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Glutathione

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Glutathione is a simple sulfur compound composed of three amino acids and the major non-protein thiol in many organisms, including plants. The functions of glutathione are manifold but notably include redox-homeostatic buffering. Glutathione status is modulated by oxidants as well as by nutritional and other factors, and can influence protein structure and activity through changes in thiol-disulfide balance. For these reasons, glutathione is a transducer that integrates environmental information into the cellular network. While the mechanistic details of this function remain to be fully elucidated, accumulating evidence points to important roles for glutathione and glutathione-dependent proteins in phytohormone signaling and in defense against biotic stress. Work in Arabidopsis is beginning to identify the processes that govern glutathione status and that link it to signaling pathways. As well as providing an overview of the components that regulate glutathione homeostasis (synthesis, degradation, transport, and redox turnover), the present discussion considers the roles of this metabolite in physiological processes such as light signaling, cell death, and defense against microbial pathogen and herbivores.

1. HISTORICAL OVERVIEW

In 1888 de Rey-Pailhade discovered that yeast cells contain a substance that he named "philothion", which reacts spontaneously with elemental sulfur to give hydrogen sulfide (reviewed by Meister, 1988a). His subsequent studies etablished the widespread presence of philothion in animal and plant tissues, and led to the conclusion that the compound contained cysteine that can undergo reversible oxidation to a disulfide form in the presence of oxygen (de Rey-Pailhade, 1928). A compound (subsequently accepted to be philothion) was extracted from muscle tissue in 1921 by Hopkins, who reported that it was autooxidizable and contained glutamic acid as well as cysteine. In the absence of a definitive composition, he suggested that "provisionally, for easy reference, the name glutathione will perhaps be admissible" (Hopkins, 1921). Hopkins' view that glutathione was a dipeptide of glutamic acid and cysteine was challenged by a report that the compound was possibly a tripeptide (Hunter and Eagles, 1927). Two years later, the development of a new procedure for preparing the compound in crystalline form confirmed that glutathione was indeed a tripeptide of glutamic acid, cysteine, and glycine (Hopkins, 1929; Figure 1). In the same year, Hopkins was awarded the Nobel Prize for this and other work on vitamins and related nutritional factors.

GSH is used here to indicate the thiol (reduced) form of glutathione while GSSG denotes the disulfide form. The term 'glutathione' is used where no distinction is drawn or both forms may be concerned.

The path to the characterization of glutathione functions in plants was opened up by another Nobel Prize laureate working on vitamins. Szent-Gyorgyi (1931) observed that cabbage leaf tissue was able to reduce the oxidized form of ascorbic acid, a process that involved the simultaneous oxidation of glutathione. Indeed, the early recognition that ascorbic acid is nearly always found in the reduced state in plants, despite the presence of terminal oxidases and oxidizing catalysts, led to the conclusion that a reducing mechanism must be present. In 1936, Hopkins and Morgan concluded that the reducing agent was glutathione (Hopkins and Morgan, 1936).

The growing recognition of the biological importance of reactive oxygen species (ROS) in the 1960's, including the discovery of superoxide, promoted renewed interest in glutathione in plants. This included placing ascorbate-glutathione interactions within a physiological context. Thus, the ascorbate-glutathione pathway was assigned a key function in the metabolism of hydrogen peroxide in the chloroplasts (Foyer and Halliwell, 1976) while glutathione became implicated in stress resistance in general (Esterbauer and Grill, 1978; reviewed by Tausz et al., 2004). At the same time, the roles of chloroplast thiols in enzyme regulation were being characterized (Wolosiuk and Buchanan, 1977). Growing attention was subsequently paid to the details of glutathione synthesis, degradation, transport and compartmentation (Rennenberg, 1982; Alscher, 1989).

With the advent of cloning and transgenic technology, the recognition of the antioxidant role of glutathione saw the production of plants with enhanced glutathione contents or reduction capac-

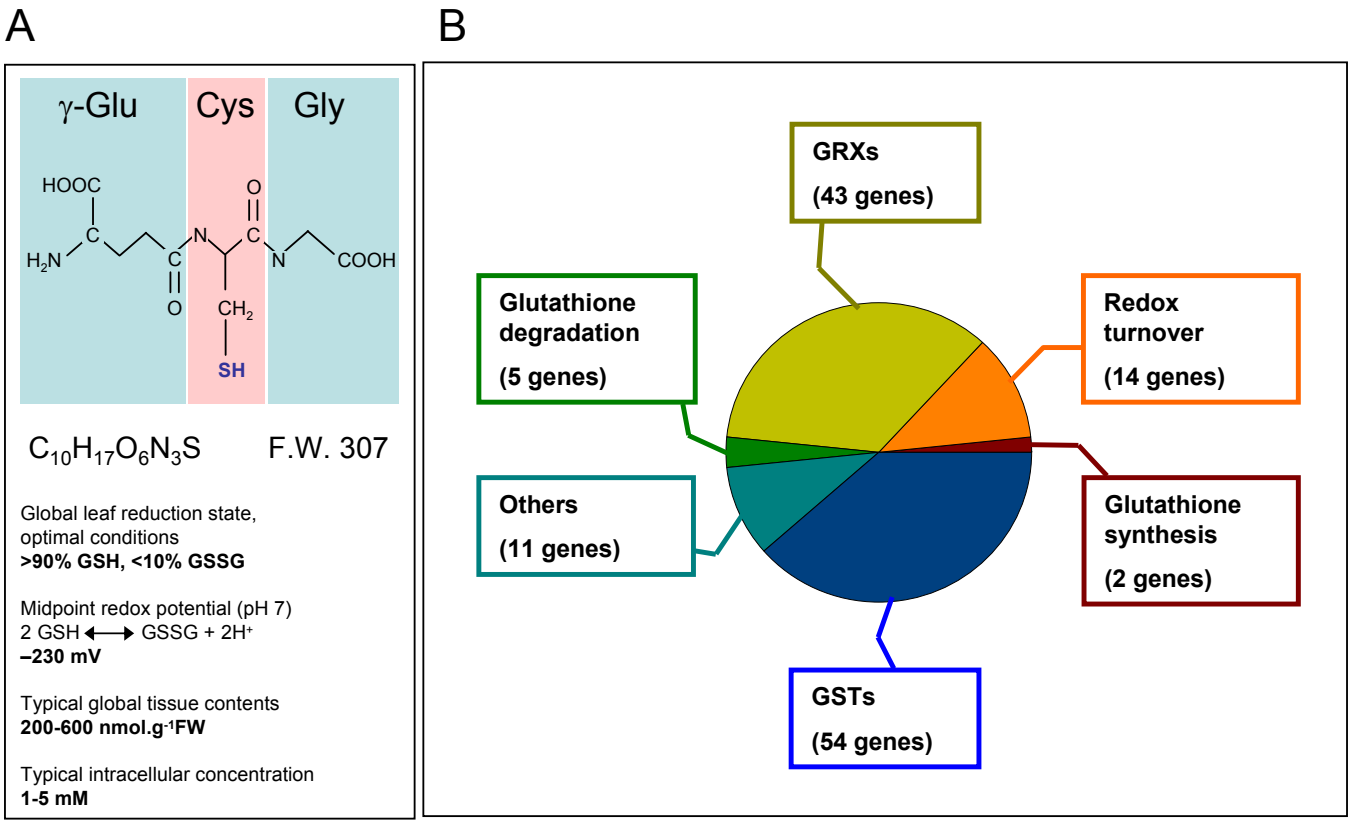

Figure 1. Glutathione: the basics.

(A) Glutathione identity card. **(B)** Simple overview of the key groups of glutathione-associated genes. The schematic composition of gene types shown in part **(B)** is based on the latest annotation on the TAIR website (www.arabidopsis.org). Note that the distribution is not intended to be exhaustive (for instance, potential or identified transporters are not included and other glutathione-dependent proteins no doubt await discovery) while annotated glutathione peroxidases are included in redox turnover, but may preferentially use thioredoxins as reductants. For a list of useful gene identities and associated information, refer to Supplementary Data 1. Information on GSTs can be found in Table 2 of Dixon and Edwards (2010).

ity (Foyer et al., 1991, 1995; Aono et al., 1993; Broadbent et al., 1995; Strohm et al., 1995; Noctor et al., 1996, 1998a,b; Creissen et al., 1999) as well as the identification of genes encoding the enzymes of glutathione synthesis and reduction in plants (May and Leaver, 1994; Creissen et al., 1995; Rawlins et al., 1995; Ullman et al., 1996). The notion that glutathione status could be involved in transmitting oxidative stress signals began to develop (Foyer et al., 1997; May et al., 1998a). Since then, analysis of Arabidopsis mutants has provided convincing demonstrations that glutathione is required for plant development (Vernoux et al., 2000; Cairns et al., 2006). Today, there is unequivocal evidence that glutathione is a multifunctional metabolite in plants that is important in redox homeostasis and signaling, as well as in defense reactions. Some of the most important of these insights have been made possible by the availability of Arabidopsis mutants and the ever-growing body of information generated by genomic and post-genomic approaches. Together, this information places this small thiol compound at the heart of the regulation of plant development and responses to the environment.

2. BIOCHEMICAL AND REDOX PROPERTIES OF GLUTATHIONE

2.1. Structure and Biological Distribution

The thiol tripeptide, glutathione (GSH; γ-L-glutamyl-L-cysteinylglycine), is found in most organisms. Linkage of the γ-carboxyl group of glutamate to the amino group of cysteine distinguishes this bond from peptide bonds in proteins. This is thought to confer stability on the molecule because it allows degradation through specific amino acid transferases (see section 5). In some organisms, such as halobacteria, glutathione can be replaced by other sulfur compounds like thiosulfate (Newton and Javor, 1985). Another example is trypanothione (N^1,N^8-bis(glutathionyl) spermidine), a compound that performs similar redox functions to glutathione in some parasitic protozoa (Fairlamb et al., 1985). In plants, glutathione typically accumulates in cells to millimolar concentrations, with tissue contents exceeding free cysteine by 10- to 50-fold.

In some plant taxa, homologous glutathione forms are found, as well as GSH. Instead of glycine, these compounds contain other C-terminal amino acids such as serine, β-alanine, or glutamate (Rennenberg, 1982; Klapheck, 1988; Klapheck et al., 1992; Meuwly et al., 1993). In legumes, in which homoglutathione (γ-Glu-Cys-β-Ala) can be found alongside GSH, the two homologs are synthesized by distinct enzymes encoded by different genes (MacNicol, 1987; Frendo et al., 2001). The path to hydroxymethylGSH (γ-Glu-Cys-Ser), which is found in cereals such as wheat (Klapheck et al., 1992), is less clear. A purified wheat glutathione synthetase (GSH-S) showed no activity against L-serine or D-serine though low activity was reported against certain other L- and D-amino acids as well as glycine (Skipsey et al., 2005a). HydroxymethylGSH could result from modifications of GSH catalyzed by enzymes with transpeptidase activity such as carboxypeptidase Y (Okumura et al., 2003). The disulfide forms of homoglutathione and γ-Glu-Cys-Ser are reducible by glutathione reductase (GR; Klapheck, 1988; Klapheck et al., 1992; Oven et al., 2001). To our knowledge, no GSH homologs have been reported in significant amounts in Arabidopsis, though analysis of purified GSH-S suggest that novel homologs may await discovery in some species (Skipsey et al., 2005a). In poplar overexpressing bacterial GSH-S, two novel peaks appeared on HPLC traces and these accumulated most strongly in plants kept in the dark, when glycine contents are low (G. Noctor, A.C.M. Arisi, L. Jouanin, C.H. Foyer, unpublished data). The chemical identity of these peaks remains unknown.

2.2. Redox Reactions

Many GSH functions are linked to reversible redox reactions of the cysteine sulfur group. Like other thiols, glutathione can undergo numerous redox reactions. Oxidized forms notably include disulfides, either with another glutathione cysteine residue to produce glutathione disulfide (GSSG) or with a different thiol to form 'mixed disulfides', as well as more oxidized forms in which the thiol group is converted to sulfenic, sulfinic or sulfonic acids (Foyer and Noctor, 2005). However, glutathione biochemistry is not restricted to these compounds. The potential for formation of an array of glutathione conjugates with endogenous and xenobiotic electrophilic species is vast (Wang and Ballatori, 1998; Dixon and Edwards, 2010). In particular, GSNO is receiving increasing attention for its physiological significance as a potential signaling molecule or as a reservoir of NO (Lindermayr et al., 2005).

Compounds able to oxidize GSH at high rates include ROS such as superoxide or the hydroxyl radical. Due to its relatively high cellular concentration, therefore, glutathione serves as a scavenger or sacrificial nucleophile. Thus, glutathione is a highly reducing chemical barrier that prevents excessive oxidation of sensitive cellular components. Many primary and secondary metabolites can act similarly as sacrificial antioxidants. However, unlike glutathione, the oxidized forms of these compounds are either not stable and/or not recycled to the reduced form at high rates. By contrast, glutathione is maintained in a predominantly reduced state by specific GRs found in the cytosol, plastids, mitochondria, and peroxisomes that have high affinity for GSSG and, particularly, NADPH (Halliwell and Foyer, 1978; Smith et al., 1989; Edwards et al., 1990; Jiménez et al., 1997; Chew et al., 2003; Kataya and Reumann, 2010). Further, glutathione oxidation occurs not only chemically but is also catalyzed by enzymes able to use GSH to reduce H_2O_2 or other peroxides to water or the corresponding alcohol (see section 7.1). Finally, the enzyme dehydroascorbate reductase (DHAR) links glutathione oxidation to ascorbate regeneration, though this reaction can also occur chemically at significant but lower rates (Foyer and Mullineaux, 1998). GSH-dependent reduction of DHA allows NADPH oxidation to be coupled to ROS removal via ascorbate and glutathione pools (Foyer and Halliwell, 1976). While plant antioxidative systems have been shown to be complex (Mittler et al., 2004) and the functions of many components remain to be defined, current information confirms that ascorbate and glutathione act together, alongside catalases, in high capacity redox-homeostatic H_2O_2-processing pathways.

2.3. How Glutathione is Measured in Plants

A range of useful methods is now available for the determination of glutathione and associated redox reactions in cells. Sensitive quantitative techniques for measuring glutathione in vitro are available by both HPLC and spectrophotometric enzymatic assay, while other methods have been developed to provide information on glutathione in situ (Figure 2). None of these measurements provide exhaustive information. In each case the data generated must be viewed within the context of the limitations imposed by the procedures employed to extract and estimate GSH:GSSG ratios in vitro or to probe glutathione pools in situ.

2.3.1. Enzymatic quantification

The most common spectrophotometric glutathione assay uses 5,5'-dithiobis(2-nitrobenzoic acid), otherwise known as DTNB or Ellman's reagent. Because this compound binds non-specifically to thiol groups, it can be used to measure protein and non-protein thiols alike. In acid extracts, from which proteins have been precipitated, this method measures total non-protein thiols (Figure 2A, bottom). The assay can provide a useful gross estimate of GSH content as this compound is usually the major non-protein thiol in most tissues. However, it should be noted that the method does not specifically measure GSH but all DTNB-reactive thiols present in the extract. It also does not detect GSSG. If NADPH and GR are include in the reaction mix (Figure 2A, middle), DNTB is continuously reduced by electrons provided by NADPH, and glutathione cycles between GSSG and GSH in an intermediate, catalytic role. This technique provides much greater sensitivity and specificity for glutathione than the end-point DTNB assay (Tietze, 1969). Without pre-treatment of extracts, the method measures both GSH and GSSG, ie, total free glutathione. The most common approach to distinguish between the two forms is to treat aliquots with a thiol reagent to remove GSH, then to assay the residual GSSG in the same way as total glutathione. Typical thiol reagents are *N*-ethylmaleimide and 2-vinylpyridine (Griffith, 1980). These methods can be readily adapted to multi-well assay in plate-readers (Queval and Noctor, 2007).

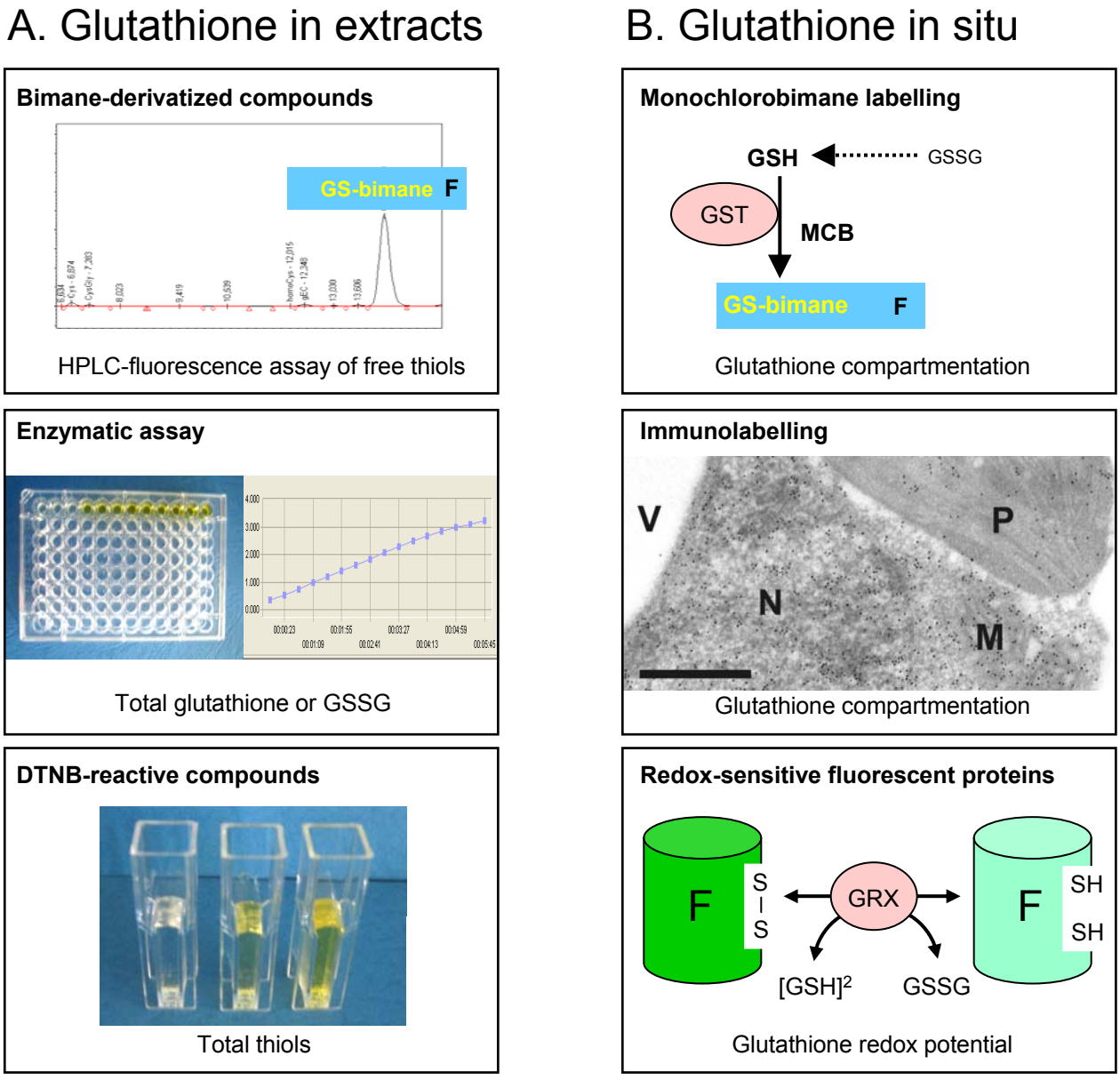

Figure 2. Some commonly used methods for measuring glutathione.

(A) Standard in vitro techniques for assaying glutathione and related thiols in protein-free extracts.

(B) Three techniques recently used to measure glutathione in situ. The image in the right middle panel was kindly provided by Bernd Zechmann (University of Graz, Austria). It shows a section through an Arabidopsis wild-type leaf mesophyll cell labelled with an anti-glutathione antibody. The black particles correspond to glutathione immunoprecipitates visible in mitochondria **(M)**, nucleus **(N)**, and plastid **(P)**, and which are much less abundant in the vacuole **(V)**. The scale bar indicates 1 µm. DTNB, 5,5'-dithiobis(2-nitrobenzoic acid). F, fluorescence. GRX, glutaredoxin. GST, glutathione *S*-transferase. MCB, monochlorobimane.

2.3.2. Liquid chromatography-based assays

The most commonly used HPLC technique is based on fluorescent labelling. Developed during the late 1970's and early 1980's (Fahey et al., 1981), this technique was first used to analyze plant extracts by the Rennenberg group (Schupp and Rennenberg, 1988; Rauser et al., 1991). It involves quantification of thiol-bimane adducts formed by treating extracts with reagents such as monobromobimane (MBB). The advantages of this sensitive technique are that the bimane derivatives have a high fluorescence yield and are quite stable (up to several days in solutions kept in the dark), and that other thiols present in the extract, such as cysteine, can be separated and measured in the same run with glutathione (Figure 2A, top). In the most common application of

the technique, aliquots are treated, prior to bimane derivatization, with a reductant such as dithiothreitol (DTT). This pre-treatment converts any GSSG present in the aliquot to GSH, and so only the total glutathione pool is determined, not the redox state. As described above for the spectrophotometric assay, GSSG can be measured by pre-treating extract aliquots with thiol reagents to remove GSH. However, this procedure is somewhat more onerous for the HPLC method because, after GSH is fixed in a complex, GSSG must subsequently be reduced to GSH prior to incubation with MBB. Approximate glutathione redox states can be estimated by simply comparing GS-bimane peak yields in aliquots from the same extract pre-treated or not with reductants like DTT, though the alkaline pH required for reaction with MBB probably causes some artefactual oxidation of extracted GSH if DTT is not present during the derivatization. This method can therefore give somewhat lower reduction states than the spectrophotometric assay (eg, Queval et al., 2011).

Other sensitive HPLC-fluorescence methods can be used based on derivatizing the primary amine group of glutathione. One example is *O*-phthaldialdehyde (OPA), a reagent that entered widespread use for HPLC analysis of amino acids in the 1980's (Lindroth and Mopper, 1979). Using OPA allows glutathione to be measured along with amino acids (Noctor and Foyer, 1998), though one drawback compared to bimane derivatization is that cysteine is not readily detectable. Other techniques applied to measuring glutathione in plants include LC-MS (Muckenschnabel et al., 2001) and capillary electrophoresis (Mendoza et al., 2008). Compared to targeted thiol analyses, however, these techniques require more specialized equipment and may also be less reproducible and quantitative. Techniques such as LC-MS are, however, among the methods of choice for identifying or analyzing GS-conjugates (eg, Brazier-Hicks et al., 2008; Mueller et al., 2008).

2.3.3 Data output

Regardless of the analytical method used to quantify glutathione in extracts, a key factor is sample preparation. The extraction procedure is crucial, and great care has to be taken to ensure that no loss of glutathione or change in redox state occurs. Best results are obtained if samples are quenched and ground in liquid nitrogen and then extracted directly into acid as the material thaws, taking care to homogenize regularly during this procedure. While some neutralization of protein-free acid supernatants is required for most of the techniques, maintaining a slightly acidic pH throughout the sample preparation minimizes GSH oxidation. Once the pH has been adjusted, extracts should be assayed as soon as possible, though from our experience GSH and GSSG are usually quite stable for a few hours in extracts kept on ice. It is not advisable to refreeze extracts, even if kept at acidic pH. For some extracts, this can lead to artefactual oxidation or degradation. As for any metabolite assay, it is advisable to check extraction efficiency, for example, with recovery experiments using known amounts of GSH or GSSG added at the stage of extraction in the presence or absence of the tissue under examination.

Measuring glutathione by enzyme assay or by HPLC-fluorescence with MBB or OPA produces comparable data for tissue contents (Noctor and Foyer, 1998; Queval and Noctor, 2007). Proper application of these methods to analysis of plants grown in controlled conditions gives typical values for leaf glutathione of the order of 200-600 nmol.g^{-1} fresh weight with the exact value depending on developmental stage and nutritional/light regime (eg, Cobbett et al., 1998; Veljovic-Jovanovic et al., 2001; Hartmann et al., 2003; Queval and Noctor, 2007). Higher values may be obtained for plants grown in less controlled conditions, eg, in crop plants growing in the field.

The overall redox state of glutathione [GSH/(GSH + 2GSSG)] in leaf extracts is around 0.9-0.95. Increases in GSSG relative to GSH are a useful indicator of oxidative stress or 'disulfide stress' (Åslund and Beckwith, 1999), and in plants can be observed in response to conditions such as ozone, pathogens, and cold, as well as deficiency in important H_2O_2-metabolizing enzymes such as catalase and ascorbate peroxidase (APX; Smith et al., 1984; Sen Gupta et al., 1991; Edwards et al., 1991; May and Leaver, 1993; May et al., 1996a; Willekens et al., 1997; Vanacker et al., 2000; Kocsy et al., 2000; Riszhky et al., 2002; Gomez et al., 2004a; Queval et al., 2007). A key property of glutathione homeostasis function is that increased oxidation, unless accompanied by appreciable consumption of GSH, is often followed or accompanied by increases in the total pool.

2.3.4. Analyzing glutathione in situ

Several methods have been developed to monitor glutathione in situ. It is important to note that these techniques measure different features of glutathione status, which may be of greater or lesser relevance depending on the information that is sought. Like in vitro analysis of glutathione, all have potential drawbacks and limitations in terms of the information they provide. In some cases, the necessary preparation imposes a stress on the sample, which could modify the factor being measured. Another caveat relates to the use of in vivo techniques under conditions different from those in which the plants are growing (eg, different actinic irradiance in studies where the rate of photosynthesis is important). Nevertheless, the availability of in situ techniques is undoubtably an important development that is adding to the repertoire of tools to analyze the complexities of glutathione homeostasis and related issues of cellular redox metabolism.

Monochlorobimane (MCB) is a less reactive analog of MBB that was used to specifically label the free GSH pool in mammalian cells in situ (Rice et al., 1986). While MBB reacts rapidly and non-specifically with thiol groups in alkaline pH conditions, the chemical reaction of MCB with glutathione is slower and accelerated by endogenous GSTs (Rice et al., 1986). In plants, in situ derivatization of the glutathione pool with MCB was used for fluorescence imaging of epidermal cells (Müller et al., 1999a,b). Subsequent studies in Arabidopsis cells and poplar leaves adapted this technique to quantify extracted GS-bimane in vitro by HPLC (Meyer et al., 2001; Hartmann et al., 2003). This approach has been used to investigate the regulation of glutathione synthesis and breakdown, as well as the compartmentation of glutathione per se (Meyer and Fricker, 2002; Hartman et al., 2003; Grzam et al., 2007; Ohkamu-Ohtsu et al., 2007b; Blum et al., 2010; Queval et al., 2011). Because GSTs are particularly abundant in the cytosol (Dixon et al., 2009), the requirement for these enzymes to obtain rapid labelling may be the most important factor contributing

to the potential compartmental specificity of the technique (Hartmann et al., 2003). Based on work in poplar, it was concluded that MCB labels only the cytosolic pool, and that this pool represents about half of total leaf glutathione (Hartmann et al., 2003). In wild-type Arabidopsis leaves, however, the entire leaf glutathione pool appeared to be accessible to MCB labelling within 2h incubation (Queval et al., 2011). Among the many factors that might explain this difference are inter-species specificity in GST compartmentalization or chloroplast glutathione transporters, variation in leaf developmental stage, or the inclusion (or not) of inhibitors during the incubation.

In situ immunolocalization has been used to examine glutathione distribution at the subcellular level by quantitative immunohistochemistry coupled to electron microscopy (Figure 2B). This approach relies on specific antibodies generated against glutaraldehyde-fixed glutathione and on the quantitative evaluation of the obtained signal by stereological methods. Antibodies that detect both GSH and GSSG have been used to label glutathione in several subcellular compartments (Zechmann et al., 2007, 2008; Kolb et al., 2010; Zechmann and Müller, 2010). Interestingly, in both Arabidopsis and tobacco, it was reported that the highest glutathione concentrations were not found in plastids but in mitochondria (Zechmann et al., 2008; Höller et al., 2010). In wild-type plants, glutathione was found to be negligible or undetectable in the vacuole, in agreement with earlier concepts that glutathione concentrations are usually low in this compartment (Rennenberg, 1982). A leaf glutathione content of 200 $nmol.g^{-1}FW$ converts to a global concentration of about 0.2 mM. Assuming low concentrations in the vacuole and apoplast, this would give a concentration of around 2 mM in other compartments, in reasonable agreement with reported values in spinach chloroplasts and the cytosol/cytoplasm of Arabidopsis cells (Foyer and Halliwell, 1976; Meyer et al., 2001). Calculations from immunolocalization studies performed in parallel with whole tissue quantification yielded estimated glutathione concentrations of 1 to 6 mM in the plastids, cytosol, and mitochondria of Arabidopsis Col-0 leaf mesophyll cells, with concentrations in the vacuole below 50 µM (Queval et al., 2011). Using non-aqueous fractionation of Arabidopsis leaves, cytosolic, plastid and vacuolar glutathione concentrations were estimated to be 3.5, 3.2 and 0.7 mM, respectively (Krueger et al., 2009). Thus, both these techniques give concentrations of the same order, regardless of potential problems such as cross-contamination between compartments or fractions during sample preparation. Differences in concentrations obtained in independent studies may also reflect differences in plant nutrition (eg, sulfur supply), which can affect the distribution of glutathione (Höller et al., 2010). Oxidative stress is among other factors that affect glutathione concentrations in a compartment-specific manner (Queval et al., 2011).

While the above two methods measure glutathione contents (and, potentially, concentrations) in vivo probes for the redox potential of glutathione have also been developed, based on fluorescent proteins containing oxidizable thiol groups. These include glutathione-specific redox-sensitive protein variants of the yellow (rxYFP) or green (roGFP) fluorescent protein (Hanson et al., 2004; Maulucci et al., 2009). These probes have been adapted for use in plants, particularly to monitor glutathione status in the cytosol (Jiang et al., 2006a; Meyer et al., 2007; Maughan et al., 2010). An important advantage of this method is that the introduced fluorescent proteins are designed to 'sense' the glutathione redox potential by equilibration of their thiol-disulfide status with that of glutathione (Figure 2B). Because the glutathione redox potential depends on GSH:GSSG and overall concentration (see section 8.1.2), these probes do not simply measure the glutathione pool size or even the oxidation state. However, as discussed further below, redox potential may be a key factor in glutathione-dependent signaling, and so these tools are providing useful information in studies of mutants or responses to stress (Meyer et al., 2007; Schwarzlander et al., 2008; Marty et al., 2009; Jubany-Mari et al., 2010). Potential constraints of these techniques are related to the limitations of optical microscopy. Most of the information generated to date has been obtained from studies of epidermal cells, and signal intensity may decrease for internal cell layers. However, such problems are less severe for the dual wavelength fluorescence ratio imaging roGFP variants (Schwarzlander et al., 2010). Because these proteins are dependent on glutaredoxin (GRX) activity, it is likely that glutathione redox potential is the major factor contributing to the signal (Meyer et al., 2007). However, recent studies have highlighted interactions between glutathione, GRX, and thioredoxins (TRX; Reichheld et al., 2007; Marty et al., 2009; Gao et al., 2010). It therefore remains to be definitively established that glutathione redox potential is the only component that is sensed by the thiol/disulfide-dependent fluorescent proteins.

3. MULTIPLE FUNCTIONS OF GLUTATHIONE

While this chapter is focused largely on glutathione homeostasis and related signaling functions, it is important to note that glutathione fulfils several essential functions in plants. Some of these roles can influence cellular glutathione status and thus the following sections briefly highlight some recent issues and key relevant points concerning the roles of glutathione in plant metabolism and defense.

3.1. Glutathione *S*-transferases

Compared to other phylogenetic groups, the GST family in plants is notable for its structural and functional diversity (see Dixon and Edwards (2010) for details of GST-encoding genes in Arabidopsis). While traditionally considered as cytosolic enzymes, work in Arabidopsis has revealed that several isoforms may localize at least partly to other compartments, including the chloroplast, peroxisome, and nucleus (Thatcher et al., 2007; Dixon et al., 2009). In many cases, the biochemical functions of specific GSTs remain to be fully elucidated. Several of these enzymes have peroxidase or DHAR activity. At least some GSTs are inducible by H_2O_2 (Levine et al., 1994; Willekens et al., 1997), and several of the Arabidopsis genes are induced by oxidative stress. For example, some GSTs of the *tau* family are strongly and rapidly up-regulated in catalase-deficient plants, and can therefore be considered useful markers for increased intracellular availability of H_2O_2 (Vanderauwera et al., 2005; Queval et al., 2007, 2009; Chaouch et al., 2010). Some GSTs that are induced by jasmonic acid (JA; Yan et al., 2007) or that have been shown to have activity against oxylipins (Mueller et al., 2008) are differentially regulated in response to H_2O_2 and/or GR deficiency (Mhamdi et al., 2010a).

Conjugation of metabolites to GSH may be important in biosynthetic and catabolic pathways (Dixon and Edwards, 2010). Recently, evidence has been presented that the reduced sulfur group in glucosinolates is derived from GSH rather than cysteine. For example, the *pad2* mutant, which is deficient in glutathione but not cysteine (Parisy et al., 2006), has decreased glucosinolate levels and impaired insect resistance (Schlaeppi et al., 2008). Moreover, LC-MS analysis of tobacco expressing Arabidopsis genes involved in the early steps of glucosinolate synthesis identified a GS-conjugate whose further metabolism was dependent on γ-glutamyl peptidase activity (Geu-Flores et al., 2009). Formation of a GS-conjugate is also part of the camalexin biosynthetic pathway (Böttcher et al., 2009).

3.2. Glyoxylase and Formaldehyde Metabolism

Glyoxalases catalyze the metabolism of potentially toxic oxo-aldehydes, the simplest of which is glyoxal, to hydroxyacids. The most common physiological substrate is thought to be methylgloxyal, which is notably formed from triose phosphate, either non-enzymically or as an intermediate in the triose-phosphate isomerase reaction (Maiti et al., 1997; Marasinghe et al., 2005). Metabolism of such oxo-aldehydes through the glyoxalase system involves conjugation to GSH, catalyzed by glyoxalase 1. A single gene (At1g11840) is annotated as a glyoxalase I homolog in Arabidopsis. Glyoxalase 2 then cleaves the glutathione to yield the corresponding free hydroxyacid (lactate in the case of methylglyoxal). Five genes are annotated to encode glyoxalase 2 in Arabidopsis (GLX2-1 to GLX2-5; At2g43430, At3g10850, At1g53580, At1g06130, At2g31350). Three of the encoded proteins are predicted to be mitochondrial enzymes that may have novel functions as well as or instead of glyoxalase 2 activity (Limphong et al., 2009). Tobacco plants overexpressing glyoxalases 1 and 2 or both glyoxalases together showed increased tolerance to both exogenous methylglyoxal and salt (Singla-Pareek et al., 2003). Similar strategies were reported to enhance salt tolerance in rice and Arabidopsis (Deb Roy et al., 2008; Singla-Pareek et al., 2008).

As well as the glyoxalase pathway, glutathione may also be involved in detoxification of formaldehyde via S-formylglutathione, producing formate which may be converted to CO2 or enter C1 metabolism (Martinez et al., 1996; Haslam et al., 2002; Achkor et al., 2003). GSHdependent formaldehyde dehydrogenase activity in Arabidopsis can result from the product of the At5g43940 gene. However, this gene encodes an enzyme with GSNO reductase activity (Sakamoto et al., 2002; Diaz et al., 2003), which may be its most important physiological function (see section 8.5).

3.3. Phytochelatins and Heavy Metal Resistance

One of the best characterized functions of glutathione is as a precursor in the synthesis of phytochelatins, which are required for responses to cadmium and other heavy metals (Grill et al., 1987, 1989; Cobbett and Goldsbrough, 2002; Rea et al., 2004). In plants and fungal species such as fission yeast, phytochelatins are produced from glutathione or homologs by phytochelatin synthase (PCS), a cytosolic enzyme (Blum et al., 2010). Two PCS genes are annotated in Arabidopsis (Ha et al., 1999; Cazalé and Clemens, 2001). The essential role of *PCS1* (At5g44070) is evidenced by its identification as the gene affected by the *cad1* mutation (Howden and Cobbett, 1992; Ha et al., 1999). *PCS2* (At1g03980) also encodes a functional enzyme but is expressed in most tissues at much lower levels than *PCS1* (Cazalé and Clemens, 2001; Blum et al., 2010). An alternative route of phytochelatin synthesis involving vacuolar carboxypeptidases has been reported in budding yeast (Wünschmann et al., 2007). Increased sensitivity to cadmium in the *cad2* mutant is caused by impaired activity of γ-glutamylcysteine synthetase (γ-ECS), the first enzyme of the glutathione biosynthesis pathway (Howden et al., 1995; Cobbett et al., 1998). This results in a glutathione content of about 25% wild-type levels.

While transgenic up-regulation of glutathione synthesis has been reported to increase heavy metal tolerance, effects of overexpressing PCS have been less clear (Zhu et al., 1999a,b; Lee et al., 2003). Attempts have been made to improve cadmium sequestration and/or tolerance by engineering leaf-specific or ectopic expression of PCS in the chloroplast (Peterson and Oliver, 2006; Picault et al., 2006). Evidence is accumulating that PCS may be involved in cellular processes other than phytochelatin synthesis (Rea et al., 2004; Clemens and Peršoh, 2009), possibly including turnover of GS-conjugates (Blum et al., 2007, 2010). Ongoing work is identifying phytochelatin-independent mechanisms of heavy metal resistance (Hugouvieux et al., 2009). The role of glutathione in heavy metal responses could be multiple, as many of these elements are considered to directly or indirectly result in enhanced cellular oxidation. Interestingly, in *Brassica napus* exposed to cadmium, phytochelatins as well as GS-cadmium complexes were found at high concentrations in the phloem whereas phytochelatin concentrations were much lower in the xylem (Mendoza-Cózatl et al., 2008).

3.4. Glutathione and Sulfur Assimilation

Cysteine is the first organic product of sulfur assimilation in plants and is notably used for synthesis of protein amino acids, either as cysteine itself or after synthesis of methionine (Leustek et al., 2000; Saito, 2000; Droux, 2004). The production of these two primary S products requires interactions with both N and C metabolism, as does the production of the tripeptide, glutathione, as discussed previously by ourselves and others (Noctor et al., 1998a,b; Kopriva and Rennenberg, 2004). Glutathione is a significant non-protein sink for reduced sulfur. Sulfur supply has been positively correlated with resistance to some pathogens, a phenomenon termed sulfur-induced resistance (SIR; Bloem et al., 2007). Although the causes underlying SIR remain to be identified, tissue contents of glutathione or precursors may be among the factors linking sulfur nutrition to the responses of plants to fungal and viral infection (Gullner et al., 1999; Bloem et al., 2007; Zechmann et al., 2007; Höller et al., 2010).

3.4.1. Glutathione regulation of sulfur assimilation

As a major storage and transport form of reduced sulfur and non-protein cysteine, glutathione has been implicated in the regulation of sulfur metabolism in Arabidopsis, as in other plants (Kopriva

and Rennenberg, 2004). Several components involved in sulfur assimilation are regulated by glutathione. Inhibition of sulfate uptake by GSH occurs in tobacco (Herschbach and Rennenberg, 1994). Similarly, glutathione has been reported to repress sulfur assimilation and uptake activities of sulfate transporters in a number of species (Buchner et al., 2004). In Arabidopsis, GSH inhibits the expression of *AST68*, a sulfate transporter, and ATP sulfurylase 1 (*APS1*), accompanied by a decrease of sulfate influx and APS activity in the roots (Lappartient et al., 1999). All three isoforms of the second enzyme of the sulfate assimilation pathway, adenosine 5'-phosphosulfate reductase (APR), seem to be particularly sensitive to inhibition by glutathione at the levels of transcripts and activity in Arabidopsis roots (Vauclare et al., 2002).

Available evidence suggests that GSH is the most important reductant for APR (Leustek, 2002). This enzyme contains a carboxyl domain with homology to TRX that appears to act as a functional GRX, as it uses GSH to reduce adenosine 5'-phosphosulfate to sulfite with apparent K_MGSH values of 0.6 to 1 mM (Bick et al., 1998). Post-translational redox control of APR (particularly APR1) marks it out as a significant control point in response to glutathione oxidation (Leustek, 2002).

3.4.2. Manipulation of sulfur assimilation and effects on glutathione

Sulfur availability limits GSH accumulation in plants (Nikiforova et al., 2003). An Arabidopsis mutant defective in the Sultr1;2 gene, encoding a high affinity sulphate transporter, has decreased levels of glutathione (Maruyama-Nakashita et al., 2003) while constitutive expression of a bacterial APR in Arabidopsis increases the contents of both cysteine and glutathione (Tsakraklides et al., 2002). Overexpression of the cysteine synthesis pathway enhances cysteine and glutathione contents in Arabidopsis and tobacco (Harms et al, 2000; Noji and Saito, 2002; Wirtz and Hell, 2007). Environmental stresses such as ozone concomitantly increase cysteine and glutathione levels, associated with the post-translational activation of APR1 (Bick et al., 2001). Genes encoding all three APRs are also induced by intracellular H_2O_2, as are cysteine, γ-EC, and glutathione (Queval et al., 2009).

4. GLUTATHIONE BIOSYNTHESIS

4.1. Enzymes, Mutants, and Inhibitors

The committed pathway of glutathione synthesis in plants has been well established for many years (Rennenberg, 1982; Noctor et al., 2002; Mullineaux and Rausch, 2005). The metabolic route is essentially similar to the pathway in most other organisms containing GSH (Meister, 1988b). From glutamate, cysteine, and glycine, two ATP-dependent enzymes produce GSH via the intermediate, γ-EC (Figure 3). Plant sequences for the single genes that encode each of these enzymes were first reported in Arabidopsis (May and Leaver, 1994; Ullman et al., 1996). The first enzyme, γ-ECS, is encoded by the *GSH1* gene (At4g23100). In wheat leaves, γ-ECS activities are strongly associated with chloroplasts (Noctor et al., 2002) and subcellular localization studies of the Arabidopsis protein have convincingly demonstrated that the enzyme is restricted to plastids (Wachter et al., 2005). In contrast, the *GSH2* gene (At5g27380) encodes two transcripts. The most abundant is the shorter form, which encodes a cytosolic GSH-S, while the longer form encodes a chloroplast-targeted protein (Wachter et al., 2005). Thus, the first step of glutathione synthesis is located in the plastid while the second step can occur in both chloroplasts and cytosol. Plastidic γ-ECS and cytosolic GSH-S are linked by γ-EC export across the chloroplast envelope and transporters have recently been described that are competent in both γ-EC and GSH transport (Maughan et al., 2010).

4.1.1. γ-ECS mutants

Knockout mutations in *GSH1* produce an embryo-lethal phenotype (Cairns et al., 2006). Less severe mutations in this gene, which produce partial decreases in glutathione contents, have been extremely useful in elucidating the functions of glutathione in plants. The *rml1* (*rootmeristemless1*) mutant has less than 5% of wild-type glutathione contents. It fails to develop a root apical meristem because the cells arrest at the G1 phase of the cell cycle (Vernoux et al., 2000). This mutant is much less affected in its shoot phenotype. By combining the *rml1* mutation with mutations in the two genes encoding dual-addressed cytosolic/mitochondrial NADPH-thioredoxin reductases (*NTRA*, At2g17420; *NTRB* At4g35460), it was shown that there is functional redundancy between glutathione and TRX systems in the control of shoot apical meristem function (Reichheld et al., 2007). Moreover, the combination of the *cad2* mutation with *ntra* and *ntrb* resulted in perturbed auxin transport and metabolism, together with a loss of apical dominance and reduced secondary root production (Bashandy et al., 2010).

Several mutations in *GSH1* decrease glutathione to a residual but still significant level (about 25 to 50% of wild-type). In themselves, these decreases do not markedly affect Arabidopsis development. They do, however, cause alterations in responses to stress and the environment. Of these mutants, *cad2* was identified by its enhanced sensitivity to cadmium, *rax1* by altered expression of *APX2* (At3g09640) and *pad2* by decreased camalexin contents and enhanced sensitivity to pathogens (Howden et al., 1995; Cobbett et al., 1998; Ball et al., 2004; Parisy et al., 2006).

4.1.2. Glutathione synthetase

As stated above, GSH-S is encoded by a single gene, *GSH2*, but alternative splicing results in localization of the gene product in both the chloroplast and cytosol (Wachter et al., 2005). While *GSH1* knockouts are embryo-lethal, *GSH2* knockout mutants are seedling-lethal, probably reflecting partial replacement of GSH functions by γ-EC, which accumulates to very high levels in these plants (Pasternak et al., 2008). The wild-type phenotype can be restored in *gsh2* by complementing the mutant with targeted expression of the enzyme to the cytosol alone (Pasternak et al., 2008). This provides further evidence that GSH can be imported from the cytosol into the plastid, consistent with radiolabelling studies of isolated wheat chloroplasts (Noctor et al., 2002).

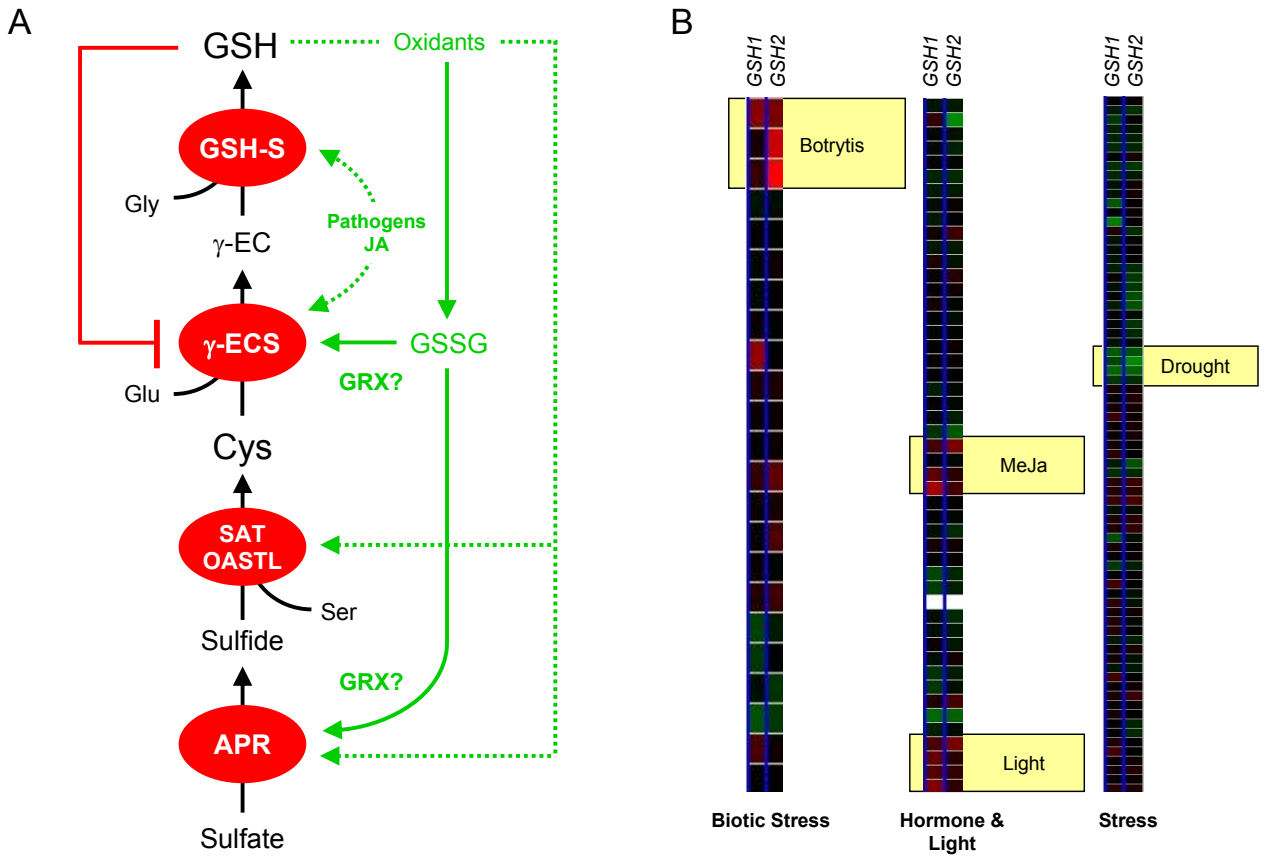

Figure 3. Pathway of glutathione biosynthesis and principal methods of regulation.

(A) Scheme showing production of glutathione from constituent amino acids and cysteine synthesis enzymes that are likely important in regulating glutathione concentration. Important regulatory mechanisms are shown as red lines (feedback inhibition) or green lines (up-regulation in response to oxidation or other factors). Green dotted lines indicate likely transcriptional activation mechanisms while solid lines indicate post-translational processes. The role of GSSG and the exact mechanisms involved in the activation of γ-ECS by thiol oxidation remain to be established. For simplicity, not all enzymes involved in sulfate reduction are shown. APR, adenosine phosphosulfate reductase. γ-ECS, γ-glutamylcysteine synthetase. GRX, glutaredoxin. GSH-S, glutathione synthetase. JA, jasmonic acid. OAS, *O*-acetylserine thiol lyase. SAT, serine acetyl transferase.

(B) Expression of *GSH1* and *GSH2* in response to a range of environmental stresses (www.genevestigator.com; Hruz et al., 2008). Examples of significant responses are highlighted. Botrytis, rosettes infected with *B.cinerea* or *B.graminis* versus uninfected rosettes. Drought, leaves from droughted plants versus watered plants. Light, light-grown seedlings compared to dark-grown seedlings. MeJa, methyl jasmonate-treated cell cultures versus solvent-treated cell cultures.

4.1.3. Buthionine sulfoximine

As well as mutants, one tool that has proved very useful in functional analysis of glutathione in plants is buthionine sulfoximine (BSO), a specific inhibitor that binds to the γ-ECS active site (Griffith and Meister, 1979). This compound is structurally homologous to methionine sulfoximine (MSO), which inhibits both γ-ECS and glutamine synthetase. The reactions catalyzed by both enzymes involve ATP-dependent ligation of an amine group (ammonia or the amino group of cysteine) to the γ-carboxy group of glutamate. Unlike MSO, BSO does not inhibit glutamine synthetase and so can be used in studies specifically focused on the role of glutathione synthesis and concentration in plants (eg, Cobbett et al., 1998; Kocsy et al., 2000; Meyer et al., 2007; Reichheld et al., 2007). Recently, a screen for BSO-insensitive Arabidopsis mutants revealed the identity of the chloroplast γ-EC and GSH transporter, called chloroquinone-like transporter (CLT; Maughan et al., 2010). These transporters are encoded by three genes: *CLT1* (At5g19380), *CLT2* (At4g24460), and *CLT3* (At5g12160/12170). This finding, taken with the available information on γ-ECS and GSH-S localization, leads to the view that the synthesis of γ-EC is restricted to the plastid but that GSH synthesis can also occur in the cytosol following γ-EC export across the plastid envelope (Wachter et al., 2005; Maughan et al., 2010).

4.2. Regulation and Manipulation of Glutathione Synthesis

The production of glutathione is regulated at multiple levels, with the most important limitations being γ-ECS activity and cysteine concentration. This has been shown by overexpression of the

two enzymes of glutathione synthesis and of enzymes involved in cysteine synthesis (Strohm et al., 1995; Noctor et al., 1996, 1998a; Creissen et al., 1999; Harms et al, 2000; Noji and Saito, 2002; Wirtz and Hell, 2007). Although these factors are considered to be the major controls, regulation is possible at other levels, such as the availability of glycine and ATP (Buwalda et al., 1990; Noctor et al., 1997; Ogawa et al., 2004). Even though the overall flux through the glutathione synthesis pathway is relatively low in comparison to primary metabolism, the multiplicity of controls suggests that the pathway is highly regulated and exquisitely sensitive to the cellular redox environment. This complexity can also be understood in terms of the different physiological functions of modified glutathione synthesis. In certain cases, such as heavy metal exposure, GSH is consumed and accelerated synthesis may be accompanied by unchanged or decreased glutathione pools. In other stresses with a strong oxidative component, increased synthesis occurs within a context of accumulating glutathione, a response that has presumably evolved to offset changes in redox potential caused by increased GSSG (see section 8.1.2).

4.2.1. Expression of glutathione synthesis genes

Relatively few conditions cause very marked induction of *GSH1* or *GSH2* transcripts. These genes are induced by JA and heavy metals (Xiang and Oliver, 1998; Sung et al., 2009) and also respond to light and some stress conditions such as drought and certain pathogens (Figure 3B). Increases in H_2O_2 availability are known to trigger accumulation of glutathione in several plant species (Smith et al., 1984; May and Leaver, 1993; Willekens et al., 1997; Queval et al., 2009). However, neither exogenous H_2O_2 nor intracellularly generated H_2O_2 cause increased abundance of *GSH1* or *GSH2* transcripts in Arabidopsis (Xiang and Oliver, 1998; Sánchez-Fernández et al., 1998; Queval et al., 2009). Some evidence has been presented that production of the γ-ECS protein is regulated at the level of translation (Xiang and Bertrand, 2000).

4.2.2. Post-translational regulation of γ-ECS

Feedback inhibition of enzyme activity by GSH has long been established in animals (Richman and Meister, 1975) and also occurs in plants (Hell and Bergmann, 1990; Noctor et al., 2002). Other types of regulation were proposed based on the failure of Arabidopsis γ-ECS to fully restore wild-type GSH levels in yeast mutants (May et al., 1998b). The tobacco enzyme was shown to be inhibited by dithiols (Hell and Bergmann, 1990), and this effect has also been described for the wheat and Arabidopsis enzymes (Noctor et al., 2002; Jez et al., 2004). The plant enzyme forms a homodimer linked by two disulfide bonds (Hothorn et al., 2006), one of which is involved in redox regulation (Hicks et al., 2007; Gromes et al., 2008). Though the exact mechanistic details are still subject to debate, activation through disulfide formation very probably contributes to the well-known up-regulation of glutathione synthesis in response to oxidative stress conditions. It remains unclear whether this mode of regulation interacts mechanistically with feedback inhibition.

4.2.3. Cysteine availability

Up-regulation of the cysteine synthesis pathway occurs concomitantly with that of GSH synthesis. Two levels of co-regulation have been described under conditions in which oxidative stress triggers accumulation of glutathione in Arabidopsis. In catalase-deficient mutants, accumulation of transcripts encoding APR and serine acetyltransferase (SAT) was observed (Queval et al., 2009). Like γ-ECS, APRs are localized in plastids, whereas the two enzymes of cysteine synthesis are found in several compartments (Droux, 2004; Kopriva and Rennenberg, 2004; Martin et al., 2005; Kawashima et al., 2005). Recent studies show that the mitochondrial SAT (encoded by *SERAT2.2*; At3g13110) makes the major contribution to cysteine synthesis under standard conditions, and that knocking out this enzyme affects flux into glutathione and leaf glutathione contents (Haas et al., 2008; Watanabe et al., 2008). However, transcripts for the chloroplast SAT (*SERAT2.1*; At1g55920) were the most strongly induced during H_2O_2-triggered up-regulation of glutathione in Arabidopsis (Queval et al., 2009).

Exposure of Arabidopsis to ozone activates at least one APR at the post-translational level through formation of a disulfide bond (Bick et al., 2001). Although much more is known about thiol-disulfide regulation mediated by TRX (Buchanan and Balmer, 2005), GRX are also important players in redox regulation (see section 8.3). One simple explanation of the link between decreased GSH:GSSG and enhanced glutathione synthesis is that increases in the chloroplast glutathione redox potential allow GRX-mediated oxidative activation of both APR and γ-ECS (Figure 3A). This would be consistent with a model for APR regulation in which the enzyme is maintained in a reduced inactive form by TRX and activated by GSSG (Leustek, 2002). Accordingly, H_2O_2-triggered accumulation of glutathione in barley is associated with appreciable increases in chloroplast GSSG content (Smith et al., 1985), and in situ analysis of glutathione in leaf mesophyll cells strongly points to a similar effect in Arabidopsis (Queval et al., 2011).

4.2.4. Overexpression of the glutathione synthesis pathway

Despite the multiplicity of controls over glutathione synthesis, plant cells can accommodate marked increases in overall glutathione content. Studies using expression of *E.coli* γ-ECS targeted to the poplar chloroplast or cytosol or to the tobacco chloroplast produced increases in leaf glutathione of up to four-fold (Noctor et al., 1996, 1998a; Creissen et al., 1999). Even higher contents were achieved by combined overexpression of γ-ECS and GSH-S (Creissen et al., 1999). Overexpression of γ-ECS in Arabidopsis also enhanced GSH, though less markedly (Xiang et al., 2001). Initial studies of several independent lines of poplar overexpressing γ-ECS showed no phenotypic effects (Noctor et al., 1998a). One of these lines has been reported to show symptoms of early leaf senescence (Herschbach et al., 2009). Clear effects of γ-ECS overexpression in tobacco included lesion formation and high levels of GSSG accumulation (Creissen et al., 1999), whereas marked negative phenotypic effects were absent in poplar and *Brassica juncea* expressing the same gene (Noctor et al., 1998a; Zhu et al., 1999a). Recently, expression of a bifunctional feedback-insensitive γ-ECS/GSH-S enzyme

from the bacterium *Streptococcus thermophilus* in tobacco has shown that the glutathione pool can be boosted by up to 20-fold without apparent marked effects on plant growth and development (Liedschulte et al., 2010). In view of the information from several recent studies (see section 8.6), these plants present a very interesting system in which to analyze responses to biotic and other stresses. Increased glutathione in plants overexpressing γ-ECS is associated with enhanced resistance to heavy metals and certain herbicides (Zhu et al., 1999a; Gullner et al., 2001). Overexpression of γ-ECS in the poplar chloroplast increased not only glutathione but also levels of several free amino acids, such as leucine, isoleucine, leucine, tyrosine, and lysine (Noctor et al., 1998a). The causes underlying these effects remain unclear, though it is interesting to note that several enzymes involved in synthesis or metabolism of some of these amino acids are among those that have been identified as potential TRX targets through redox protemics approaches (Montrichard et al., 2009).

Compared to boosting γ-ECS capacity, overexpression of GSH-S has less marked effects on glutathione contents. No effect of substantial increases in GSH-S activity was observed in poplar (Foyer et al., 1995; Noctor et al., 1998a), though enhanced synthesis of GSH was induced when γ-EC was supplied to leaf tissue from the transformants (Strohm et al., 1995). Thus, GSH-S may become limiting when γ-EC supply is increased. *Brassica juncea* lines overexpressing GSH-S showed improved resistance to cadmium (Zhu et al., 1999b). Expression of a soybean homoGSH-S was used as part of an interesting strategy to confer herbicide resistance in tobacco, which is sensitive to the herbicide, fomesafen (Skipsey et al., 2005b). When expressed together with a homoGSH-preferring GST from soybean, which is fomesafen-tolerant, enhanced resistance was observed in the transformed tobacco lines (Skipsey et al., 2005b).

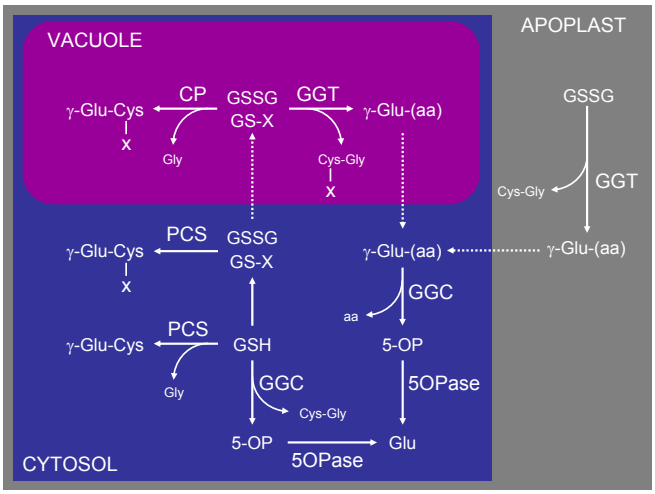

Figure 4. Possible routes of glutathione breakdown in Arabidopsis.

For clarity, the term γ-EC, used elsewhere in this chapter, is replaced here by γ-Glu-Cys. Emphasis is placed on the localization of the initiating reactions. For simplicity possible downstream reactions are not always shown (eg, GGT action on γ-Glu-Cys conjugates). Only the transpeptidation reaction catalyzed by GGT is shown, but these enzymes could also catalyze hydrolysis of GSH to Glu and Cys-Gly. aa, amino acid(s). CP, carboxypeptidase. GGC, γ-glutamyl cyclotransferase. GGT, γ-glutamyl transpeptidase. 5-OP, 5-oxoproline. 5-OPase, 5-oxoprolinase. PCS, phytochelatin synthase. X, *S*-conjugated compound or second moiety of disulfide.

5. GLUTATHIONE BREAKDOWN

Overall rates of glutathione catabolism in Arabidopsis leaves have been estimated at about 30 nmol.g^{-1} FW h^{-1} (Ohkamu-Ohtsu et al., 2008), suggesting that some pools of glutathione could turn over several times daily. In mammals, degradation of glutathione to dipeptides and amino acids is part of the γ-glutamyl cycle (Meister, 1988b). This involves hydrolysis or transpeptidation of GSH at the plasmembrane by γ-glutamyl transpeptidase (GGT), and intracellular metabolism of γ-glutamyl amino acid derivatives by γ-glutamyl cyclotransferases (GGC) and 5-oxoprolinase (5-OPase) to produce free glutamate. These enzyme activities were characterized in tobacco cells by Rennenberg and co-workers (Rennenberg et al., 1981; Steinkamp and Rennenberg, 1984; Steinkamp et al., 1987). Two GGTs purified from tomato were shown to have broad substrate specificity against GSH, GSSG and GS-conjugates and to localize to the cell wall or outer surface of the plasmalemma (Martin and Slovin, 2000). Both showed K_MGSH values of about 0.1 mM (Martin and Slovin, 2000), consistent with their localization in a compartment where GSH concentrations are relatively low (Vanacker et al., 1998; Ohkamu-Ohtsu et al., 2007a). Studies in maize and barley also detected GGT activity in the apoplast (Masi et al., 2007; Ferretti et al., 2009). Interestingly, the pH optimum of the enzyme detected in barley roots was reported to be higher than seven, which suggests that the activity could increase in conditions that induce alkalinisation of the apoplast (Ferretti et al., 2009). Recent studies in Arabidopsis have significantly extended our knowledge in this area while also revealing the potential complexity of the breakdown of GSH, GSSG, and GS-conjugates (Figure 4). Key outstanding questions are cellular/tissue specificities and activities against GSH compared to GS-conjugates or GSSG, which can be considered as a glutathione-glutathione conjugate.

5.1. γ-Glutamyl Transpeptidase

Four genes were predicted to encode GGT in Arabidosis (Storozhenko et al., 2002). One of these sequences (*GGT1*; At4g39640) was identified in a screen of Arabidopsis cDNAs that render yeast tolerant to the thiol-oxidizing agent, diamide (Kushnir et al., 1995). Overexpression of this GGT in tobacco caused an increase in extracellular GGT activity (Storozhenko et al., 2002). The *GGT2* sequence (At4g39650) is contiguous and highly similar to *GGT1* and is also localized outside the cell, though its expression is largely confined to young siliques whereas *GGT1* is more widely expressed (Ohkamu-Ohtsu et al., 2007a; Destro et al., 2010). However, root GGT activity can be enhanced by incubation with GSH and comparative analysis of *ggt1* and wild-type plants suggests that GGT2 may contribute to this inducible activity (Destro et al., 2010). Unlike animal GGT, GGT1 and GGT2 are probably bound to the cell wall rather than anchored to the plasmalemmma (Martin et al., 2007; Ohkamu-Ohtsu et al., 2007a). Knockout mutants for GGT1 show a phenotype of decreased growth and early

senescence, linked to accumulation of GSSG (Ohkamu-Ohtsu et al., 2007a). Thus, one function of extracellular GGTs may be to counteract oxidative stress or to salvage excreted GSSG (Ohkamu-Ohtsu et al., 2007a; Ferretti et al., 2009). A third GGT, called GGT4 (At4g29210), originally called GGT3 by Ohkamu-Ohtsu et al. (2007a,b), is localized in the vacuole where its major role is considered to be the metabolism of GS-conjugates (Grzam et al., 2007; Martin et al., 2007; Ohkamu-Ohtsu et al., 2007b). The fourth sequence (At1g69820), now called GGT3, is considered unlikely to encode a functional GGT but transcripts have been detected in some tissues (Martin et al., 2007; Destro et al., 2010).

5.2. Glutathione Breakdown by Other Enzymes

While several studies have focused on GGTs, a recent analysis suggests that these enzymes may not be the major route for GSH breakdown in plants (Ohkamu-Ohtsu et al., 2008). γ-Glutamyl peptides such as those that result from GGT activity, can be degraded to 5-oxoproline by GGC activity. No gene has yet been identified for GGC in Arabidopsis but a single gene (*OXP1*; At5g37830) shows high similarity to animal 5-OPase and the encoded protein is likely found in the cytosol (Ohkamu-Ohtsu et al., 2008). Knockout *oxp1* mutants accumulate high levels of 5-oxoproline, and this accumulation is not affected in triple *oxp1 ggt1 ggt4* mutants that are deficient in the major GGT activities as well as 5-OPase (Ohkamu-Ohtsu et al., 2008). Based on these and other observations, it was proposed that cytosolic GGC, rather than vacuolar or extracellular GGT, is the major enzyme initiating GSH degradation and that this route could be particularly important in the intercellular transport function of GSH (Ohkamu-Ohtsu et al., 2008).

Another cytosolic route for glutathione degradation could involve PCS, which may be particularly important in GS-conjugate breakdown (Blum et al., 2007, 2010). In Arabidopsis leaf discs, no evidence was found that PCS contributes to metabolism of GS-conjugates, though this route might become significant when PCS is activated by heavy metals (Grzam et al., 2006). Work in Arabidopsis suspension cells suggests that cytosolic PCS is a potentially significant route for GS-conjugate metabolism, and the relative importance of the different pathways could be dependent on tissue or cell type (Blum et al., 2007; Brazier-Hicks et al., 2008). Yet another route of glutathione degradation would involve cleavage of the glycine by a carboxypeptidase activity (Steinkamp and Rennenberg, 1985). A vacuolar carboxypeptidase activity has been described that degrades GS-conjugates in barley (Wolf et al., 1996). Although not shown in Figure 4, other proteins may also metabolize GSH, GSSG, or GS-conjugates. These include proteins such as GGP1, an enzyme with a putative glutamine amidotransferase domain that has been implicated in the removal of the Glu residue from a GS-conjugate during glucosinolate synthesis (Geu-Flores et al., 2009). In addition to GGC, Arabidopsis genes remain to be identified for the vacuolar carboxypeptidase and for dipeptidases that could cleave Cys-Gly. In conclusion, there is still no consensus on the compartmentation of glutathione degradation. Most attention continues to be paid to apoplastic and vacuolar routes. A third possibility involves cytosolic degradation of GS-conjugates although evidence for appreciable accumulation of these compounds in this compartment is lacking.

6. COMPARTMENTATION AND TRANSPORT

6.1. Intercellular Transport

Glutathione synthesized in the chloroplast and cytosol is transported between cells and organs. Together with *S*-methylmethionine, glutathione is considered to be a major form of organic sulfur translocated in the phloem (Herschbach and Rennenberg, 1994, 1995; Bourgis et al., 1999; Mendoza-Cózatl et al., 2008). Tobacco cells and broad bean protoplasts are able to import both GSH and GSSG (Schneider et al., 1992; Jamaï et al., 1996). One group of proteins that could be responsible for transport across the plasmalemma is the oligopeptide transporter (OPT) family. First identified in yeast, where the protein is named HGT1, these proteins are able to transport GSH, GSSG, GS-conjugates as well as other small peptides (Bourbouloux et al., 2000). Heterologous expression in yeast of a homologous *B. juncea* gene, *BjGT1*, showed that the protein was competent in glutathione uptake (Bogs et al., 2003) while a rice homolog, OsGT1, can transport GSH, GSSG, and GS-conjugates (Zhang et al., 2004). In Arabidopsis, there are nine annotated *OPT* genes (Koh et al., 2002). OPT6 (Atg27730) may be able to transport GS-conjugates and GS-cadmium complexes as well as GSH and GSSG (Cagnac et al., 2004). Another study reported that GSH but not GSSG was transported by OPT6 (Pike et al., 2009). Consistent with its proposed role in long-distance transport, the *OPT6* gene is highly expressed in the vasculature (Cagnac et al., 2004). However, OPT6 does not seem to be a glutathione-specific transporter: it is active against a broad spectrum of peptides, including peptides that may have signaling functions in plants (Pike et al., 2009). Knockout *opt6* mutants are aphenotypic, suggesting gene redundancy (Pike et al., 2009).

6.2. Transport Between the Plastid and the Cytosol

Direct analysis of ^{35}S-GSH uptake by wheat chloroplasts suggested the existence of components able to translocate glutathione across the inner chloroplast envelope (Noctor et al., 2002). The recent identification of the CLT family of transporter proteins that are targeted to the plastid envelope and that transport both γ-EC and glutathione (Maughan et al., 2010) is consistent with the exclusive location of γ-ECS in the plastid and the major location of GSH-S in the cytosol (Figure 5; Wachter et al., 2005). The triple *clt1 clt2 clt3* knockout has depleted cytosolic GSH and enriched chloroplastic GSH (Maughan et al., 2010). The first of these transporters was identified in a screen for mutants resistant to BSO. There is as yet no direct evidence that the CLT transports the inhibitor itself. However, its closest homolog, the chloroquinone transporter in the malaria parasite *Plasmodium*, has recently been shown to bind BSO (Martin et al., 2009). Chloroplast uptake of GSH by this transporter family likely explains the observation that *gsh2* knockout mutants can be rescued by expression of GSH-S in the cytosol alone (Pasternak et al., 2008).

6.3. Transport into the Nucleus and Mitochondria

A significant proportion of cellular GSH has been detected in the nucleus using in situ immunolocalization techniques (Zechmann

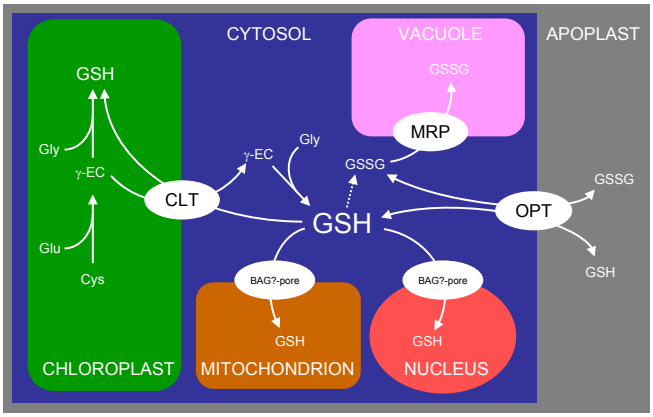

Figure 5. Subcellular compartmentation of glutathione synthesis and glutathione transport.

BAG, Bcl2-associated anathogene. CLT, chloroquinone-like transporter. MRP, multidrug resistance-associated protein. OPT, oligopeptide transporter.

et al., 2008). Indeed, glutathione is recruited into the nucleus early in the cell cycle in both mammals and plants (Markovic et al., 2007; Diaz-Vivancos et al., 2010a). This has led to the concept of a nuclear redox cycle that occurs during mitosis in which GSH moves into the nucleus during G1, promoting oxidation of the cytosol and enhanced glutathione accumulation (Diaz-Vivancos et al., 2010b). In Arabidopsis cells growing in culture, GSH localization in the nucleus at G1 is accompanied by enhanced levels of ROS and lowering of the oxidative defense shield, as reflected by transcriptomic changes (Diaz-Vivancos et al., 2010a). The accumulated GSH is divided between the daughter cells, in which the process begins again. These observations suggest the presence of proteins in plants that are able to alter the permeability of nuclear pores, facilitating GSH sequestration in the nucleus. While in plants these proteins remain to be identified, in mammalian tissues it is considered that the anti-apoptotic factor Bcl-2 is a crucial component regulating GSH transport into the nucleus, as it is in mitochondria (Voehringer et al., 1998). As noted above, high concentrations of glutathione have been detected in leaf mitochondria (Zechmann et al., 2008), despite the absence of evidence for GSH synthesis in this organelle. It is thus tempting to suggest that pore-regulating proteins operate to control the mitochondrial and nuclear GSH concentration in a manner similar to that observed in animal cells (Figure 5).

6.4. Transport into the Vacuole

GS-conjugates are known to be transported into the vacuole by tonoplast MRP (Multidrug Resistance-associated Protein) transporters of the ABC (ATP-Binding Cassette) type (Martinoia et al., 1993; Rea, 1999; Foyer et al., 2001). Barley vacuoles rapidly take up GSSG but GSH is taken up much more slowly (Tommasini et al., 1993). Arabidopsis MRPs can transport GSSG as well as GS-conjugates when heterologously expressed in yeast (Lu et al., 1998). Vacuolar import of GSSG may play a role in maintaining cytosolic glutathione redox status (Tommasini et al., 1993). Recent observations in Arabidopsis suggest that vacuolar accumulation of GSSG is a physiologically important part of the response to oxidative stress (Queval et al., 2011). Glutathione was increased about four-fold in catalase-deficient *cat2* mutants (mainly as GSSG), but most of this increase was accounted for by changes in chloroplast and vacuolar glutathione, with the cytosolic and nuclear glutathione pools remaining relatively stable (Queval et al., 2011). The K_MGSSG for uptake into barley vacuoles was found to be 0.4 to 0.6 mM (Tommasini et al., 1993) while values for AtMRP1 (At1g30400) and AtMRP2 (At2g34660) were reported to be 219 and 73 μM, respectively (Lu et al., 1998). In the absence of stress conditions, cytosolic GSSG concentrations may be well below these values (see section 8.1). This suggests that the activity of MRP proteins will be stimulated in response to stress-induced increases in cytosolic GSSG accumulation and function to dampen such increases. Further, specific MRPs are induced by oxidative stress (Sánchez-Fernández et al., 1998) and also in Arabidopsis mutants that accumulate high amounts of GSSG (Mhamdi et al., 2010a).

7. REDOX TURNOVER

The global tissue glutathione pool is largely reduced under optimal conditions. Divergence from this highly reduced state is an indicator of stress. However, it should be noted that certain compartments, such as the endoplasmic reticulum, are maintained in an oxidized state (Hwang et al., 1992; Enyedi et al., 2010), as are also some cell types (eg, those of the root quiescent center) or dormant tissues like seeds (Kranner and Grill, 1996; Kranner et al., 2002; Kranner et al., 2006). Decreases in GSH:GSSG may be produced by compromised regeneration of GSH, direct reaction with ROS and other oxidants, or the activities of several types of peroxidases. The principal enzymes involved in these reactions are now discussed.

7.1. Peroxidation Reactions

There is a close relationship between intracellular H_2O_2 availability and the status of the glutathione pool. This is most notably evident from the effect of inhibiting leaf catalase activities, either pharmacologically or genetically. While such inhibition causes modest or undetectable increases in H_2O_2 contents, it triggers a marked perturbation of both the glutathione pool size and redox state (Smith et al., 1984; May and Leaver, 1993; Willekens et al., 1997; Noctor et al., 2002; Riszhky et al., 2002; Queval et al., 2007, 2009; Chaouch et al., 2010). The effects of increased intracellular H_2O_2 availability appear to be quite specific to glutathione, and do not involve readily detectable changes in the status of other major antioxidants or redox couples (Mhamdi et al., 2010a).

Apart from catalase, various peroxidases can metabolize H_2O_2. Since the 1970's the predominant view has been that the major antioxidative H_2O_2-metabolizing peroxidases in plants are APXs, rather than GSH-dependent peroxidases, which are important in mammalian cells. However, GPXs have also been characterized in plants (Eshdat et al., 1997). Unlike the animal enzymes, plants GPXs are selenium-independent, because their catalytic action depends on cysteine rather than selenocysteine

(Eshdat et al., 1997). Plant GPXs can use both GSH and TRX as reductants (Herbette et al., 2002) but TRX is the more efficient reducing substrate (Iqbal et al., 2006). Thus, the enzymes can functionally be considered to be peroxiredoxins (PRX) rather than GPXs (Navrot et al., 2006). Several of these genes, such as *GPX6* (At4g11600), are induced by oxidative stress (eg, Mhamdi et al., 2010a). In addition to possible antioxidant functions, GPXs may be involved in redox signal transduction in plants (Miao et al., 2006), as they are in yeast (Delauney et al., 2002). They have also been described as playing a role in plant immune responses as well as photooxidative stress (Chang et al., 2009). Unlike APX, these enzymes can reduce both H_2O_2 and organic peroxides with similar efficiency (Iqbal et al., 2006; Navrot et al., 2006). Compared to APX (Asada, 1999), the catalytic rate and affinity for H_2O_2 of GPXs are rather low. This lends support to the notion that the main physiological roles of these enzymes is in regulating the concentrations of lipid peroxides that are poorly or not metabolized by catalase or APX, or in peroxide sensing.

Athough annotated plant GPXs use TRXs more efficiently than GSH, at least two other types of enzyme could be important in peroxidation of glutathione (Figure 6). Certain GSTs show GSH-dependent peroxidase activity against H_2O_2 and organic peroxides (Wagner et al., 2002; Dixon et al., 2009). Work in poplar has shown that glutathione could also be involved in peroxide metabolism though GRX-dependent PRX (Rouhier et al., 2002). It has been reported in yeast that GRXs can also directly reduce peroxides to the corresponding alcohol (Collinson et al., 2002).

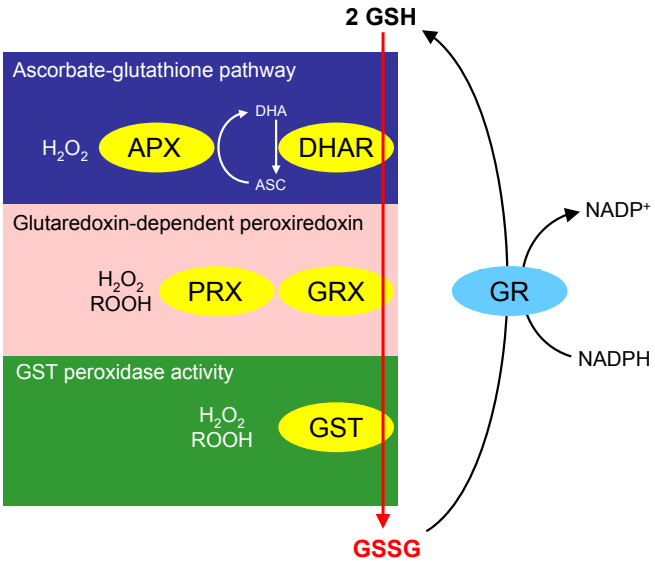

Figure 6. Major links between glutathione and peroxide metabolism.

The scheme is intentionally simplified. Oxidation of glutathione could also be caused, directly or via ascorbate, through direct chemical reactions with compounds such as superoxide. APX, ascorbate peroxidase. ASC, ascorbate. DHA(R), dehydroascorbate (reductase). GR, glutathione reductase. GRX, glutaredoxin. GST, glutathione *S*-transferase. PRX, peroxiredoxin. ROOH, organic peroxide.

7.2. Ascorbate Regeneration

The classic route by which GSH oxidation is coupled to H_2O_2 metabolism is the ascorbate-glutathione pathway (Foyer and Halliwell, 1976). In this pathway, GSH contributes to ascorbate regeneration by reducing DHA, either chemically or via enzymes called DHA reductases, which constitute a class of GSTs (Dixon et al., 2002). Overexpression studies have confirmed the role of DHAR in maintaining a high tissue ascorbate redox state, and have highlighted the roles of GSH-dependent ascorbate pools in a number of physiological responses, eg, the control of stomatal closure (Chen et al., 2003).

It remains unclear whether direct GSH peroxidation or oxidation by DHA is the major route for H_2O_2-dependent glutathione oxidation (Figure 6). Some relevant physiological evidence comes from analysis of catalase-deficient plants which, as noted above, are useful systems for probing the potential functions of reductive H_2O_2-metabolizing pathways in vivo (Dat et al., 2001; Mhamdi et al., 2010c). The major leaf catalase isoform in Arabidopsis is CAT2 (At4g35090; Queval et al., 2007; Mhamdi et al., 2010c). In Arabidopsis *cat2* knockouts, *DHAR2* (At1g75270), which is predicted to encode a cytosolic enzyme, is induced along with cytosolic *APX1* (At1g07890; Mhamdi et al., 2010a). This observation is consistent with studies of Arabidopsis *apx1* knockouts (Davletova et al., 2005), and suggests that the cytosolic ascorbate-glutathione pathway is important in metabolizing H_2O_2 originating in other compartments. However, tobacco plants deficient in both catalase and cytosolic APX show a less marked phenotype than the parent lines (Rizhsky et al., 2002), whereas Arabidopsis double *cat2 gr1* mutants have a more severe phenotype than *cat2* single mutants (Mhamdi et al., 2010a). While a more direct comparison of interactions between these components is required, the above results seem to be at odds with the notion that GR activity is solely required for the classical ascorbate-glutathione pathway. If this were the case, APX and GR1 deficiency should not produce what appear to be opposite effects in a catalase-deficient background. This and other observations of Mhamdi et al. (2010a) provide in vivo evidence that the GR-glutathione system could be important in ascorbate-independent H_2O_2 metabolism (eg, through some GSTs) as well as for the ascorbate-glutathione pathway. Further, such observations are consistent with specificity of redox functions for ascorbate and glutathione, as discussed elsewhere (Noctor et al., 2000; Potters et al., 2002; Foyer and Noctor, 2011).

7.3. Glutathione Reductase

7.3.1. Genes and Subcellular Localization

The high cellular GSH:GSSG ratio is maintained by GRs, which are flavoproteins with high affinity for both GSSG and NADPH (Halliwell and Foyer, 1978; Edwards et al., 1990). Classical fractionation studies described high GR activities in chloroplasts (Foyer and Halliwell, 1976) but GR has also been detected in the cytosol, mitochondria, and peroxisomes (Edwards et al., 1990; Rasmusson and Møller, 1990; Jiménez et al., 1997; Stevens et al., 2000; Romero-Puertas et al., 2006). Two genes encode GR in Arabidopsis. The first, *GR2* (At3g54660) encodes an enzyme corresponding to the pea GR that was the first protein shown to be dual-addressed to

plastids and mitochondria (Creissen et al., 1995). Dual localization of the gene product in these two compartments has also been demonstrated in Arabidopsis (Chew et al., 2003). Cytosolic GR activity is encoded by *GR1* (At3g24170) but proteomic analysis suggested that this gene also encodes peroxisomal GR activity (Kaur et al., 2009). It has recently been shown that import of GR1 into the peroxisomes occurs through the Peroxisomal Targeting Sequence 1 (PTS1) pathway, though targeting efficiency was considered to be relatively weak (Kataya and Reumann, 2010). From results obtained in pea, it seems that the peroxisomal enzyme represents a relatively minor part of the GR1 gene product (Romero-Puertas et al., 2006), which overall accounts for 30 to 60% of the total leaf enzyme activity in Arabidopsis (Marty et al., 2009; Mhamdi et al., 2010a). Available information therefore suggests that GR1 is mainly targeted to the cytosol, though an intriguing question concerns the physiological significance of its localization in the peroxisomes, which may be important sources of ROS and related signals (Foyer and Noctor, 2003; del Río et al., 2006; Nyathi and Baker, 2006; Mhamdi et al., 2010c).

7.3.2. Overexpression Studies

Glutathione reductase has been overexpressed in a number of plant species. In all cases the reported effects of enhanced GR activity in the chloroplast or cytosol are relatively modest, indicating that under many conditions, activity is sufficient to maintain glutathione pools and function (Aono et al., 1993; Broadbent et al., 1995; Foyer et al., 1991, 1995; Korneyev et al., 2005; Ding et al., 2009). However, increases in GR activity do increase the reduction state of the ascorbate pool (Foyer et al., 1995). These observations are entirely consistent with efficient coupling of the reactions of the ascorbate-glutathione pathway. Nevertheless, as noted above, there are specific situations such as increased availability of H_2O_2 where the glutathione pool becomes preferentially oxidized, suggesting that GR may become limiting for GSSG reduction. This could be a key property of pathway regulation, allowing altered glutathione status to transmit signaling information, and is discussed further below (section 8.1.1).

7.3.3. Mutagenesis Studies in Arabidopsis

Arabidopsis T-DNA mutants for *GR2* are embryo-lethal (Tzafrir et al., 2004), presumably reflecting the crucial importance of maintaining appropriate glutathione reduction states in plastids, mitochondria, or both, at least during certain developmental stages. In contrast, *gr1* knockout mutants are aphenotypic, despite a significant decrease in both total extractable GR activity and GSH:GSSG ratios (Marty et al., 2009; Mhamdi et al., 2010a). The aphenotypic nature of *gr1* mutants is due to the 'back-up' GSSG regeneration activity by the cytosol-located NTR-TRX system (Marty et al., 2009). The TRX system also replaces GR in insects and yeast (Kanzok et al., 2001; Meyer et al., 2008). Triple mutants in which both NTRs and GR1 are knocked out show a male-sterile phenotype, demonstrating the requirement for at least one of the systems for plant survival (Marty et al., 2009). Further, it was reported that *gr1* mutants were little affected in abiotic stress responses, and no increase in sensitivity to exogenously supplied H_2O_2 was observed (Marty et al., 2009). Based on this study, the specificity of *GR1* or *NTR-TRX* function with regard to glutathione reduction remained unclear. However, when the *gr1* mutation is introduced into the *cat2* background, in which intracellular H_2O_2 places an increased load on regeneration of GSH, the resulting double mutants show a much exacerbated phenotype compared to *cat2* (Mhamdi et al., 2010a). This effect is linked to dramatic oxidation and accumulation of glutathione in *cat2 gr1*, while the *gr1* mutation is associated with compromised responses to pathogens and altered expression of genes involved in defense hormone signaling (Mhamdi et al., 2010a). These observations strongly suggest that, although not required for growth in controlled environment conditions, *GR1* plays a specific role in stress, particularly biotic stress. They also underline the importance of the compartmentation of H_2O_2 production in the analysis of potential roles of specific components such as GR1 in redox homeostasis and stress responses. Further, transcriptomic patterns suggest that GR-GRX systems may function largely independently of TRXs in oxidative stress signaling, at least at the level of transcript abundance (Mhamdi et al., 2010a). However, the results of Marty et al. (2009) suggest that, under conditions in which GSSG accumulates, the resulting engagement of the NTR-TRX system could be one mechanism potentially contributing to TRX oxidation and signaling.

8. GLUTATHIONE IN REDOX SIGNALING

While thiol-disulfide redox signaling through the chloroplast TRX system was well established by the end of the 1970's, attention has been focused on glutathione-linked redox signaling only more recently (Foyer et al., 1997; May et al., 1998a). Both for TRX and for glutathione, essential information was generated by the sequencing and annotation of the Arabidopsis genome. This advance revealed the great number and diversity of TRXs and GRXs (Laloi et al., 2001; Lemaire, 2004; Lemaire et al., 2007; Meyer et al., 2008; Rouhier et al., 2008; Rouhier, 2010), and was the starting-point for the ongoing characterization of glutathione-mediated redox signaling systems in plants.

8.1. Glutathione Redox Potential and NADPH: Theoretical Considerations and Potential Importance in Signaling

A key outstanding issue is to what extent GR activity ensures that the glutathione and NADP(H) couples are in thermodynamic equilibrium (at the same redox potential). At cytosolic glutathione concentrations of 2 to 5 mM (Meyer et al., 2001; Queval et al., 2011), and an NADP(H) redox potential of -320 mV (ie, $NADP^+$:NADPH = 1), redox equilibrium would require the GSH:GSSG ratio to be in the range of 10^5 to 10^6. In plant tissues such as leaves in the absence of stress, measured GSH:GSSG ratios in extracts are much lower, typically about 20 (eg, Queval and Noctor, 2007; Mhamdi et al., 2010a), while intracellular ratios in other organisms are often cited as being in the range of 10^2 to 10^3 (eg, Trotter and Grant, 2002). Chung et al. (1991) proposed that GSH inhibition of GR prevents the two redox couples from reaching equilibrium. Another possibility is that global cellular or tissue measurements, while a useful indicator of redox perturbation, do not precisely

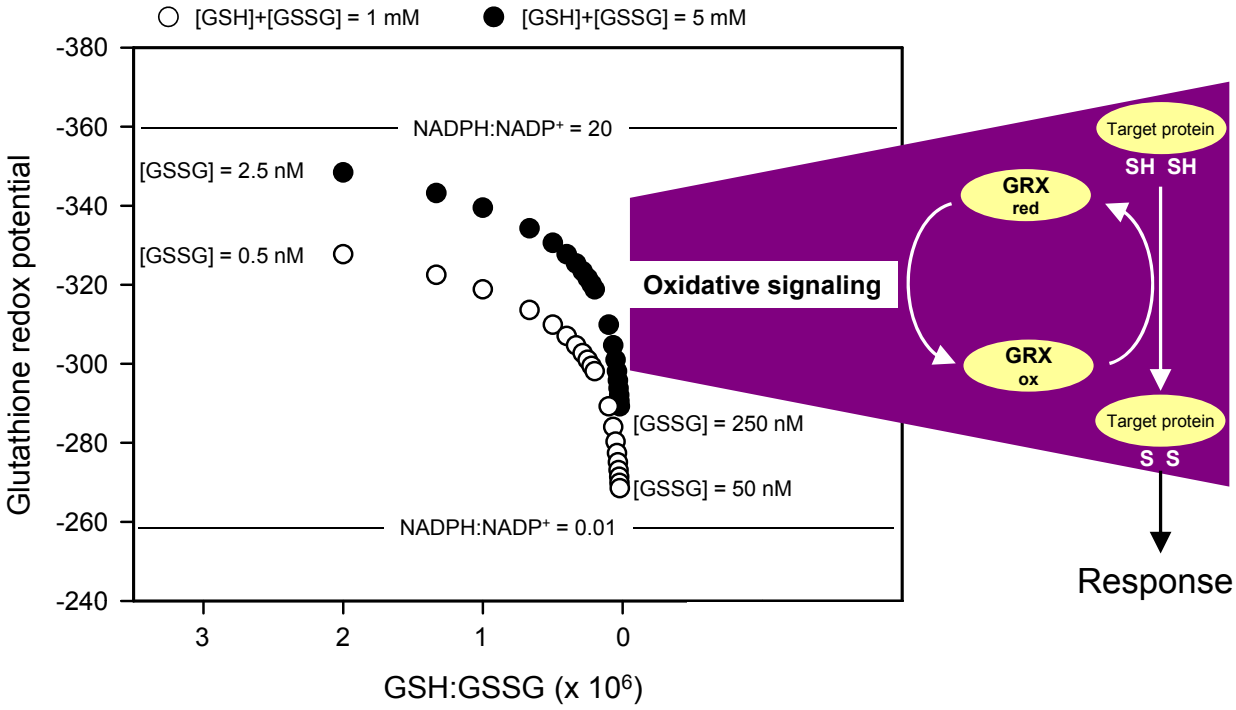

Figure 7. Scheme of glutaredoxin(GRX)-dependent oxidative signaling potentially mediated by changes in glutathione redox potential. The relationship between glutathione redox potential and GSH:GSSG is shown for two total glutathione concentrations, assuming a midpoint potential of -230 mV and constant pH. NADPH:NADP+ ratios at two glutathione redox potentials are shown assuming thermodynamic equilibrium between NADP(H) and glutathione. The horizontal lines on the graph indicate a redox potential span of about 100 mV corresponding to NADPH:NADP+ values of 20 (top) to 0.01 (bottom). GRX_{red}, reduced glutaredoxin. GRX_{ox}, oxidized glutaredoxin. For further discussion, see text.

reflect GSH:GSSG ratios in certain intracellular compartments, and there is accumulating evidence that this is indeed the case.

8.1.1. Insights from studies with redox-sensitive GFP

In vivo roGFP studies suggest that the glutathione redox potential in wild-type Arabidopsis in the absence of stress is lower than -300 mV in the cytosol and in other compartments (Meyer et al., 2007; Schwarzlander et al., 2008; Jubany-Mari et al., 2010). Given that the cytosolic NADPH:NADP+ ratio is not far removed from one (Igamberdiev and Gardeström, 2003), these redox potential values suggest that the two couples are close to redox equilibrium in this compartment. Such a situation could confer high sensitivity on the signaling function of glutathione redox potential mediated through GRX-dependent changes in protein thiol-disulfide status. A change in redox potential of about 50 mV is sufficient to significantly alter the balance between oxidized and reduced forms of TRX-regulated proteins (Setterdahl et al., 2002). For example, an increase in redox potential from -350 to -300 mV converts the TRX-regulated chloroplast glucose-6-phosphate dehydrogenase 1 (At5g35790) from almost completely inactive to active (Née et al., 2009).

The relationship between glutathione redox potential and redox state is shown in Figure 7. Knockout mutants for cytosolic NADP-isocitrate dehydrogenase (At1g65930) do not show a significant difference in whole leaf NADP+:NADPH ratios, but the absence of this enzyme does modify the GSH:GSSG ratio under conditions of increased H_2O_2 availability (Mhamdi et al., 2010b). This is consistent with the notion that changes in NADP reduction state that are undetectable in whole tissues may be sufficient in vivo to allow significant changes in glutathione redox potential, and hence signal amplification. Such effects may be of physiological significance, especially if localized to specific cells in certain conditions, eg, during plant responses to pathogens. Given the low K_M of GR for NADPH (Smith et al., 1989; Edwards et al., 1990) compared to likely cytosolic NADPH concentrations (around 150 µM: Igamberdiev and Gardeström, 2003), this could occur through adjustment of reactant concentrations in the NADP-glutathione equilibrium rather than kinetic limitation of GR activity by NADPH.

The above discussion suggests that glutathione oxidation during stress is unlikely to be caused by kinetic limitation of GR by reductant but that decreases in NADPH:NADP+ could impact thermodynamically on the glutathione pool. Moreover, a substantial body of literature data shows that the extractable activity of GR is the lowest of the classical ascorbate-glutathione pathway enzymes. Given that enzymes other than DHAR may also contribute to GSH oxidation under oxidative stress conditions (Figure 6), the relatively low capacity of GR may be important to allow GSSG accumulation to fulfil signaling functions. This may involve gradual uncoupling of ascorbate and glutathione reduction states as flux through the pathway accelerates. This notion is consistent with the behaviour of the two pools observed in several studies of catalase-deficient plants.

8.1.2. The significance of glutathione concentration

A potentially important factor in redox signaling is glutathione concentration. This is because the actual glutathione redox potential is related to $[GSH]^2$:GSSG (Mullineaux and Rausch, 2005; Meyer, 2008). Thus, if the GSH:GSSG ratio remains unchanged, decreases in glutathione concentration in themselves cause the redox potential to increase, ie, become more positive (Figure 7). The cytosolic thiol/disulfide redox potential as detected by roGFP was more positive in both *cad2* and *gr1* mutants (Meyer et al., 2007; Marty et al., 2009), underscoring the contributions of concentration (decreased in *cad2*) and GSH:GSSG (decreased in *gr1*) to the measured redox potential. Quantification of the glutathione redox potential in triple *clt1 clt2 clt3* mutants, perturbed in chloroplast envelope glutathione transport, revealed an increase of about 20 mV in the cytosol while the plastidial potential was not affected (Maughan et al., 2010). Decreased reduction state of GRXs caused by a more positive redox potential could explain observations in plants that both lack NTR and are deficient in glutathione (Reichheld et al., 2007; Bashandy et al., 2010).

Given that glutathione concentration can clearly increase in response to decreases in GSH:GSSG, it is unclear why the GSH:GSSG ratio does not seem to increase to compensate for decreases in glutathione concentration. One possibility is that the GSH:GSSG ratio is already extremely high in some compartments, as discussed above, and so cannot be increased further in response to decreases in glutathione concentration. Because the K_M GSSG values of GRs have been determined to be in the 10-50 µM range (Smith et al., 1989; Edwards et al., 1990), this may partly reflect a limitation over redox equilibrium with NADPH at very low GSSG concentrations.

8.2. Glutathione in the Regulation of Plant Development

Cell identity has a profound influence on signaling processes that govern cell fate (Jiang et al., 2006b) and responses to abiotic stress (Dinneny et al., 2008). Several lines of evidence demonstrate that glutathione status is important in plant development. The most convincing has come from analysis of the phenotypes of GSH-deficient Arabidopsis mutants, which has shown that glutathione has critical functions in embryo and meristem development (Vernoux et al., 2000; Cairns et al., 2006; Reichheld et al., 2007; Frottin et al., 2009; Bashandy et al., 2010). Other evidence has been provided by studies of cell proliferation using cell cultures or analysis of quiescence in meristems (Kerk and Feldman, 1995; Potters et al., 2002; 2004; Diaz Vivancos et al., 2010a,b). Glutathione status has also been implicated in influencing the regeneration efficiency of somatic embryos (Belmonte et al., 2005). The role of glutathione in the cell cycle has recently been reviewed (Diaz-Vivancos et al., 2010b).

8.3. Glutaredoxins

Thiol-disulfide exchange reactions are a fundamental mechanism of regulation of enzyme activity in plants (Buchanan and Balmer, 2005). While several chloroplast enzymes are regulated in this way by TRX, GRX may also couple glutathione redox potential to changes in protein thiol-disulfide status (Figure 7). Compared to many other organisms, terrestrial plants contain a large GRX family. These genes were classified by Lemaire (2004) into subfamilies depending on predicted active site motifs, with additional GRX or similar sequences described more recently (Navrot et al., 2006; Rouhier et al., 2006; Meyer et al., 2008). Based on a four amino acid motif at the active site sequence, the encoded proteins are grouped into 'classical' CxxC/S GRX (class I), 'monothiol' CGFS GRX (class II), and CC-type GRX (class III), with the last sub-class being specific to land plants (Lemaire, 2004; Rouhier, 2010). While specific nomenclatures have been proposed for some of these GRXs (Ziemann et al., 2009; Rouhier, 2010), much remains to be discovered regarding their functions and those of related sequences that have been described in *Arabidopsis*.

8.3.1. Class I and Class II Glutaredoxins

The activities of class I GRXs include thiol-disulfide exchange, (de)glutathionylation, and regeneration of PRX and methionine sulfoxide reductase (MSR; Rouhier et al., 2002, 2006; Zaffagnini et al., 2008; Tarrago et al., 2009; Gao et al., 2010). A DHAR activity has long been known for GRX (Wells et al., 1990) and appears to be specific to this type, but its physiological importance remains unclear. Members of both classes of GRX have been shown to play roles in assembly of iron-sulfur clusters (Rouhier et al., 2007; Bandyopadhyay et al., 2008), though this activity is most strongly associated with class II GRX (Rouhier, 2010).

Arabidopsis class II GRXs have been identified as interacting with ion channels or as involved in oxidative stress responses (Cheng and Hirschi, 2003; Cheng et al., 2006). The plastidic glutaredoxin CXIP1 (AtGRXcp1; At3g54900) is able to rescue the sensitivity of yeast GRX5 mutants to H_2O_2 and Arabidopsis mutants for this GRX have slightly increased sensitivity to exogenous H_2O_2 (Cheng et al., 2006). Manipulation of the expression of a tomato GRX with a CGFS active site in both Arabidopsis and tomato also modulates the antioxidative system and alters stress tolerance (Guo et al., 2010). Expression of a GRX from an arsenic-hyperaccumulating fern was also reported to increase thermotolerance in Arabidopsis (Sundaram and Rathinasabapathi, 2010).

As discussed further below, both class I and class II GRX have been shown to be competent in protein deglutathionylation in vitro (Gao et al., 2009a). Although members of the CGFS (class II) sub-family are sometimes referred to as 'monothiol' GRX, in at least some cases catalytic activity involves formation of an intramolecular disulfide bond with a second cysteine located distally from the GRX active site motif. These types of GRX may be dependent on TRX reductase rather than GSH for their reduction (Zaffagnini et al., 2008; Gao et al., 2009a).

8.3.2. Class III Glutaredoxins

Compared to the other GRX, relatively little is known about the biochemistry of the plant-specific class III GRX (CC-type or ROXY GRX). However, certain members of this class are the best characterized GRX in terms of physiological function, with three members implicated in plant development and defense. *GRX480*/*ROXY19* (At1g28480) overexpression represses the JA

marker gene, *PDF1.2* (Ndamukong et al., 2007) while *ROXY1* (At3g02000) and *ROXY2* (At5g14070) are involved in petal and anther development (Li et al., 2009). Both of these functions involve interaction with TGA transcription factors. Complementation experiments showed that the rice orthologs of *ROXY1* or *ROXY2* can rescue the defect in petal development in Arabidopsis *roxy1* mutants, as can *GRX480* (Li et al., 2009; Wang et al., 2009). Overexpression of Arabidopsis or rice *ROXY1* in Arabidopsis also increased susceptibility to *Botrytis cinerea* (Wang et al., 2009). This biochemical redundancy suggests that the key factor that determines the specificity of Class III GRX function may be temporal and/or spatial expression patterns (Ziemann et al., 2009). Both this notion and the proposed roles of this type of GRX in ROS signaling receive support from the analysis of plants with perturbations in glutathione status (Mhamdi et al., 2010a). H_2O_2-triggered alteration of glutathione status in *cat2* and *cat2 gr1* mutants modifies the abundance of transcripts encoding four class III GRXs but not other GRX types. These genes included *GRX480* as part of a generalized effect on JA-associated genes, while *ROXY1* or *ROXY2* expression were not affected (Mhamdi et al., 2010a).

8.4. Protein *S*-Glutathionylation

Glutathione can form stable mixed disulfide bonds with protein cysteine residues (protein *S*-glutathionylation or thiolation). *S*-Glutathionylation is a ubiquitous redox-sensitive and reversible modification of cysteinyl residues that can directly regulate the activity of the affected protein. Isolated reports of protein *S*-glutathionylation in plants have appeared over the years, eg, for seed acyl carrier protein (Butt and Ohlrogge, 1991), but it is only over the last decade that systematic attention has been paid to the significance and potential of this process. Elucidation of this phenomenon remains technically challenging, but several techniques have been used to identify target proteins (Ito et al., 2003; Dixon et al. 2005; Michelet et al., 2005, 2008 ; Zaffagnini et al., 2007; Holtgrefe et al., 2008; Gao et al., 2009a). To date, the information derived is largely qualitative and probably preferentially detects relatively abundant proteins. Further, the in vivo significance of the identified modifications remains in many cases to be established.

An intriguing finding is that f-type TRX is glutathionylated on a cysteine residue external to the active site, decreasing its activity against target proteins such as plastidial NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Michelet et al., 2005). The activity of another TRX-independent isoform of GAPDH is also decreased by glutathionylation (Zaffignini et al., 2007). Therefore, in conditions leading to glutathionylation in the chloroplast, the activities of both types of GAPDH are likely to be decreased, either directly or indirectly, through glutathionylation of TRX f. These observations point to a new layer of complexity in thiol-disulfide regulation of chloroplast metabolism. Interestingly, some of the proteins regulated by TRXs and glutathione, including the glutathionylated GAPDH isoform, are known to associate into complexes, eg, with CP12, in a TRX-dependent manner (Howard et al., 2008).

Recent studies also point to thiol-disulfide regulation of metabolism outside the chloroplast. The activity of the mitochondrial enzyme, glycine decarboxylase (GDC), is sensitive to oxidative stress (Taylor et al., 2002), and this enzyme has recently been shown to be inhibited by thiol modifications on several cysteine residues (Palmieri et al., 2010). Given that NADH production through this enzyme is highly active during photorespiration, this mechanism may act together with other thiol modifications, such as those that regulate mitochondrial alternative oxidase activity (Vanlerberghe et al., 1995; Gelhaye et al., 2005), as part of redox regulation related to ROS production by the respiratory electron transport chain. Glycolytic NAD-dependent GAPDH is a cytosolic enzyme that is also partly associated with mitochondria and the nucleus (Giegé et al., 2003; Anderson et al., 2004). This enzyme is inhibited by oxidation (Hancock et al., 2006), and has been identified as a glutathionylated protein (Dixon et al., 2005; Holtgrefe et al., 2008).

The in vivo mechanisms underlying *S*-glutathionylation remain to be unequivocally established. They may involve thiol-disulfide exchange between protein thiol groups and GSSG, perhaps catalyzed by certain GRX, occur through conversion of cysteine thiol groups to thiyl radicals or sulfenic acids, or be mediated by GSNO (Dixon et al., 2005; Gao et al., 2009b; Holtgrefe et al., 2008; Palmieri et al., 2010). Several possible routes of deglutathionylation are also possible, notably including GRX-dependent monothiol and dithiol mechanisms (Gao et al., 2009b). Biochemical analysis of *Chlamydomonas* proteins has shown that some class I and class II GRX are competent in deglutathionylation reactions, though there appears to be specificity within both groups (Zaffagnini et al., 2008; Gao et al., 2009a, 2010). Some class II GRXs appear to catalyze deglutathionylation through a monothiol mechanism, the intermediate glutathionylated GRX likely being resolved by formation of an intramolecular disulfide between the active site Cys and a partially conserved C-terminal Cys. This disulfide was shown to possess a low redox potential and to be reduced by thioredoxin reductase rather than by GSH (Zaffagnini et al., 2008). For some class II GRXs, the reductant of the intermediate glutathionylated GRX is TRX, not GSH, and the dithiol mechanism involves a disulfide bond with a Cys residue distal from the active site motif (Zaffagnini et al., 2008). Monothiol deglutathionylation mechanisms include CPYC-dependent reactions involving only the active site Cys that is closer to the N-terminus, as well as deglutathionylation via a poplar GRX with a CSYS active site (Couturier et al., 2009). GRX-catalyzed deglutathionylation through a monothiol mechanism is also likely part of the catalytic cycle of Arabidopsis MSRB1 (At1g53670) whereas MSRB2 (At4g21860) is regenerated through a TRX-dependent dithiol mechanism (Tarrago et al., 2009). While TRXs are not considered to be directly active against mixed disulfides, a role for yeast TRX in deglutathionylation has recently been suggested (Greetham et al., 2010).

Although several class III GRX showed modified transcript abundance in response to disulfide stress in Arabidopsis, no other type of GRX or TRX was affected, even when GSSG accumulation was very marked (Mhamdi et al., 2010a). This suggests either that transcriptional control of these types of proteins is not an important part of the response or that accumulation of GSSG is not in itself sufficient to induce significant protein *S*-glutathionylation.

8.5. Protein *S*-Nitrosylation and GSNO Reductase

Another glutathione-linked modification of protein cysteine residues is *S*-nitrosylation (Lindermayr et al., 2005; Romero-Puertas et al., 2008; Lindermayr and Durner, 2009). GSNO may be an

important physiological NO donor, for example, in the regulation of NPR1, where nitrosylation of proteine cysteine residues acts antagonistically to reduction by TRX (Tada et al., 2008). Inhibition of a methionine adenosyltransferase by GSNO-triggered *S*-nitrosylation has been proposed to be an important regulatory mechanism in ethylene synthesis (Lindermayr et al., 2006), while modification of PRX by *S*-nitrosylation has been implicated in the regulation of ROS signaling (Romero-Puertas et al., 2007).

Cytosolic GAPDH can be *S*-nitrosylated as well as *S*-glutathionylated (Lindermayr et al., 2006). Similar to the GDC P protein studied in leaf mitochondrial extracts (Palmieri et al., 2010), cysteine groups on purified GAPDH were found to undergo both modifications after GSNO treatment (Holtgrefe et al., 2008; Palmieri et al., 2010). Both for GDC and GAPDH, the modifications were associated with a loss of activity. The inhibition of GDC by thiol modifications adds to the growing awareness of the potential importance of photorespiration-linked redox reactions in plant stress responses (Foyer et al., 2009; Petershansel et al., 2010) while oxidative inhibition of GAPDH could contribute to regulating relative fluxes through the oxidative pentose pathway and glycolysis during stress (Holtgrefe et al., 2008).

Specific NO-producing enzymes in plants remain to be fully characterized, but GSNO reductase could be influential in regulating GSNO-dependent reactions. This enzyme is encoded by a single gene in Arabidopsis (At5g43940), and catalyzes the NADH-dependent reduction of GSNO to *S*-aminoglutathione, which then decomposes to ammonia and free GSH (Sakamoto et al., 2002; Feechan et al., 2005; Díaz et al., 2006; Barroso et al., 2006; Lee et al., 2008; Chen et al., 2009). As well as interactions with NO, glutathione has been implicated in the possible signaling functions of carbon monoxide in *Medicago sativa* (Han et al., 2007).

8.6. Glutathione in Environmental Responses

8.6.1. Light Signaling

Evidence for the role of glutathione in irradiance-dependent signaling came from the identification of the Arabidopsis *rax1* mutant. This line contains less than 50% wild-type glutathione contents, and shows enhanced constitutive expression of the high light-induced gene, *APX2* (Ball et al., 2004). Interestingly, overexpression of a tomato CGFS-type (class II) GRX in Arabidopsis also enhances expression of *APX2* (Guo et al., 2010). Several studies in Arabidopsis and other species point to a potential link between growth daylength, light signaling and glutathione status (Becker et al., 2006; Queval et al., 2007; Bartoli et al., 2008). Genetic evidence of a link between glutathione and photoreceptor signaling has been provided by identification of arsenic-tolerant mutants, called *ars4* and *ars5* (Sung et al., 2007). While *ars4* was shown to be a phytochrome A (*phyA*) mutant (Sung et al., 2007), *ars5* is affected in a component of the 26S proteasome (Sung et al., 2009). Mutants for *phyA* showed increased resistance to BSO as well as to arsenic (Sung et al., 2007), and *ars5* had enhanced levels of glutathione when exposed to arsenic, accompanied by increased *GSH1* and *GSH2* transcripts (Sung et al., 2009).

8.6.2. Biotic Interactions, Salicylic Acid, and Phytoalexin Synthesis

It is well established that exogenous GSH acts similarly to fungal elicitors in activating the expression of defense-related genes (Wingate et al., 1988; Dron et al., 1990) including *PATHOGENESIS-RELATED1* (*PR1*; Gomez et al., 2004b; Senda and Ogawa, 2004). Moreover, accumulation of glutathione is triggered by pathogen infection (Edwards et al., 1991; May et al., 1996a), and this can involve characteristic transient changes in glutathione redox state (Vanacker et al., 2000; Parisy et al., 2006). Similar changes have also been reported following exogenous application of the defense-related hormone salicylic acid (SA), or biologically active SA analogs (Mou et al., 2003; Mateo et al., 2006; Koornneef et al., 2008). Glutathione perturbation in catalase-deficient *cat2* is also linked to hypersensitive response (HR)-like lesions as part of a wide spectrum of defense responses that are conditionally induced in this line (Chaouch et al., 2010).

Thiol-disulfide status is clearly involved in the regulation of the SA-dependent NPR1 pathway (Déspres et al., 2003; Mou et al., 2003; Rochon et al., 2006; Tada et al., 2008). It was proposed that SA-dependent *PR1* induction requires reduction of disulfide bonds of the oligomeric form of NPR1 that could be mediated by TRX or GRX in vivo (Mou et al., 2003) A more recent study reported that monomerization of the NPR1 protein to the active form was catalyzed by TRX but that glutathione is involved through GSNO-dependent nitrosylation of NPR1 monomers (Tada et al., 2008). The reoligomerization was postulated to be necessary to prevent protein depletion (Tada et al., 2008). The identification of NPR1 as a target of *S*-nitrosylation underpins the involvement of GSH in plant disease resistance, in which protein *S*-nitrosylation is considered to be a key post-translational modification. The *Atgsnor1* mutant for the enzyme GSNO reductase has compromised resistance to both virulent and avirulent pathogens (Feechan et al., 2005).

Further evidence that glutathione is involved in defense against biotic stress came from the studies of *Arabidopsis* mutants altered in GSH synthesis or metabolism. Studies on the effect of GSH deficiency have reported conflicting results. Analysis of responses of *cad2* to virulent and avirulent strains of the oomycete *Peronospora parasitica* and the bacterium *Pseudomonas syringae* did not reveal compromised resistance (May et al., 1996b) but subsequent studies of *cad2* and *rax1-1* reported increased susceptibility to avirulent strains of *P. syringae* (Ball et al., 2004). Triple mutants defective in glutathione transport across the chloroplast envelope showed decreased expression of *PR1* and resistance to the oomycete *Phytophthora brassicae* (Maughan et al., 2010). The *pad2* mutant is deficient in the *Arabidopsis* phytoalexin camalexin (Glazebrook and Ausubel, 1994) and shows enhanced susceptibility to various bacterial, fungal and oomycete pathogens as well as to insects (Ferrari et al., 2003; Parisy et al., 2006; Schlaeppi et al., 2008). The affected gene in *pad2* was identified as *GSH1* and the line was shown to have GSH contents that were lower than those in *cad2* (Parisy et al., 2006). A comparative study of the allelic mutants *rax1*, *cad2*, and *pad2* revealed an inverse correlation between resistance to *P. brassicae* and glutathione contents, though only *pad2* was markedly affected in camalexin contents and its response to *P. syringae* (Parisy et al., 2006). These observations suggest that there is a critical glutathione status below which the accumula-

tion of pathogen-defense related molecules and consequently disease resistance, is impaired. This level could vary according to conditions and pathogen specificity. In the case of camalexin synthesis, it is not yet clear whether GSH acts as a regulatory component or as a precursor of the thiazole ring, or both (Parisy et al., 2006; Glawischnig, 2007; Nafisi et al., 2007). However, knockout mutants for *GR1*, which are not glutathione-deficient but which have a decreased GSH:GSSG ratio, accumulate less SA than wild-type and show increased sensitivity to virulent *P. syringae* (Mhamdi et al., 2010a). This observation suggests that glutathione redox potential could be one factor that determines some of the effects reported in glutathione-deficient mutants.

The components that link changes in glutathione status to pathogen responses remain for the most part unidentified. As well as GRX, GSNO, and *S*-glutathionylation reactions, GSTs may play important roles (Lieberherr et al., 2003; Sappl et al., 2004; Davoine et al., 2006). Induction of GSTs by SA and other hormones has been systematically studied (Sappl et al., 2009). An active process occuring at the pathogen entry site consists of conjugation of GSH to insect-deterrent indole glucosinolate hydrolysis products to mediate the accumulation of other antifungal glucosinolate compounds (Bednarek et al., 2009). An activation of both GST transcripts and protein activity was also observed in cryptogein-induced tobacco HR together with the conjugation of oxylipins to GSH. This was suggested to be an HR-linked event with a signaling role during plant defense (Davoine et al., 2006).

Another plant-microbe interaction in which GSH plays a significant role is symbiosis between legumes and nitrogen-fixing rhizobia, which involves the production of nodules. These organs contain high levels of both GSH and homoglutathione, and deficiency in these molecules inhibits nodule formation (Frendo et al., 2005; Pauly et al., 2006).

8.6.3. Glutathione and Cell Death

It has been proposed that the glutathione redox state is a key determinant of cell death and dormancy (Kranner et al., 2006). According to this model, an increase in redox potential above a threshold reducing value necessarily causes death and/or growth arrest. Increased glutathione leaf contents triggered by γ-ECS overexpression in the tobacco chloroplast triggered lesion formation and induced *PR* gene expression (Creissen et al., 1999). In tobacco deficient in catalase, lesions also appear under conditions that cause accumulation of GSSG (Chamnongpol et al., 1996; Willekens et al., 1997). Double Arabidopsis knockouts for catalase and GR1 (*cat2 gr1*) show dramatic oxidation of glutathione (more than 2 µmol GSSG g^{-1} FW, representing 95% of the leaf glutathione pool). While *cat2 gr1* undergoes leaf bleaching when grown in long days, this effect is associated with repression rather than stimulation of SA-dependent pathogen responses (Mhamdi et al., 2010a), even though a wide range of these responses is associated with the less marked glutathione oxidation observed in *cat2* single mutants (Chaouch et al., 2010). Moreover, H_2O_2-triggered lesions on the leaves of *cat2* can be reverted by blocking SA synthesis through the isochorismate pathway or by treating leaves with *myo*-inositol, but in neither case does the suppression of cell death correlate with less oxidized tissue glutathione status (Chaouch and Noctor, 2010; Chaouch et al., 2010). Glutathione perturbation is equally or more marked in *cat2 gr1* in short day as in long day growth conditions, but neither bleaching nor necrotic lesions are observed in the double mutant in short days (Mhamdi et al., 2010a). These observations suggest that perturbation of glutathione status is not in itself sufficient to cause bleaching or cell death. Accumulation of GSSG in *cat2* grown from seed in air is associated with a stunted phenotype, possibly related to the roles of thiols in the plant cell cycle, growth and development (Reichheld et al., 1999, 2007; Vernoux et al., 2000). However, significant GSSG accumulation is also observed in *gr1* knockout mutants, which are aphenotypic (Marty et al., 2009; Mhamdi et al., 2010a). Together with the recent study of Liedschulte et al. (2010), the available data suggest that high levels of glutathione (as GSH or GSSG) can be tolerated by plants and are not in themselves sufficient to trigger cell death pathways. As well as cellular tolerance of shifts in glutathione status, subcellular compartmentation could be a key issue.

One explanation of the difference between the effects of overexpressing monofunctional γ-ECS and bifunctional γ-ECS/GSH-S in the tobacco chloroplast is that lesions are caused by γ-EC accumulation. This metabolite can accumulate to high levels when γ-ECS is overexpressed alone, either in the cytosol or the chloroplast (Noctor et al., 1996, 1998a; Creissen et al., 1999). Support for this idea comes from the observation that tobacco overexpressing both γ-ECS and GSH-S had an ameliorated phenotype compared to tobacco expressing the first enzyme alone (Creissen et al., 1999). Differences in γ-EC accumulation may therefore explain the differences between the studies of Creissen et al. (1999) and Liedschulte et al. (2010). However, the extent of γ-EC accumulation in tobacco overexpressing the bifunctional bacterial enzyme has not yet been documented, and there are several lines of evidence that argue against this interpretation. The first is that γ-EC was increased rather than decreased in tobacco overexpressing both enzymes of glutathione synthesis (Creissen et al., 1999). Second, abolition of photorespiratory glycine production by high CO_2 enhances γ-EC accumulation in poplar expressing *E.coli* γ-ECS in the cytosol or chloroplast. In this condition, γ-EC can accumulate to more than 1 µmol.g^{-1} FW without triggering lesions (Noctor et al., 1997, 1999). Third, H_2O_2-activated glutathione accumulation in Arabidopsis *cat2* plants is accompanied by increased γ-EC contents and this does not in itself promote lesions (Queval et al., 2009). Currently available information suggests that neither total glutathione nor GSSG accumulation necessarily induces cell death in tobacco, poplar or Arabidopsis, and that further work is required to establish the significance of γ-EC accumulation in lesion formation. Despite this, there is a clear though apparently complex link between glutathione status and SA-dependent cell death.

8.6.4. Jasmonic Acid and Responses to Herbivores

While glutathione status is clearly implicated in SA signaling, several strands of evidence point to an equally influential role in JA signaling. The first indication of a link was provided by Xiang and Oliver (1998), who reported JA induction of transcripts encoding enzymes of GSH synthesis and GR. Subsequent work has shown

that other antioxidative genes can be induced by JA (Sasaki-Sekimoto et al., 2005) while this hormone has been implicated, together with thiol status, in resistance to selenite (Tamaoki et al., 2008). Overexpression of *GRX480* represses JA-triggered induction of the defensin, *PDF1.2* (Ndamukong et al., 2007) and SA-dependent repression of this marker gene is correlated with changes in glutathione status and opposed by inhibition of glutathione synthesis (Koornneef et al., 2008).

Analysis of *cat2*-deficient plants provided some evidence that H_2O_2 may oppose JA-regulated gene expression (Vanderauwera et al., 2005). A comparative analysis of *cat2* and *gr1* knockouts suggests that the H_2O_2-JA interaction is daylength-conditioned and may be at least partly mediated by glutathione status (Mhamdi et al., 2010a). Based on JA- or oxylipin-dependent expression patterns (Yan et al., 2007; Mueller et al., 2008), the peroxidatic or conjugation activities of GSTs against electrophilic metabolites (Wagner et al., 2002; Davoine et al., 2006), and the known interaction of CC-type GRX with transcription factors (Ndamukong et al., 2007; Li et al., 2009), glutathione may influence JA signaling through several GSTs and GRX. Specific genes within the GST family and the class III GRX subfamily show modified expression in response to changes in glutathione status in *cat2* and *gr1* mutants (Mhamdi et al., 2010a). Interestingly, GSNO reductase transcripts are decreased by both wounding and JA (Díaz et al., 2003).

Resistance to some insects is compromised in the glutathione-deficient *pad2* mutant and this is linked to decreased glucosinolates (Schlaeppi et al., 2008). The effect could be reverted by GSH treatment but not DTT, possibly suggesting that it is linked to a requirement for GSH in biosyntheses rather than mediated via redox changes (Schlaeppi et al., 2008). This is consistent with studies in tobacco engineered to express the Brassica glucosinolate pathway (Geu-Flores et al., 2009). It therefore seems that changes in glutathione status could impact functionally on JA-dependent stress responses through both a redox signaling effect, possibly mediated by GRX, and a requirement for GSH in glucosolinate synthesis.

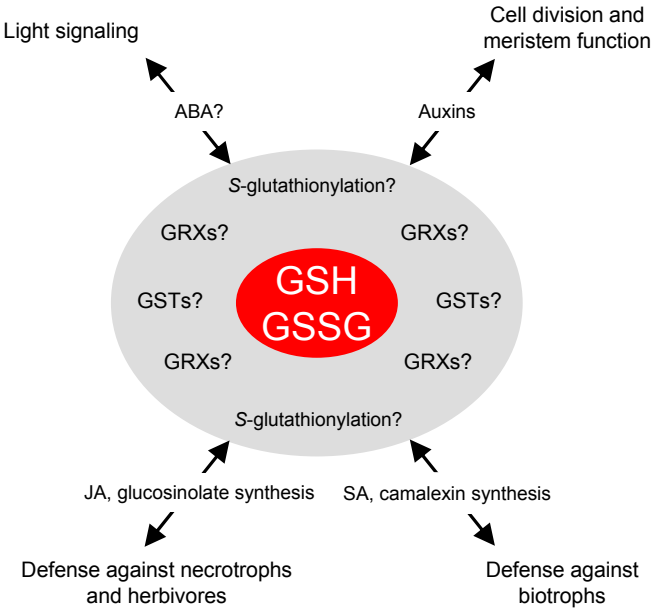

Figure 8. Hormone-dependent growth and stress responses affected by glutathione status.

ABA, abscisic acid. JA, jasmonic acid. SA, salicylic acid. Other abbreviations as defined in other legends.

9. CONCLUSION AND PERSPECTIVES

The list of cellular processes that are dependent on or influenced by glutathione in plants continues to grow. Probably the most exciting development over the last decade is the emergence of glutathione as an important signaling molecule. It is now clear that glutathione status can interact with several phytohormone-dependent pathways (Figure 8). The view of signal transduction operating in isolation through linear pathways is gradually being replaced by one that takes fuller account of cellular complexity. Arguably, the integration of signal transduction mechanisms in plants can be seen in at least some cases as analogous to metabolic control theory, where no single component is ever fully dominant. From a physiological 'whole plant' perspective, it is perhaps in this context that major redox buffers like glutathione act as an interface between the environment and the cell. Through its interactions with sulfur and nitrogen metabolism, with ROS and ascorbate via peroxidases and DHAR, with NADPH via GR, with protein thiol-disulfide groups through GRX, and with signaling metabolites and hormones via GSTs, glutathione is an outstanding candidate as a central "hub" molecule whose status can modulate other signaling pathways.

Many of the most significant breakthroughs have been made possible by studies of glutathione-deficient Arabidopsis mutants. While there is a clear requirement for glutathione in antioxidant metabolism as well as specific features of abiotic stresses involving detoxification of some heavy metals and xenobiotics or other potentially toxic compounds, current information concerning biotic stress points to at least two ways in which glutathione availability influences responses in Arabidopsis (Figure 8). The first is as a co-factor or sulfur source for the production of compounds such as camalexin and glucosinolates. The second involves a signaling function related to glutathione redox status and/or concentration. Regarding the second, a key issue is the importance of changes in glutathione status that may be monitored as glutathione redox potential (thermodynamic signaling). However, it is still unclear whether the redox potential of glutathione is a principal player or whether other factors such as modified abundance of specific glutathione-dependent proteins or reactive glutathione derivatives are more important. To date, documented changes in glutathione redox potential, whether in mutants or in stress conditions, are of the order of 20-30 mV, and it remains to be established whether these are sufficient to greatly modify the biological activity of dependent proteins. A related issue is whether the processes affected in studies of glutathione-deficient mutants simply reflect the requirement for a threshold glutathione concentration. Or are these processes also modified by dynamic changes in glutathione that can be induced by the environment or at specific developmental stages or in specific cells?

Despite the insights from studies of Arabidopsis mutants over the last decade, the components that link glutathione to phytohormone signaling remain in many cases to be identified. Although the signaling scheme shown in Figure 7 is based on a classical thiol-disufide exchange reaction, novel types of redox regulation may await discovery, eg, involving the plant-specific class III GRX. An important question concerns the relationship between the biological activity of these proteins and glutathione redox potential or concentration. Similarly, much remains to be described concerning the regulation of the intracellular distribution of glutathione and glutathione-dependent proteins.

While recent studies have established redundancy between glutathione and other thiol systems such as TRX, the physiological significance of this overlap remains to be resolved. Another issue is protein *S*-glutathionylation. The inventory of potential targets is currently being described, as are the mechanisms potentially regulating glutathionylation and deglutathionylation. An even more challenging task will be to elucidate the functional impact of such changes, both in terms of their quantitative influence on the biological activity of the affected protein, as well as their fractional control over the integrated network of subcellular and cellular regulation. The relative importance of the various mechanisms regulating glutathione synthesis in different conditions also remains to be established, as does the integration of these controls with changes in transport and degradation.

Research effort is gradually turning toward species of obvious agronomic importance. Part of the knowledge generated on glutathione in Arabidopsis may not be completely transferable to other species (eg, because of specificity of defense pathways). Nevertheless, our view is that much of the information generated from studies in Arabidopsis is generic, ie, it concerns cellular processes that cut across taxonomic boundaries. Apart from the well documented advantages of Arabidopsis as a model system, the plethora of specific genetic resources and tools now available should ensure that this species continues to feature strongly in studies on the roles of glutathione and glutathione-dependent processes in plant function.

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