Reduced glutathione (GSH) is the most abundant low-molecular weight thiol in plant cells. It accumulates to high concentrations, particularly in stress situations. Because the pathway of GSH synthesis consists of only two enzymes, manipulation of cellular glutathione contents by genetic intervention has proved to be relatively straightforward. The discovery of a new bacterial bifunctional enzyme catalysing GSH synthesis but lacking feedback inhibition characteristics offers new prospects of enhancing GSH production and accumulation by plant cells, while the identification of γ-glutamyl cysteine and glutathione transporters provides additional possibilities for selective compartment-specific targeting. Such manipulations might also be used to affect plant biology in disparate ways, because GSH and glutathione disulphide (GSSG) have crucial roles in processes as diverse as the regulation of the cell cycle, systemic acquired resistance and xenobiotic detoxification. For example, depletion of the total glutathione pool can be used to manipulate the shoot:root ratio, because GSH is required specifically for the growth of the root meristem. Similarly, chloroplast γ-glutamyl cysteine synthetase overexpression could be used to increase the abundance of specific amino acids such as leucine, lysine and tyrosine that are synthesized in the chloroplasts. Here we review the aspects of glutathione biology related to synthesis, compartmentation and transport and related signalling functions that modulate plant growth and development and underpin any assessment of manipulation of GSH homeostasis from the viewpoint of nutritional genomics.

Abbreviations – γ-EC, γ-glutamyl cysteine; γ-ECS, γ-glutamyl cysteine synthetase; APR, adenosine 5’-phosphosulphate reductase; BS, bundle sheath; BSO, 1-buthionine-SR-sulfoximine; CAT, catalase; CDK, cyclin-dependent kinase; ER, endoplasmic reticulum; FW, fresh weight; GGT, γ-glutamyl transpeptidase; GPX, glutathione peroxidase; GR, glutathione reductase; GRX, glutaredoxin; GS, glutathione-S; GSH, reduced glutathione; GSH-S, glutathione synthetase; GSNO, S-nitrosoglutathione; GSSG, glutathione disulphide; GST, glutathione S-transferase; GS-X, glutathione conjugates; Hgt1P, high-affinity glutathione transporter; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; mBBr, monobromobimane; MRP, multidrug-resistant transporter; NPR1, non-expressor of PR proteins 1; OPT, oligopeptide transport proteins; PC, phytocheletin; PCD, programmed cell death; PMF, proton motive force; PR, pathogenesis related; QTL, quantitative trait loci; RNS, reactive nitrogen species; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; TGA, a group of bZIP (basic leucine repeat)-type transcription factors; TRX, thioredoxin.
Introduction

The thiol tripeptide glutathione \( \gamma\text{-Glu-Cys-Gly; reduced glutathione (GSH)} \) does not immediately come to mind in considerations of human nutrition. However, this ubiquitous tripeptide thiol is a vital intracellular and extracellular protective antioxidant against oxidative/nitrosative stresses, which play a key role in the control of many human diseases. GSH is also important in immunity modulation in animals as well as in remodelling of the extracellular matrix, apoptosis and mitochondrial respiration in disease. Mammalian cancer cells often accumulate GSH, where there is also a metabolic switch to glycolysis rather than TCA cycle as a major energy source (Pozuelo Rubio et al. 2004). Interactions of GSH with antiapoptotic factors such as Bcl-2 in cancer cells have been linked to radiation and multidrug resistance (Ortega et al. 2003). However, because high GSH concentrations are essential for both antioxidant and immune defence systems in mammals, tissue GSH levels can be regulated, particularly in malnourished patients through diet and nutrition before therapeutic treatments (Bray and Taylor 1993).

Plants make abundant amounts of glutathione, and its synthesis and accumulation in either the chloroplast or cytosolic compartments of the plant cell can be enhanced or decreased relatively easily by genetic engineering approaches (Strohm et al. 1995, Creissen et al. 1996, Noctor et al. 1998a, b, Zhu et al. 1999, Xiang et al. 2001). Ectopic expression of \( \gamma\text{-glutamyl cysteine synthetase (}\gamma\text{-ECS, also called glutamate cysteine ligase)} \) (Noctor et al. 1996) and certain enzymes of sulphur assimilation pathway (Harms et al. 2000) or glutathione reductase (GR) (Foyer et al. 1991) in transgenic plants results in substantial increases in leaf glutathione. Glutathione contents have been increased by at least five-fold the values of wild-type plants without negative effects (Noctor et al. 1998b, 2002a). Only one report to date has indicated that an enhanced capacity to generate and accumulate the intermediate \( \gamma\text{-glutamyl cysteine (}\gamma\text{-EC)} \) can produce negative effects (Creissen et al. 1999). The increased capacity to generate glutathione and enhance cellular glutathione pools leads to higher rates of sulphur assimilation, modified amino acid metabolism and enhanced stress tolerance (Noctor et al. 1998a, b). Transformed poplar trees with an increased capacity to synthesize and accumulate glutathione are currently being used in the field for bioremediation purposes to purify and restore soils polluted by human activities (Peuke and Rennenberg, 2005a, b). Genetic engineering approaches have led to an improved understanding of how compartment-specific alterations in glutathione synthesis affect metabolism and defence. Similarly, the analysis of mutants deficient in glutathione has greatly advanced current concepts of how GSH and glutathione disulphide (GSSG) regulate cell signalling and development. Chemical inhibition of GSH synthesis using 1-buthionine-S, R-sulfoximine (BSO), a specific inhibitor of \( \gamma\text{-ECS (Griffith and Meister 1979, Griffith 1982, Maughan and Cobbett 2003)} \), has been widely used to study the effects of GSH depletion in plants (Hell and Bergman 1990, Vernoux et al. 2000). Analysis of Arabidopsis thaliana T–DNA insertion and EMS mutants selected by their ability to grow on BSO concentrations that are inhibitory to the wild-type has indicated that different mechanisms can confer BSO resistance (Maughan 2003).

Since its initial discovery in yeast over 125 years ago, glutathione has been shown to have a prodigious number of critical functions (Fig. 1). GSH is a powerful reducing agent and is a key player in the plant redox-state (Noctor and Foyer 1998, Noctor et al. 2002a). Here we will focus on how manipulation of GSH synthesis and metabolism has advanced current concepts of glutathione homeostasis and function, concentrating on aspects related to cell signalling, development and defence that may be of importance in any consideration of nutritional genomics involving glutathione. The characteristics and functional importance of putative thiol-based reactive oxygen species (ROS) sensors and associated redox-signalling cascades have recently been discussed

Fig. 1. The functions of glutathione in plants linked by essential signalling functions that integrate plant growth, development and defence processes.
(Foyer and Noctor 2005). In this context, we will concentrate here on the implications for the plant of modulating glutathione homeostasis.

Like ascorbate, glutathione limits the lifetime of ROS and lipid peroxide signals. Moreover, GSH alters calcium signalling in plants (Gomez et al. 2004a) and participates in the calcium-dependent pathways of ROS-signal transduction (Rentel and Knight 2004). Through oxidation to GSSG, other signalling cascades can be initiated through thiol-disulphide exchange and thiolation. Moreover, the interaction between GSH and nitric oxide, catalysed by the enzyme formaldehyde dehydrogenase (also known as class III alcohol dehydrogenase), is also important in signal transduction as S-nitrosoglutathione (GSNO) is thought to be a stable NO transport form (Liu et al. 2001). The formaldehyde dehydrogenases, which also catalyse the NAD-dependent formation of S-formylglutathione and S-hydroxymethyl glutathione, are regulated by wounding and salicylic acid (SA) (Diaz et al. 2003). Hence, glutathione is directly implicated in the coordinated control of signalling by ROS and reactive nitrogen species (RNS) that determines cell fate in situations such as pathogen attack.

It is important to note that thioredoxins (TRXs), glutathione and lipid peroxides all target the same protein residues, allowing us to envisage a dynamic signalling network where proteins are modulated by these three signalling systems according to their respective concentrations and the associated enzymes such as glutaredoxins (GRXs).

Control of plant growth and development

Glutathione regulation is important in a number of key processes associated with plant growth and development. These include the following:

The cell cycle and plant development

Current knowledge of how cell division is controlled and how it interacts with different aspects of plant development such as morphogenesis, architecture and growth rate has greatly increased over the last 5 years. Cell-cycle regulation involves components that respond to signals from the external environment as well as intrinsic developmental programmes, and it ensures that DNA replication occurs at high fidelity within the constraints of prevailing environmental conditions (Dewitte et al. 2003, de Jager et al. 2005). Cyclin-dependent kinases (CDKs) play a central role in cell-cycle regulation, with negative (KRP proteins) and positive (D-type cyclins) regulators acting downstream of environmental inputs at the G1 checkpoint (Dewitte and Murray 2003, de Jager et al. 2005). The activity of the CDK/cyclin complexes is regulated by a network of regulatory mechanisms including transcription, proteolysis, phosphorylation/dephosphorylation, interaction with regulatory proteins and intracellular trafficking.

In Arabidopsis, the embryo cells of the dry seed are arrested in the G1 phase of the cell cycle. Germination is initiated by water uptake and the resumption of metabolism and cell division. Nitric oxide is a potent regulator of germination in dormant seeds such as those of Arabidopsis (Bethke et al. 2004, 2006). Germination is stimulated by the presence of environmental nitrate from which NO can be synthesized via nitrate reductase. The relatively low abundance of GSH within the dry seed means that GSH has either to be regenerated from oxidized or bound forms upon water uptake or that it has to be synthesized de novo during germination to achieve the high levels present in seedlings. Low GSH levels in the dry seed and during germination may prevent excessive formation of GSNO, allowing sufficient free NO to be present to stimulate the germination process. However, it is also possible that NO accumulation serves as a trigger for GSH synthesis during germination.

While it is well established that the G1 checkpoint is very sensitive to environmental conditions, it has only recently become clear that this control might be exerted through oxidation–reduction (redox) regulation and associated components, particularly glutathione and ascorbate, whose concentrations and redox state influence G1 progression. Glutathione and ascorbate exert independent controls on the plant cell cycle (Potters et al. 2004).

Numerous interactions between the plant and environment take place at the root/soil interface. Monobromobimane (mBBr) has been widely used to detect GSH in vivo. Application of this GSH-localization system to Arabidopsis roots shows that GSH accumulates in the vascular system and root tip (Fig. 2A). The relatively high concentration of GSH in root tips, where actively dividing cells are found (Fig. 2A), suggests that growing tissues have a requirement for GSH. Indeed, studies using the GSH-deficient ROOTMERistemLESS1 mutant (rm1) which has less than 2% of the wild-type GSH levels have shown that without GSH root meristem cells arrest in G1 (Cheng et al. 1995, Vernoux et al. 2000). This mutation only affects post-embryonic root development, and while overall the roots are shorter, the cell files and types are not aberrant. It is important to note that in contrast to the root meristem, the shoot meristem is able to produce all the aboveground organs with development and timing...
that are comparable with the wild-type despite very low GSH levels.

The rml1 phenotype can also be induced chemically by treating cells or seedlings with BSO to lower GSH levels (Fig. 2B–G). This causes the cells in the root meristem to stop dividing, and, like the root meristem cells of rml1, they arrest in the G1 phase (Fig. 2F,G). These results suggest that GSH plays a key role in potentiating signals permitting cell-cycle progression and hence root growth. Similarly, root nodule formation is prevented when GSH synthesis is blocked by addition of BSO, suggesting that like the root meristem, the nodule meristem is unable to develop in the absence of GSH (Frendo et al. 2005). While the absolute content of GSH and the GSH : GSSG ratio clearly influence cell-cycle progression, the targets of glutathione action are unknown at present. In mammalian systems, this regulation includes transcription of the retinoblastoma gene product as well as its activity, which function directly downstream of G1 activators (Yamauchi and Bloom 1997).

Given the importance of glutathione redox status in regulating cell division, it is perhaps not surprising that the GSH : GSSG ratio also regulates embryogenesis in plants (Belmonte and Yeung 2004, Belmonte et al. 2005) as it does in animals. Application of GSH in the second half of embryo development results in precocious germination, while application of GSSG improved somatic embryogenesis (Belmonte and Yeung 2004, Belmonte et al. 2005). The redox status of the embryo glutathione pool delineates specific stages of embryo development both in vivo and in vitro. A high GSH/ GSSG ratio is essential for cell division and proliferation during the initial stages of embryo development, but later in development, the redox status of the glutathione pool decreases (Belmonte et al. 2005). This metabolic shift in cellular glutathione redox status appears to be required for continual embryonic development, and proper acclimation of storage product deposition is possibly facilitated through interactions with hormones such as abscisic acid. Zygotic embryos are held in G1 until germination-specific d-type cyclins promote cell division (Mausubelel et al. 2006). It is thus tempting to suggest that redox controls may act upstream of germination-specific cell-cycle regulation in a fashion similar to the regulation of division in the root apical meristem.

Because GSH is a modulator of general plant development, it is not surprising that it has also been implicated in senescence processes (Ogawa et al. 2002). One specific mode of action affects the activity of the ubiquitin-dependent 26S proteasome. The activity of this protein degradation pathway may be influenced by cellular redox status in plants as it is in animals, where high GSH/GSSG ratios impede the binding of target proteins (Hochstrasser 1998, del Pozo et al. 1998, Theriault et al. 2000, Sangerman et al. 2001).

**Plant defence and systemic acquired resistance**

GSH is a transcriptional regulator. It enhances the DNA-binding activity of NFkB, a transcription factor that is central to the regulation of the oxidative stress response in animal systems. Few homologues of major animal or microbial redox-sensitive elements and factors have been reported in plants to date apart from the activator
protein-1 (a transcription factor controlling response to oxidative stress) and the antioxidative responsive element that is present in the maize catalase (CAT) promoter. However, cytosolic thiol-disulphide status reactions are crucial in plant innate immune responses. GSSG accumulation is often observed in close conjunction with cell death and systemic acquired resistance (SAR) responses resulting from exposure to pathogens (Vanacker et al. 2000) or abiotic stresses (Gomez et al. 2004a). RNS are also important in the plant-pathogen responses, and the strong interaction between GSH and NO in signal transduction process, as GSNO for example, suggests that mutual upregulation of these signalling components may occur in challenged cells.

An increase in tissue GSH contents activates the expression of PR genes through the non-expressor of PR protein 1 (NPR1) pathway (Mou et al. 2003, Gomez et al. 2004a). The NPR1 protein is part of the mechanism through which SA triggers SAR to pathogens. While many aspects of NPR1 function with regard to binding of the TGA transcription factors and movement to the nucleus to facilitate SAR are not fully understood, this glutathione-modulated ankyrin repeat protein is one of the key regulators of SA-dependent gene expression (Cao et al. 1994, Delaney et al. 1995). Such studies indicate a strong relationship between SA and glutathione homeostasis in stress signalling. Increased oxidation resulting from exposure to stress stimulates glutathione accumulation. SA enhances this response presumably by increasing oxidation possibly through inhibitory effects on CAT and ascorbate peroxidase.

Cell death and the GSH/GSSG redox couple

The many varied functions of GSH and importance of the GSH : GSSG redox couple as a sensitive signal for cellular events necessitates strict GSH homeostasis. In animal cells, substantial evidence implicates redox potential as an important factor determining cell fate. All aerobic organisms maintain thiols in the reduced (–SH) state. GSH : GSSG ratios of 100:1 are typical values for animal and plant cells. Departures from high values for animal and plant cells. Departures from high GSH : GSSG ratios result in PCD (reviewed in Schaef er and Buettner 2006). Low GSH : GSSG ratios enhance translation of γ-ECS (Xiang and Oliver 1998). Significant deviations from high GSH : GSSG ratios result in PCD (reviewed in Noctor et al. 2002a, b). The significance of alterations to the GSH : GSSG ratio can be quantified using the Nernst equation through which the redox potential of a given redox couple can be calculated: $E = E^\circ + \frac{RT}{nF} \ln \left( \frac{[GSH]}{[GSSG]} \right)$. This allows a quantitative appreciation of the impact of changes in total glutathione content on the redox state of the cell. The activity of GR is essential in maintaining the high cellular GSH : GSSG ratios. Ectopic expression of bacterial GR in the chloroplast or cytosol of transgenic plants increased the GSH : GSSG ratio and gave added protection against some stresses (Foyer et al. 1991, 1995, Aono et al. 1993, 1995, Creissen et al. 1996).

Plants that are deficient in CAT activity have also been used to investigate oxidative signalling resulting from decreased H$_2$O$_2$ detoxification (Vandenabeele et al. 2002, 2004). CAT-deficient plants are particularly interesting as overall levels of H$_2$O$_2$ accumulation are relatively small, but there is a drastic increase in tissue glutathione levels (Smith et al. 1984, Smith et al. 1989) together with a low GSH : GSSG ratio (Noctor et al. 2002b). CAT-deficient plants have been used to identify a complete inventory of genes induced or repressed in H$_2$O$_2$-induced PCD, which appears as clusters of dead palisade parenchyma cells along the leaf veins when these plants are exposed to high light (Vandenabeele et al. 2004). The stimulation of GSH synthesis and modified GSH : GSSG ratios are intrinsic features of ozone and H$_2$O$_2$-dependent signalling. Thus, GSSG and thiolation may be important in adding specificity to the signal and/or promoting second messenger signal transduction in these pathways (Rentel and Knight 2004, Foyer and Noctor 2005).

Transport systems also fulfil important roles in determining the GSH : GSSG ratios of individual cell compartments (Foyer et al. 2001). Transport of GSSG into the vacuole by the MRP transporters or across the plasmalemma (Jamai et al. 1996) contributes to GSH : GSSG homeostasis in the cytosol. GSH degradation may also be an important factor (Storonzenko et al. 2002). However, the γ-glutamyl cycle, which contributes to GSH metabolism in animals, remains to be
confirmed in plants (Storozhenko et al. 2002). Overexpression of one putative component, \( \gamma \)-glutamyl transferase (GGT) in Arabidopsis, did not alter the GSH : GSSG ratio or total tissue glutathione contents (Storozhenko et al. 2002).

Thiol-disulphide exchange reactions are a universal mechanism for perception of redox perturbation. In addition, S-glutathionylation or thiolation is a further crucial mechanism in perception of altered glutathione redox state. Protein glutathionylation is a widespread response to oxidizing conditions. It is becoming established as an important mechanism for reversible modification of protein cysteinyl residues in plants as it is in animals. While relatively few thiolated proteins have been identified to date, those that have been characterized include important metabolic enzymes such as sucrose synthase and acetyl CoA carboxylase and defence enzymes such as dehydroascorbate reductase (Dixon et al. 2005). Thiolation can have a number of effects on proteins, but it primarily protects cysteinyl residues from irreversible oxidation to their sulfenic and sulphonic acid derivatives. The process is reversed by specific GRXs and/or TRXs, but much remains to be characterized concerning these regulatory systems (Noctor in press). Both types of redox-active proteins are encoded by quite large gene families in plants (Lemaire 2004). While relatively few plant thiolation targets have been identified to date, many as yet unidentified targets are likely also to be thio-redoxin targets, which are becoming increasingly well characterized (Buchanan and Balmer 2005).

**Glutathione as a sink for reduced sulphur and regulator of sulphur assimilation**

Current evidence suggests that GSH has a role in sulphur homeostasis. GSH is an abundant low-molecular weight thiol and hence a potentially important sulphur sink as well as a predominant transported form of reduced sulphur (reviewed in Hell 1997). This model predicts that excess sulphur will be stored in GSH and will be released when the cell experiences sulphur deficiency or requires increased sulphur availability. Important mechanistic support came from studies which show GSH is transported long distances in the phloem and xylem (Rennenberg et al. 1979). Furthermore, GSH is transported to developing tissues from maize scutella (Rauser et al. 1991). Herschbach et al. (2000) showed that not only is GSH found in the phloem and xylem but also that GSH levels control sulphate loading into the xylem. Together with cysteine, glutathione forms part of the repertoire of signals that modulate sulphate uptake and assimilation (Kopriva and Rennenberg 2004). GSH accumulation in cells has feedback effects on the pathway of primary sulphur assimilation (Kreuzwieser and Rennenberg 1998). The addition of GSH to Arabidopsis roots caused a decrease in adenosine 5’ phosphosulphate reductase (APR) activity and transcript abundance (Vauclare et al. 2002) as well as in ATP sulphurylase activity (Lappartient et al. 1999). Long distance GSH transport has also been suggested to decrease sulphur assimilation (Lappartient and Touraine 1996) and poplar (Herschbach et al. 2000). A simple regulatory mechanism has been proposed with regard to the regulation of demand-driven sulphur assimilation by positive signals such as 3-oxoacylserine and negative signals such as GSH (Kopriva and Rennenberg 2004).

**Effects of manipulation of GSH synthesis on metabolism and development in mutant and transgenic plants**

Glutathione is synthesized in both photosynthetic and non-photosynthetic cell types. However, green leaves are often the major organs of GSH production and export, and chloroplasts contain high (3–5 mM) GSH concentrations (Foyer and Halliwell 1976, Smith et al. 1985). However, certain non-green cell types such as trichomes also have a very high capacity to produce and accumulate glutathione (Gutiérrez Alcalá et al. 2002). Variable proportions of leaf glutathione are localized in the chloroplasts, but values are generally considered to be between 30 and 40% of the total (Klapheck et al. 1987) with 1–4 mM in the cytosol (Noctor et al. 2002a).

In nearly all organisms studied to date, GSH is synthesized from constituent amino acids in a two-step ATP-dependent reaction sequence (Fig. 3) catalysed by \( \gamma \)-ECS and glutathione synthetase (GSH-S) (Hell and Bergmann 1988, 1990, Meister 1988). With the exception of a novel enzyme from Streptococcus agalactiae that catalyses both activities (Janowiak and Griffith 2005), \( \gamma \)-ECS and GSH-S enzymes have been identified as separate proteins and gene products from numerous eukaryotes and gram-negative prokaryotes. The \( S. \ agg \) alactiae enzyme has several features that are worthy of note. This is not only the first bifunctional \( \gamma \)-ECS-GSH-S enzyme to be isolated but is also the first GSH synthesis enzyme system to be identified from a gram-positive organism (Janowiak and Griffith 2005). The \( \gamma \)-ECS-GSH-S sequence encodes an 85 kDa protein, which when purified exhibits GSH-S activity with a similar specific activity to that of \( \gamma \)-ECS. It has similar \( K_m \) values for cysteine and ATP to the Escherichia coli \( \gamma \)-ECS, but the streptococcal \( \gamma \)-ECS-GSH-S has a lower affinity for glutamate than the \( E. \ colii \) GSH-S. This may be...
explained by the fact that gram-positive bacteria maintain exceptionally high glutamate levels (of up to 100 mM). The GSH-S domain has a relatively small molecular mass (31 kDa) with an amino acid sequence more related to D-Ala-ligase than GSH-S (Janowiak and Griffith 2005). It is important to note GSH inhibits neither the γ-ECS nor the GSH-S activity of the streptococcal γ-ECS-GSH-S, allowing S. agalactiae to maintain much higher GSH concentrations than for example E. coli, despite the fact that the overall γ-ECS activity is relatively lower. It is interesting to note that S. agalactiae lack catalase and therefore rely heavily on GSH-based enzymes for peroxide detoxification (Janowiak and Griffith 2005).

The E. coli genes encoding γ-ECS and GSH-S have been used extensively in genetic engineering approaches to enhance glutathione contents in plants (Noctor et al. 1998a, Zhu et al. 1999). Targeting of the bacterial γ-ECS and GSH-S to either the chloroplast or cytosol has led to marked increases in enzyme activity. Increases in γ-ECS but not GSH-S led to large constitutive increases in leaf glutathione (Noctor et al. 1996, 1998a, Creissen et al. 1999). Moreover, glutathione was also increased in xylem sap, phloem exudates and roots (Herschbach et al. 2000). The identification of a γ-ECS-GSH-S enzyme that facilitates glutathione synthesis in S. agalactiae suggests that similar bifunctional enzymes might occur in other gram-positive bacteria where glutathione synthesis was thought to be relatively uncommon. These bifunctional γ-ECS-GSH-S enzymes might be extremely useful in future plant genetic engineering approaches to manipulate glutathione for nutritional purposes. Similarly, specific homologues of glutathione could be introduced for example by ectopic expression of enzymes such as homoglutathione synthetase.

The endogenous plant γ-ECS and GSH-S enzymes display a high degree of sequence homology. In A. thaliana, γ-ECS is encoded by a single gene, GSH1, with a plastid target signal (May and Leaver 1994). A recent analysis using a γ-ECS-GFP reporter system has indicated that most of, if not all, the A. thaliana γ-ECS protein is located in the chloroplasts (Wachter et al. 2005). Targeting information relative to GSH-S suggests that it is present both in chloroplasts and the cytosol. The chloroplast and cytosol thus cooperate in de novo GSH synthesis, the chloroplast acting as the source of γ-EC for both chloroplastic and cytosolic GSH production.
These observations are surprising given that earlier studies localized γ-ECS and GSH-S activities in the cytosol as well as the chloroplasts (Klapheck et al. 1987, Hell and Bergmann 1988, 1990). Moreover, the studies with transgenic plants have shown that GSH synthesis can be increased by targeting the synthetic enzymes to either the chloroplastic or the cytosolic compartments (Noctor et al. 1996, 1998a, Creissen et al. 1999). However, kinetic analysis has revealed that the chloroplast and cytosolic isoforms have similar properties (Noctor et al. 1996, 1998a, Creissen et al. 1999). More work is required to fully rationalize the molecular genetic evidence with the biochemical observations.

The targeting information indicating that γ-EC is largely synthesized in the chloroplasts (Wachter et al. 2005) has implications for glutathione homeostasis. Firstly, at least some of γ-EC produced in the chloroplast must be exported to the cytosol, as most of the GSH-S is located in this compartment. Secondly, overexpression of γ-EC in the chloroplast enhanced not only leaf γ-EC and glutathione contents, but it also increased the abundance of amino acids synthesized in the chloroplasts such as valine, leucine, isoleucine, lysine and tyrosine (Noctor et al. 1998a). It is interesting to note that increases in these amino acids was specific to chloroplastic γ-ECS overexpression and that the degree of enhancement occurred in almost direct proportion to increases in γ-EC and glutathione (Noctor et al. 1998a).

GSH metabolism is more complex in C4 species such as maize than in C3 species such as Arabidopsis, tobacco and poplar. In maize leaves, cysteine synthesis is exclusively localized in the bundle sheath (BS) tissues (Burgener et al. 1998). γ-ECS and GSH-S transcripts and proteins are similar in abundance in maize leaf mesophyll (M) and BS cells, and they were also found in the epidermis and stomatal guard cells (Gomez et al. 2004b). It is important to note however that an earlier study found that GSH-S activity primarily in the M cells (Burgener et al. 1998).

A number of GSH-deficient A. thaliana mutants have been described in the GSH1 locus. The analysis of γ-ECS-deficient mutants such as rml, cad and rax has provided useful information concerning the effects of glutathione depletion in plants (Cheng et al. 1995, Howden et al. 1995, Vernoux et al. 2000, Ball et al. 2004). The mutants have rather different characteristics. For example, the cad2-1, which has 15–30% of wild-type GSH, and the rax1-1, which has 50% of wild-type GSH, have different leaf transcriptome profiles (Ball et al. 2004). It is possible that the GSH1 gene products are themselves involved in signal transduction.

The extensive literature concerning the effects of manipulation of γ-ECS and GSH-S activities in plants has already been reviewed (Noctor et al. 1998a, Noctor et al. 2002a). The major factors controlling GSH accumulation in plants as in animals are abundance of γ-ECS and the availability of cysteine (Noctor et al. 1996, 1998a, 1998b, 2002a, Xiang and Oliver 1998, Xiang and Bertrand 2000, Xiang et al. 2001). Overexpression of enzymes such as γ-ECS (Noctor et al. 1996), GR (Foyer et al. 1991, 1995, Aono et al. 1993, 1995) or serine acetyltransferase (Harms et al. 2000) enhances tissue GSH contents. However, there are few attempts to date to examine the effects of simultaneous expression of these enzymes. Increased tissue glutathione in transformed plants has resulted in largely beneficial effects (Foyer et al. 1995, Strohm et al. 1995, Noctor et al. 1996, 1998a, Pilon-Smits et al. 1999, Zhu et al. 1999). The marked chlorotic phenotype produced by chloroplastic E. coli γ-ECS overexpression in transformed tobacco is unusual and may result from perturbed signal transduction rather than direct effects of GSH or γ-ECS accumulation per se (Creissen et al. 1999).

Some of the effects of ectopic γ-ECS expression are illustrated in Fig. 4 using transformed Arabidopsis seedlings produced by Cobbett et al. (1998). The high level of GSH1 transcripts observed in the transformed Arabidopsis plants relative to controls (Fig. 4A) is accompanied by increased total leaf glutathione contents (Fig. 4B). Moreover, the enhanced capacity for GSH synthesis arising from the increased expression of γ-ECS results in a much lower effect of BSO in terms of its ability to deplete tissue glutathione (Fig. 4B). The pronounced positive effect of GSH1 overexpression and higher tissue GSH availability on Arabidopsis root growth is illustrated in Fig. 4C. It is interesting to note that in the presence of BSO, the wild-type plants have a similar phenotype to the rm1 mutant as illustrated by the absence of a post-embryonic root. However, enhanced γ-ECS activity (o/e line) alleviates this inhibition of root development, presumably due to the higher capacity for GSH synthesis even in the presence of BSO, allowing the root to grow.

Sulphur uptake by the roots of transformed bacterial γ-EC-expressing poplar trees was enhanced to meet the requirements of increased demand for sulphur caused by enhanced GSH synthesis (Herschbach et al. 2000). However, the glutathione contents of untransformed and transformed poplar leaf discs were increased substantially by incubation with cysteine, particularly in the light. This suggests that cysteine supply remains a key limiting factor for glutathione synthesis even when γ-ECS activity is increased (Strohm et al. 1995, Noctor et al. 1996, 1997). The leaf cysteine levels were maintained at values similar to the wild-type in transgenic poplars with high γ-ECS activities, but the activities of
enzymes involved in sulphate reduction and assimilation were not increased (Noctor et al. 1998a, b, Hartmann et al. 2004). Similarly, microarray analysis of plants undergoing sulphate starvation did not reveal effects on \( \gamma \)-ECS or GSH-S transcripts suggesting little coordination of the pathways of GSH synthesis in response to sulphur availability (Hirai et al. 2003, Maruyama-Nakashita et al. 2003). Hence, to remove metabolic limitations in cysteine supply, it will be essential to consider solutions that modulate sulphur assimilation to further enhance the capacity of plant tissues to synthesize and accumulate glutathione. For example, modulation of oxylipin signalling may be one approach, as methyl jasmonate stimulates transcription of both sulphur assimilation and GSH synthesis genes (Harada et al. 2000).

Regulation of glutathione synthesis and accumulation

There is very little literature information available on the coordinate regulation of expression of \( GSH1 \) and \( GSH2 \) in plants. It is worth noting the very high abundance of \( GSH2 \) transcripts in the quiescent centre of \( Arabidopsis \) roots, compared with surrounding cells (Nawy et al. 2005), and it may be that a high level of GSH is required in these stem cells for antioxidant defence, because quiescent centre cells are essentially devoid of reduced ascorbate.

\( GSH1 \) and \( GSH2 \) mRNAs are increased by conditions that require enhanced GSH abundance for metabolic functions such as heavy metal sequestration. Neither GSH nor GSSG exercise much control over \( GSH1 \) and \( GSH2 \) transcription (Xiang and Oliver 1998). Similarly, while \( \text{H}_2\text{O}_2 \) and CAT inhibitors increase tissue GSH, they did not affect \( GSH1 \) or \( GSH2 \) transcript abundance (Xiang and Oliver 1998). Exposure to oxidative stress increased \( \gamma \)-ECS activity and glutathione in \( Arabidopsis \), but \( GSH1 \) mRNA abundance was unchanged. In contrast, exposure to heavy metals such as cadmium and copper increases \( GSH1 \) and \( GSH2 \) transcripts (Schäfer et al. 1998, Xiang and Oliver 1998). While SA does not regulate \( GSH1 \) or \( GSH2 \) transcripts, jasmonic acid (JA)

![Fig. 4. Effect of \( \gamma \)-glutamyl cysteine synthetase (\( \gamma \)-ECS) overexpression in \( Arabidopsis \) thaliana. Ten-day-old transgenic \( A. \) thaliana (Cobbett et al. 1998) seedlings were compared with the wild-type. The effects of constitutive \( \gamma \)-ECS expression (\( \gamma \)-ECS o/e) are demonstrated by semi-quantitative RT-PCR (A) tissue reduced glutathione (GSH) contents (B) and the stimulatory effect of the resultant high GSH levels on seedling root growth (C). The higher activities of \( \gamma \)-ECS activity in the transgenic plants leads to greater GSH abundance in that persists even in the presence of 0.7 mM 1-buthionine-5R-sulfoximine (C).]
has a marked stimulatory effect (Xiang and Oliver 1998). Methyl jasmonate caused a rapid transient increase in transcripts encoding sulphur assimilation enzymes and GSH1 and GSH2 mRNA levels in Arabidopsis (Harada et al. 2000). 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 H$_2$O$_2$ or modified GSH/GSSG ratios in de-repressing translation of the existing mRNA (Xiang and Oliver 1998). Stress-induced increases in glutathione, such as those observed in plants deficient in catalase, have shown that glutathione accumulation is preceded or accompanied by a marked decrease in the reduction state of the pool (Smith et al. 1984, Willekens et al. 1997). 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 H$_2$O$_2$ or changes in the GSH/GSSG ratio (Xiang and Bertrand 2000). A reoxy-sensitive 5’UTR-binding complex is thus suggested to control γ-ECS mRNA translation in A. thaliana (Xiang and Bertrand 2000).

It has long been accepted that post-translational regulation of γ-ECS through end-product inhibition by GSH may control tissue GSH concentrations (Hell and Bergmann 1990, Schneider and Bergmann 1995, Jez et al. 2004). Moreover, γ-ECS activity is inhibited by thiols in plant extracts (Noctor et al. 2002a) as well as in the purified recombinant enzyme (Jez et al. 2004). Such feedback control of γ-ECS activity by GSH concentrations is easy to understand in terms of the exclusive localization of this enzyme in the chloroplast, where localized GSH accumulation in the limited space of the organelle would cause a specific feedback limitation on γ-ECS activity, until excess GSH could be exported to the cytosol.

Post-translational control of γ-ECS activity remains a possibility (May et al. 1998), but protein phosphorylation similar to that reported in animals (Sun et al. 1996) has not yet been found in plants. In animals, a smaller regulatory subunit increases the catalytic potential of the larger catalytic subunit by alleviating the effects of feedback control (Huang et al. 1993, Mulcahy et al. 1995). A recent analysis in human cells has shown that a number of enzymes affecting glutathione metabolism and breakdown are targets for of 14-3-3 proteins (Pozuelo Rubio et al. 2004), and this may be also the case in plants. Because the precise pathway of glutathione degradation in plants remains poorly resolved, and many important plant enzymes are controlled by interactions with 14-3-3 proteins (DeLille et al. 2001), this type of regulation of GSH synthesis and/or degradation is a possibility that merits further investigation.

**GSH homeostasis-transport**

The physical separation of the rate-limiting step of GSH synthesis catalysed by γ-ECS from the second reaction step suggests that γ-ECS and GSH transport have fundamental roles in glutathione homeostasis. Recent data has implicated oligopeptide transport proteins (OPTs) in GSH transport. However, none of the nine AtOPTs has a high affinity for GSH transport, and the cellular localizations of these proteins have not been resolved. Chloroplastic transporters are particularly important, as they are essential for the export of γ-ECS and GSH produced within the chloroplast. However, none of the known transporters that have been implicated in GSH/GSSG transport have been shown to be localized in the chloroplast despite the fact that recent data suggests that the chloroplast may be the major site of γ-ECS production. One or more high-affinity γ-ECS/GSH/GSSG transporters targeted to the chloroplast may be expected, as illustrated in Fig. 3. Preliminary biochemical experimentation has shown that GSH is transported into isolated wheat chloroplasts. Noctor et al. (2002a) observed that 35S-labelled GSH was taken up by chloroplasts in a time-dependent linear fashion. There appeared to be two independent systems for uptake with at least one of those being an active transport system. This was inferred by observing linear uptake of labelled GSH up to 20–30 μM GSH showing saturation at a concentration of 100–200 μM, which was followed by an increase in the rate of uptake until a concentration of 1 mM was reached. From these initial experiments, there is evidently great potential for modulating GSH concentration by transport in response to an altered demand for GSH caused by either the enzymes of the cytosol or ascorbate-glutathione cycle in the chloroplast.

GSH transport has not only been observed at the chloroplast membrane. Early experiments with heterotrophic tobacco cells suggested that GSH could be transported across plasma membranes of cells (Schneider et al. 1992). In this study, both high-affinity (Km 18 μM) and low-affinity (Km 780 μM) GSH transport systems were identified. Other biochemical studies indicated that some transporters had preferences for specific GSH metabolites. For example, the bean plasma-lemma transporter exhibited a significantly greater affinity for GSSG compared with GSH (Jamaï et al. 1996). Taken together, the biochemical data from chloroplasts and plasma membrane preparations suggest that there is diversity in the GSH transport mechanisms.
To date, the best-characterized glutathione transporters are all members of the peptide transporter superfamily. Early research in yeast identified the Yeast Cadmium Factor (YCF1) protein as a glutathione-S-conjugate (GS-conjugate) transporter (Szczypka et al. 1994; Falcon-Perez et al. 1999). YCF1 is a vacuolar membrane protein that transports Cd\textsuperscript{2+}•GSH\textsubscript{2} and consequently is essential for Cd\textsuperscript{2+} tolerance in yeast. YCF1 is a member of the ABC-type transporters similar to the multiple drug-resistance transporters (MRPs) in humans (Decottignies and Goffeau 1997). A common feature of many MRP proteins is the ability to transport GS-conjugates and, in some cases, cotransport of GSH and drugs (Rappa et al. 1997). MRP homologues are also found in plants (Kolukisaoglu et al. 2002). In Arabidopsis, there are 14 genes (and one putative pseudogene) encoding MRP transporters (Kolukisaoglu et al. 2002, Martinoia et al. 2002). Among these, only AtMRP1, AtMRP2, AtMRP3 and AtMRP5 have been characterized. Transport of GS conjugates was observed for AtMRP1, AtMRP2 and AtMRP3 and is consistent with the phenotype observed in an AtMRP5 insertion mutant (Lu et al. 1997, Lu et al. 1998, Tommasini et al. 1998, Gaedeke et al. 2001, Liu et al. 2001). More recently, AtMRP3 has been implicated in Cd detoxification, as it is upregulated by Cd treatment which suggests that it may be transporting GSH-Cd or PC-Cd complexes (Bovet et al. 2005). However, the ability of the MRP proteins to transport GSH and GSSG is variable. For example, while only AtMRP2 and AtMRP4 exhibited competitive inhibition of GS-conjugate transport by GSH, all four showed competitive inhibition in the presence of oxidized glutathione (GSSG). Because GSSG can be considered a GS-conjugate, this is consistent with the MRP proteins being predominantly GS-conjugate transporters. However, the observed GSSG transport may also suggest that they play an important role in removing oxidized glutathione as a mechanism to help maintain the redox poise of the cell (Foyer et al. 2001).

A second peptide transporter belonging to a different family of peptide transporters from yeast has provided further insights into GSH transport. A high-affinity glutathione transporter (Hgt1p) was identified using the hgt1 mutant of Saccharomyces cerevisiae which was unable to transport GSH (Bourbouloux et al. 2000). The Hgt1p protein is a member of the oligopeptide transporter (OPT) family (Stacey et al. 2002). In Arabidopsis, there are nine OPT orthologues of the yeast OPT proteins (Hgt1p and Opt2p) (Koh et al. 2002). All of the OPT proteins are believed to be integral membrane proteins transporting small peptides (3–5 amino acids) (Koh et al. 2002). Heterologous expression of the Arabidopsis OPT genes in S. cerevisiae has confirmed that most are able to transport small peptides, while only some exhibit glutathione transport capabilities and none exhibit high-affinity transport similar to Hgt1p (Foyer et al. 2001, Koh et al. 2002). Thus, although glutathione transport has been demonstrated in plant cells, no GSH-specific transporters, like Hgt1p, have been identified. Thus, while considerable movement of GSH/GSSG has been observed in biochemical studies, little is known about the factors responsible for this transport.

Conclusions and perspectives: prospects for manipulation of glutathione metabolism for improved nutritional quality

The ubiquity of GSH and its critical functions regulating plant growth and as an important sulphur sink make GSH an attractive target for manipulation to improve crops for enhanced nutritional value as well as improved crop growth, sustainability, predictability and vigour. Fortunately, plant biology now has an extensive toolbox available to help achieve this aim. Many of the genes found in model plant systems such as Arabidopsis have counterparts in important crop species, and hence it is expected that much of the knowledge on GSH gained in laboratory systems will be readily transferable to important crop varieties. In addition, plant cell cultures could be used as a cheaper cell synthetic factory for the production of γ-EC, GSH, GSSG and related homologues than the yeast system that is currently used for GSH production. Glutathione has potential for use in the food industry, for example as a natural food additive with flavour-enhancing properties. In whole plants, there is likely to be a ceiling on the enhanced glutathione concentrations that can be achieved, set by the level of feedback inhibition on GSH synthesis within the chloroplast. However, this ceiling GSH content may vary between plant species, because it is known that some alpine plants hyperaccumulate other antioxidants particularly ascorbate, and the same may be true of glutathione. There are essentially no metabolic arguments suggesting that GSH hyperaccumulation in plants is impossible, because GSH accumulation is simply a matter of compartmentation, such that the site of GSH accumulation is separate from that of synthesis, preventing feedback inhibition. This separation could easily be achieved in plant cell culture systems where GSH synthesis and export were enhanced such that GSH and related peptides were transported into the culture medium from which they could be continually harvested. Moreover, because the bifunctional streptococcal γ-EC-GSH-S shows no feedback inhibition by GSH, plant cells transformed to
express this enzyme may be able to maintain much higher GSH concentrations than those expressing the plant or E. coli enzymes.

Manipulating GSH should affect three main phenotypic aspects (1) enhanced crop resilience and growth; (2) improved innate immune responses and basal resistance to pathogen attack and (3) increasing sulphur/cysteine content in crop plants. A number of studies have demonstrated the feasibility of enhancing plant glutathione synthesis and accumulation. Such plants have been used successfully for phytoremediation purposes and to reclaim arable land. All studies to date have shown that increasing the GSH content of plants concomitantly increases the total organic sulphur content. Comparatively, the manipulation of GSH partitioning, both throughout the plant and between cellular compartments, has lagged behind, because no high-affinity GSH transporters have been identified to date. The identification of these will be a key step in combining and interpreting current results in GSH research. Moreover, while GSH transport mutants will allow for a more detailed dissection of signalling and stress pathways, they will also provide for future targeted nutriomics approaches where glutathione might be enriched in specific cell types or in discrete compartments of the plant cell.

The fully sequenced genomes of species such as Arabidopsis and rice together with new breeding technologies and large scale ‘omics’, systems biology approaches will allow increasingly rapid advances in understanding and applications. For example, predictability of crop yield and hence income to the farmer and reliable sources for the food industry are severely hampered by environmental factors. Collectively, the environmental abiotic stresses restrict plant vigour and create a ‘yield gap’. This is the difference between the theoretical maximum or yield potential of the crop and the actual yield achieved by the farmer. Over the last 50 years, plant breeders have improved the yield potential, but the yield gap remains, and it is forecast to increase as the world climate becomes less predictable. In measurements made under field conditions, maize leaf glutathione contents correlate well with chilling resistance and yield. One factor determining the sensitivity of maize to both short-term and long-term chilling is the restriction over glutathione reduction and cycling between the maize leaf BS and M cell types (Gomez et al. 2004b). By understanding how glutathione is used by the plant in acclimation responses to environmental cues at the molecular level, we can use this information to widen the range in which crop plants grow and produce crops that perform closer to their theoretical maximum. The quantitative trait loci (QTL) analysis in plants such as maize is essential to determine chromosome regions carrying genes which control GSH accumulation and stress tolerance and other important traits in crops. Mapping experiments at field sites have allowed us to identify two major QTL for the total amount of glutathione in maize, with a confidence interval of about 30 cM. To date, little information on QTL for glutathione has appeared in the literature. Detailed fine maps of QTL regulating glutathione accumulation under field conditions in different plant species will be useful not only to the scientific community but also to plant breeders and associated industries.

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