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INTRODUCTION AND MECHANISM GLUTATHIONE REDUCTASE IN PLANT STRESS TOLERANCE

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ABSTRACT
Glutathione reductase (GR) is an essential enzyme which plays an important role in the Ascorbate Glutathione metabolic pathway for the oxidative tolerance in plants. It acts as an anti-oxidant enzyme preserving a reduced intracellular environment and protection of cellular macromolecules like DNA, proteins and lipids. During exposure to the biotic stresses, the differential modulation of GR in plants has been widely implicated in the significance of antioxidants as the major components of plant defense operations. How the stress factor affects the plant leaves and what is the role of GR as a defense mechanism to protect the plants from these stresses, will be studied in this review in a gross detail. This review lays emphasis on the overview about structure, localization, biosynthesis and mechanism of GR from the abiotic stress for exposed crop plants and also points-out unexplored aspects in the current context. It draws inspiration from a number of observations and research papers by scientists across the molecular biology world to understand and assert that GR is a vital enzyme without which a plant's survival is difficult and impossible. We hope that this review would enlighten the reader to think broadly about the plant's life cycle and importance of GR as a performance enhancing-agent in plants to help maintain the balance between plant and animals in the environment.

Key words: GR, GSSG, H_2O_2, ROS, Plant Stress

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Introduction
Glutathione reductase [EC 1.6.4.2] was first time introduced by E. Racker in 1955. In GR, he identified NADPH as the primary electron donor (Rao *et al.*, 2008). FAD and the thiol group are also present and act as an initial mechanism for this enzyme. The scientists explain that GR which is present in plant source might be less sensitive to NADPH from *E. coli* and other sources. The deduced GR amino acid sequence shows 41 to 48% identity with those of *Escherichia coli*, pea, and *Arabidopsis thaliana* GR (Jiang *et al.*, 1995).

Now a days, whole world suffer from the different types of environmental problem which generates a harmful effect on living system. Plants and animals are facing difficulty in survival due to the change in the environmental conditions which causes the abiotic and biotic stress in plants and animals. The world population is increasing day by day and certainly after 10 years it will increase upto a billion of population. On the other hand, there is decrease in crop productivity worldwide due to the environmental stress, which results in a damage to biomolecules such as lipids, proteins, amino acids, and DNA of plant cells. Glutathione acts as an antioxidant in plants. It consists of antioxidant enzyme GR which causes synergistic actions in scavenging free radicals. There has been growing evidence over the past three decades showing that malnutrition (e.g., dietary deficiencies of protein, selenium, and zinc) or excess of certain nutrients (e.g., iron and vitamin C) give rise to the oxidation of bimolecules and cell injury (Fang *et al.*, 2002). Reactive oxygen species (ROS) are being produced as byproducts of usual metabolic pathways in plants. ROS damage the cell structure and lead to formation of free radicals like H_2O_2, OH, ROOH, and ROO^- (Jiang *et al.*, 2001). Due to this there is a loss of the growth function and productivity of the plants. It is necessary to increase the food production for the living environment by minimizing these environmental stresses or protect the plant cell from the stress and enable it to tolerate the damaging of the lipids, proteins and nucleic acid. It is necessary to save crops from excessive stress so as to enable the production of transgenic plants in a better way for their perfect cellular function and regulation activity (Bita *et al.*, 2013). It is very important to compare the response of other enzymes like as SOD, APX, MDHAR, GPX etc with that of GR in order to be used as an anti-oxidative protection system in plants (Kaminski *et al.*, 2012). In this review, we have mainly emphasized the antioxidant enzyme i.e., GR (GR) which play a major role in AsA-GSH cycle to protect the plants. It increases the cell growth, functions and regulations of plants and directly removes the free radicals like OH, O_2 from the plant leaves like Indian mustard (Thirupathi *et al.*, 2011). GR was introduced in the Indian mustard and it was concluded that it reduces the heavy metal like cadmium which act as an effective inhibitors of photosynthesis and reduce the chlorophyll content in plants (Ouzounidou *et al.*, 1997). GR is also introduced in the *E. coli gor* gene and it is first and last enzyme in the ASA-GHS pathway which is responsible for the detoxification of the H_2O_2 in a green leaves (Foyer *et al.*, 1994). However there is a visible change in the distribution of the activity between the different isoenzyme forms of GR.

In GR oxidized glutathione (GSSG) and NAD (P) H, there are important substrate to protect the plant leaves. These antioxidant enzymes convert the oxidized glutathione (GSSG) to a reduced form of glutathione (GSH) to balance the ratio of GSH/GSSG in the abiotic stress (Trivedi *et al.*, 2013). In the studies of the genetically engineered plants, the activity of GR is firstly reported for the improvement and large scale production of the crop plants. GR enzyme is used for transferring the *gor* gene of *Eschericha coli* to tobacco plant and expressing the cytosolic tobacco cells (Foyer *et al.*, 1991). Furthermore, the roles of the amino-acid which are involved in the synthesis of GR enzyme to protect the transgenic plants and control of oxidative stress have also been discussed.

What is Glutathione?

Glutathione is found mainly in both eukaryotes and prokaryotes or gram-negative bacteria. Glutathione is ubiquitous low molecular weight compound and is a major pool of non-protein reduced sulphur (Kunert *et al.*, 1993). Glutathione act as a storage and transport form of reduced sulphur such as SO_2 and H_2S. Glutathione is the thiol tripeptide function in the plant which is constituted by three amino acids namely glutamate (glu), cysteine (cys) and glycine (gly) and is represented by the formula Y-L-glutamycl-L-cysteiyl-glycine in a plant tissue (Volodymyr, 2012). In peroxidase enzyme, Glutathione act as cofactor to detoxify peroxides and attack on biological molecules which is generated by oxygen radicals. The functional group of thiol GSH exist either in reduced form or an oxidized form with disulphide between two identical molecules (Rouhier *et al.*, 2008). GHS confer its biological properties and make it crucial metabolite to perform multiple function including growth and development. GSH is an essential component of the cell's scavenging system for reactive oxygen compound. Glutathione is present in millimolar concentration in plant tissue and it is regarded as one of the major determinants of cellular redox homeostasis (Alfonso. *et al.*, 2006). It is important in both abiotic and biotic stress in plants for the growth, development and protection of the crops. Glutathione is under tight homeostatic control both intra and extracellular. A dynamic balance is maintained between GSH Synthesis, it's recycling from GSSG/oxidized Glutathione and its utilization (Chavan *et al.*, 2005). The homeostatic Glutathione redox cycle attempts to keep Glutathione repleted as it is being consumed.

What is GR [E.C 1.6.4.2]

GR plays a very essential role in balancing the ratio of ROS and protecting the cell from the abiotic stress in eukaryotes and prokaryotic organisms (Yousuf *et al.*, 2012). GR is the water soluble enzyme found in mitochondria, chloroplasts, perixosomes and cytosol. GR has been purified from different tissue of plant species like leaves or root of plants (e.g. *Arabidopsis thaliana*, *orzyza sativa*, *Brassica junncea*). GR is a ubiquitous flavoenzyme that convert the oxidized glutathione GSSG to the reduced two molecules of GSH and is associated with oxidation of NADPH (Countour *et al.*, 2000). GR is the regenerating enzyme for the Asocorbate Glutathione cycle which removes hydrogen peroxide (H_2O_2) (Maria *et al.*, 2005). GR plays an important role when oxidative stress like salinity, drought, UV radiation, high light intensity, chilling and herbicides occurs (Ali and Alqurainy). Under these conditions, consumption of NADPH between GR and photo reductive carbon cycle enzyme take place. Mostly GR is a homo-dimmer of 100-120 kda, and its sub unit size range is between 53kda and 59kda (Wingsle., 1989; Anderson *et al.*, 1990; Edwards *et al.*, 1990; Madamanchi *et al.*, 1992). GR appears to be ubiquitous in the biosphere and helps in the protection of plants from the oxidative stress. GR is present in millimolar concentrate in leaves (7-9) where it is generally reduced (Foyer *et al.*, 1991). Several function of GR includes the detoxification of herbicides, the removal of toxic derivatives of oxygen in ASA-GSH cycle, the induction of enzyme and its contribution in Sulphur metabolism and the regulation of genes expression (Mohammad *et al.*; 2012). GR act as a potential enzyme for the defense system to protect the plants, its activity has been found in *Brassica junncea, Arabidopsis thalliana, Traticum aestivum, C. annuum* against the heavy metals like cadmium (Leo *et al.,* 2002). GR helps in monitoring the Glutathione level and Cadmium present in genetically modified GR plants as compared with wild types plants for the function of chloroplast (Zhu *et al*.; 1991). The glutathione system follows a general physiological environmental stress-response to protect the plants. Alternatively, if the organism is unable to adjust to the changes in the environmental condition, it is possible that there is loss of the glutathione synthesis in plants and causes an oxidative stress. Tausz *et al.,* 2004 found that an initial stress response was related to changes in the glutathione redox state, whereas acclimation was marked by increased glutathione concentrations, increased related enzyme activities, and/or a more reduced redox state of glutathione. The latter was interpreted as overcompensation leading to enhanced regeneration of glutathione. Decaying of the GR makes a strong effect upon stress that is related to progressive degradation and oxidation of the glutathione pool. A time-course analysis, which has rarely been done in the published literature, showed this sequence of events. When apple trees were subjected to progressing drought, the initial response was a slight oxidation of the glutathione pool, followed by increase in glutathione concentrations, because GR acts as a stress marker for the antioxidative defense system.

Structure of GR

Structure of GR signifies that a flavin adenine dinucleotide obliged homodimer is present. GR has been purified and well characterized from the leaves of several plant species such as *Spinacia oleracea, Pisum sativum*, and *Zea mays* (Mahan *et al.,* 1987). GR is present in form of homodimers with a molecular mass ranging from 100 to 150 kDa, which consist one FAD per monomer. If the thiols group of GR is absent, it exhibits in form of dimmer and tetramers or larger form. In cellular conditions these larger forms show catalytic activity. Glutathione and its product, balance the enzyme in its dimeric and tetramers forms (Yousaf *et al.,* 2012). Although most of the GR exists as homodimer, they are also found as monomer in *Chalmydomonas reinharditii* (Takeda *et al.,* 1993) and heterodimer in *Pisum sativum* and *Zea mays*. GR consists of dimeric form and may be further changed into tetramers or even higher crowded states and the type of higher order state is pH and temperature-dependent rather than products. This mechanism is used in the catalytic activity of GR and their regulation (Rao *et al.,* 2008). Some organisms, for instance, *Drosophila* and *Trypanosomes* do not posses GR, and hence reduction of Glutathione is accomplished by either the thioredoxin or the trypanothione system (Kanzok *et al.,* 2001). These isoforms of GR are found to be restorative, by different environmental signals or perform diverse functions under adverse conditions and help in single transduction (Sharma *et al.,* 2012).

Biosynthesis of GR

GR is found in both Gram-negative bacteria, prokaryotes and eukayrotes but it is rarely found in Gram-positive bacteria (Griffith *et al.,* 2005). In the biosynthesis of the GSH, plant genes are transferred in eukaryotes from bacterial cell through the ancestry of mitochondria (Copely *et al.,* 2002). GSH represent the non-protein pool which diminish the sulphur (Kunert *et al.,* 1993). The reduced form of GSH is a bipeptide thiol with the formula y-gals-cys-gly, the biosynthesis of GSH consist three amino acids constituents namely glutamine, cystein and glycine in their metabolic pathway which consists two sequential ATP dependent reactions (Meister *et al.,* 1983). These ATP-dependent reactions are catalyzed by two enzymes: gamma glutamyl-cystein synthetase and glutathione synthetase (Meister, 1995). Both enzymes have different function, gamma glutamyl-cystein synthetase catalyzes the formation of a peptide bond between y-carboxylate of glutamate and cystein (u-cys) and the glutathione synthetase catalyze the subsequent formation of peptide bond between the cysteinyl carboxylate of y-clutamylcysteine(y-EC) and the amino group of glycine. All these single step reactions require hydrolysis of ATP that occur in chloroplastic and non-chloroplastic compartment which is found in both photosynthetic and non-photosynthetic tissue for the production of the peptide bond (Foyer *et al.,* 2002) . It increases the GSH content in plants through which several plant species produced the analogous tripeptide derivatives of y-glutamyl cystein in place of GSH. The biosynthesis of the glutathione occurs in two ATP-dependent steps. In the first step, Y-Glu-cys synthetase catalyzes the linking of cystein and glutamate to form gamma-Glutamyl cystein and in second step glycine is linked to the C-terminal end of gamma-glutamyl cystein to form final product. All this data gives the information about the structure, function, localization and regulation of GR in plants. The function of GSH has been identified be the storage form of reduced sulphur (Nector *et al.,* 2011). In the regulation of plants, GSH help in the inter-organ sulphur allocation, it also acts as a regulator of gene expression (Wingate *et al.,* 1998). GR a flavo-protein oxidoreductase NAD(P)H-dependent cellular enzymatic antioxidant helps in regulation of cellular redox environment (Gill *et al.,* 2012). GR converts oxidized GSSG to reduced GSH which help in balancing the ratio of GSH/GSSG under abiotic stress (Schaffer *et al*., 2001). GSSG consist of two GSH linked by disulphide bridge which can convert back to GSH by GR. GR helps in maintaining GSH pool which is crucial for the active function of proteins. The presence of cystein, the active site of GR actually catalyzes the NADPH dependent reduction of GSSG to GSH. Presently two GSH synthetase (GS) groups namely prokaryotic and eukaryotic forms have been reported that function as tetrameric and dimeric enzymes respectively synthesis functionally terameric and dimeric enzyme (Galant *et al.,* 2009). A putatively gly-rich loop was reported to modulate activity of Arabidopsis GS (Oliver *et al.,* 1997) which was identified by Galant *et al.,* 2009 as a part of the active site formation. The eukaryotic homologous work in site directed mutagenesis and ligand binding analysis.

Subcellular localization of GR in plants

GR is mainly located in chloroplast, mitochondria (Edwards *et al.,* 1990; Madamanchi *et al.,* 1992; Creissen *et al.,* 1995; Jimenez *et al.,* 1997; Rudhe *et al.,* 2004) but it is also found in perixosomes (Jimernez *et al.,* 1997, Delrio *et al.,* 2002a). These plant organelles consists two enzymes APX and MDAR in an Ascorbate glutathione cycle which occurred in the peroxisomal membrane whereas GR and DAR occurred in the peroxisomal matrix (Jimenez *et al.,* 1998; del Rio *et al.,* 2002a). Glutathione is synthesized in both the chloroplast and cytosol of plant leaves. GR is mainly localised in the stroma of chloroplast because chloroplast has a capacity for Ascorbate transport in an Ascorbate glutathione cycle (Anderson *et al*., 1983). The protein products of these nuclear-encoded plastid genes are targeted to the chloroplast organelle (Ahmad, *et al*., 2012). Chloroplast and cytosol protect the leaves from the oxidative stress and other harmful compounds through the redox link between the two compartments by the reduction of H_2O_2 free radicals. Transit peptide sequences located in the N-terminus of these proteins facilitate the transfer from the cytosol, where they are synthesized, to the chloroplast organelle (Schein *et al*., 2001). Both cytosolic and chloroplastic isoforms carry the active site, whereas specific cytosolic domain can solely be found in cytosolic isoforms.

Mitochondria consist of GR which rapidly scavenges O_2 free radicals from the oxidative stress. Mitochondrial sources are signified by electron transport chains (Andras *et al.,* 2012). Mitochondria imperilments lead to activation of nuclear gene.

In the cellular location of GR, most of the molecular property of perixosome is unknown but GR present in perixosome is recognised by the polyclonal antibody raised against total GR from pea leaves *Pisum sativum* (Francisco *et al.,* 1991). The GR isoenzyme present in perixosome has been purified by immune gold electron microscopy (Nyathi *et al.,* 2006). The important character of perixosomal GR is brought about by abiotic stress condition such as high light, darkness, high temperature, heavy metal toxicity.

Mechanism of GR

It is a part of a group called flavoenzymes and has an important disulfide group where one mole of NADPH is necessary to decrease GSSG to GSH for every mole of GSSG (Ithayaraja., 2011). Regarding GR catalytic mechanisms, it is to be noted here that the availability of substrate largely control GR redox interconversions where in comparison to the reduced GR more stability is shown by the oxidized GR form which can tolerate divalent metal ions including $Zncl_2$, $Cucl_2$ and $Fecl_2$ (Rao *et al.,* 2008). The catalytic mechanism involves two steps: first, the flavin moiety is reduced by NADPH, the flavin is oxidized and a redox-active disulfide bridge is reduced to produce a thiolate anion and a cystein. The decrease in GSSG via thiole disulfide interchange reactions is involved in the 2nd step (Ghisla *et al*., 1989). If the reduced enzyme is not reoxidized by GSSG, a reversible inactivation may happen. In the process of the reduction of GSSG to GSH, GR acts in a ping-pong manner in which the NADPH binds and converts a hydride to FAD, then leaves before di-glutathione binds. In addition, GR controls GSH/GSSG ratio and supplies GSH for GPX and DHAR. The GPX and DHAR convert H_2O_2 and DHA into H_2O and AsA, respectively. Although GR gets the reduction power from NADPH, H^+, it dissipates this energy and, in turn, enhances $NADP^+$/NADPH, H^+ ratio (Gill *et al.,* 2012)

Role of GR

GR is one of the potential enzymes of the enzymatic antioxidant system which sustain reduced status of GSH and play the important role in maintaining the sulfhydryl group (Yousuf *et al.,* 2012). GR has been used in the transgenic to provide the plants with tolerance against the oxidative stress (Foyer *et al.,* 1994). GR plays an important role in maintaining function and protecting from the oxidative stress in abiotic and biotic stress. It can also act as scavenger for hydroxyl radical, singlet O_2 and various electrophiles. Recent research has highlight a regulatory role of glutathione is influencing the expression of many gene which are important in the plant responses to both abiotic and biotic stress (Mullineaux *et al.,* 2005). GR play an important role in metabolism and it is also involved in the detoxification of xenobiotic and protection against heavy metal toxicity as a source of reductant in enzyme reaction (Mohammad *et al.,* 2012). It is also involved in the effects of growth and development of plants, regulation of plastidic and nuclear gene expression (Chi *et al.,* 2008). GR is also involves in resistance to pathogen infection and tolerance to environmental disruption that promote oxidative stress. GR also participate in transport and regenerate antioxidant and vitamin E and C to their reactive forms (Rahman., 2007). The ratio of GSSG/GSH present in cell is a key factor in properly maintaining the oxidative balance from the cell (Montserrate *et al*.; 2009). Glutathione participate in both enzymatic and non-enzymatic reaction as an antioxidant, in conjugation reaction as a nucleophile and in disulphide exchange with other thiol compound and thiol group in proteins (Deponte., 2013). GR plays an important role in cell defense against reactive oxygen metabolites by maintaining the cellular reduce GSH pool available to various tissues (Magdalena *et al.,* 2012). GR also plays the important role in resistance to oxidative stress caused by photo inhibition (Aono *et al.,* 1995) and parquet (Aono *et al.,* 1995). The metabolism of plant cell under oxidative stress is generally characterised by an increase formation of ROS.

Significance of GR

GR is key regulator of redox state of the plant cell managing development and stress tolerance response. In plant GSH protein namely thiodoxin (TRX), glutaredoxin (GPX) and GR are organised in complex multigene families (Belin *et al*., 2014). GR plays the important role in ROS detoxification, regeneration and stress tolerance in plants and has the ability to alter the redox state of important components of the electron transport chain. GR is important for stress tolerances in the recycling of GSH and maintenance of GSH/GSSG ratio in plant cell (Pang *et al*., 2010). Increased GR activity in metal, metalloids, salinity and drought stresses has been widely perceive in many plant species like *T.aestivum, Z.mays, carcumis sativum* and *Phaseolus aureus* under high temperature. Role of GR in plant adaptation and signalling during chilling and cold has also been reviewed (Kocsy *et al.,* 2000). GR is an essential enzyme which reduces glutathione disulphide to the sulfhydryl form GSH by the NADPH dependent reduction, which is an important cellular antioxidant system. The component of glutathione redox cycle, GR, plays important role in the protection of the cell from the toxic effects of reactive oxygen species (Tandogan *et al.,* 2010). Due to its significance the enzyme has been purified from a number of animals, plants and microbial.

Degradation of Glutathione

GR catalyses the reduction of GSSG to GSG using NADPH as electron donor. The structure and function of these enzymes and its gene is given by Mullineaux and Creissen, 1996. In transgenic plants, with over expression of GR under normal and stress condition an increase of the glutathione level occurred in plants which increase glutathione activity in the cytosol, plastids or combination of plastids and mitochondria (Jozefczak *et al.,* 2012). Direct attack by free radicals and other oxidative agents can also deplete Glutathione. It can be speculated that by cycling, GSSG may not be lost as a consequence of thiol disulphide exchange reaction or by degradation. Alternatively increase cycling of glutathione may feedback in some way to stimulate an increase in its biosynthesis (Biswas *et al.,* 2009).

In 2003, Chen et al., observed that related to the increase of GSH in GR over expressing plants, an ectopic over expression of dehydroascorbate reductase in tobacco and maize not only increased the level of redox state of ascorbic acid but also induced an approximately two fold increase of GSH level and an increase GSH/GSSG ratio. Removal of glutathione from cell or organs and its transport to another part of the plant may also be a means of modulating glutathione levels in a given cell type .When we increase glutathione which is present in leaves and leaflet of plant and it enhance the activity of Y-ECS and it evidenced that the endogenous activity of this enzymes to control the accumulation of GSH due to these there is no change in GSH poplars and the over expression of Glutathione synthase (Oliver *et al.*, 2001).

$$2GSH \leftrightarrow GSSG + 2H^{+} + 2e^{-}$$

Conclusion

This review is largely attributed to the enzyme GR and its capacity to protect plants and leaves from ROS which adversely affect the growth, development, food production, formation of transgenic plants and overall performance of daily activities of plants. GR helps the plants to work in their full capacity to produce food so as to enable maximum production. Large production of food by plants is very critical for the mankind since population is increasing at a growing rate day by day. Food which is produced by plants is required by all as their necessary dietary supplement and without GR this high amount of food formation is not possible by plants. GR plays an important role in ROS detoxification, GSH regeneration and managing the development and stress response program. GR acts as an important enzyme and as an anti oxidative component in ascorbate glutathione cycle and it saves its thiol group to protect against the free radicals. GR converts the oxidised glutathione into reduced glutathione which influences the expression of many genes which are important in protecting the plants from abiotic and biotic stress. It is present in every cell organelles like mitochondria, cytosol, chloroplast etc. of plant leaves to constantly protect it from free radicals and oxidative stress. GR is also used as an active agent to tolerate the oxidative stress in genetically modified plants. GR increases the immunity capacity of plants to fight all adverse situations. So we can safely conclude that without GR plant organelles are expoed to several hazards and their performance becomes dull and slow, that GR is very necessary in today's atmosphere to maintain high crop yield for mass population.

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