 2011; Hasanuzzaman et al., 2011a). In contrast, an increase in MDHAR activity due to long-term drought stress was observed in diploid hybrid which was accompanied by higher AsA and lower DHA content compared to their parents (Gao et al., 2009). Transgenic tobacco plants overexpressing the *AtMDHAR1* gene showed enhanced stress tolerance in terms of higher net photosynthetic rates under O_3 , salt and osmotic stress and greater PSII effective

quantum yield and a lower level of H_2O_2 under O_3 and salt stresses (Eltayeb et al., 2007). Additionally, Kavitha et al. (2010) showed that tobacco plants overexpressing the *AmMDHAR* gene showed higher MDHAR and APX activity. The transgenic lines showed an enhanced redox state of AsA and reduced level of lipid peroxidation denoting the vital role of MDHAR in AsA regeneration and oxidative stress tolerance.

11.3 Dehydroascorbate reductase (DHAR)

Oxidation of AsA by APX leads to the formation of a short-lived MDHA radical, which is converted to AsA by MDHAR or disproportionates nonenzymatically to AsA and dehydroascorbate (DHA). DHA is recycled to AsA by DHAR, which requires GSH as the reductant (Chen et al., 2003). Mottova et al. (2003) reported that salt stress significantly induced DHAR activity and a higher AsA and AsA/DHA ratio in salt-tolerant cultivar whereas the activity remained unchanged in a salt-sensitive tomato cultivar accompanied by higher oxidative damage. Mung bean and rapeseed seedlings subjected to salt and heavy metal stress showed a significant decrease in DHAR activity (Hossain et al., 2010; Hossain et al., 2011; Hasanuzzaman et al., 2011a). However, a sharp increase in DHAR activity was observed in rice shoot tissues under mild drought stress but the activity decreased under severe drought stress (Sharma & Dubey, 2005). Heat (55°C) altered DHAR activity in tobacco BY-2 cells, showing an important phenomenon in maintaining redox balance when MDHAR activity decreased significantly. The increase in DHAR activity could be a sort of feedback regulation mechanism occurring to improve AsA regeneration from DHA when AsA depletion occurs at its production sites due to enhanced ROS (Locato et al., 2009). Therefore, DHAR may optimize the utilization of the still available AsA in a cellular organelle that is strongly subjected to oxidation by the overproduced ROS. Transgenic tobacco plants overexpressing the *cytDHAR* gene exhibited higher DHAR activity, a higher level of AsA and ascorbate redox state and exhibited enhanced tolerance to O_3 -, drought-, salt-, and PEG-induced oxidative stresses (Eltayeb et al., 2006). Wang et al. (2010) showed that *DHAR* overexpression in *Arabidopsis thaliana* increased AsA and GSH levels and their redox state relative to WT showed oxidative stress tolerance induced by high light and high temperature (40°C). Therefore, increasing AsA content through enhanced ascorbate recycling could limit the deleterious effects of environmental oxidative stress.

11.4 Glutathione reductase (GR)

GR is a flavoprotein oxidoreductase that catalyzes the reduction of GSSG to GSH by utilizing NADPH. The adaptive behaviors of salt-tolerant and -sensitive genotypes suggest that GR plays a significant role in maintaining the glutathione redox state under oxidative stress. Salt-tolerant plant species showed an increase in GR activity in response to salt stress whereas in the sensitive genotypes the activity decreased or remain unchanged (Shalata et al., 2001; Sekmen et al., 2007). In our recent study, mung bean and rapeseed seedlings showed a decrease in GR activity under a high level of salt and Cd stress (Hossain et al., 2010; Hasanuzzaman et al., 2011a, 2011b). A drought-tolerant rice genotype significantly increased GR activity and antioxidant metabolites such as AsA and GSH when the level of H_2O_2 decreased. However, in the susceptible genotype the activity of GR decreased as the level of GSH and AsA decreased. Selote & Khanna-Chopra (2004) proposed that during water stress an integrated antioxidant defense system, including the AsA-GSH cycle, in

developing panicles, is an important factor related to enhanced spikelet fertility in the drought-resistant rice genotype under upland conditions. Similarly, a drought-tolerant cultivar (Zarina) showed a sharp increase (≈ 3 -fold) in GR activity under mild drought stress (Sánchez-Rodríguez et al., 2010). Recently, Martret et al. (2011) reported that co-expression of DHAR+GR or GR+GST in tobacco chloroplasts exhibit altered metabolism and improved abiotic stress tolerance. The level of AsA and GSH was significantly increased in both the double transformants (DHAR+GR and GR+GST). In response to chilling stress, the H_2O_2 content increased nearly 3-fold in WT plants whereas the double transgenic plants reduced H_2O_2 levels more efficiently than WT or single gene transformants.

11.5 Catalase (CAT)

CATs, the first antioxidant enzymes to be discovered and characterized, are predominantly localized in the peroxisomes and glyoxysomes for scavenging H_2O_2 . Purev et al. (2010) reported a significant induction of the *PgCat1* gene in response to various stimuli such as heavy metals, plant hormones, osmotic agents, UV and chilling stress. Salt-tolerant *P. maritima* and *L. pennellii* showed an increase in CAT activity in response to salt stress (Mittova et al., 2003; Sekmen et al., 2007) indicating that an increase in CAT activity may be an adaptive response to overcome oxidative stress. However, a decrease in CAT activity in response to various stresses was also reported (Sharma & Dubey, 2005; Hossain et al., 2010; Hossain et al., 2011; Hasanuzzaman et al., 2011a). The *Cat1*-deficient mutant under a high light regime showed leaf necrosis and was unable to maintain glutathione in the reduced state when exposed to elevated light indicating that the recycling of GSH is defective because of a general shortage of reducing power for the AsA-GSH cycle to allow continuous recycling of the substrates (Mhamdi et al., 2010). A 'gain-of-function' study also revealed the importance of the CAT gene in plant stress tolerance. Overexpression of *E. coli* catalase gene (*katE*) in Indica rice (*Oryza sativa* cv. BR5), conferred tolerance to salt stress; transformed plants formed flowers and produced normal seeds (Moriwaki et al., 2008).

11.6 Glutathione peroxidase (GPX)

GPXs are key enzymes of the antioxidant network in plants present in different subcellular organelles. Their principal activity is thought to catalyze the reduction of H_2O_2 , organic hydroperoxides (ROOH) and lipid hydroperoxides to H_2O and alcohol using GSH and/or other reducing equivalents (Foyer & Noctor, 2011). Most identified plant GPX genes were shown to have high homology to the mammalian phospholipid hydroperoxide glutathione peroxidases (PHGPX), which have a higher affinity to lipid hydroperoxides than to H_2O_2 . However, at least two plant PHGPXs probably represent novel isoforms of TRX peroxidase, which are generally more active against H_2O_2 than lipid peroxides (Foyer & Noctor, 2005b). The specific expression pattern of PHGPX in salt-induced tolerant foxtail millet seedlings suggests that its product plays a crucial role in the defense reaction against salt-induced oxidative damage (Sreenivasulu, 2004). In addition, gene expression and the activity of GPX were found to increase in response to salt, drought and heavy metal stresses (Mittova et al., 2003; Hossain & Fujita, 2010; Hossain et al., 2011). Despite this, transgenic plants overexpressing GPX were more tolerant to oxidative stress caused by treatments with H_2O_2 , MV, and environmental stress conditions, such as chilling, salinity and drought (Gaber et al., 2006) indicating the potential physiological role of GPX in higher plants against oxidative stress.

11.7 Glutathione S-transferases (GSTs)

GSTs are a superfamily of multifunctional enzymes best known for their role in enzymatic detoxification of xenobiotics. GST acts by catalyzing the conjugation of GSH with electrophilic, often hydrophobic toxic compounds to form derivatives that can be secreted from the cell, sequestered in the vacuole, or catabolized (Dixon & Edwards, 2010). In addition, plant GSTs also provide protection against oxidative stress induced by abiotic stresses and oxidants (Fujita & Hossain, 2003a, 2003b; Hossain et al., 2006a, 2006b; Dixon & Edwards, 2010). Functioning as GPX and DHAR, plants GSTs can catalyze the reduction of hydroperoxides to less harmful alcohols and safeguard protein function from oxidative damage and maintain redox homeostasis by regenerating AsA from DHA (Dixon & Edwards, 2010). Due to its high GSH content, we used pumpkin as a model plant and studied the induction pattern and role of pumpkin GSTs in oxidative stress tolerance and detoxification, by exposing pumpkin seedlings and callus to different types of stresses viz. environmental stresses (dehydration, high and low temperatures), hormones such as 2,4-D and methyl jasmonate (MJ), aldehydes and alcohols including α,β -unsaturated carbonyl compounds, heavy metals including Cd, Mn, Cr and As, and antioxidants and oxidants. High temperature (42°C) and dehydration induced pumpkin GSTs to different degrees (Hossain & Fujita, 2002; Fujita & Hossain, 2003a). Pumpkin GSTs are significantly induced by α,β -unsaturated carbonyl compounds, saturated chain aldehydes and alcohols (Fujita & Hossain, 2003b). However, α,β -unsaturated aldehydes were the most effective inducers and their potency is related to the Michael acceptors reaction. Pumpkin GSTs were also induced by heavy metals, different antioxidants and oxidants (Hossain et al., 2006a). *CmGSTF1* was not responsive to all the applied stresses except for MJ (Fujita & Hossain, 2003b; Hossain et al., 2006a). Induction of *CmGSTF1* by MJ alone is therefore indicative of the possibility of involvement of pumpkin GSTs in developmental processes. Transgenic tobacco plants overexpressing the *PjGSTU1* gene showed drought tolerance and Green Fluorescent Protein (GFP) fusion studies revealed the presence of *PjGSTU1* in the chloroplast of transgenic plants which was correlated with its role in ROS removal (George et al., 2010).

11.8 Ascorbate (AsA)

AsA is one of the most abundant and powerful antioxidants in plant cells. It is an integral weapon in the defense against ROS generated by various abiotic and biotic stresses in different subcellular organelles and the apoplast. AsA has the ability to donate electrons in a number of cellular redox reactions and serves as a major cellular redox regulatory antioxidant (Smirnoff, 2000). AsA can directly quench $^1\text{O}_2$, $\text{O}_2^{\bullet-}$ and $\bullet\text{OH}$ and regenerate α -tocopherol from α -chromanoxyl radical thereby providing protection to membranes (Thomas et al., 1992). It is the substrate of APX, which is a critical component of the AsA-GSH cycle for H_2O_2 detoxification. A wealth of evidence suggests that stress-resistant plants are up-regulated or maintained higher AsA levels than stress-sensitive plants (Shalata et al., 2001; Mittova et al., 2003). In our recent study with mung bean, rapeseed and wheat seedlings, the level of AsA decreased in response to salt stress (Hossain et al., 2011; Hasanuzzaman et al., 2011a, 2011b). AsA level increased in a drought-tolerant rice genotype (N22) while in the susceptible genotype (N118) its levels decreased (Selote & Khanna-Chopra, 2004). However, decrease in AsA content in response to drought and heavy metal stresses was reported (Sharma & Dubey, 2005; Sečenji et al., 2010; Hossain et al., 2010). Zhang et al. (2011) showed that transgenic tomato plants overexpressing the GDP-Mannose

3',5'-epimerase (an important enzyme of the ascorbate biosynthesis pathway) gene (*SIGME*) exhibited a significant increase in total AsA content in different plant organs and showed enhanced oxidative stress tolerance induced by MV. The transgenic plants showed a higher survival and growth rate under chilling and salt stress. They proposed that improved stress tolerance was closely related to higher endogenous AsA content that increased the ability to scavenge ROS.

11.9 Glutathione (GSH)

The thiol tri-peptide GSH is one of the major antioxidant and redox buffers in plants found abundantly in all cell compartments. GSH takes part in the control of H_2O_2 levels through the AsA-GSH cycle (Foyer & Noctor, 2005a). It can also function directly as a free radical scavenger by reacting with 1O_2 , $O_2^{\bullet-}$, and $^{\bullet}OH$ (Larson, 1988). It is also involved in the transfer and storage of sulfur and in the detoxification of heavy metals where phytochelation (PC) derived from GSH forms heavy metal complexes. Additionally, GSH has been associated with several growth- and development-related events in plants, including cell differentiation, cell death and senescence, pathogen resistance and enzymatic regulation (Ogawa, 2005). Up-regulation of the GSH level is of pivotal importance, because it induces the signal transduction and defense against ROS and MG which is achieved through different pathways with various control points (Fig. 3) which include orchestrated activation of genes encoding enzymes related with GSH and AsA (Hossain & Fujita, 2011). A sharp increase in GSH content was found in mung bean, rapeseed and wheat seedlings subjected to salinity and Cd stress (Hossain & Fujita, 2010; Hossain et al., 2010, 2011; Hasanuzzaman et al., 2011a). A desiccation-tolerant plant (*Boea hygrosopica*) showed an almost 50-fold increase in GSH content under dehydration stress (Sgherri et al., 1994). Additionally, a drought-tolerant rice genotype showed an increase in GSH content while in the susceptible genotype the GSH content decreased (Selote & Khanna-Chopra, 2004). Ivanova et al. (2010) found that overexpression of γ -glutamylcysteine synthetase gene (*gsh1*) in the cytosol led to a 2-fold increase of foliar GSH content. Biomass accumulation of WT poplar hybrid decreased in heavy metal-contaminated soil by more than 30-fold, whereas transformants showed a 2-fold decrease. Thus, poplars overexpressing γ -ECS in the cytosol were more tolerant to heavy metal stress.

12. Coordinated role of ascorbate and glutathione during oxidative stress tolerance

Glutathione and ascorbate co-operation is the key for the cellular redox homeostasis in the antioxidative AsA-GSH cycle as well as redox regulation of signaling pathways, gene expression and plant metabolism. Positive correlations between high contents of AsA and GSH and higher activities of AsA and GSH utilizing and regenerating enzymes in inducing stress tolerance have frequently been found. Correlative response of AsA and GSH and other antioxidant enzymes were observed in wheat seedlings subjected to salt stress. The fresh and dry weights of salt-stressed seedlings were significantly reduced, but least reduced in the tolerant cultivar (H 168). The salt stress treatment caused a temporary increase in the activities of SOD, APX, GR and CAT. The increases were consistent in the tolerant genotypes, but mostly stopped or even inverted in the susceptible cultivar. Importantly, the AsA and GSH contents increased significantly in the tolerant cultivar but decreased in the susceptible cultivars. It can, therefore, be concluded that higher AsA and

GSH content and antioxidative enzymes conferred salinity tolerance of the resistant cultivars (El-Bastawisy, 2010). Liu et al. (2009) showed that mild oxidative shock induced by exogenous PQ pretreatment in cucumber leaves modifies the functioning of AsA and GSH utilizing and regenerating enzymes and showed drought-induced oxidative stress tolerance. Drought stress and PQ pretreatment increased the activities of SOD, CAT, GPX, APX, DHAR, MDHAR, GR, and non-enzymatic antioxidants such as AsA and GSH in leaf tissues. However, PQ-pretreated drought-stressed seedlings resulted in higher activities of those enzymes and non-enzymatic antioxidants such as AsA and GSH and AsA/DHA and GSG/GSSG ratios compared to seedlings subjected to drought stress without a pretreatment. Subsequently, Lin et al. (2011) further showed that simultaneous induction of both AsA and GSH content and their metabolizing enzymes by PQ pretreatment increased the tolerance to salt-induced oxidative stress in cucumber leaves. Salt stress significantly increased the activities of SOD, APX and GR but decreased the activities of CAT, GPX, MDHAR, DHAR and AsA accompanied by higher $O_2^{\bullet-}$, H_2O_2 and MDA levels. However, PQ pretreated salt-stressed seedlings maintained higher activities of SOD, GPX, MDHAR, DHAR, GR as well as AsA, GSH, AsA/oxidized ascorbate, GSH/GSSG ratios, accompanied by lower levels of $O_2^{\bullet-}$, H_2O_2 and MDA.

Xu et al. (2010) showed the H_2O_2 -induced upregulation of AsA and GSH metabolism in inducing Al-induced oxidative stress tolerance in wheat seedlings. Al stress increased the $O_2^{\bullet-}$ and H_2O_2 level leading to more predominant lipid peroxidation, programmed cell death, and inhibited root elongation in both Al-tolerant and -sensitive genotypes. Al-stress increased the activities of SOD, POD, CAT, MDHAR, DHAR, GR, GPX and AsA and GSH content and their redox state. However, Al-stress seedlings pretreated with H_2O_2 showed higher SOD, POD, CAT, MDHAR, DHAR, GR, GPX activities and AsA and GSH content and their redox state than non-treated Al-stressed seedlings. Importantly, antioxidant capacity was more enhanced in the Al-sensitive genotype than in the tolerant one. Therefore, H_2O_2 pretreatment makes the plant more tolerant to Al-induced oxidative stress by inducing AsA and GSH levels and their metabolizing enzymes.

13. Simultaneous expression of two or more transgenes related to AsA- and GSH-metabolism in plants and abiotic stress tolerance

Only few recent studies in plants explored how transgenic plants overexpressing multiple genes related to AsA and GSH metabolism showed better tolerance against abiotic oxidative stress by co-regulation of their antioxidant machinery. Zhao et al. (2009) studied the co-expression of GST and CAT gene in transgenic plants under Cd and heat stress and in combination with heat and Cd stress conditions to understand the influence on other function-linked components of the antioxidant defense system, including AsA-GSH cycle enzymes and metabolites such as AsA, GSH and their redox state. Transgenic plants under Cd stress and combined stress (Cd and heat) conditions showed a sharp increase in CAT, GST, APX, MDHAR, DHAR, GR activities and maintained a higher ascorbate and glutathione redox state, a higher photosynthetic rate and a lower level of H_2O_2 and chloroplast destruction under stress. Their results denote that co-expression of GST and CAT ultimately affects the AsA-GSH cycle and coordinates up-regulation of AsA-GSH pathway enzymes rendering the plants more tolerant to Cd and heat-induced oxidative stress. Additionally, tobacco plants overexpressing three antioxidant enzymes (CuZnSOD, APX and DHAR) showed greater tolerance to oxidative stress than double transgenic

(CuZnSOD and APX) or WT plants (Lee et al., 2007). Transgenic plants overexpressing three antioxidant genes had higher DHAR activity, and higher ratios of AsA to DHA, and GSSG to GSH compared to double transgenic (CuZnSOD and APX) plants.

Consequently, Ahmad et al. (2010) found that transgenic plants overexpressing SOD, APX and choline oxidase (*codA* gene, gene for betaine synthesis) in naturally betaine non-accumulator plants (potato) showed enhanced protection against oxidative stress as indicated by lower levels of H₂O₂ compared with double transgenic (SOD+APX) and non-transgenic plants after MV-mediated oxidative stress. Additionally, transgenic plants overexpressing three genes synergistically enhanced tolerance to salt and drought stresses by maintaining higher activities of SOD, APX and CAT and betaine level. These results are strongly coherent with the findings of our studies on exogenous proline- and betaine-induced oxidative stress tolerance in mung bean seedlings (Hossain & Fujita, 2010; Hossain et al., 2010, 2011). Additionally, transgenic tobacco plants overexpressing *cytSOD* or *cytAPX* alone, or in combination, enhanced tolerance to drought-induced oxidative stress. The most striking observation was that the transgenic plants overexpressing both enzymes showed higher MDHAR, DHAR, GST, POX and CAT activity in addition to SOD and APX activity in the soluble fractions. Moreover, an increase in the activity of some antioxidant enzymes (APX, SOD & POD) was also observed in the chloroplastic fraction of the transgenic plants. These results indicate that co-regulation among the antioxidant enzymes in different subcellular organelles is also vital to obtain substantial tolerance against oxidative stress (Faize et al., 2011).

The tolerance mechanism of oxidative stress in response to various abiotic stresses was investigated in transgenic potato tubers overexpressing D-galacturonic acid reductase gene (*GalUR*) with enhanced accumulation of AsA (Hemavathi et al., 2010). Enhanced activity of SOD, CAT, APX, DHAR and GR were observed in transgenic potato tubers subjected to various abiotic stresses induced by MV, NaCl and ZnCl₂. The ascorbate redox state (AsA:DHA) and ratio of reduced to oxidized glutathione (GSH:GSSG) were significantly higher in transgenic tubers than in WT tubers. Therefore, transformation of one gene related to ROS metabolism can have a substantial influence on other enzymes; moreover, AsA-producing transgenic plants modulate glutathione metabolism because they are interdependent in keeping the AsA-GSH cycle fully functional and thereby reducing oxidative stress (Foyer & Noctor, 2011). Dixit et al. (2011) noted that overexpression of even one gene can have a profound influence on other antioxidant enzymes in plants. Tobacco plants overexpressing the GST gene (*TvGST*) showed better Cd tolerance, as indicated by lower Cd accumulation and lipid peroxidation, than WT plants. Most importantly, the transgenic plants showed significantly higher SOD, GST, GPX, APX and CAT enzyme activities under Cd stress than WT plants. Their results further proved that co-regulation among antioxidant enzymes is essential to maintain the correct balance between overproduction of ROS and their scavenging to keep them at the required levels to execute their signaling function and to improve oxidative stress tolerance.

14. Involvement of AsA/DHA, GSH/GSSG ratios in abiotic stress response, redox regulation and signaling

During abiotic stress-driven oxidative stress, higher plants have the ability to sense and translate ROS signals into specific cellular responses. Additionally, ROS have the ability to oxidize redox-sensitive proteins directly or indirectly through the use of molecules like

AsA and GSH. AsA and GSH are united together through redox flux and coordinate their action during the metabolism of ROS (Foyer & Noctor, 2005a, 2005b, 2011). Compartment-specific variations in AsA/DHA and GSH/GSSG ratios may have a substantial significance in redox signaling. The ascorbate redox state in the apoplast is critically important in a number of stress responses such as in the control of guard cell signaling, stomatal movement and plant growth (Chen et al., 2003). Under stressful conditions AsA oxidation to DHA takes place and, in turn, this molecule can modulate plant responses to stress (Lopez-Carbonell et al., 2006). DHA is believed to signal the redox state of the apoplastic environment, and hence to allow the cell to perceive stress in the environment. DHA accumulation in the apoplast may trigger the arrest of cell growth (Latowski et al., 2010). Moreover, DHA may act as a potential factor in signaling pathways. Reversible modification of specific proteins by DHA could be important in cell signaling. Unlike ascorbate, the redox potential of glutathione is a function of both the GSH/GSSG ratio and the concentration of GSH. According to the Nernst equation the glutathione redox state is a second order of function of GSH concentration (Kuźniak, 2010). The redox state of the GSH/GSSG couple is altered under abiotic stress conditions because two molecules of GSH are converted to GSSG through oxidation. Conditions that trigger the accumulation of GSSG often also lead to a subsequent increase in total glutathione content and are related to stress-induced changes in H₂O₂ content. Stress-induced changes in the H₂O₂ content and GSH/GSSG ratio have a central role in signaling due to their effects on transcription, translation and post translational modification of proteins and metabolic processes (Neill et al., 2002; Szalai et al., 2009). Unfortunately, the role of the glyoxalase pathway in regulating GSH concentration and the glutathione redox state is often neglected. However, our recent studies showed that simultaneous induction of glyoxalase pathway enzymes (Gly I and Gly II) and GR by exogenous chemical treatment (proline, betaine, Se and NO) increased the GSH content and the GSH/GSSG ratio (Hossain & Fujita, 2010; Hossain et al., 2010, 2011; Hasanuzzaman et al., 2011a, 2011b) and several studies conducted in a number of plant species under abiotic stress conditions have elucidated the fact that a high GSH/GSSG and/or AsA/DHA ratio sustained by increased GSH and AsA or decrease of GSSG and DHA may be key element for efficient protection against abiotic oxidative stress.

15. Intimate relationship between GSH-dependent MG detoxification system and AsA- and GSH-based ROS detoxification system: clues from stress-tolerant and transgenic plants

Some of the most exciting advances in understanding, sensing and response networks of abiotic stress tolerance by using stress-tolerant, stress-sensitive and transgenic plants lead to a cross talk between the AsA- and-GSH dependent ROS detoxification system and the GSH-dependent MG detoxification system in counteracting abiotic stress-induced oxidative damage (Fig. 3). In deciphering the molecular insights of salinity-induced oxidative stress tolerance in transgenic tobacco overexpressing glyoxalase pathway enzymes, Yadav et al. (2005b) first revealed and discussed the interconnection of GSH-based ROS and MG metabolism in plants. Transgenic plants overexpressing both Gly I and II genes showed better antioxidative protection under salt stress (200 mM NaCl) while WT plants suffered from serious oxidative stress. Furthermore, transgenic plants reflect higher basal antioxidant enzyme activities such as APX, GR, GST and GPX, although the activities of these enzymes

increased sharply when salt stress was imposed. Based on their findings it can be concluded that glyoxalase transgenic plants showed enhance salinity tolerance by regulating multiple biochemical pathways and also by having multiple functions, including: (i) prevention of excessive accumulation of MG, which could deplete GSH; (ii) maintenance of higher antioxidative activities of AsA-GSH cycle enzymes that regulate the level of H_2O_2 and ascorbate and glutathione redox ratios; (iii) maintenance of higher activities of GST and GPX, which utilize GSH in degrading lipid peroxide, organic hydroperoxide and H_2O_2 ; (iv) protection of non-enzymatic antioxidants from oxidative and antioxidative enzymes from inactivation through advanced glycation. These four mechanisms hold true for various abiotic stresses and recent studies further demonstrated that co-ordinated induction of both detoxification pathways showed enhance abiotic stress tolerance in different plant species and cultured cells (see later in the next section).

El-Shabrawi et al. (2010) further pointed out the interaction among glyoxalase and ROS detoxification systems while identifying biochemical markers for enhanced salt tolerance in two rice cultivars differing in salt tolerance. Analysis of non-enzymatic antioxidants and their redox state (AsA, DHA, AsA/DHA, GSH, GSSG and GSH/GSSG) and the antioxidant and glyoxalase pathway enzyme activities (SOD, APX, CAT, GPX, GR, POX, Gly I and Gly II) and isozyme expression of antioxidant enzymes depicts that the salt-tolerant cultivar Pokkali maintained higher enzymatic activities – reflected by isozyme analysis – and showed oxidative stress tolerance – as indicated by a lower level of H_2O_2 and oxidative DNA damage – than the salt-sensitive cultivar (IR64) suggesting that Pokkali possesses a more efficient antioxidant defense system to cope with salt-induced oxidative stress. Furthermore, Pokkali exhibited a higher GSH/GSSG ratio and a higher AsA/DHA ratio than IR64. Their results showed that fine modulation of AsA and GSH metabolism and regulation of ROS via the antioxidant and glyoxalase systems and higher proline content in the tolerant rice cultivar allowed it to show better tolerance against salinity-induced oxidative stress. Based on the above findings we therefore infer that ROS and MG metabolism are tightly correlated and that a plant induces both pathways in response to abiotic stress tolerance.

Fig. 3. Schematic illustration of possible metabolic interaction of AsA- and GSH-based antioxidative system and GSH-based glyoxalase system in plant cells (modified from Hossain et al., 2011). Dotted lines indicate non enzymatic reactions. For further discussion, see text.

16. Induction of abiotic stress tolerance in plants by simultaneous induction glyoxalase system and AsA- and GSH-based ROS detoxification system through exogenous chemical treatments

Abiotic stress tolerance is a multigenic trait and acquired tolerance must be a cumulative result of different multiple metabolic pathways and genes. However, up-regulation of at least two detoxification pathways (ROS and MG) provides substantial tolerance against abiotic oxidative stress (Hoque et al., 2008; Kumar & Yadav, 2009; Hossain & Fujita, 2010; Hossain et al., 2010, 2011; Hasanuzzaman et al., 2011a, 2011b). Although a close relationship between MG and ROS metabolism was first described by Yadav et al. (2005b) in a transgenic system, later on, Hoque et al. (2008) further showed experimental evidence of a close relationship among ROS and MG detoxification systems in tobacco (*N. tabacum* L. cv. BY-2) cells by applying exogenous proline and betaine under salt stress (200 mM NaCl) conditions. Salt stress increased protein oxidation, contents of thiol, disulfide, GSH and GSSG, and the activity of GST and Gly II enzymes, but decreased the redox state of both thiol-disulfide and glutathione, and the activity of GPX and Gly I enzymes involved in ROS and MG detoxification. Exogenous application of proline or betaine (20 mM) resulted in reduced protein oxidation, and in an increase in the glutathione redox state and activity of GPX, GST and Gly I under salt stress. In previous studies, Hoque et al. (2007) showed that exogenous proline and betaine increased the activity of APX, DHAR and GR under salt stress (200 mM NaCl). These results suggest that exogenous application of proline or betaine rendered the plants more tolerant to salinity-induced oxidative damage by modulating antioxidant and glyoxalase systems. Consequently, Kumar and Yadav (2009) reported that simultaneous induction of both MG and ROS detoxification by exogenous proline and betaine induces cold tolerance in *Camellia sinensis* (L.) O. Kuntze. MG and lipid peroxidation levels increased in tea bud (youngest topmost leaf) in response to cold stress (4°C) whereas this increase did not occur when tea bud was exposed to proline and betaine (25 mM). Exposure of tea bud to proline and betaine helped to maintain the thiol/disulfide ratio and enhanced the activities of GST and GR during cold stress. Furthermore, both proline and betaine showed a protective effect on Gly I and activated Gly II. Finally, they concluded that proline and betaine might provide protection against cold stress by regulating the formation of MG and lipid peroxidation and by activating or protecting some antioxidant and glyoxalase pathway enzymes.

A similar correlated regulation of glyoxalase and antioxidant pathways in inducing heavy metal tolerance was also observed in mung bean (*Vigna radiata* cv. Binamoog-1) seedlings (Hossain et al., 2010). Seven-day old seedlings were subjected to 1 mM CdCl₂ for 48 h with or without proline and betaine. Cadmium stress caused a profound increase in GSH and GSSG content, while the AsA content decreased with a sharp increase in H₂O₂ and lipid peroxidation (MDA). APX, GST, GPX, and Gly I activities increased in response to Cd stress while the activity of CAT, MDHAR, DHAR, GR and Gly II decreased. Exogenous application of proline and betaine (5 mM) showed an increase in GSH and AsA content, maintained a high GSH/GSSG ratio and increased the activity of APX, DHAR, MDHAR, GR, GST, GPX, CAT, Gly I and Gly II involved in ROS and MG detoxification systems more than the control and most Cd-stressed plants, with a concomitant decrease in GSSG content, H₂O₂ and MDA levels. These findings suggest that both betaine and proline provide protection against Cd-induced oxidative stress by reducing H₂O₂ and MDA levels and by

increasing the antioxidant defense and MG detoxification systems. Recently, we further demonstrated that coordinated induction of antioxidative and glyoxalase defense systems by using exogenous proline and betaine protects mung bean seedlings from salinity-induced oxidative damage (Hossain et al., 2011). Salt stress (200 mM NaCl, 48 h) caused a sharp increase in GSH and GSSG content while the GSH/GSSG ratio and AsA content decreased. The GR, GPX, GST and Gly II activities increased in response to salt stress while the MDHAR, DHAR, CAT and Gly I activities decreased sharply with an associated increase in H₂O₂ and MDA. Contrarily, salt-stressed seedlings pre-treated with proline or betaine (5 mM proline or betaine, 24 h) showed an increase in AsA, GSH content, GSH/GSSG ratio and maintained higher activities of APX, DHAR, GR, GST, GPX, CAT, Gly I and Gly II involved in ROS and MG detoxification systems than untreated control seedlings and most salt-stressed plants with a simultaneous decrease in GSSG content, H₂O₂ and MDA levels. These results further coincide with our previous results denoting that simultaneous induction of both detoxification pathways make the plant more tolerant to salinity-induced oxidative stress. A similar concomitant induction of ROS and MG detoxification systems by exogenous selenium (Se) pretreatment were reported in rapeseed (*Brassica napus* cv. BINA sharisha 3) seedlings under salt stress (Hasanuzzaman et al., 2011a). Twelve-day-old seedlings were supplemented with Se (25 µM Na₂SeO₄) and salt (100 and 200 mM NaCl) separately and in combination. The AsA content in leaves decreased significantly with increasing salt stress. The amount of GSH and GSSG increased as the level of salt stress increased, while the GSH/GSSG ratio decreased. The activity of APX and GST increased with an increase in salt concentration while GPX activity increased only at moderate salt stress (100 mM NaCl). GR activity remained unchanged at 100 mM NaCl but it was decreased under severe (200 mM NaCl) salt stress. The activities of MDHAR, DHAR, CAT, Gly I, and Gly II decreased following the imposition of salt stress, whereas a sharp decrease in these activities was observed under severe salt stress (200 mM NaCl) with higher H₂O₂ and MDA levels. Importantly, Se-supplemented salt-stressed seedlings showed an increase in AsA and GSH contents, GSH/GSSG ratio, and the activities of APX, MDHAR, DHAR, GR, GST, GPX, CAT, Gly I, and Gly II more than the control and in most cases than seedlings subjected to salt stress without Se supplementation. Additionally, Se-supplemented salt-stressed seedlings showed lower oxidative damage as indicated by lower H₂O₂ and MDA levels. These results suggest that the exogenous supplementation of Se induces salt stress-induced oxidative stress tolerance in rapeseed seedlings by enhancing their antioxidant defense and MG detoxification systems.

The latest findings by Hasanuzzaman et al. (2011b) further prove that modulation of the glyoxalase and ROS detoxification systems by exogenously applied SNP (an NO donor) improved oxidative stress tolerance of wheat (*Triticum aestivum* L. cv. Pradip) seedlings subjected to salt stress (150 and 300 mM NaCl, 4 d). The AsA content decreased in leaf tissues in response to salt stress while the GSH and GSSG contents and the GSH/GSSG ratio increased as the level of salt stress increased. GST activity increased in response to salt stress while APX, MDHAR, DHAR, CAT and GPX activities remained unchanged. GR, Gly I and Gly II activities decreased following the imposition of salt stress with a concomitant increase in the levels of H₂O₂ and MDA. Furthermore, salt-stressed seedlings pretreated with NO (1 mM SNP, 24 h) showed an increase in the AsA and GSH contents and the GSH/GSSG ratio as well as the activities of MDHAR, DHAR, GR, GST, GPX, Gly I, and Gly II compared to seedlings subjected to salt stress without pretreatment. The authors concluded that NO-

induced coordinated induction of AsA- and GSH-based ROS and MG detoxification systems make the plant more tolerant to salinity-induced oxidative stress. The evidence described above clearly illustrates that the appropriate induction of a detoxification system (both ROS and MG) renders the plant more tolerant to various abiotic stresses in a synergistic manner by efficient regulation of both AsA and GSH levels and their utilizing and regenerating enzymes.

17. Conclusion and future perspective

MG and ROS are clearly emerging as leitmotifs in plant life, being involved in most physiological responses to stress as well as developmental processes (Paulus et al., 1993; El-Shabrawi et al. 2010; Hossain & Fujita, 2011). Imbalances in metabolic processes due to abiotic or biotic stresses or certain genetic defects may lead to increased accumulation of ROS and MG, forming a potential threat for plant growth and survival. Usually a dynamic balance has to be maintained between ROS and MG generation and scavenging in order to guarantee normal plant growth. The glyoxalase system and AsA- and GSH-based antioxidant systems play a central role in regulating ROS and MG levels in plants. It is now clearly evident that ROS regulate a complex signal transduction network within plant development and its response and adaptation to both biotic and abiotic stressors although signaling roles of MG in higher plants is scarce. Considerable progress has been made over the last few years in understanding how plants protect themselves against MG and ROS while several genes encoding the components of both MG and ROS detoxification systems have been cloned, characterized and used in the construction of transgenic lines. Although gene manipulation seems to be a sound approach to counteract oxidative or MG stress, attempts to improve stress tolerance, particularly by manipulation of a single antioxidant gene or either Gly I or Gly II genes, have seen limited success because of the need for a balanced interaction of protective enzymes and other metabolites of MG and ROS detoxification systems (Yadav et al., 2005b; Lee et al., 2007; Martret et al., 2011). Several recent studies using enzyme protectants (proline and betaine) or a signaling molecule (NO) also proved that simultaneous induction of different components of both MG and ROS detoxification pathways showed substantial tolerance to abiotic oxidative stress. Inhibition of one component of the glyoxalase system or ROS detoxification system strongly influence the activity of other enzymes or metabolites and thereby lead to deterioration of the system because both systems unite together through a multifunctional redox molecule (GSH). Therefore, it is important to clarify of the bottlenecks affecting the performance of both glyoxalase and ROS detoxification systems under various abiotic stresses in the future. Complete elucidation of MG metabolism by integration of proteomics and metabolomics, and dissecting its signaling roles by using model plant species would be worthwhile research to improve multiple abiotic stress tolerance. Pyramiding both H₂O₂ and MG detoxifying genes in one genetic background and to study their consequence in stress response and tolerance will also be a fascinating future area of study. However, a major gap exists in our understanding about how plants sense MG and oxidative stress in different subcellular compartments and how this stress signal is transduced, thus activating large-scale and coordinated expression of different enzymes and metabolites of their detoxification pathways. A complete understanding of the interaction between ROS, MG and plant hormones and transcription factors (Sasaki-Sekimoto et al., 2005; Takatsume et al., 2006) and components of ROS and MG detoxification pathways in different subcellular

compartments will reveal more subtle regulatory roles of both detoxification systems in abiotic stress tolerance. In addition, identification of master regulators that control stress response activation will accelerate the process to improve and strengthen plant fitness to changing climates.

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19. References

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World population is growing at an alarming rate and is anticipated to reach about six billion by the end of year 2050. On the other hand, agricultural productivity is not increasing at a required rate to keep up with the food demand. The reasons for this are water shortages, depleting soil fertility and mainly various abiotic stresses. The fast pace at which developments and novel findings that are recently taking place in the cutting edge areas of molecular biology and basic genetics, have reinforced and augmented the efficiency of science outputs in dealing with plant abiotic stresses. In depth understanding of the stresses and their effects on plants is of paramount importance to evolve effective strategies to counter them. This book is broadly divided into sections on the stresses, their mechanisms and tolerance, genetics and adaptation, and focuses on the mechanic aspects in addition to touching some adaptation features. The chief objective of the book hence is to deliver state of the art information for comprehending the nature of abiotic stress in plants. We attempted here to present a judicious mixture of outlooks in order to interest workers in all areas of plant sciences.

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