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ISBN 978-3-319-66681-5 ISBN 978-3-319-66682-2 (eBook)
https://doi.org/10.1007/978-3-319-66682-2

Library of Congress Control Number: 2017958440

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Glutathione (γ-glutamyl-cysteinyl-glycine) is a ubiquitously distributed sulfur-containing antioxidant molecule that plays key roles in the regulation of plant growth, development, and abiotic and biotic stress tolerance. It is one of the most powerful low-molecular-weight thiols, which rapidly accumulates in plant cells under stress. Recent in-depth studies on glutathione homeostasis (biosynthesis, degradation, compartmentalization, transport, and redox turnover) and the roles of glutathione in cell proliferation and environmental stress tolerance have provided new insights for plant biologists to conduct research aimed at deciphering the mechanisms associated with glutathione-mediated plant growth and stress responses, as well as to develop stress-tolerant crop plants. Glutathione has also been suggested to be a potential regulator of epigenetic modifications, playing important roles in the regulation of genes involved in the responses of plants to changing environments. The dynamic relationship between reduced glutathione (GSH) and reactive oxygen species (ROS) has been well documented, and glutathione has been shown to participate in several cell signaling and metabolic processes, involving the synthesis of protein, the transport of amino acids, DNA repair, the control of cell division, and programmed cell death. Two genes, *gamma-glutamylcysteine synthetase (GSH1*) and *glutathione synthetase (GSH2)*, are involved in GSH synthesis, and genetic manipulation of these genes can modulate cellular glutathione levels. Any fluctuations in cellular GSH and oxidized glutathione (GSSG) levels have profound effects on plant growth and development, as glutathione is associated with the regulation of the cell cycle, redox signaling, enzymatic activities, defense gene expression, systemic acquired resistance, xenobiotic detoxification, and biological nitrogen fixation. Being a major constituent of the glyoxalase system and ascorbate-glutathione cycle, GSH helps to control multiple abiotic and biotic stress signaling pathways through the regulation of ROS and methylglyoxal (MG) levels. In addition, glutathione metabolism has the potential to be genetically or biochemically manipulated to develop stress-tolerant and nutritionally improved crop plants. Although significant progress has been made in investigating the multiple roles of glutathione in abiotic and biotic stress tolerance, many aspects of glutathione-mediated stress responses require additional research.

The main objective of this volume is to explore the diverse roles of glutathione in plants by providing basic, comprehensive, and in-depth molecular information for advanced students, scholars, teachers, and scientists interested in or already engaged in research that involves glutathione. Finally, this book will be a valuable resource for future glutathione-related research and can be considered as a textbook for graduate students and as a reference book for frontline researchers working on glutathione metabolism in relation to plant growth, development, stress responses, and stress tolerance.

As editors of this volume, we are highly thankful to our experienced and well-versed contributors, who cordially accepted our invitation to write their chapters. We would also like to extend our thanks to Dr. Kenneth Teng and the editorial staff of Springer New York, who enabled us to initiate this book project. We believe that the information covered in this book will make a sound contribution to this fascinating area of research.

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Murcia, Spain — Pedro Diaz-Vivancos
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Contents

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Mohammad Anwar Hossain is a professor in the Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh. He received his B.Sc. in agriculture and M.S. in genetics and plant breeding from Bangladesh Agricultural University, Bangladesh. He also received an M.S. in agriculture from Kagawa University, Japan, in 2008 and a Ph.D. in abiotic stress physiology and molecular biology from Ehime University, Japan, in 2011 through a Monbukagakusho scholarship. In November 2015, he moved to Tokyo University, Japan, as a JSPS postdoctoral researcher to work on isolating low-phosphorus stress-tolerant genes/QTLs from rice. He has over 50 peer-reviewed publications on important aspects of plant physiology and breeding, plant nutrition, plant stress responses and tolerance mechanisms, and exogenous chemical priming-induced abiotic oxidative stress tolerance. He has edited five book volumes, including this one, published by CRC Press, Springer, and Elsevier. He is a professional member of the International Metabolomics Society, Bangladesh Society of Genetics and Plant Breeding, Bangladesh Association for Plant Tissue Culture and Biotechnology, and Seed Science Society of Bangladesh.

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Masayuki Fujita is a professor in the Department of Plant Science, Faculty of Agriculture, Kagawa University, Kagawa, Japan. He received his B.Sc. in chemistry from Shizuoka University, Shizuoka, and his M.Agr. and Ph.D. in plant biochemistry from Nagoya University, Nagoya, Japan. His research interests include physiological, biochemical, and molecular biological responses based on secondary metabolism in plants under biotic (pathogenic fungal infection) and abiotic (salinity, drought, extreme temperatures, and heavy metals) stresses; phytoalexin, cytochrome P450, glutathione *S*-transferase, and phytochelatin; and redox reaction and antioxidants. He has over 150 peer-reviewed publications and has edited 2 books.

Lam-Son Phan Tran is head of the Signaling Pathway Research Unit at RIKEN Center for Sustainable Resource Science, Japan. He obtained his M.Sc. in biotechnology in 1994 and Ph.D. in biological sciences in 1997 from Szent Istvan University, Hungary. After doing his postdoctoral research at the National Food Research Institute (1999–2000) and the Nara Institute of Science and Technology of Japan (2001), in October 2001, he joined the Japan International Research Center for Agricultural Sciences to work on the functional analyses of transcription factors and osmosensors in *Arabidopsis* plants under environmental stresses. In August 2007, he moved to the University of Missouri-Columbia, USA, as a senior research scientist to coordinate a research team working to discover soybean genes to be used for genetic engineering of drought-tolerant soybean

plants. His current research interests are the elucidation of the roles of phytohormones and their interactions in abiotic stress responses, as well as translational genomics of legume crops with the aim to enhance crop productivity under adverse environmental conditions. He has published over 110 peer-reviewed papers with more than 80 research and 30 review articles and contributed 8 book chapters to various book editions published by Springer, Wiley-Blackwell, and the American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America. He has also edited nine book volumes, including this one, for Springer and Elsevier.

Chapter 1
Chemistry, Biosynthesis, and Antioxidative Function of Glutathione in Plants

Wilma Sabetta, Annalisa Paradiso, Costantino Paciolla, and Maria Concetta de Pinto

Abstract Glutathione, a tripeptide constituted by glutamate, cysteine, and glycine, is an abundant metabolite that functions as a master regulator of intracellular redox homeostasis. Under optimal conditions, glutathione is mostly present in the reduced form (GSH), with a free thiol group. The link of two molecules of GSH, via a disulfide bond, leads to the formation of glutathione disulfide (GSSG). GSH can be oxidized, directly or indirectly, by reactive oxygen species, working as a scavenger that prevents excessive oxidation of cellular environment. GSH can also react with different thiols to form mixed disulfides. These reversible redox reactions are responsible for many GSH functions. GSH biosynthesis is dependent on the activity of the two ATP-dependent enzymes γ-glutamylcysteine synthetase and glutathione synthetase, encoded, respectively, by the nuclear *GSH1* and *GSH2* genes. The first step of GSH biosynthesis occurs in the plastids, while the second step can take place in both plastids and cytosol. The use of different *gsh1* mutants and *GSH1* overexpressing plants has helped to shed light on the multiple roles of GSH in plant growth, development, and response to changing environment. The maintenance of a high GSH/GSSG ratio is crucial for many physiological functions, and a decrease in this ratio can be utilized as an indicator of oxidative stress. The GSH/GSSG ratio also acts as an important regulator of several mechanisms involved in plant development and in plant stress response. In addition to redox state, also GSH concentration and its subcellular distribution are central factors controlling redox homeostasis and signaling.

Keywords Antioxidant • Cell compartments • Glutathione • Glutathione disulfide • Redox signaling • Redox state

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M.A. Hossain et al. (eds.), *Glutathione in Plant Growth, Development, and Stress Tolerance*, https://doi.org/10.1007/978-3-319-66682-2_1

Contents

1 Introduction

Glutathione (GSH) is a ubiquitous low-molecular-weight thiol in eukaryotes (Meister and Anderson 1983). This multifaceted molecule plays a number of key roles in plant biology. GSH is a product of sulfur metabolism, and, being mobile through long-distance transport, it also represents a storage form of reduced sulfur, since it can be remobilized in case of need (Rennenberg 2001). Additionally, GSH, like cysteine, is able to work as an important signal involved in the modulation of sulfate uptake and assimilation (Kopriva and Rennenberg 2004).

GSH plays a crucial role in different phases of plant life cycle; it is involved in embryo and meristem development (Cairns et al. 2006; Vernoux et al. 2000), as well as in pollen germination and in the development of flower primordia (Zechmann et al. 2011; Hatano-Iwasaki and Ogawa 2012; Gulyas et al. 2014). GSH mediates important cellular processes, like cell cycle progression and programmed cell death (Diaz-Vivancos 2010a, b; Kranner et al. 2006). Through the action of glutathione S-transferases (GSTs), GSH is also involved in the detoxification of different toxic compounds, such as xenobiotics, herbicides, and air pollutants (Cummins et al. 2011). Moreover, being the substrate for phytochelatin synthesis, GSH plays a key role in the detoxification of heavy metals (Freeman et al. 2004).

GSH is considered one of the most important cellular antioxidants, since it is able to scavenge directly or indirectly reactive oxygen species (ROS), which are unavoidable by-products of aerobic metabolism. Thus, GSH is a key metabolite in plant responses to biotic and abiotic stresses, in which it serves to remove ROS and, therefore, to limit the extent of oxidative damages (Foyer and Noctor 2005). However, it has been shown that GSH, through interactions with stress hormones, can also be implicated in the strengthening of ROS signals in plants (Han et al. 2013a, b). Therefore, it is conceivable that GSH is not only a simple antioxidant, but, being involved in the control of redox-sensitive proteins, it is able to couple changes in intracellular redox state to development/defense responses of plants, through the ROS-dependent signaling pathways (Foyer and Noctor 2005, 2016; Paciolla et al. 2016).

2 Glutathione Chemistry

GSH is a tripeptide consisting of glutamate, cysteine, and glycine (γ-glutamylcysteinylglycine). The linkage of the γ-carboxyl group of glutamate to the amino group of cysteine renders this bond different from peptide bonds found in proteins and gives stability to the molecule, which cannot be degraded by amino peptidases but requires specific carboxypeptidase and/or γ-glutamyl transpeptidase (Steinkamp and Rennenberg 1985; Wolf et al. 1996; Martin and Slovin 2000; Storozhenko et al. 2002). Some plants also possess GSH homologs in which glycine is substituted by other amino acids, as in the cases of homoglutathione (γ-Glu-Cys-β-Ala) in legumes and hydroxymethylglutathione (γ-Glu-Cys-Ser) in cereals (Klapheck 1988; Klapheck et al. 1992; Meuwly et al. 1993).

Due to the low molecular weight and to the presence of several hydrophilic groups, namely, two carboxylic groups, one amine and one thiol, GSH is a highly water-soluble compound.

The thiol group of the cysteine, being the most important chemically reactive group of GSH, is responsible for the biological and biochemical activity of this tripeptide: it permits redox reactions, as well as reactions of nucleophilic displacement. In the reactions with free radicals, GSH donates hydrogen atoms and produces the thiyl radical, which can also be formed by subtraction of one electron from the thiolate anion by photoionization or metal ions (Wonisch and Schaur 2001). Thiyl radicals, adequately stable and poorly reactive with other hydrogen donors, can dimerize and lead to the formation of glutathione disulfide (GSSG). Apart from GSSG, oxidized forms of glutathione comprise disulfides with other thiols to form "mixed disulfides" and more oxidized forms of the thiol group (Foyer and Noctor 2005). The thiol group of GSH can also act as a nucleophile reacting with a wide spectrum of electrophiles. In this case, it will not lead necessarily to disulfide formation but rather to the formation of GS-conjugate with various compounds. These reactions are important for detoxification of endogenous or xenobiotic compounds (Wang and Ballatori 1998; Dixon and Edwards 2010). Indeed, the GS-conjugates are usually transported by ABCC (subclass C of the ABC transporters) proteins, which are ATP-dependent pumps, to the vacuole, where the amino acids of GSH can be recycled (Martinoia et al. 1993; Lu et al. 1998; Grzam et al. 2006). GSH can also react with nitric oxide (NO), with the formation of nitrosoglutathione (GSNO), a molecule that is receiving increasing consideration for its role as a possible signaling molecule and/or as a NO reservoir (Lindermayr et al. 2005).

The reactions of thiolate-disulfide exchange seem to be due to a nucleophilic displacement on sulfur, in a similar way to those occurring in other nucleophilic displacements (Wonisch and Schaur 2001). The thiol-disulfide exchange reactions of glutathione are important in mediating the reversible oxidation/reduction of the

redox-sensitive proteins and therefore play a key role in maintaining cellular redox state (Foyer and Noctor 2005). In addition, GSH also participates in posttranscriptional protein modification through S-glutathionylation, which consists in the formation of a stable mixed disulfide between GSH and a protein thiol. In this way, GSH protects proteins from irreversible modifications that can be induced by oxidation (Noctor et al. 2012).

2.1 Glutathione Oxidation

Chemical oxidation of GSH is strongly dependent on its deprotonation to the thiolate form and, consequently, can be influenced by pH changes. Since the *pKa* of the GSH thiol is about 9.0, approximately only 1% of GSH will be deprotonated in the cytosol (pH 7.2) and a lower percentage of the thiolate will occur in acidic compartments, such as vacuole or apoplast. Consistently, in chloroplasts glutathione's reactivity will be increased in the light, when the photosynthetic electron transport leads to stroma alkalinization (Rahantaniaina et al. 2013 and references therein).

ROS are able to chemically react with GSH, leading to its oxidation (Fig. 1.1). However, the reaction's rate between singlet oxygen and GSH (2×10^6 M^{-1} s^{-1}) is lower than that occurring between this reactive species and other antioxidants, such as tocopherols and carotenoids. On the other hand, GSH reacts quickly with hydroxyl radical (8.1×10^9 M^{-1} s^{-1}), even if this oxidant reacts very fast with many other metabolites, such as ascorbate (ASC) and sugars that have higher cellular concentrations. The direct reaction of GSH with H_2O_2 is very slow (0.9 M^{-1} s^{-1}). Therefore, superoxide, which reacts with GSH at a rate similar to other antioxidants (7×10^5 M^{-1} s^{-1}), seems to be the major ROS contributing to un-catalyzed production of GSSG in vivo (Rahantaniaina et al. 2013 and references therein).

Dehydroascorbate (DHA), the stable product of ASC oxidation, is also able to directly oxidize GSH at significant rates (Fig. 1.1), which are higher at pH 8 than at pH 7. In addition, DHA reductases (DHARs) can catalyze the oxidation of GSH to reduce DHA to ASC (Fig. 1.1). In this way, DHARs provide a link between ascorbate and glutathione pools and allow GSH to take part, indirectly, in H_2O_2 reduction that finally relies on electrons derived from NAD(P)H and/or ferredoxin. In the ascorbate-glutathione (ASC-GSH) pathway, ASC peroxidase reduces H_2O_2 to water at the expense of ASC producing monodehydroascorbate; this last is an unstable product that can dismutate to ASC and DHA, which can then be reduced, chemically or by DHARs, by GSH with the simultaneous production of GSSG (Foyer and Halliwell 1976).

GSH oxidation can also depend on the activity of specific peroxidases (Fig. 1.1). Some GSTs, enzymes involved in the formation of a covalent bond between the sulfur atom of GSH and an electrophilic compound, can use GSH to reduce organic hydroperoxides (Wagner et al. 2002; Dixon et al. 2009; Dixon and Edwards 2010; Cummins et al. 2011). A number of GSTs have both conjugase

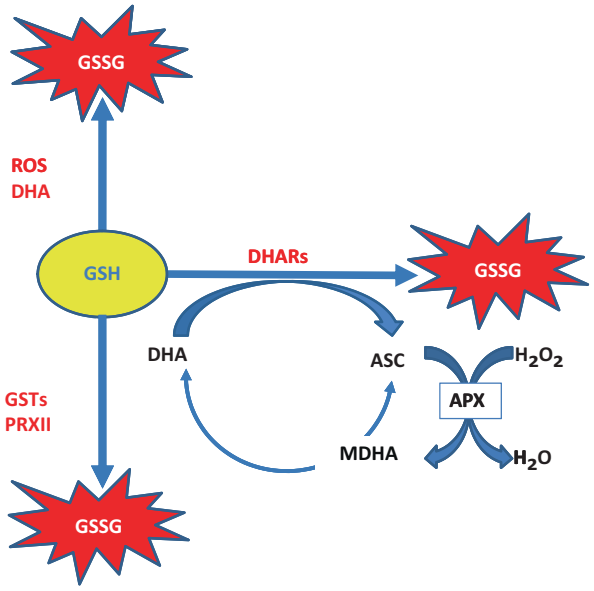

Fig. 1.1 Principal reactions involved in glutathione (GSH) oxidation. GSH can be chemically oxidized by reactive oxygen species (ROS) and dehydroascorbate (DHA). DHA reductases (DHARs) can also oxidizes GSH to regenerate ascorbate (ASC), allowing GSH to take part, indirectly, in H_2O_2 reduction. In this pathway, ASC peroxidase (APX) reduces H_2O_2 to water at the expense of ASC producing monodehydroascorbate (MDHA); this last is an unstable product that can dismutate to ASC and DHA. DHA can then be reduced by DHARs, producing glutathione disulfide (GSSG). Finally, GSH can be also oxidized by the peroxidase activity of glutathione *S*-transferases (GSTs) and type II peroxiredoxins (PRXII). More details are given in the text

and peroxidase activities. The GSTs of the lambda class, which have an active-site cysteine, could generate GSSG by catalyzing the reduction of small molecules or by the deglutathionylation of cysteine residues of proteins (Dixon et al. 2002; Dixon and Edwards 2010).

Several type II peroxiredoxins (PRXs), which are thiol peroxidases that can reduce both H_2O_2 and other organic peroxides, can oxidize GSH, through the action of glutaredoxins (Rouhier2010); on the other hand, GSH peroxidase (GPX), which belongs to the PRX family, contrary to what previously believed, acts as thioredoxin-dependent peroxidase and not as GSH-dependent peroxidase (Iqbal et al. 2006; Navrot et al. 2006).

Other enzymes could be responsible for GSSG formation. For instance, GSNO reductase can produce GSSG from GSH and GSNO (Sakamoto et al. 2002); adenosine phosphosulfate reductase, a key chloroplastic enzyme involved in sulfate reduction, employs GSH as electron donor (Bick et al. 1998); the activity of a plant methionine sulfoxide reductase may need GSH oxidation, which can occur via glutaredoxins (Tarrago et al. 2009).

3 Glutathione Biosynthesis

The biosynthetic pathway of GSH has been characterized in several organisms, and seems to act through a conserved chemical way (Rennenberg 1980; Meister 1988). In higher plants as in animals, GSH biosynthesis occurs in two ATP-dependent steps through the sequential action of γ-glutamylcysteine synthetase (γ-ECS) and GSH synthetase (GS). In the first reaction, γ-ECS, also known as glutamate-cysteine ligase, catalyzes the synthesis of the intermediate γ-glutamylcysteine (γ-EC) from glutamate and cysteine (May and Leaver 1994; Jez et al. 2004; Musgrave et al. 2013). The γ-carboxylate group of glutamate is initially phosphorylated by ATP and subsequently subjected to the nucleophile attack of the amino group of cysteine. The second reaction is catalyzed by GS that, similarly to the γ-ECS, first forms an acylphosphate intermediate at the C-terminal of γ-EC and then, displacing inorganic phosphate, links the amino groups of a glycine to produce GSH (Jez and Cahoon 2004; Herrera et al. 2007; Musgrave et al. 2013). Both enzymes are encoded by single-copy genes, called *GSH1* for γ-ECS (May and Leaver 1994) and *GSH2* for GS (Rawlins et al. 1995; Ullmann et al. 1996), which possess alternate transcription start sites, thus leading to either plastid-targeted or cytosolic proteins (Wachter et al. 2005). Immune-electron microscope analyses in *Arabidopsis* leaves have permitted to sub-localize the γ-ECS to the chloroplasts and the GS both into plastids and cytosol (Hell and Bergman 1988, 1990; Preuss et al. 2014). Activity assays of both enzymes, in cytosol and chloroplast fractions of *Arabidopsis* and wheat leaves, have revealed the 82% of γ-ECS total activity in the chloroplast and the 69% of GS total activity in the cytosol (Noctor et al. 2002a). Thereby, the subcellular localization of these two enzymes in plants makes GSH biosynthesis a compartmentalized process and supports the idea of a specific movement of biosynthetic intermediates between organelles and cytosol. Consistently, most of the neo-synthesized chloroplastic γ-EC moves to the cytosol as substrate of cytosolic GS (Pasternak et al. 2008).

Functional studies on plant lines carrying severe mutations at the *GSH1* and *GSH2* genes have allowed to establish the molecular basis for the comprehension of γ-ECS and GS roles in numerous species. Knockout mutations of these genes result in lethal phenotypes in different eukaryotes, indicating that GSH biosynthesis is essential for cell life (Grant et al. 1996; Kim et al. 2005). In particular, the knockout of the *Arabidopsis* GSH1 gene causes lethality at the embryo stage (Cairns et al. 2006), while *GSH2*-deficient lines show delay in the development, associated with death at the seedling stage (Pasternak et al. 2008). This difference appears to reflect the likely replacement of the missing GSH with the γ-EC intermediate, which accumulates to high levels in *gsh2* mutant plants. Additionally, these plants can partially restore the wild-type phenotype with an increase of the cytosolic γ-ECS activity (Pasternak et al. 2008), thus supporting the evidence of transport of GSH and its biosynthetic intermediates from cytosol to plastids (Noctor et al. 2002a). Less severe mutations, causing reduced GSH levels, have also been obtained by forward genetics approaches. In particular, some *gsh1* mutants are worth mentioning: the root-meristem-less1 (*rml1*) mutant, with only the 5% of wild-type GSH amount, is

Table 1.1 Effects on plant development and response to environmental stress of GSH deficiency in different glutathione biosynthetic mutants

GSH biosynthetic mutants	GSH content	Effects on plant development and response to environmental stress	References
Rml1 (root meristemless1)	3% of wt	Impairment in initiation and maintenance of cell division during post-embryonic root development.	Cheng et al. (1995) Vernoux et al. (2000)
Zir 1 (zinc tolerance induced by iron 1)	15% of wt	Seedlings smaller in size than the wild type. High sensitivity to Zn excess and no Fe-mediated Zn tolerance.	Shanmugam et al. (2012)
Pad2-1 (phytoalexin-deficient 2-1)	22% of wt	Enhanced susceptibility to various pathogens due to low accumulation of antimicrobial defenses and alteration in SA-dependent pathway. Wilting of leaves and downregulation of various stress-responsive genes under combined cold and osmotic stress.	Parisy et al. (2007) Dubreuil-Maurizi et al. (2011) Kumar et al. (2015)
Cad2-1 (cadmium sensitive 2-1)	15–45% of wt	Under Cd stress, deficiency of phytochelatins and consequent heavy metals sensitivity Moderate susceptibility to pathogens.	Howden et al. (1995) Cobbett et al. (1998) Parisy et al. (2007)
Rax1-1 (regulator of APX2 1-1)	20–50% of wt	Constitutive expression of stress-inducible APX2 Under photooxidative stress, altered expression of a wide set of defense-related genes	Ball et al. (2004)

unable to develop root apical meristems (Cheng et al. 1995; Vernoux et al. 2000); less drastic reductions, up to the 50% of total glutathione, do not reveal evident phenotypic aberrations but cause alterations in stress signal transduction and response, such as high sensitivity to cadmium in the *cad2* mutants or to pathogens in the *pad2* mutants (Howden et al. 1995; Cobbett et al. 1998; Parisy et al. 2007). The level of GSH content and the altered response in the development and in the response to environmental changes in different GSH biosynthetic mutants are summarized in Table 1.1.

3.1 Molecular Characteristics of γ-ECS and GS

Numerous studies performed in several organisms have permitted to better understand the structure, mechanism of action, and regulation of the enzymes involved in GSH biosynthesis.

On the basis of sequence analysis from multiple species, three main distinct families of γ-ECSs belonging to the plants and γ-protobacteria group, the α-protobacteria

group, and the non-plant eukaryotes group (mammals, yeasts, trypanosomes) have been identified (May and Leaver 1994). Despite sharing a putative distant ancestor, common structural motifs and very similar catalytic mechanism of action, insignificant sequence homology has been found between the three groups (Copley and Dhillon 2002). In particular, the cloning of γ-ECS from different plant species has highlighted the strong sequence diversity with the mammalian and microbial counterparts (Frendo et al. 1999; Wu et al. 2009). Plant γ-ECSs share more structural and functional similarity with yeast γ-ECSs than with the heterodimeric γ-ECSs found in several eukaryotes or with the monomeric γ-ECS of *Escherichia coli* (Seelig et al. 1984; Fraser et al. 2002; Hibi et al. 2004; Biterova and Barycki 2009). In plant γ-ECSs, two magnesium ions in the active site increase the reactivity of the γ-phosphate group of ATP and assist the right orientation of the glutamate γ-carboxylate, thus stabilizing the resulting γ-glutamyl-phosphate intermediate. The cysteine binding site, adjacent to the glutamate binding site, undergoes a significant conformational change upon ligand binding, thus becoming reactive for cysteine attack. A close arginine, in a highly conserved position, usually provides the transition state of this reaction and the final peptide bond formation (Biterova and Barycki 2009).

GSs are members of the ATP-grasp superfamily, consisting of ligases that form amide bonds in peptides after translation (Li et al. 2003; Dinescu et al. 2004). They are usually characterized by the ATP-grasp binding site, with two α-helices and β-sheets, and an active site with high specificity for the substrate. A phylogenetic analysis of members of the GS family from several species allowed to infer the genetic distance and thereby to collocate plant GSs close to yeast GSs, sharing the 40% sequence homology (Wang and Oliver 1997). In contrast to the bacterial GSs that are homo-tetramers, mammalian, plant, and yeast GSs have been identified as homodimers, composed of two identical subunits linked by disulfide bonds (Gushima et al. 1983; Yamaguchi et al. 1993).

The heterologous GS protein, obtained by the overexpression of a cDNA of *Arabidopsis thaliana* GS in bacteria and yeast cells, shares high sequence similarity to *GSH2* products from other species and is characterized by the presence of an extremely conserved glycine-rich domain, close to the carboxy-terminus, typical of the eukaryotic GS family (Rawlins et al. 1995; Ullmann et al. 1996; Wang and Oliver 1997; Galant et al. 2009). A deep structural characterization of the *Arabidopsis* GS revealed the presence, in the active site, of three specific regions, involved in the bonds of ATP, magnesium, and both γ-EC and GSH (Herrera et al. 2007). Experiments of site-directed mutagenesis permitted to identify the role of 15 specific amino acids in GS active site, showing the sensibility of plant GS even to minor amino acid changes in the active site and suggesting that the ATP and the γ-EC binding can enhance reciprocally (Herrera et al. 2007).

As other members of the ATP-grasp family, plant GSs are able to use different amino acids during the tripeptide formation (Skipsey et al. 2005). The replacement of glycine with β-alanine, serine, or glutamate has been observed in several crop species that, unlike animals, can synthesize alternative forms of GSH. Homoglutathione, the most known analog of GSH, characterized by the

presence of a β-alanine instead of glycine, is synthesized by homoglutathione synthetases (hGSs). From a structural point of view, plant hGSs are generally quite similar to human, yeast, and *Arabidopsis* GSs, also confirming the invariant amino acidic composition in the binding sites for ATP, Mg^{2+}, and γ-EC (Gogos and Shapiro 2002; Galant et al. 2009). The preference of β-alanine versus glycine is crucially determined by the active site of hGS. The active site of both enzymes is structurally composed by a lid domain, a glycine-rich loop, and an alanine-rich loop. The first two are critical for ATP binding and are responsible for the major conformational changes, while the alanine-rich loop can interact with glycine in the GS structures or with β-alanine in the hGS structure (Gogos and Shapiro 2002; Galant et al. 2009).

3.2 Regulation of Glutathione Biosynthesis

Cysteine availability is one of the most important factors affecting GSH biosynthesis. Indeed, a constitutive enhancement in GSH content can be achieved by the supply of exogenous cysteine or by the increase of enzymes involved in cysteine synthesis, which are, in turn, influenced by the availability of reduced sulfur (Buwalda et al. 1988, 1990; Harms et al. 2000; Noji and Saito 2002; Wirtz and Hell 2007). Treatments of leaves and roots of spinach and maize with excess of exogenous cysteine caused a significant GSH increase (Buwalda et al. 1988, 1990; Farago and Brunold 1994). Cysteine concentration presumably regulates GSH biosynthesis independently by the amount of γ-ECS, as shown in leaf discs of both wild-type and transformed poplar lines overexpressing the bacterial γ-ECS (Noctor et al. 1996). The cysteine control on the γ-ECS activity can prevent an excessive GSH production and regulate the cysteine metabolism itself (Buwalda et al. 1988; Rennenberg 1995). In addition to cysteine, glycine and ATP availability can also affect GSH production (Buwalda et al. 1990; Noctor et al. 1997; Ogawa et al. 2004).

Changes in *GSH1* and *GSH2* expression, which can influence γ-ECS and GS levels, represent also a possible way to regulate GSH synthesis. Indeed, heavy metals, jasmonic acid, and oxidative stress seem to activate the expression of both genes, which also respond to high light and other kind of stress (Xiang and Oliver 1998; Sung et al. 2009).

The production of plants overexpressing bacterial *GSH1* and *GSH2* genes has provided important insights into the regulation of GSH biosynthesis and metabolism. Leaves of transformed poplar lines overexpressing the cytosolic bacterial γ-ECS contain higher levels of cysteine, γ-EC, and total glutathione than those measured in leaves of untransformed plants (Noctor et al. 1996; Arisi et al. 1997). A more substantial increase in GSH has been obtained targeting the bacterial γ-ECS to the chloroplast (Noctor et al. 1998). The overexpression of the same bacterial γ-ECS in *Arabidopsis* and Indian mustard leads to a twofold increase of the GSH content, without evident phenotypes or general physiological perturbations (Xiang et al. 2001; Zhu et al. 1999b). A considerably greater increase in GSH content has been

obtained by the overexpression into tobacco plants of a bifunctional protein with both γ-ECS and GS activities, isolated from *Streptococcus*, suggesting that GS activity can be limiting when γ-EC is made available (Liedschulte et al. 2010; Noctor et al. 2012). Overexpression of *GSH 2* gene from *Escherichia coli* in the cytosol or in the chloroplast enhances GS activities but leaves unchanged GSH content (Strohm et al. 1995; Foyer et al. 1995; Noctor et al. 1998). On the other hand, the overexpression of the same gene in presence of cadmium, which increases γ-EC intermediate, stimulates GSH biosynthesis (Zhu et al. 1999a). The rate-limiting effect of GS for GSH biosynthesis during stress conditions has been also demonstrated in tobacco plants overexpressing a soybean GS, which confers tolerance to the Fomesafen herbicide (Skipsey et al. 2005).

However, among the two biosynthetic enzymes, γ-ECS is generally thought to play a key role in regulating GSH biosynthesis. The γ-ECS activity is limited by the availability of free cysteine and ATP and is tightly feedback modulated by the end product itself. As in mammals, a feedback inhibition of γ-ECSs by GSH is a useful way to modulate glutathione homeostasis in plants, especially under conditions in which this tripeptide is rapidly consumed (Hell and Bergmann 1990; Noctor et al. 2002a). Moreover, rapid transcriptional activation and post-translational modifications of γ-ECS ensure the strict control of intracellular GSH levels (Hell and Bergmann 1990; May et al. 1998; Noctor et al. 2002a; Jez et al. 2004). A fine post-translational activation of *Arabidopsis* γ-ECS occurs through changes in its redox state. Indeed, the reversible formation of a disulfide bond makes γ-ECS more active (Jez et al. 2004). In this way, under oxidizing conditions γ-ECS is activated in parallel with the increased demand for GSH. As the GSH level increases, the more reduced intracellular environment causes an inactivation of γ-ECS, thus providing an efficient and rapid switch mechanism for the control of GSH biosynthesis (Hicks et al. 2007). The active enzyme in the oxidized status works as a dimer with two intermolecular disulfide bonds located at specific cysteine sites (Cys178-Cys398 and Cys341-Cys356); in a reducing environment, these bonds are disrupt and the enzyme comes back to the less active monomeric form. The first of the two disulfide bonds seems to be essential for the dimer formation, since experiments of site-direct mutagenesis of these cysteine block both the *Arabidopsis* and *Brassica juncea* γ-ECSs in the monomeric form (Hothorn et al. 2006; Hicks et al. 2007). The γ-ECS monomer/dimer transition by disulfide linkages is very common in the plant kingdom and seems to be related to the sub-compartmentalization of GSH biosynthesis in the chloroplast (Gromes et al. 2008).

4 Importance of Glutathione in the Redox Regulation

GSH, being involved directly or indirectly in the removal of ROS, like other numerous metabolites, can work as an antioxidant. However, it is interesting to note that the antioxidant and signaling functions of GSH are interdependent, since both require enzymes such as GSTs and PRXs that reduce H_2O_2 or other organic

peroxides through thiol-mediated pathways (Noctor et al. 2012). The uniqueness of GSH as antioxidant and signaling molecule is also due to its high abundance and low redox potential, as well as to an ubiquitous distribution in plant cells.

4.1 Glutathione Redox State

A key feature of the cellular glutathione pool is its high reduction state. The glutathione pool is maintained predominantly in a reduced state by glutathione reductases (GRs), whose activities depend on the key electron carrier, NAD(P)H. GRs are flavoproteins with high affinity for both GSSG and NAD(P)H (Halliwell and Foyer 1978; Edwards et al. 1990). GR activities have been found in chloroplasts, cytosol, mitochondria, and peroxisomes (Foyer and Halliwell 1976; Edwards et al. 1990; Rasmusson and Møller 1990; Jiménez et al. 1997; Stevens et al. 2000; Romero-Puertas et al. 2006). In *Arabidopsis*, two genes encoding dual-targeted GRs have been identified. *GR1* encodes for the cytosolic and peroxisomal GRs and is responsible for the 30–60% of the total leaf enzymatic activity (Marty et al. 2009; Kataya and Reumann 2010; Mhamdi et al. 2010). *GR2* encodes an enzyme that is targeted to plastids and mitochondria (Chew et al. 2003). Loss of function of *GR1* determines only modest GSSG accumulation in leaf tissue, probably due to the supporting GSSG-reducing activity of cytosolic NAD(P)H-thioredoxin (TRX) systems (Marty et al. 2009). On the other hand, mutants for *GR2* are embryo-lethal (Tzafrir et al. 2004).

Under optimal conditions, total tissue glutathione pool is mostly reduced; GSH/GSSG ratios in leaves are usually no less than 20:1 (Noctor et al. 2012). However, this is an average value, and these ratios might be higher or lower depending on the specific considered compartments (Meyer et al. 2007; Queval et al. 2011). Indeed, some compartments, such as the endoplasmic reticulum and vacuoles as well as some cell types, like cells of the quiescent center, or dormant tissues, like seeds, are maintained in a more oxidized state (Hwang et al. 1992; Enyedi et al. 2010; Queval et al. 2011; Kranner and Grill 1996; Kranner et al. 2006).

In absence of stress, the glutathione redox potential, related to $[GSH]^2/[GSSG]$, determined by in vivo studies with the redox-sensitive GFPs (roGFPs), is lower than −300 mV in the cytosol, and similar values have been reported in nuclei (Meyer et al. 2007; Jubany-Mari et al. 2010; Schnaubelt et al. 2015). This low glutathione redox potential suggests that GSSG concentrations should be in the nanomolar range, in contrast with the analyses conducted on the entire tissues, in which, usually, GSSG concentrations are reported in the micromolar range (Noctor et al. 2012 and references therein). It could be possible that the low redox potential reported in the cytosol is due to the sequestration of GSSG in other compartments, such as the endoplasmic reticulum, vacuole, or apoplast, where glutathione reduction capacity is relatively low (Noctor et al. 2012). Under optimal conditions, the preservation of a very low glutathione redox potential in the cytosol could permit the initiation of oxidative signaling, with a quite low accumulation of GSSG, which can be perceived

by sensitive thiol proteins, as significant changes in redox potential (Noctor et al. 2012, 2013). It has been suggested that changes in glutathione redox potential of about 50 mV could be sufficient to alter the balance between oxidized and reduced forms in thiol-disulfide status of sensitive TRX-regulated proteins (Setterdahl et al. 2003; Noctor et al. 2012). The glutathione redox potential is a primary component controlling relations between oxidative signals and sensitive protein targets, and it can be affected not only by changes in GSH/GSSG ratio but also by absolute GSH concentration (Meyer et al. 2007). For instance, the cytosolic redox potential, measured by roGFP, is more oxidizing in *GR*-deficient (*gr1*) mutants in which the GSH/GSSG ratio but not the glutathione concentration is decreased, as well as in GSH-deficient *cad2* mutant (Meyer et al. 2007; Marty et al. 2009). An increase in the redox potentials of both cytosol and nuclei has been also shown in wild-type plants in which a buthionine sulphoximine (BSO)-dependent GSH depletion has been obtained (Schnaubelt et al. 2015). However, it is interesting to note that changes in redox potential caused by altered GSH/GSSG ratio or by GSH depletion affect different signaling pathways. For instance, in the *Arabidopsis* catalase-deficient (*cat2*) mutant, in which the GSH/GSSG ratio is altered, GSH has a significant role in the induction of the oxidant-dependent salicylic acid and jasmonic acid signaling pathways (Han et al. 2013a, b). In addition, the *cat2* mutants grown under high light show decreased expression of auxin synthesis genes (Gao et al. 2014). On the other hand, in plants with low GSH levels, such as the *rml1-1* mutant, the profile of stress-responsive salicylic acid and jasmonic acid-dependent genes is not altered, while changes in transcript linked to altered hormone responses occur (Schnaubelt et al. 2015).

Deviation from the high reduced state of glutathione can take place in different conditions where oxidant production occurs. Various biotic or abiotic stresses, affecting the rate of ROS production and/or ROS removal, can change glutathione redox state (Gupta et al. 1991; Vanacker et al. 2000; Gomez et al. 2004). Many studies carried out with plants in which enzymes involved in the H_2O_2 removal had been inhibited show a strict link between high accumulation of H_2O_2 and changes in glutathione redox state (May and Leaver 1993; Willekens et al. 1997; Noctor et al. 2002b; Rizhsky et al. 2002; Queval et al. 2007, 2009; Chaouch et al. 2010). For instance, in *Arabidopsis cat2* mutants the transfer from elevated CO_2 conditions to air causes oxidation of the leaf glutathione pool within hours, which is accompanied in the subsequent days by total glutathione accumulation (Queval et al. 2009). In the same mutants under photorespiratory conditions, a moderate rate of endogenous H_2O_2 production causes a strong decrease in the GSH/GSSG ratio of the whole leaf (Queval et al. 2012).

Under oxidative conditions, GSSG accumulation may be explained as the net result of oxidation processes which overcomes, even if only slightly, the capability of glutathione reduction (Noctor et al. 2013; Fig. 1.2). GR1 activity is necessary under oxidative stress, since it has been shown that in *cat2 gr1* double mutants, deficient in both the major leaf catalase and GR1, accumulation of GSSG is massively increased compared with the parent lines (Mhamdi et al. 2010). However,

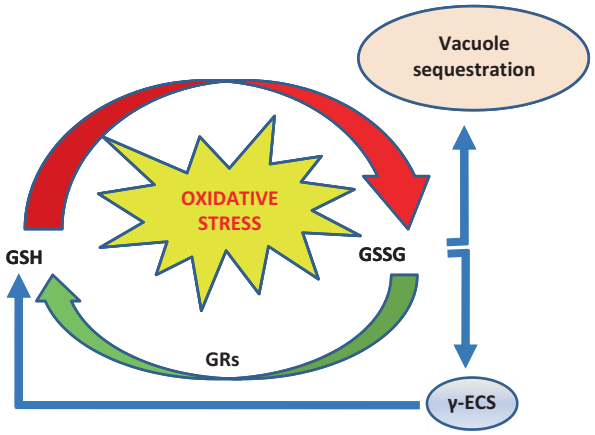

Fig. 1.2 Changes in glutathione content and redox state under oxidative stress. Under stress conditions, the oxidative processes overcome the capability of glutathione reduction, due to glutathione reductases (GR). The increase in glutathione disulfide (GSSG) content stimulates γ-glutamylcysteine synthetase (γ-ECS), leading to an increase of total glutathione pool. On the other hand, the excessive accumulation of GSSG in sensitive subcellular compartments, such as cytosol and nuclei, is avoided by its compartmentalization in vacuoles. More details are given in the text

since GR has a Michaelis constant (Km) for GSSG of 10–50 μM (Smith et al. 1989; Edwards et al. 1990), it is possible that a kinetic limitation of this enzyme occurs (Noctor et al. 2012).

Often under stress conditions, GSSG accumulation in plants is not accompanied by a decrease in GSH, whose levels remain quite constant, rather by an increase in total glutathione pool that seems to be principally due to GSSG accumulation (Smith et al. 1984; Willekens et al. 1997; Mhamdi et al. 2010). This behavior can be explained by new synthesis of GSH and compartmentalization of GSSG (Noctor et al. 2012, 2013; Fig. 1.2).

An increase in GSH neosynthesis can occur during oxidative stress, as a result of activation of cysteine and GSH production at transcriptional and posttranslational levels (Hicks et al. 2007; Gromes et al. 2008; Queval et al. 2009). In addition, a considerable quantity of the GSSG generated by oxidative stress can be accumulated in the vacuole (Queval et al. 2011) by ABCC transporters (Martinoia et al. 1993; Lu et al. 1998). New GSH biosynthesis is necessary for GSSG accumulation and successive sequestration; indeed, introducing a *cad2* mutation in the *cat2* background, GSSG accumulation is inhibited (Han et al. 2013a; Noctor et al. 2013).

The decrease in GSH/GSSG ratio, measured in whole cell extracts, although a merged value of GSH/GSSG ratios in different cell compartments, represents a valuable marker for oxidative stress, because GSSG accumulation in specific compartments, such as the vacuole, is dependent on its increase in other compartments (Noctor et al. 2013).

4.2 Glutathione Abundance and Distribution in Plant Cells

GSH is the most abundant low-molecular-weight thiol in plant tissues, and it generally accumulates to millimolar concentrations (Queval et al. 2011; Koffler et al. 2013). In the *Arabidopsis* gametophyte, cytosol, plastids, nuclei, and mitochondria contain similar amounts of GSH (Zechmann and Russell 2011). On the other hand, in the sporophyte GSH content can vary significantly among different cell compartments. In roots and leaves of different plant species, GSH has been localized with the highest contents in mitochondria, followed by nuclei, peroxisomes, cytosol, and plastids (Zechmann and Müller 2010). In the center of *Arabidopsis* old leaves the calculated GSH concentrations, by quantitative immunoelectron microscopy, vary from 14.8 mM in mitochondria, 6.4 mM in nuclei, 4.5 mM in the cytosol, to 4.4 mM in peroxisomes (Koffler et al. 2013; Zechmann 2014). Concentrations of about 1 mM have been observed in chloroplasts of *Arabidopsis* leaves (Queval et al. 2011; Koffler et al. 2013). On the other hand, very low levels of GSH (0.03–0.08 mM) have been found in vacuoles and, with this technique, GSH is undetectable in the apoplast (Queval et al. 2011; Koffler et al. 2013). Although mitochondria have the highest GSH concentrations, in mesophyll cells of *Arabidopsis* leaves, cytosol and chloroplasts, having the greater volumes, contain 50% and 30%, respectively, of total GSH (Queval et al. 2011).

In the next paragraphs, the role and the importance of GSH and its redox state in different plant cell compartments will be discussed.

4.2.1 Cytosolic Glutathione

The cytosolic glutathione redox potential, as previously said, is negative and quite stable. The control of the redox state in this compartment is favored by the continuous reduction of GSSG by GR and/or by the sequestration of GSSG in the vacuole. Thus, the sequestration keeps GSSG very low in this sensitive compartment and guarantees suitable but not extreme accumulation during oxidative stress (Hartmann et al. 2003). Consistently, different stresses are able to render the cytosolic environment more oxidized (Meyer et al. 2007; Jubany-Mari et al. 2010). The cytosol, although not directly involved in ROS production, plays a key role in the integration of redox signals (Foyer and Noctor 2016; Paciolla et al. 2016). The importance of cytosolic GSH in the signaling events occurring in abiotic and biotic stress response has been confirmed by the use of plants with mutation in the genes coding for proteins required for the transport of γ-EC and GSH across the plastid envelope membranes. These mutants (*clt1clt2clt3*) have an altered partitioning of GSH between plastid and cytosol, with a clear decrease in the cytosolic GSH content. The *clt1clt2clt3* mutants show enhanced sensitivity to cadmium and to the fungal pathogen *Phytophthora brassicae*, and are not able to activate a correct pathogen defense, defecting in the salicylic acid-dependent expression of pathogenesis-resistance protein (Maughan et al. 2010).

4.2.2 Glutathione in Chloroplasts and Peroxisomes

GSH in chloroplast has an important function in the organelle's protection from possible oxidative damages caused by ROS (Pietrini et al. 2003). Indeed, during stress conditions that induce stomata closure, such as excess light, high salinity, and drought, which induce high ROS production in chloroplasts (Asada 2006; Golan et al. 2006; Pospisil 2012), GSH accumulates not only in the stroma but also in the thylakoid lumen (Heyneke et al. 2013; Zechmann 2014). During oxidative stress occurring in *cat2* mutants, in which the initial increase in H_2O_2 production is extrachloroplastic, a strong accumulation of GSSG occurs in the chloroplast (Queval et al. 2011). This GSSG accumulation could be dependent on an import from the cytosol or, more probably, on GSH oxidation within the chloroplast (Noctor et al. 2013). However, independently by the mechanism involved, GSSG accumulation in the chloroplasts may influence not only the thiol-dependent reactions in this compartment but also the synthesis pathways contributing to the regulation of total glutathione content (Noctor et al. 2013). On the other hand, insufficient content of GSH in chloroplasts, permitting ROS accumulation, leads to cell death (Doyle et al. 2010). In plants subjected to biotic stress, compartment-specific changes in GSH content can occur. For instance, at the beginning of *Botrytis cinerea* and *Pseudomonas syringae* infections in *Arabidopsis* plants, GSH accumulates in chloroplasts, whereas, at later stages, depletion of GSH in chloroplasts leads to ROS accumulation and progression of disease symptoms (Großkinsky et al. 2012; Simon et al. 2013). The GSH decline in the chloroplast, despite the active GSH synthesis, may be due to its transport in other cellular compartments (Noctor et al. 2002a; Noctor et al. 2013).

Peroxisomal GSH behaves in a similar way to the chloroplastic one. Indeed, during the first phase of pathogen infection, peroxisomes function as GSH accumulators, whereas the GSH decrease in the successive stages contributes to the induction of necrotic lesions (Großkinsky et al. 2012; Simon et al. 2013). In tomato plants infected with *B. cinerea*, the decline in GSH content in peroxisomes has been linked to the pathogen-induced senescence of leaves (Kuźniak and Sklodowska 2001). On the other hand, stress conditions that favor photorespiration and H_2O_2 production in peroxisomes lead to GSH accumulation in this cell compartment (Miller et al. 2010; Hernández et al. 2013).

4.2.3 Mitochondrial Glutathione

Mitochondrial glutathione is strongly reduced (Schwarzlander and Finkemeier 2013) and, as the chloroplastic one, is involved in the direct and indirect removal of ROS, protecting membranes, proteins, as well as DNA (Foyer et al. 2004; Green et al. 2006; Rhoads et al. 2006). However, mitochondrial GSH seems to be necessary for correct plant development. Indeed, the biosynthetic mutants of *Arabidopsis pad2-1*, which show the same level of mitochondrial GSH of the wild-type plants and up to 91% of GSH decrease in other cell compartments (Zechmann et al. 2008),

have a normal phenotype when grown in non-stressed conditions (Parisy et al. 2007). On the other hand, the *rml1* mutant, with a 96–98% drop in the GSH levels in all the compartments and with the highest GSH depletion in mitochondria (Zechmann and Müller 2010), forms extremely short roots and small shoots and leaves (Cheng et al. 1995; Vernoux et al. 2000). The importance of mitochondrial GSH for survival has been demonstrated using the *pad2-1* mutants, which under short-term excess light show a huge increase only in mitochondrial GSH (Heyneke et al. 2013). The depletion of mitochondrial GSH, occurring in *Nicotiana tabacum* plants infected with tobacco mosaic virus (TMV) and in *Arabidopsis* plants infected with *B. cinerea*, is accompanied with the development of necrotic lesions (Király et al. 2012; Simon et al. 2013). A drop in total GSH contents and accumulation of GSSG in mitochondria has been also observed in the pathogen-induced senescence of tomato plants (Kuźniak and Sklodowska 2001). Thus, the depletion of mitochondrial GSH seems to promote ROS accumulation and to be responsible for the initiation of programmed cell death (Zechmann 2014).

4.2.4 Nuclear Glutathione

Nuclei of non-stressed leaves, after mitochondria, show the highest concentration of GSH (Koffler et al. 2013), which co-localizes with DNA (Diaz-Vivancos et al. 2010a). Changes in nuclear redox balance of nuclei may cause DNA damages, which could induce mutations and eventually cell death (Diaz-Vivancos et al. 2010b).

When GSH synthesis is impaired, as occurs in the *rml1-1* mutants or in wild-type seedlings treated with BSO, GSH depletion, in the cytosol as well as in nuclei, arrests cell cycle in the roots; the decrease in GSH modulates the expression of genes involved in cell cycle control (Schnaubelt et al. 2015). Accordingly, it has been proposed that high levels of GSH in nuclei during G1 phase represent an essential strategy to permit cell cycle progression (Diaz-Vivancos et al. 2010a, b). In particular, in proliferating cells in the G1 phase, GSH is recruited and sequestered in the nucleus, leading to a strong depletion of the cytoplasmic GSH pool, and a concomitant decrease in transcripts linked to oxidative signaling and stress tolerance (Markovic et al. 2007;Diaz-Vivancos et al. 2010a). The subsequent changes in cytosolic redox state trigger GSH synthesis, with a subsequent increase in the total glutathione pool, which occurs before the disappearance of nuclear envelope. Successively, the cytoplasmic and nuclear GSH pools become in equilibrium (Markovic et al. 2007; Pellny et al. 2009; Diaz-Vivancos et al. 2010a, b). The retention of GSH within the nucleus causes an arrest of cell cycle at the S/G2 phases (Locato et al. 2015).

High levels of GSH in nuclei play important roles in the protection of sensitive nuclear components, such as DNA and proteins, but are also involved in the regulation of the expression of genes involved in the activation of plant defense (Han et al. 2013a, b; García-Giménez et al. 2013). Consistently, an increase in nuclear GSH content is a common event during pathogen attack (Király et al. 2012; Großkinsky et al. 2012; Simon et al. 2013). It is possible that nuclear GSH accumulation, after

pathogen infection, can function as a signal to increase total GSH contents (Zechmann 2014). Accordingly, in TMV-infected tobacco plants, as well as in *Arabidopsis* plants infected with *P. syringae* and *B. cinerea*, the increase in nuclear GSH is followed by a strong accumulation of GSH in chloroplasts and cytosol (Király et al. 2012; Großkinsky et al. 2012; Simon et al. 2013).

4.2.5 Glutathione in Other Cell Compartments

Vacuoles, under non-stress conditions, have very low concentrations of GSH (Queval et al. 2011; Koffler et al. 2013), probably for the presence in this compartment of carboxypeptidases involved in its degradation (Steinkamp and Rennenberg 1985; Wolf et al. 1996). However, as discussed above, under oxidative stress, GSSG sequestration in this compartment can function as a protective mechanism involved in the control of cytosolic redox potential (Queval et al. 2011; Noctor et al. 2013). Moreover, vacuoles also function as a sink for GSH conjugates. For instance, cadmium can form complexes with GSH, which are then transported into vacuoles (Van Belleghem et al. 2007). GS-conjugates, successively, could be degraded in this compartment by the action of carboxypeptidase and γ-glutamyl transpeptidase (Steinkamp and Rennenberg 1985; Wolf et al. 1996; Grzam et al. 2007).

In the apoplast in basal conditions, GSH content is very low and sometimes under the level of detection (Vanacker et al. 1998, 2000; Zechmann et al. 2008; Tolin et al. 2013). Also in this case, these low GSH levels can be explained by the presence of the γ-glutamyl transpeptidases, GGT1 and GGT2, located in the cell wall and in the plasma membrane, which degrade GSH (Martin and Slovin 2000; Storozhenko et al. 2002;Ferretti et al. 2009). Consistently, in *Arabidopsis ggt1* mutants the level of apoplastic GSH is similar to the chloroplastic one (Tolin et al. 2013). It has been proposed that GSH content and redox state in the apoplast are involved in sensing and signaling environmental stress (Tolin et al. 2013). For instance, fungal infections in barley plants cause GSH accumulation in apoplast. Moreover, the apoplastic glutathione pool becomes more oxidized during the hypersensitive response (Vanacker et al. 1998, 2000).

Endoplasmic reticulum contains glutathione essentially as GSSG, which is needed in order to create an appropriate environment for disulfide bridges formation and proper folding of proteins (Hwang et al. 1992; Enyedi et al. 2010). In *Arabidopsis gsh2* mutants the accumulation of γ-EC and the low GSH levels have a negative impact on protein folding occurring in this compartment (Au et al. 2012).

5 Conclusions

Over the last decades, many experimental evidences have shown that GSH has a key role as antioxidant and that it is an irreplaceable player in the control of cellular redox state. The maintenance of a high GSH/GSSG ratio is crucial for many physiological functions, and a decrease in this ratio can be utilized as an indicator of

oxidative stress. Different reactions could contribute to GSH oxidation during oxidative stress, modifying its redox state (Fig. 1.1). GSH oxidation can occur by chemical reactivity of the thiol group with ROS and DHA or can be catalyzed by specific enzymes. The oxidation of GSH by DHARs makes a link between ASC and GSH pools and allows GSH to take part, indirectly, in H_2O_2 reduction. On the other hand, GSH oxidation by some GSTs and type II PRXs renders the antioxidant and signaling functions of GSH interdependent. Indeed, GSH, by participating in thiol-disulfide exchange, is also involved in the control of ROS-dependent signaling. Consistently, the GSH/GSSG ratio functions as an important regulator of several mechanisms involved in plant development and in plant response to environmental changes (Rahantaniaina et al. 2013; Foyer and Noctor 2016). Even under oxidative stress, the avoidance of an excessive oxidation in various sensitive cell compartments, like cytosol and nuclei, is given by the mutual aid of different mechanisms, among which are de novo GSH synthesis, GSSG reduction, and GSSG sequestration in opportune cell compartments, such as the vacuole (Fig. 1.2). Thus, not only the concentration and the redox state but also the subcellular distribution of GSH are central factors controlling redox homeostasis and signaling, which act as key actors in influencing the outcome of plant responses to environmental changes.

Recently, it has been shown that the depletion of the cytosolic GSH in the *Arabidopsis clt1clt2clt3* triple mutants, which negatively affects biotic stress tolerance (Maughan et al. 2010), has no effect on the decrease in leaf area induced by abiotic stress (Schnaubelt et al. 2013). Given the different responses of plants to the changes in GSH concentration and redox state in different cell compartments, in the near future it will be crucial to understand the specificity of these changes in response to distinct environmental stresses and at different stages of plant development.

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