Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling

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Abstract

Glutathione has numerous roles in cellular defence and in sulphur metabolism. These functions depend or impact on the concentration and/or redox state of leaf glutathione pools. Effective function requires homeostatic control of concentration and redox state, with departures from homeostasis acting as signals that trigger adaptive responses. Intercellular and intracellular glutathione pools are linked by transport across membranes. It is shown that glutathione can cross the chloroplast envelope at rates similar to the speed of biosynthesis. Control of glutathione concentration and redox state is therefore due to a complex interplay between biosynthesis, utilization, degradation, oxidation/reduction, and transport. All these factors must be considered in order to evaluate the significance of glutathione as a signalling component during development, abiotic stress, or pathogen attack.

Key words: Chloroplasts, γ-glutamylcysteine synthetase, glutathione, maize, plant–pathogen interaction, transporter, Triticum aestivum, wheat, Zea mays.

Introduction

Glutathione (γ-Glu-Cys-Gly) is a multifunctional metabolite in plants (Fig. 1). It is a major reservoir of non-protein reduced sulphur, and has crucial functions in cellular defence and protection. Glutathione reacts chemically with a range of active oxygen species (AOS), while enzyme-catalysed reactions link GSH to the detoxification of H2O2 in the ascorbate–glutathione cycle. Importantly, GSH protects proteins against the denaturation that is caused by oxidation of protein thiol groups during stress. All these functions involve the oxidation of the thiol group, principally to form glutathione disulphide (GSSG). Cellular GSH:GSSG ratios are maintained by glutathione reductase (GR), a homodimeric flavoprotein that uses NADPH to reduce GSSG to two GSH. Like all other aerobic organisms, plants maintain cytoplasmic thiols in the reduced (–SH) state because of the low thiol-disulphide redox potential imposed by millimolar amounts of glutathione, which acts, therefore, as a thiol buffer. Although transient disulphide bonds do occur during the catalytic cycle of some enzymes, stable protein disulphide bonds are relatively rare except in quiescent tissues such as seeds. The multiple roles of GSH within the cell, together with the stability of GSSG, may make this redox couple ideally suited to information transduction. The GSH/GSSG ratio is likely to be far more influential in the control of gene expression and protein function than the absolute size of the glutathione pool. This review specifically addresses the key factors involved in glutathione homeostasis (synthesis, sinks, partitioning, and transport) that are central to the putative roles of this compound in signalling.

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Abbreviations: AOS, active oxygen species; CAT, catalase; chl chlorophyll; CHS, chalcone synthase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; EC, γ-glutamylcysteine; ECS, γ-glutamylcysteine synthetase; FW, fresh weight; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSH-S, glutathione synthetase; GSSG, glutathione disulphide; GST, glutathione S-transferase; JA, jasmonic acid; PGA, 3-phosphoglyceric acid; SA, salicylic acid; SAR, systemic acquired resistance.

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In addition to its antioxidant functions, glutathione is a precursor of phytochelatins and a substrate for glutathione-S-transferase (GST; Fig. 1). Over and above its function in the ascorbate–glutathione cycle, glutathione acts as a direct electron donor to peroxides in reactions catalysed by glutathione peroxidase (GPX). The animal GPX is a selenoprotein that detoxifies H$_2$O$_2$ at high rates. By contrast, plant GPXs are not constitutive but are induced in response to stress. They do not contain selenium and only catalyse GSH-dependent reduction of H$_2$O$_2$ at rates which are very low compared with the high rates of H$_2$O$_2$ generation in plants (Foyer and Noctor, 2000). In plants, ascorbate peroxidase (APX) and catalase (CAT) are predominant in the detoxification of H$_2$O$_2$ while GPXs are more important in other areas of oxidant metabolism, including the removal of lipid and alkyl peroxides (Eshdat et al., 1997). The only clear demonstration of GPX targeting thus far has shown direction to the chloroplast (Mullineaux et al., 1998). In addition to their role in conjugation, GSTs can use GSH to reduce peroxides (Cummins et al., 1999). Transgenic tobacco lines overexpressing plant GST/GPX were reported to show enhanced antioxidant capacity and substantial improvement in seed germination and seedling growth under stress (Roxas et al., 1997). GSTs form a large, heterogeneous family of proteins that share the defining characteristic of catalysing the nucleophilic attack of the sulphur atom of GSH (or homologue) on the electrophilic centre of their substrates. They are therefore responsible for the removal of compounds that are potentially genotoxic or cytotoxic by virtue of their reaction with electrophilic sites in DNA, RNA and proteins. It has become clear, however, that the function of GSTs is not limited to these reactions: GSTs also seem to be involved in a ‘ligandin’ function important, for example, in the anthocyanin synthesis pathway (Marrs, 1996). It may be that certain GSTs function as flavonoid-binding proteins as suggested recently for AN9, a GST required for efficient anthocyanin export from the cytosol in petunia (Mueller et al. 2000). Consistent with this is the

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Fig. 1. Glutathione biosynthesis and interacting processes in plant cells. γ-EC, γ-glutamylcysteine; γ-ECS, γ-glutamylcysteine synthetase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulphide; GSH-S, glutathione synthetase; GST, glutathione S-transferase.
observation that the anthocyanin content of Arabidopsis leaves correlated with GSH content in plants with modified capacity for GSH biosynthesis (Xiang et al., 2001).

While it has long been accepted that glutathione is essential for vigour, it has only recently been recognized that this tripeptide cannot be functionally replaced, except perhaps by one of its homologues. The rnl1 mutant of Arabidopsis, which is deficient in γ-ECS and contains no detectable glutathione, has a marked phenotype with an absence of root development and a small shoot system, and can survive only in tissue culture supplied with GSH (May et al., 1998a). Similarly, transgenic Arabidopsis with less than 5% wild-type leaf glutathione contents were shown to be significantly decreased in size and biomass, and were more sensitive to environmental stress (Xiang et al., 2001). A strong correlation has also been demonstrated beween root GSH content and the capacity of the cells in the root apical meristem to proliferate (May et al., 1998a). However, although a 96% reduction of shoot glutathione contents was associated with shorter roots in transformed Arabidopsis, the decrease in root length was only of the order of 40% (Xiang et al., 2001).

All these recent developments underline the importance of the control of glutathione concentration and redox state in plant cells. This review discusses the many advances made over the last decade in the understanding of glutathione biosynthesis, and also begins to address the key issue of compartmentation and related transport processes. These processes, complex and poorly defined, are discussed here in the light of new data obtained in the authors’ laboratory.

Biosynthesis of glutathione

Enzymes and genes

The pathway of glutathione biosynthesis is well established and is similar in plants, animals and microorganisms. In two ATP-dependent steps, catalysed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GSH-S), the constituent amino acids are linked to form the complete tripeptide (Fig. 1). The N-terminal peptide bond linking glutamic acid to cysteine in GSH is unusual in that glutamic acid is linked via the γ- rather than the α-carboxyl group. The low activities of γ-ECS and GSH-S in plants, and the complexities of the procedures for enzyme extraction and assay, have precluded extensive purification and kinetic characterization. Consequently, much of the current knowledge of their structure, regulation and function has been gleaned from molecular techniques and plant transformation. It is clear that the two-step reaction sequence occurs in both chloroplastic and non-chloroplastic compartments and is found in photosynthetic and non-photosynthetic tissues (Foyer and Noctor, 2001). A gene encoding γ-ECS, here denoted as gsh1, was originally cloned from Arabidopsis thaliana by complementation of an E. coli mutant deficient in this enzyme (May and Leaver, 1994). Heterologous expression of the Arabidopsis γ-ECS in a yeast mutant recovered only 10% of the GSH measured in the wild-type yeast (May and Leaver, 1994). This discrepancy provoked much speculation concerning the identity of the cloned gene, but further complementation studies have now confirmed that this gene does indeed encode a protein with true γ-ECS activity (May et al., 1998a).

Functional complementation of an E. coli mutant deficient in GSH-S activity was also used to clone the Arabidopsis thaliana gene for this enzyme, which is denoted here as gsh2 (Rawlins et al., 1995). The ability of several plant species to make homologues of glutathione depends on the specificity of the synthetases involved. Specific legume GSH-Ss use either glycine to form GSH or β-alanine to form homoglutathione. Recent work in Medicago truncatula suggests that separate genes encode GSH-S and homoglutathione synthetase (hGSH-S) and that the divergence in specificity has arisen by gene duplication after the evolutionary divergence of the Leguminosae (Frendo et al., 1999). The two genes are very homologous and are found on the same fragment of genomic DNA. In a consideration of the distribution of the biosynthetic enzymes in legume nodules, Becana et al. have suggested that γ-ECS is plastidic, hGSH-S is cytosolic and GSH-S isoforms exist in both the cytosol and mitochondria in several legume species (Becana et al., 2000).

Regulation of biosynthesis

Glutathione synthesis is controlled primarily by γ-ECS activity and cysteine availability: As in animals, the activity of γ-ECS limits the rate of glutathione synthesis in plants under most conditions. Consistent with this notion is the observation that the cad-2 Arabidopsis mutant, which has a mutation in the gsh1 gene, has only one-third of the tissue glutathione contents of the wild-type (Cobbett et al., 1998). Antisense suppression of γ-ECS in Arabidopsis also causes substantial decreases in leaf glutathione (Xiang et al., 2001). A key role for γ-ECS in controlling the rate of glutathione synthesis is supported by the increases in extractable enzyme activity in tissues treated with cadmium (Rüegsegger and Brunold, 1992). Most tellingly, overexpression of an E. coli γ-ECS but not GSH-S, in poplar or tobacco substantially increases leaf glutathione contents (Strohm et al., 1995; Noctor et al., 1996, 1998; Creissen et al., 1999), as does homologous overexpression of the Arabidopsis γ-ECS (Xiang et al., 2001).
Bacterial genes encoding γ-ECS and GSH-S have been introduced into poplar, mustard and tobacco (Strohm et al., 1995; Foyer et al., 1995; Noctor et al., 1996, 1998; Zhu et al., 1999; Pilon-Smits et al., 1999; Creissen et al., 1999). Over-expression, with targeting of the bacterial enzyme protein to either the chloroplast or cytosol, led to marked increases in enzyme activity. Increases in γ-ECS, but not GSH-S, not only led to constitutive increases in leaf glutathione (up to 400%: Noctor et al., 1996, 1998; Creissen et al., 1999) but glutathione was also increased in xylem sap, phloem exudates and roots (Herschbach et al., 2000). Of particular note is the observation that the cysteine pool was not depleted by the increased demand for thiols, but was even slightly enhanced in response to increased γ-ECS activities, pointing to co-ordinate regulation of cysteine synthesis and glutathione synthesis. This observation is supported by leaf thiol contents in plants homologously over-expressing the plant γ-ECS (Xiang et al., 2001). Despite enhanced GSH contents in the phloem of poplar, sulphur uptake by the roots was markedly enhanced to meet the requirements of increased demand for sulphur (Herschbach et al., 2000). Nevertheless, incubation of leaf discs with cysteine increased glutathione contents substantially in untransformed and transformed poplars, particularly in the light, suggesting that cysteine supply remains a key limitation (Strohm et al., 1995; Noctor et al., 1996, 1997).

The dipeptide produced by the γ-ECS reaction is present at very low levels in most untransformed plants. In the poplars overexpressing γ-ECS, however, γ-EC was greatly increased. In some conditions, this was attributable to insufficient availability of Gly (Noctor et al., 1997). Even when Gly was abundant, however, γ-EC was still much higher than in untransformed plants, reflecting a shift in control from γ-ECS to GSH-S, whether the bacterial γ-ECS was present in the cytosol or chloroplast (Noctor et al., 1998). This suggested that overexpression of both enzymes together would increase the potential for constitutive enhancement of tissue glutathione contents even further than that achieved by γ-ECS overexpression alone. This effect was observed when tobacco lines overexpressing each of the biosynthetic enzymes from *E. coli* were crossed to produce hybrids over-producing both enzymes although, surprisingly, γ-EC contents were found to be higher in the hybrid lines than in those lines overexpressing γ-ECS alone (Creissen et al., 1999). The marked phenotype produced by chloroplastic γ-ECS overexpression in tobacco complicates the interpretation of these results. By contrast, a phenotype linked to chloroplastic γ-ECS overexpression was not observed in poplar (Noctor et al., 1998) or *Brassica juncea* (Zhu et al., 1999; Pilon-Smits et al., 1999), except that these transformed plants were more, rather than less, stress tolerant. Similar results have been reported in transformed *Arabidopsis* overexpressing the endogenous γ-ECS (Xiang et al., 2001). The above evidence demonstrates that the most important factors controlling plant glutathione are the activity of γ-ECS and the availability of cysteine, and recent work suggests that these two factors may be co-ordinated (H Rennenberg, personal communication). The *in vivo* activity of γ-ECS is determined by control at multiple levels, and these are discussed in the following sections.

**Control of transcription and translation of γ-ECS and GSH-S:** Studies in animals, particularly on cancer cells challenged with chemotherapeutic agents, have shown that transcription of the γ-ECS gene is regulated by protein factors and by conserved antioxidant response elements upstream of the coding sequence (Foyer and Noctor, 2001). Relatively little is known about the co-ordinate regulation of expression of gsh1 and gsh2 in plants, but it is clear that GSH and GSSG per se exercise little or no control over transcription (Xiang and Oliver, 1998). Similarly, H2O2 did not affect transcript abundance. The abundance of gsh1 and gsh2 transcripts was increased by cadmium in *Brassica juncea* (Schäfer et al., 1998) and by both cadmium and copper in *Arabidopsis* (Xiang and Oliver, 1998). Jasmionic acid (JA) also increased gsh1 and gsh2 transcripts and a common signal transduction pathway may be involved (Xiang and Oliver, 1998). Interestingly, JA is involved in the control of glucosinolate synthesis, activation of which can represent a significant increase in sulphur demand in the Brassiceae (Doughty et al., 1995). Although transcript abundance was increased by heavy metals and JA, oxidative stress was required for the translation of the transcripts, implicating regulation at the post-transcriptional level and a possible role for factors such as H2O2 or modified GSH/GSSG ratios in de-repressing translation of the existing mRNA (Xiang and Oliver, 1998). It is interesting to note that among the most spectacular increases in glutathione are those observed when plants deficient in CAT are placed in conditions favouring photosorption (Smith et al., 1984; Willekens et al., 1997), where the accumulation in total glutathione is accompanied by a marked decrease in the reduction state of the pool. A similar response was elicited by exposing poplar leaves to ozone (Sen Gupta et al., 1991). The 5′untranslated region (5′UTR) of the gsh1 gene was found to interact with a repressor-binding protein that was released upon addition of H2O2 or changes in the GSH/GSSG ratio (Xiang and Bertrand, 2000). A redox-sensitive 5′UTR-binding complex is thus suggested to control γ-ECS mRNA translation in *A. thaliana* (Xiang and Bertrand, 2000).

**Post-translational control of γ-ECS:** Post-translational regulation of γ-ECS through end-product inhibition by
GSH is a crucial factor in controlling GSH concentration in animals and plants. Covalent modification may also be influential. There is some evidence to suggest that rat γ-ECS is regulated by protein phosphorylation (Sun et al., 1996) but this has not yet been found in studies on γ-ECS from plants. May et al. concluded that protein factors are involved in post-translational control of γ-ECS and are required for full activity (May et al., 1998b). The failure of the plant enzyme to operate ectopically was explained by the absence of such endogenous plant factors (May et al., 1998a, b). In the animal enzyme system, a smaller regulatory subunit acts to increase the catalytic potential of the larger catalytic subunit by increasing its $K_i$ value for GSH and decreasing the $K_m$ for glutamate, thereby alleviating feedback control and allowing the enzyme to operate under in vivo conditions (Huang et al., 1993). It is nevertheless clear that even in the absence of the smaller subunit, the large catalytic subunit is capable of effective catalysis, since overexpression of this polypeptide alone yielded increased glutathione levels in transfected human cells (Mulcahy et al., 1995). The highest glutathione contents were, however, obtained by dual overexpression of both subunits (Mulcahy et al., 1995).

While protein factors have not been identified in plants, and there is as yet no evidence for control of γ-ECS by phosphorylation, several enzymes in plants are controlled by interactions between phosphorylation status and factors such as 14-3-3 proteins, regulatory components found in several compartments of the plant cell (DeLille et al., 2001). This interaction inactivates enzymes such as nitrate reductase, but also confers stability against proteolytic attack. It is as yet unclear whether this type of regulation might be important in glutathione synthesis in plants, but it is perhaps worth considering a potential role in the post-translational control of glutathione synthesis. Non-linearity of the specific activity of γ-ECS with protein concentration has been alluded to previously (Hell and Bergmann, 1990). As part of a study on the intracellular distribution of GSH metabolism in wheat (see below), γ-ECS activity was measured in unfractionated leaf extracts and in purified chloroplasts. In both cases, specific activity increased substantially as protein was increased (Fig. 2). At the highest amounts of protein in the assay, the activity in wheat leaf extracts was very similar to that reported previously (approximately 0.5 nmol mg$^{-1}$ protein min$^{-1}$: cf. Fig. 2 and Hell and Bergmann, 1990). In chloroplast extracts, the activity showed an almost linear increase with protein and, at the highest protein concentration, attained values that were more than 4-fold higher than those found in whole leaf extracts (Fig. 2). On a chlorophyll basis, maximal γ-ECS activity was approximately twice as high in chloroplast extracts as in whole leaf extracts. These effects are unlikely to be due to low-molecular-weight effectors since similar results were obtained whether or not extracts were desalted prior to assay.

γ-ECS from whole tobacco and parsley cells has been shown to be subject to inhibition by GSH, in a manner that is competitive with respect to glutamate (Hell and Bergmann, 1990; Schneider and Bergmann, 1995). The $K_{iGSH}$ was 0.27–0.45 mM at a glutamate concentration of 10–20 mM (Hell and Bergmann, 1990). Figure 3 shows...
Table 1. γ-ECS activity extracted from purified chloroplast shows similar sensitivity to inhibitors to the activity from whole leaves

Activities are given in nmol mg\(^{-1}\) protein min\(^{-1}\) (% activity in the absence of inhibitors in brackets). Extracts and inhibitor were co-incubated for 15 min prior to the initiation of the reaction. Chloroplasts extracted as in Table 2. γ-ECS assayed as in Fig. 2. The ratio of soluble protein to chlorophyll in the isolated chloroplasts was approximately half that of whole leaf extracts. BSO, buthionine sulfoximine.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Enzyme activity (% control)</th>
<th>Leaves</th>
<th>Chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>0.58 (100)</td>
<td>1.07 (100)</td>
<td></td>
</tr>
<tr>
<td>1 mM GSH</td>
<td>0.54 (93)</td>
<td>0.90 (84)</td>
<td></td>
</tr>
<tr>
<td>10 mM GSH</td>
<td>0.14 (24)</td>
<td>0.03 (3)</td>
<td></td>
</tr>
<tr>
<td>1 mM BSO</td>
<td>0.11 (18)</td>
<td>0.14 (13)</td>
<td></td>
</tr>
</tbody>
</table>

that the enzyme extracted from purified wheat chloroplasts, assayed at a constant protein concentration, was also sensitive to GSH. γ-ECS from wheat chloroplast and whole leaf material showed a similar sensitivity to inhibition by GSH and by buthionine sulfoximine (Table 1). It would appear, therefore, that chloroplastic and extrachloroplastic isoforms possess similar regulatory properties, although this notion can only be definitely confirmed by biochemical studies of the extrachloroplastic enzyme(s). Given the likely difficulties of isolating cytosolic γ-ECS through classical techniques, purification through heterologous expression of a cloned cDNA offers the best approach to this question.

Intercellular compartmentation of glutathione synthesis

The implications of heterogeneity of cells with regard to glutathione metabolism have only recently become the focus of research effort. Apart from the pioneering studies of Rennenberg (see below), little information on this question had appeared in the literature. Given that the sensitivity of certain cells may be explained by their lack of adequate glutathione production or recycling, this question is key.

Compartmentation in C\(_3\) leaves: Glutathione is not produced at equivalent rates by all tissues or, indeed, by all cells within a tissue. Of particular note is the high capacity for GSH biosynthesis in some types of trichomes. These specialized unicellular or multicellular structures on the epidermis can have a protective function in excreting toxic compounds such as cadmium. The trichomes found on the stem and leaf surface of various A. thaliana ecotypes, show much higher expression of enzymes involved in the synthesis of cysteine and GSH and have GSH contents 2–3 times higher than the surrounding basal and epidermal cells (Gutierrez-Alcala et al., 2000). The evidence for GSH, GSSG and GS-conjugate transport systems on the plasma membrane associated with systemic transport has recently been discussed (Foyer et al., 2001) and hence these systems will not be elaborated upon here further.

Compartmentation in maize leaves: An extreme example of the differential intercellular partitioning of glutathione metabolism is observed in maize. In common with other plants that show ‘Kranz anatomy’ and C\(_4\) photosynthesis, maize leaves have two photosynthetic cell types whose functions are very different. The enzymes of the Benson–Calvin cycle, which are very sensitive to redox regulation, are localized in bundle sheath chloroplasts. In contrast to the mesophyll, the bundle sheath cells have very low amounts of photosystem II, ferredoxin and ferredoxin-NAD\(^+\) reductase and, therefore, a lower capacity for the photochemical production of reducing power. \(\text{H}_2\text{O}_2\) was found only in the mesophyll compartment in optimal growth conditions (Douli et al., 1997) but accumulated in the bundle sheath cells at low temperatures (Pastori et al., 2000a). Following exposure to cold stress, oxidative damage was found almost exclusively in the bundle sheath (Kingston-Smith and Foyer, 2000).

Recent evidence suggests that the sensitivity of maize leaves to chilling-induced precocious senescence is related to the ability to synthesize and regenerate GSH (Kocsy et al., 2000) and to the absence of antioxidant generation and recycling capacity in the bundle sheath cells (Kingston-Smith and Foyer, 2000). Like DHAR, GR is localized only in the leaf mesophyll cells. By contrast, other antioxidant enzymes are either restricted to the bundle sheath cells (APX and superoxide dismutase) or are found to be approximately equally distributed between the two cell types (CAT and MDHAR; Douli et al., 1997; Pastori et al., 2000a). The exclusive localization of GR activity in the mesophyll cells may be explained by the comparative lack of reductant in the bundle sheath cells (Douli et al., 1997). Because of their low water-splitting capacity, bundle sheath cells may not generate sufficient NADPH to support the reduction of GSSG and DHA. GSSG and DHA produced in the bundle sheath tissues must, therefore, be transported to the mesophyll tissues to be reduced.

The absence of GR from the maize bundle sheath is due to post-transcriptional regulation, since GR transcripts are found in both cell types (Pastori et al., 2000b). Cysteine is synthesized in the bundle sheath whereas GSH-S activity is located predominantly in the mesophyll cells (Burgener et al., 1998). It appears, therefore, that glutathione is synthesized in the cells where GR is present and that the bundle sheath relies on the mesophyll for both the synthesis of glutathione and the reduction of GSSG. cDNAs corresponding to maize γ-ECS and GSH-S mRNA have recently been isolated. The 1664 bp nucleotide sequence obtained for γ-ECS mRNA
(EMBL Nucleotide Sequence Database Acc. No. AJ302783) consists of a 38 nt 5' untranslated region that precedes the first ATG, a 1317 nt open reading frame encoding 437 amino acids and a 309 nt 3' untranslated region. No transit peptide could be identified from the amino acid sequence and analysis with the PSORT program showed high probability of a cytosolic localization. The 1608 bp nucleotide sequence of the mRNA isolated for GSH-S (EMBL Nucleotide Sequence Database Acc. No. AJ302784) presents an open reading frame between nucleotides 278 and 1510 that encodes a peptide of 409 amino acids, with no transit peptide. Southern blot analysis indicates that the gene encoding GSH-S is present as a single copy. Screening of a BAC library taken by isolating intact chloroplasts from wheat leaves. Although highly intact wheat chloroplasts can be prepared by lysis of protoplasts prepared via enzymatic digestion (Edwards et al., 1978), this method is of questionable suitability for the study of the distribution of stress-linked components because the isolation procedure itself affects AOS production and the antioxidant system (Ishii, 1987; Papadakis et al., 2001). Therefore, the development of a more direct and rapid method for the isolation of intact wheat chloroplasts was sought, using the mechanical homogenization of leaf tissue. While mechanical homogenization is easily applied to species with soft leaf tissue (notably spinach and pea), the literature contains very few reports of the successful use of this technique in the isolation of wheat chloroplasts, probably because fragments of fibrous bundle-sheath strands are released during tissue homogenization and cause rupture of the fragile chloroplasts. Nevertheless, by using young leaves, it was possible to develop a method producing an adequate yield (0.5–1 mg chlorophyll) of predominantly intact chloroplasts (Table 2). This method allowed chloroplasts to be liberated and separated from the homogenate in under 5 min and to be purified in less than 30 min. Given that all leaf antioxidative enzymes, including chloroplastic isoforms, have thus far been shown to be nuclear-encoded, this rapid isolation technique should avoid possible artefactual changes in enzyme distribution. The chloroplasts were photosynthetically competent, as judged by their high rates of electron transport (Table 2). Although unable to catalyse CO2-dependent O2 evolution at appreciable rates, the chloroplasts were competent in O2 evolution when supplied with 3-phosphoglyceric acid (Table 2). This indicates activity of the phosphate translocator and at least two enzymes of the Calvin cycle, as well as retention of nucleotides (NADP(H), adenylates). The chloroplast volume (Table 2) was within the range of that measured in other species (Heldt, 1980).

Expressed with respect to chlorophyll, NADP-GAPDH activity in the wheat chloroplasts was similar to that measurable in the unfractionated homogenate (Table 3). The latency of this activity in the chloroplast preparation was greater than 80%. While a large part of GR activity was located in the chloroplasts, the proportion of DHAR associated with the organelle was rather

**Glutathione homeostasis and signalling**

Intracellular compartmentation of glutathione metabolism

Intercompartmental variations (e.g. chloroplast versus cytosol, apoplast versus cytosol) in glutathione concentration and redox state may be crucial in signalling. By the end of the 1970s, it was known that spinach chloroplasts contained high concentrations of glutathione (Foyer and Halliwell, 1976) and that photosynthetic tobacco cells exported glutathione into the culture medium much faster than did heterotrophically grown cells (Bergmann and Rennenberg, 1978). It was subsequently shown that GSH is translocated from source leaves in the phloem (see references in Herschbach et al., 2000). As discussed above, γ-ECS and GSH-S activities were shown to be located both inside and outside the chloroplast (Klapheck et al., 1987; Hell and Bergmann, 1988, 1990) and the ability of photosynthetic cells to synthesize GSH in chloroplastic and cytosolic compartments was confirmed by overexpression studies (Noctor et al., 1996, 1998; Creissen et al., 1999). Together, these observations indicate that photosynthetic cells are likely to be able to export chloroplastically-produced glutathione from the cell. Studies have been conducted of 35S-GSH uptake into tobacco cells and bean leaf protoplasts (Schneider et al., 1992; Jamaï et al., 1996), and a GS-conjugate transporter is known to operate at the tonoplast (Foyer et al., 2001), but nothing is known about glutathione transport across the chloroplast envelope. The following sections present results that have recently been obtained on compartmentation and transport, and these findings are discussed within the context of published literature data in order to evaluate the role of different processes in the control of the intracellular distribution of glutathione metabolism.

**Distribution of glutathione metabolism between the chloroplast and the rest of the cell**

Because of the agronomic importance of wheat, the compartmentation of glutathione metabolism was undertaken by isolating intact chloroplasts from wheat leaves. Although highly intact wheat chloroplasts can be prepared by lysis of protoplasts prepared via enzymatic digestion (Edwards et al., 1978), this method is of questionable suitability for the study of the distribution of stress-linked components because the isolation procedure itself affects AOS production and the antioxidant system (Ishii, 1987; Papadakis et al., 2001). Therefore, the development of a more direct and rapid method for the isolation of intact wheat chloroplasts was sought, using the mechanical homogenization of leaf tissue. While mechanical homogenization is easily applied to species with soft leaf tissue (notably spinach and pea), the literature contains very few reports of the successful use of this technique in the isolation of wheat chloroplasts, probably because fragments of fibrous bundle-sheath strands are released during tissue homogenization and cause rupture of the fragile chloroplasts. Nevertheless, by using young leaves, it was possible to develop a method producing an adequate yield (0.5–1 mg chlorophyll) of predominantly intact chloroplasts (Table 2). This method allowed chloroplasts to be liberated and separated from the homogenate in under 5 min and to be purified in less than 30 min. Given that all leaf antioxidative enzymes, including chloroplastic isoforms, have thus far been shown to be nuclear-encoded, this rapid isolation technique should avoid possible artefactual changes in enzyme distribution. The chloroplasts were photosynthetically competent, as judged by their high rates of electron transport (Table 2). Although unable to catalyse CO2-dependent O2 evolution at appreciable rates, the chloroplasts were competent in O2 evolution when supplied with 3-phosphoglyceric acid (Table 2). This indicates activity of the phosphate translocator and at least two enzymes of the Calvin cycle, as well as retention of nucleotides (NADP(H), adenylates). The chloroplast volume (Table 2) was within the range of that measured in other species (Heldt, 1980).

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*Glutathione homeostasis and signalling*
Table 2. Characterization of chloroplasts isolated mechanically from young wheat leaves

Data for chloroplasts from pea leaves, isolated by a similar protocol, are shown for comparison. Values are means ± SD of 23 (wheat) and three (pea) independent chloroplast preparations. (Table 2) must therefore be treated as lower limits for the chloroplast allocation within photosynthetic cells: true values would be higher if a significant fraction of enzyme is also present in non-photosynthetic cells.

Table 3. Distribution of enzymes associated with glutathione metabolism between the chloroplastic and extra-chloroplastic compartments of young wheat leaves

Table 4. Chloroplasts from wheat leaves retain only a small proportion of thiols present in the leaf

Thiols were extracted from chloroplasts and leaves by acid extraction into 0.1 M HCl, 1 mM EDTA and analysed by reverse-phase HPLC with fluorimetric detection of monobromobimane derivatives (by a method modified from Noctor and Foyer, 1998). Contents are expressed in nmol mg⁻¹ chl (means ± SD of three independent leaf extracts or chloroplast preparations). hmGSH, hydroxymethyl glutathione (γ-Glu-Cys-Ser).
this notion. First, a higher chloroplast glutathione concentration (63–81 nmol mg⁻¹ chl) was reported in spinach, representing a mean concentration of 3.5 mM (Foyer and Halliwell, 1976). Second, a higher proportion of glutathione was also found in chloroplasts isolated non-aqueously from barley (Smith et al., 1985). Here, more than 50% of leaf glutathione was recovered in the chloroplasts, although this method may lead to overestimation due to adhesion of extrachloroplastic material to the isolated chloroplasts. Using marker enzymes to correct for this artefact, a value of 35% leaf glutathione in pea chloroplasts isolated in non-aqueous media was calculated (Klapheck et al., 1987). By contrast, the same article reported that chloroplasts prepared in aqueous media from pea protoplasts retained only 5% of glutathione present in the parent protoplasts (Klapheck et al., 1987). When isolated in aqueous media, pea chloroplasts had glutathione contents of 7–22 nmol mg⁻¹ chl, values very similar to those we found in wheat (cf. Table 3). Moreover, another study in pea found only 10% of the leaf glutathione in percoll-purified chloroplasts (Bielawski and Joy, 1986). It was concluded that pea chloroplasts lose glutathione during extraction in aqueous media, probably due to leakage to small molecules during the isolation (Klapheck et al., 1987).

Another possibility is the operation of transporters (see below). Whatever the processes responsible for low contents following aqueous isolation, it seems clear that chloroplasts from wheat, like those from pea, lose glutathione and other thiols during this method of extraction while retention of glutathione is higher in spinach chloroplasts. Interestingly, it was reported that, expressed on a chlorophyll basis, the glutathione content of pea protoplasts was similar to that of pea leaves (Klapheck et al., 1987). By contrast, mesophyll protoplasts have been purified from wheat (authors’ unpublished results) that have high photosynthetic activity (100–170 μmol O₂ mg⁻¹ chl h⁻¹ in the presence of NaHCO₃ but that contain only approximately 30% of the initial leaf glutathione content (c. 60 and 200 nmol mg⁻¹ chl in protoplasts and leaves, respectively).

Table 5 presents a summary of the principal literature data on the chloroplast complement of glutathione, together with values for the four enzymes that were measured in wheat. In the leaves of dicotyledonous species, γ-ECS and GSH-S have been found to be more or less equally divided between chloroplastic and non-chloroplastic compartments, with a somewhat higher proportion of γ-ECS found in the chloroplast (Table 5). The effects of protein concentration on γ-ECS activity (Fig. 2) prevented estimating the distribution of this enzyme in wheat leaves, but it seems clear that, whatever the precise distribution, a higher proportion of this enzyme is found in the wheat chloroplast than GSH-S.

### Table 5. Literature data for the fraction of tissue glutathione and associated enzymes found in plastids

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Species and tissue</th>
<th>% in plastid</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-ECS</td>
<td>Pea leaves</td>
<td>72b</td>
</tr>
<tr>
<td></td>
<td>Spinach leaves</td>
<td>61b</td>
</tr>
<tr>
<td></td>
<td>Maize roots</td>
<td>46b</td>
</tr>
<tr>
<td>GSH-S</td>
<td>Cultured tobacco cells</td>
<td>24a</td>
</tr>
<tr>
<td></td>
<td>Pea leaves</td>
<td>48b, 47–69b</td>
</tr>
<tr>
<td></td>
<td>Spinach leaves</td>
<td>58b</td>
</tr>
<tr>
<td></td>
<td>Maize roots</td>
<td>8b</td>
</tr>
<tr>
<td>GR</td>
<td>Pea leaves</td>
<td>52–75b, 77b, 77b</td>
</tr>
<tr>
<td></td>
<td>Spinach leaves</td>
<td>80b, 67b</td>
</tr>
<tr>
<td></td>
<td>Spinach leaves</td>
<td>9b, 28b</td>
</tr>
<tr>
<td></td>
<td>Pea leaves</td>
<td>65b</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Pea leaves</td>
<td>10b, 5b, 35b</td>
</tr>
<tr>
<td></td>
<td>Barley leaves (chloroplasts isolated in non-aqueous media)</td>
<td>50–65b</td>
</tr>
</tbody>
</table>

In nmol chloroplast protein⁻¹ min⁻¹, maximal activities were 2.3 (γ-ECS: Fig. 2) and 0.23 (GSH-S). This is surprising considering that overexpression studies strongly suggest that the first enzyme exerts the major limitation on synthesis of GSH in both cytosolic and chloroplastic compartments (Strohm et al., 1995; Noctor et al., 1996, 1998). However, studies of spinach chloroplasts, and of barley and pea chloroplasts isolated non-aqueously, indicate a chloroplastic GSH concentration close to 5 mM (Foyer and Halliwell, 1976; Smith et al., 1984; Klapheck et al., 1987) which would be sufficient to inhibit the chloroplastic γ-ECS by about 90% (Fig. 3) at 10–20 mM Glu, a likely chloroplastic concentration of this amino acid (Winter et al., 1994). It is perhaps worth noting that analysis of maize roots also found only a small fraction of GSH-S in the plastid (Table 5). The distribution of these enzymes could be dependent on developmental stage. Even though the wheat leaves used here were photosynthetically competent, they were nevertheless young. Literature data suggest, perhaps, that in more mature leaves, GSH-S is more strongly associated with the chloroplast (Table 5).

The data presented in Table 2 for GR are in agreement with literature studies (Table 5). In leaves, the bulk of GR activity is found in the chloroplast whereas root plastids may contain a lower proportion of the total cellular activity (Table 5). About 20% of the pea leaf activity was associated with the cytosol (Edwards et al., 1990). Lower GR activities have been reported in isolated mitochondria and peroxisomes (Edwards et al., 1990; Jimenez et al., 1997). Of the four enzymes discussed here, GR is by far...
the best characterized at the gene level. The first gene sequence encoding plant GR was isolated from pea, shown to encode a product with an N-terminal sequence characteristic of chloroplast-targeting sequences (Creissen et al., 1992), and later the product was found to be targeted to both chloroplasts and mitochondria (Creissen et al., 1995). Subsequently, cDNAs for other isoforms have been isolated from various species. Multiple activity bands have been identified in protein extracts from pea and spinach (Edwards et al., 1990; Foyer et al., 1991).

Less is known about the compartmentation of DHAR. The data in wheat (Table 3) give a value intermediate between those found in spinach and pea (Table 5). Because various enzymes can catalyse GSH-dependent reduction of dehydroascorbate as a ‘secondary’ reaction, the presence of a specific DHAR activity in chloroplasts has been the subject of controversy (Foyer and Mullineaux, 1998). Very recently in spinach, however, a chloroplast DHAR has been purified and a corresponding gene cloned and sequenced (Shimaoka et al., 2000). The authors reported a stromal DHAR activity of 34 μmol mg⁻¹ chl h⁻¹ (Shimaoka et al., 2000). Since DHAR activities from leaf tissue are typically 100–400 μmol mg⁻¹ chl h⁻¹, this would also represent a fairly small proportion associated with the chloroplast, although part of the high extrachloroplastic activity may be attributable to proteins other than a specific DHAR. It is interesting that GR/DHAR ratios appear to be markedly higher in the chloroplast than outside this organelle: on the basis of differential effects on the ascorbate and glutathione redox states observed in transgenic and mutant plants, it was suggested that DHAR activity in the chloroplast may be too low to couple the ascorbate and glutathione pools effectively (Noctor et al., 2000). In a recent theoretical article, the first attempt to model flux through the chloroplast Mehler-peroxidase and ascorbate–glutathione cycles is reported (Polle, 2001). It was concluded that chloroplastic DHAR is likely to be unimportant in chloroplast redox cycling, but that the chemical reduction of DHA by GSH would be sufficiently fast to allow an effective ascorbate–glutathione cycle (Polle, 2001). It is nevertheless clear that the redox states of the leaf ascorbate and glutathione pools can vary independently: decreased expression of the chloroplastic protein 2-cys peroxiredoxin led to a more oxidized ascorbate pool without effect on the highly reduced glutathione pool (Baier et al., 2000). Both thermodynamic and kinetic considerations predict that glutathione should be oxidized before ascorbate (Noctor et al., 2000; Polle, 2001), and this is indeed observed in plants deficient in CAT (see below). The factors that may allow DHA to accumulate in the presence of abundant GSH have been discussed, and include microcompartmentation within the chloroplast (Noctor et al., 2000; Polle, 2001). Polle’s model is an excellent first step towards the comprehensive evaluation of oxidant processing and antioxidant cycling within the chloroplast, and presents or reinforces several important conclusions concerning the importance of non-enzymatic reduction and the likely location of ‘rate-limitations’ (Polle, 2001). Such approaches are likely to be very useful in understanding the response of chloroplast-derived stress to leaf physiology. Nevertheless it is noted that the common assumption that the chloroplast is the predominant site of AOS production in leaf cells may not be valid under many conditions (Noctor et al., 2002), and that it cannot be excluded that the chloroplast antioxidative system may sometimes be subject to oxidative loads of both chloroplastic and extra-chloroplastic origin.

**Transport of glutathione across the chloroplast envelope**

It has previously been reported that incubation of wheat chloroplasts with 35S-labelled GSH at 1 and 100 μM resulted in time-dependent uptake that was linear for at least 15 and 8–10 min, respectively (Noctor et al., 2000). To characterize the uptake process, rates were measured over the first 5 min following addition of 35S-GSH, within which time uptake was linear at all concentrations between 1 μM and 1 mM (Fig. 4). Regression analysis

![Fig. 4](image)
was used to calculate rates: fitted lines gave a positive intercept on the y-axis (Fig. 4), probably indicating a rapid binding to the external surface of the chloroplast followed by a constant rate of uptake. At low concentrations (1–50 μM) the time-dependent uptake results in an accumulation of glutathione to a calculated chloroplast concentration of up to 5-fold the external concentration. Given that these external concentrations represent values at least 10-fold lower than the internal chloroplast concentration before addition of labelled GSH (c. 0.5 mM; see above), the data suggest active uptake of external glutathione. This conclusion is supported by the concentration dependence of GSH uptake (Fig. 5).

Uptake was linear up to 20–30 μM GSH then showed saturation at around 100–200 μM GSH followed by a further increase in rate up to a concentration of 1 mM (Fig. 5). The results suggest that at least two systems are able to take up GSH across the chloroplast envelope, one showing half-saturation at around 30–50 μM GSH with a maximal capacity of approximately 0.6–0.8 nmol mg\(^{-1}\) chl min\(^{-1}\) and a second with lower affinity and higher capacity. Transport at 50 μM GSH was not affected by added ATP or by light (data not shown). No difference in uptake was observed if the possible oxidation of GSH was countered by the presence of NADPH and yeast GR in the external medium. Although direct uptake of the disulphide form has not been examined, the presence of GSSG at 10 and 500 μM significantly inhibited uptake of GSH at 10, 50 and 500 μM. At a constant GSH concentration of 50 μM, inhibition by GSSG was half-maximal at approximately 0.4 mM (Fig. 6). These data suggest that the two forms of glutathione can be transported by common systems, although GSH appears to be preferred. GSSG was also reported to inhibit GSH uptake by bean protoplasts (Jamai et al., 1996), but not by tobacco cells (Schneider et al., 1992). It is conceivable that the weak effect of GSSG in wheat chloroplasts (Fig. 6) could be due to an effect on \(^{35}\)S-GSH concentration rather than an effect on the transport process itself. However, mixtures of GSSG and GSH are relatively stable at neutral pH so an effect of GSSG on the chemical GSH concentration is unlikely given the brevity of each incubation. It is possible, nonetheless, that thiol-disulphide exchange reactions result in conversion of \(^{35}\)S-GSH to \(^{35}\)S-GSSG, particularly when GSSG is in excess of GSH. Here, it is worth noting that Schneider reported no effect of 0.25 mM GSSG on the uptake of \(^{35}\)S-GSH (50 μM) into tobacco cells during a 2 h incubation at pH 6.0 (Schneider et al., 1992). Although this study’s experiments were conducted at pH 7.6, which is likely to be more conducive to thiol-disulphide exchange, all solutions were prepared immediately before assay and the incubation period used (5 min) was much shorter than those employed previously in studies of glutathione uptake across the plasmalemma. Nevertheless, it remains unclear whether the effect of GSSG is mediated at the level of transport.

The capacity of the high-affinity wheat chloroplast glutathione uptake system was 40–60 nmol mg\(^{-1}\) chl h\(^{-1}\)
while, at physiological GSH concentrations (1 mM), the uptake rate was about 200 nmol mg⁻¹ chl h⁻¹ (Fig. 5). By applying a modified but similar method, it was not possible to detect significant uptake into wheat mesophyll protoplasts within 60 min, at either pH 7.6 or pH 5.5. This contrasts with the literature report of significant GSH uptake into broad bean protoplasts, where transport was shown to be due to a single saturable phase with \( K_m \) 0.4 mM and \( V_{max} \) of 2.1 nmol 10⁷ protoplasts min⁻¹ (Jamaï et al., 1996). This difference may be attributable to differences in methodology or to inactivation of the wheat plasmalemma translocator during protoplast isolation. It is worth comparing maximum GSH transport rates across the plasmalemma with those measured in wheat chloroplasts. A mean chl content per wheat protoplast of 11.8 ± 2.0 pg (\( n = \)five independent protoplast preparations) was measured. Assuming a similar chl content, a rate of 2.1 nmol×10⁷ broad bean protoplasts min⁻¹ converts to around 1 nmol mg⁻¹ chl h⁻¹, though this capacity is unlikely to be reached at physiological extracellular GSH concentrations. In photoheterotrophic tobacco cells, as in wheat chloroplasts, two phases were identified in kinetic characterization of GSH uptake (Schneider et al., 1992). A high-affinity system displayed a \( K_m \) of 18 μM and a capacity of 19–20 nmol g⁻¹ dry weight min⁻¹, while at higher concentrations a second phase was observed with \( K_m \) 780 μM and a capacity of c. 170 nmol g⁻¹ dry weight min⁻¹ (Schneider et al., 1992). The physiological significance of the second system is unclear. Given a chl content in the region of 10–20 mg g⁻¹ dry weight, high-affinity transport by the tobacco cells would proceed at about 60–120 nmol mg chl⁻¹ h⁻¹. The capacity of wheat chloroplast transport is therefore intermediate between those previously measured in photoheterotrophic tobacco cells and broad bean protoplasts.

The bean protoplast plasmalemma transports GSSG at higher rates than GSH (Jamaï et al., 1996), consistent with a predominant physiological role in the recovery of glutathione oxidized in the apoplast, where GR activity is low or nil. Further work is required to identify possible chloroplast envelope transporters which might preferentially transport GSSG. It remains unclear whether GSSG and GSH are transported by the same system at the plasmalemma. The two species had distinct kinetics, GSH showing a single saturable phase with \( K_m \) 0.4 mM whereas GSSG showed two saturable phases (Jamaï et al., 1996). Protoplast transport of the two species were similarly dependent on pH and both resulted in decreased acidification of the extracellular medium in leaf pieces (Jamaï et al., 1996).

A high-affinity glutathione transporter has recently been cloned from yeast, with a \( K_m \) value close to that of the high-affinity system characterized in tobacco cells and wheat chloroplasts (54 μM: Bourbouloux et al., 2000). Negligible inhibition was observed with other peptides or amino acids, but glutathione conjugates and GSSG both competed significantly with GSH. A database search of amino acid sequences identified homologues from other organisms, including five from Arabidopsis, which had 38–51% identity with the cloned yeast gene (Bourbouloux et al., 2000).

Although glutathione concentrations in the vacuole are thought to be low, it is clear that the compound enters via transport of conjugates. Transport from the cytosol to the vacuole occurs via a Mg-ATP glutathione-S-conjugate transporter which is up-regulated along with GSTs upon exposure to xenobiotics (Martinoia et al., 1993; Li et al., 1995). The glutathione S-conjugates formed with anthocyanin and medicarpin are also transported into the vacuole by a specific glutathione S-conjugate transporter (Li et al., 1995), where they are further metabolized (Marrs, 1996; for further discussion of vacuolar uptake, see Foyer et al., 2001).

A key question concerning glutathione transporters is whether they catalyse net transport. Many plastid envelope translocators involved in primary C and N metabolism generally catalyse strict exchange reactions (e.g. phosphate translocator, dicarboxylic acid transporter, glutamate transporter, adenylate translocator). An obvious candidate for exchange with glutathione is not apparent, and further work is required to resolve this question. If an exchange mechanism is not involved, a unidirectional transport of glutathione would presumably require energy input. Evidence was obtained that uptake of both GSH and GSSG into bean protoplasts was driven by the proton gradient across the plasmalemma (Jamaï et al., 1996). On the other hand, the rate of GSH uptake into photoheterotrophic tobacco cells by the high-affinity system decreased as the pH was lowered from 7.0 to 5.0 (Schneider et al., 1992). The pH gradient across the chloroplast envelope is smaller than that across the plasmalemma and is very light-dependent (Werdan et al., 1975). The mechanism of chloroplast envelope transport of glutathione requires further investigation. Another interesting question is whether specificity to glutathione, observed in plasmalemma transport (Jamaï et al., 1996; Bourbouloux et al., 2000), is conferred by the Glu-Cys \( \gamma \)-carboxy peptide bond, or whether the transporter recognizes the thiol group. The work of Schneider et al., which reported inhibition by pretreatment of the cells with the alkylating reagent iodoacetate but not by the presence of GSSG during the uptake assay, suggests that the transport may involve thiol-binding (Schneider et al., 1992). On the other hand, the results of Jamaï et al. suggest that the characteristic N-terminal peptide link may be crucial (Jamaï et al., 1996). It is interesting to note, however, that the cloned yeast transporter showed little activity with \( \gamma \)-EC (Bourbouloux et al., 2000).
Glutathione homeostasis: the relationship between concentration and redox state

It is an interesting question whether many plant processes are subject to strict homeostasis in the sense in which this term is applied to mammalian systems. Plant metabolism is generally less insulated from environmental variation and, as a result, has evolved considerable flexibility and redundancy. Two independent properties of any given pool of glutathione are obvious: its concentration and its redox state. The first, although subject to regulation at multiple levels (notably, as far as homeostasis is concerned, by end-product feedback inhibition), can vary considerably. Leaf glutathione concentrations fluctuate seasonally, diurnally, and are influenced by plant nutrition, particularly sulphur availability. Earlier evidence that glutathione acts homeostatically in sulphur metabolism, being synthesized primarily in the leaves then translocated in the phloem to regulate sulphate uptake at root level, has been succeeded by a more complex picture that glutathione acts homeostatically in sulphur metabolism, particularly sulphur availability. Earlier evidence that glutathione acts homeostatically in sulphur metabolism, being synthesized primarily in the leaves then translocated in the phloem to regulate sulphate uptake at root level, has been succeeded by a more complex picture that glutathione acts homeostatically in sulphur metabolism, particularly sulphur availability.

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It is clear from this equation that changes in glutathione redox potential do not occur if GSH/GSSG remain constant, even if the total concentration of the pool changes dramatically. Hence, the total glutathione concentration and its redox state may be independent parameters, from a chemical point of view. However, signalling initiated by changes in the redox state may lead to up-regulation of glutathione synthesis and, hence, increases in the total concentration. A likely redox potential for the chloroplastic glutathione redox couple is −0.23 V (Foyer and Noctor, 2000). The glutathione redox potential of animal cells has been estimated to vary from about −0.24 V in actively dividing cells to approximately −0.17 V in cells undergoing apoptosis (Schafer and Buettner, 2001).

Maintenance of homeostasis in the face of all the metabolic demands placed on the glutathione pool involves a complex interplay between synthesis, degradation, transport, storage, oxidation–reduction, further metabolism and catabolism as plants respond to environmental, developmental and nutritional cues. Redox cycling is much faster than synthesis, transport or degradation. However, the antioxidant systems ensure that any changes in glutathione redox state are relatively slow. Although it is difficult to know the rate of redox turnover of glutathione in vivo (though see Polle, 2001, for a thorough analysis of flux through the chloroplast antioxidative system), plants deficient in CAT display probably the best documented and most striking perturbation of the glutathione pool. Low activities of the major leaf peroxisomal form of this enzyme mean that the copious amounts of H$_2$O$_2$ generated in C$_3$ plants in photosynthesis must be metabolized by an alternative route. This imposes a marked increase in the oxidative load on the photosynthetic cell which, ultimately, results in cell death and necrosis (Smith et al., 1984; Willekens et al., 1997). For a limited period of a few days, however, barley CAT mutants can cope with photorespiratory H$_2$O$_2$ without showing deleterious effects on either photosynthesis or phenotype (Smith et al., 1984; Noctor et al., 2000). Their ability to do so presumably reflects enhanced engagement of other pathways of H$_2$O$_2$ detoxification, notably the ascorbate–glutathione cycle, as evidenced by the sustained oxidation of glutathione accompanied by increases in the total glutathione pool. In the barley mutant, the net accumulation of GSSG 4 d after transfer to air was around 0.7 μmol g$^{-1}$ FW (Smith et al., 1984). Very similar effects are observed in transformed tobacco deficient in CAT (Willekens et al., 1997). At least in barley, H$_2$O$_2$ does not accumulate to any great extent within this time (Noctor et al., 2001). Since the mutant has negligible leaf CAT activity and rates of H$_2$O$_2$ generation via photorespiration can be estimated relatively easily, a rough estimate can be made of the relationship between redox cycling and net oxidation of the glutathione pool. In a recent study of this mutant (Noctor et al., 2002), results very similar to those of Smith et al. were obtained (Smith et al., 1984). These experiments were carried out at 250 μmol quanta m$^{-2}$ s$^{-1}$, at which a typical rate of ribulose-1,5-bisphosphate oxygenation in barley leaves is around 180 μmol g$^{-1}$ FW h$^{-1}$ (Noctor et al., 2002; for comparison, typical whole leaf GR capacity measured under standard conditions is about 70 μmol g$^{-1}$ FW h$^{-1}$). At this rate, the photorespiratory process generates approximately 10 mmol H$_2$O$_2$ g$^{-1}$ FW.
in 4 d with a 14 h light period. The proportion of H₂O₂ detoxification that involves redox turnover of the glutathione pool is difficult to estimate. Polle concluded that in the chloroplast this value was probably low and the proportion of H₂O₂ detoxification that involves redox turnover of the glutathione pool is difficult to estimate. Polle concluded that in the chloroplast this value was probably low and that oxidation of glutathione by chemical reaction with superoxide was equally if not more important than GSH oxidation by DHA (Polle, 2001). Outside the chloroplast, however, in CAT-deficient plants, glutathione may be much more closely involved in H₂O₂ detoxification: (1) in the illuminated leaves of C₃ plants, under many conditions, the major source of AOS production outside the chloroplast is the direct production of H₂O₂ by glycollate oxidase activity; (2) although monodehydroascorbate (MDHA) reductase activity is found outside the chloroplast, ferredoxin-dependent regeneration of ascorbate from MDHA is not possible; (3) in most cases, as discussed above, extrachloroplastic DHAR activities are higher than those within the chloroplast; (4) extrachloroplastic GPX,GST activities may make a more substantial contribution to H₂O₂ processing outside the chloroplast. Even if it assumed that, when CAT activity is negligible, only 1–10% of the H₂O₂ generated in photorespiration drives oxidative turnover of glutathione (either directly or via the ascorbate-glutathione couple), the net accumulation of GSSG is still only around 0.07–0.7% of the rate of redox turnover of the glutathione pool. This figure will be lower if a higher proportion of H₂O₂ detoxification involves engagement of the glutathione pool. It is evident that accumulation of GSSG can result from small imbalances in the rates of oxidation and reduction of glutathione. Although rates of synthesis and transport are probably around two orders of magnitude slower than redox cycling (Fig. 7), they are likely to be much closer to the imbalance between reduction and oxidation, and could therefore exert some influence on glutathione redox state in a given compartment.

The data of Smith et al. suggest that the changes in glutathione concentration and redox observed in the barley mutant occur both within and outside the chloroplast (Smith et al., 1985). Generation of H₂O₂ within the peroxisome is therefore capable of influencing the chloroplastic glutathione pool. Whether the changes in chloroplastic glutathione redox state are due to movement of H₂O₂ into the chloroplast or transport of GSSG from the cytosol is unclear. The increase in the glutathione pool presumably results from up-regulation of synthesis, perhaps through effects on translation of γ-ECS and/or GSH-S, as discussed above. It is worth considering the factors that determine the intracellular distribution of glutathione (Fig. 7). Although the capacity of chloroplast transport is in the same range as the maximum extractable activities of the enzymes that catalyse glutathione synthesis, these data suggest that

![Diagram](image-url)

**Fig. 7.** Control of the intracellular concentration of glutathione in leaf cells. For synthesis, transport and redox cycling, an estimate of likely rates in vivo is indicated. GSH, reduced glutathione; GSSG, glutathione disulphide.
the transporter would operate much closer to its maximum capacity than the enzymes under in vivo conditions. Under most conditions, the enzymes probably work at considerably less than half maximal capacity because of kinetically limiting substrate concentrations and competitive inhibition of γ-ECS by glutathione. Increased glutathione in one compartment will (a) counteract further glutathione synthesis through feedback inhibition and (b) favour transport of glutathione out of that compartment, if net transport does occur. These processes would tend to equalize glutathione concentrations between chloroplast and cytosol, and compartment-specific increases would require sustained differential increases in the expression of enzyme isoforms. The extent to which compartment-specific changes occur is unclear, but there are several cytosolic processes (e.g. phytochelatin synthesis, GST activity) for which an increased synthesis of chloroplastic glutathione would seem inappropriate. However, it may well be that chloroplastic synthesis contributes to the supply of glutathione to these processes via transport across the envelope. As noted above, emerging evidence points to the existence of glutathione transporters with similar kinetic properties on different membranes of the leaf cell. Perhaps the physiological significance of these transporters is to minimize perturbations of glutathione homeostasis that would otherwise result from differential demands in distinct compartments.

Catabolism of glutathione could also impact on glutathione homeostasis. Three routes of breakdown, involving GSH, GSSG and glutathione-S-conjugates, are possible and each pathway may fulfil an essentially different function. Catabolic destruction of GSSG may serve as a detoxification process. GSSG is involved in thiolation reactions forming mixed disulphides with proteins in conditions of oxidative stress. Since this process inactivates many biosynthetic enzymes the presence of a large GSSG pool is not compatible with many metabolic reactions; catabolism of GSSG would essentially return the system to pre-stress homeostasis. Catabolism of GSH, on the other hand, largely concerns the mobilization of cysteine, for example, during seed storage protein synthesis or during periods of sulphur deprivation. This requires the successive breakage of the two peptide bonds.

Glutathione catabolism is well characterized in animals, failure of this process resulting in death (Meister, 1988). Transpeptidases, which catalyse the reversible hydrolysis of the N-terminal peptide bond, initiate catabolism by removing the γ-linked Glu from GSH, GSSG, glutathione conjugates, and other peptides. The Glu moiety is either hydrolysed or donated to an amino acid acceptor or even to another GSH molecule. The second step in catabolism is less well characterized. The Cys=Gly bond is not unique to the glutathione tripeptide and several enzymes, including aminopeptidase M and Cys=Gly dipeptidase, are able to hydrolyse the bond (Meister, 1988). The transpeptidases are part of the γ-glutamyl cycle and as such are involved in amino acid transport in some tissues (Meister, 1988). The γ-glutamyl moiety is metabolized by a γ-glutamylcyclotransferase to oxo-proline which is subsequently converted to glutamate by oxo-prolinase. Homologous activities are also present in plants (Rennenberg et al., 1981; Steinkamp et al., 1987; Steinkamp and Rennenberg, 1984). In addition, however, a carboxypeptidase exists which is able to remove Gly as the first step of degradation, leaving γ-EC (Steinkamp and Rennenberg, 1985). These enzymes are cytosolic but, more recently, a vacuolar carboxypeptidase has been identified that cleaves the Gly moiety from glutathione-S-conjugates (Wolf et al., 1996): it is thus possible that cleavage of conjugated glutathione in the vacuole may be a major route of catabolism in certain conditions. Moreover, since GSSG can be considered as a glutathione-S-glutathione conjugate, transport of GSSG by the vacuolar conjugate transporter may play a role in removing this species from the cytosol. The failure to detect significant accumulation of glutathione-S-conjugates in vacuoles suggests that they are rapidly catabolized in this compartment (Marrs, 1996).

Glutathione and signalling

The GSH/GSSG couple is well suited to the role of redox sensor, indicative of the general cellular thiol-disulphide redox balance, and producing profound effects on metabolism and gene expression. Regulation of gene expression by GSH and GSSG may be specific, i.e. these compounds may be irreplaceable by other redox components. Alternatively, reported effects may reflect general changes in the cellular redox state, which are known to regulate gene expression in both prokaryotes and eukaryotes.

The glutathione pool is an important redox component in plant cells. Changes in intracellular glutathione status may, therefore, be expected to have important consequences for the cell, through modification of the metabolic functions associated with glutathione-regulated genes. In animal cells redox regulation of the transcription factor NFκB involves glutathione. This regulation is important for T cell function since glutathione augments the activity of T cell lymphocytes (Suthanthiran et al., 1990). Application of exogenous glutathione can elicit changes in the transcription of genes encoding cytosolic Cu,Zn superoxide dismutase and GR in tobacco and pine (Hérouart et al., 1993; Wengle and Karpinski, 1996) and 2-cys peroxiredoxins in Arabidopsis (Baier and Dietz, 1997). Glutathione-inducible hypersensitive elements have been identified in the proximal region of the chalcone synthase (CHS) promoter (Dron et al., 1988).
Glutathione has been implicated in defence reactions against biotic stresses. Marked changes in the glutathione pool, such as those shown in Fig. 8, occur during the hypersensitive response to pathogen attack. In barley resistant to powdery mildew a transient decrease in the leaf GSH/GSSG ratio, linked to H$_2$O$_2$ accumulation around the mesophyll cells immediately below the attacked epidermal cell, precedes the increase in the total leaf glutathione pool (Fig. 8). Although pathogen-induced increases in the intracellular concentration of glutathione and GSH-dependent induction of phenylalanine ammonia lyase and CHS have been demonstrated (Wingate et al., 1988; Vanacker et al., 2000), it is unlikely that GSH is the primary signal responsible for the increase in phytoalexins following pathogen attack. Using an artificial precursor of glutathione

![Fig. 8. Induction of H$_2$O$_2$ and glutathione during the hypersensitive response in the leaves of resistant barley, in response to attack by the fungus *Blumeria graminis*. The time-course of H$_2$O$_2$ accumulation in the attacked Alg-R leaf epidermal cells and in the underlying mesophyll was measured as the appearance of brown coloration due to staining with diaminobenzadine. Cell death in epidermal cells was estimated by the accumulation of autofluorogenic phenolic compounds. Specimens were stained post-fixation with aniline blue to show fungal structures. Micrographs were obtained using transmitted white light (14.00–18.00 h) or incident blue-violet light (20.00–24.00 h). H$_2$O$_2$ was first detected 14 h after inoculation in mesophyll cells underlying the attacked epidermal cell, at the same time as marked changes in the leaf glutathione pool were observed. A transient decrease in the GSH/GSSG ratio preceded an increase in total leaf glutathione. These were specific to glutathione, the total leaf ascorbate content and Asc/DHA ratio remaining constant throughout pathogen attack. HR, hypersensitive response. (For other details see Vanacker et al., 2000).]
biosynthesis, l-oxothiazolidine-4-carboxylate, to increase intracellular thiol concentrations it was shown that enhanced intracellular GSH concentrations alone did not induce phytoalexin synthesis (Edwards et al., 1991). It was concluded that changes in the intracellular glutathione concentration in response to pathogen attack were too slow to be consistent with the initiation of the elicitation response. In interactions between powdery mildew and oat or barley, however, induction of glutathione precedes maximal induction of transcripts for phenylpropanoid metabolism (Zhang et al., 1997; Vanacker et al., 2000). There are differences in the signal transduction pathways for elicitation of CHS transcription by fungal elicitor and glutathione (Choudhary et al., 1999), suggesting that increases in glutathione are not primarily responsible for the elicitation of the defence response. A role for glutathione in subsequent parts of the signal transduction pathway is nevertheless possible.

H2O2-mediated orchestration of gene expression may be central to the ability of plants to elicit antioxidative defences in response to abiotic and biotic threats and hence develop pre-emptive cross-tolerance. Similarities between the oxidative stress caused by ozone fumigation and pathogen-induced responses suggest common elements in signal transduction routes involving SA, JA and ethylene (Rao and Davis, 1999). Transformed tobacco plants, deficient in the H2O2-scavenging enzyme CAT, also show symptoms that are linked to the activation of pathways involved in apoptosis, such as the induction of pathogenesis-related (PR) proteins (Chamnongpol et al., 1996). Hydrogen peroxide-mediated induction of glutathione has been demonstrated in different systems (May and Leaver, 1993). It is apparent that the symptoms which develop in CAT-deficient plants subjected to prolonged exposure to photorespiratory conditions do not simply result from chemical damage. Rather, they resemble a precocious senescence, a regulated shutdown of leaf cell function that involves components common to signal transduction in response to pathogen attack (Chamnongpol et al., 1996; Takahashi et al., 1997). A central player is the phenolic molecule SA, but disruption of glutathione homeostasis may also be a vital piece of the signalling jigsaw (Fig. 8).

AOS modulate nitric oxide signalling in the hypersensitive response, leading to cell death in the region close to the pathogen attack (Delledonne et al., 1998) and systemic acquired resistance (SAR) in surrounding tissues. SAR involves the pre-emptive deployment of gene expression to modify cell metabolism to cope with future attacks. Increases in the SA concentration are observed at the site of infection, and to a lesser extent at remote sites. H2O2 and SA (and perhaps also glutathione) are potential systemic messengers carrying information concerning attack to unchallenged plant tissues. Of the complex array of antioxidants found in plant cells, glutathione alone shows strong induction and rapid accumulation in response to pathogen attack (Fig. 8; Edwards et al., 1991; Vanacker et al., 2000). The effect on biosynthesis is specific to glutathione and not a general effect on the synthesis of low molecular weight antioxidants. It appears to be a universal response in plants faced by pathogen attack or environmental stress, where the antioxidant defences are temporarily overwhelmed by an oxidative burst or by the accumulation of AOS as a result of impaired metabolism.

The signalling mechanisms involved in induction of GSH biosynthesis during pathogen attack are unknown. As noted earlier, H2O2 increases tissue glutathione contents, whereas JA increases the transcript abundance of the enzymes of GSH synthesis, but does not affect GSH concentration (Xiang and Oliver, 1998). Pathogen-induced increases in glutathione in the cells surrounding the site of attack could have two possible roles in defence. First, they would increase protection from excessive damage caused by the accumulation of AOS during the oxidative burst, which could occur chemically or through increased substrate availability for enzymes such as GSTs and GPXs. Second, changes in the redox state and concentration of glutathione may be an essential secondary messenger mediating the signalling effects of hydrogen peroxide (Foyer et al., 1997; May et al., 1998a).

Crucial to signal transduction processes associated with defence responses appears to be the interaction between SA, H2O2 and glutathione (Rao and Davis, 1999). There is also evidence that glutathione is involved in the regulation of cell division (May et al., 1998a). In plants, as in animals, cell growth and death responses appear to be coupled. Morphogenesis involves differential cell division and cell expansion in response to positional cues generated during development by cell-to-cell communication. At maturity, tissue homeostasis can be influenced by the action of growth regulators, which act singly or in combination. Growth and development are also influenced by cell death, which is required for processes such as the formation of tracheary elements, the release of mature pollen, the selective elimination of organs during embryogenesis and, in some species, during flower development. Damaged cells that are not eliminated via programmed cell death can proliferate and form tumours. Glutathione is necessary for the cell to enter the G1 phase, the pre-mitotic phase of the cell cycle in which the cell is capable of responding to extracellular stimuli that determine whether it will enter the S phase, or enter quiescence, differentiation or death (May et al., 1998a).

Like GSH, GSSG may initiate or potentiate signalling cascades. GSSG can regulate GR expression as the pea GR gene contains a putative GSSG binding site (Creissen et al., 1992). A second, potentially more important mechanism of GSSG action, involves the spontaneous oxidation of protein sulphhydryl groups to form mixed
disulphides, a reaction termed thiolation. While disulphide bonds form spontaneously through a chemical reaction, re-reduction in vivo requires the intervention of a protein (e.g. GR, thioredoxin, protein-disulphide isomerase). Hence, some disulphide bonds are transient, but others can be very long-lived. Reversible disulphide bond formation has long been recognized as an important mechanism of modulating protein activity. It has only recently, however, been shown that this mechanism could also be a crucial initial signalling event. The formation of such intramolecular disulphide bonds within proteins alters their configuration and biological activity (Demple, 1998). Reversible protein thiolation protects essential thiol groups on key proteins from irreversible inactivation during oxidative stress and also plays an important regulatory role in controlling metabolism, protein turnover and gene transcription (Foyer and Noctor, 2001).

There are many examples of proteins that undergo thiolation in animals, but relatively few have been described in plants. Thiolation has been found, for example, to activate microsomal GSTs (Dafre et al., 1996), while thiolation of proteins such as phosphotyrosine-specific protein phosphatases may also mediate signal transduction pathways that initiate key stress responses (Fordham-Skelton et al., 1999).

Thiolation may be a particularly important phenomenon in seed development. Some key seed proteins such as acyl carrier protein are known to be thiolated in the latter stages of seed development and dethiolated during imbibition (Butt and Ohlrogge, 1991). Dry seeds often have a higher glutathione content than other tissues (Klapheck, 1988; Kranner and Grill, 1996) but much of the pool is present as GSSG. The seed GSH/GSSG ratio not only controls protein function but is considered to regulate protein synthesis as well, in a manner similar to that observed in mammalian cells (Kranan and Grill, 1996), since low GSH/GSSG ratios block protein synthesis and prevent germination (Fahey et al., 1980). As soon as germination starts GSSG and protein-thiols are re-reduced and protein synthesis and function are re-established. Thiolation of proteins in the dry seeds could have three functions: firstly, if oxidation of critical cysteine residues marks proteins for degradation, thiolation will protect both the protein and glutathione from degradation; secondly, it could modulate protein activity by interfering with protein cysteine residues; thirdly, it could be involved in signal transduction associated with the quiescent state. In addition, low GSH/GSSG ratios (like low ascorbate/DHA ratios; de Pinto et al., 1999) block or delay cell division.

Conclusions

The concentration and redox state of intracellular glutathione pools depends on the complex interplay of numerous factors. Glutathione redox state is remarkably constant, but extreme oxidative stress leads to oxidation of the pool, as observed during ozone exposure or pathogen attack or in plants with low CAT activities (Smith et al., 1984; Sen Gupta et al., 1991; Willekens et al., 1997; Vanacker et al., 2000; Noctor et al., 2001). Oxidation in these cases is accompanied by increases in total glutathione. During drought, however, oxidation of glutathione is not always accompanied by increases in the pool size (Smirnoff, 1993). The redox state of glutathione will depend on the balance between oxidative processes and the in vivo GR activity. GR activity may well reflect reductant supply as much as enzyme capacity, particularly, perhaps, outside the chloroplast. Interestingly, however, chloroplastic overexpression of either plant or bacterial GR can increase the leaf GSH:GSSG ratio and mitigate damage due to certain stresses (Foyer and Noctor, 2001). Furthermore, several studies have shown a correlation between GR activity and the absolute size of the glutathione pool.

The operation of translocators may minimize intercompartmental fluctuations in glutathione concentration and redox state, and allow different compartments to co-operate in glutathione synthesis, redox turnover and degradation. The results presented here on uptake of GSH into the chloroplast are preliminary, and an understanding of the influence of chloroplast envelope transporters will require further characterization of this process. Transport of other thiols such as Cys and γ-EC is worthy of investigation, particularly in view of the differential intracellular distribution of γ-ECS and GSH-S observed in tissues such as young wheat leaves. Another important question is the origin of mitochondrial glutathione and the possibility of a transporter located on the inner mitochondrial membrane. Identification of genes encoding plant transporters will be facilitated by the recent cloning of a yeast glutathione transporter (Bourbouloux et al., 2000).

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