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REVIEW ARTICLE

Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants

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Abstract

Crucial roles in sulphur metabolism and plant defence have been described in recent years for the tripeptide thiol glutathione. In spite of this, the metabolism of glutathione and its response to stress conditions remained only partly understood. In many plants, one of the major difficulties in studying the control of glutathione synthesis is the low extractable activities of the enzymes involved. Consequently, several groups have exploited transformation technology using genes for the enzymes of glutathione synthesis or reduction. This approach has allowed the production of plants with systematically enhanced levels of glutathione (up to 4-fold higher than untransformed controls) and has permitted numerous insights into the control of glutathione synthesis or reduction state and its interaction with other areas of primary or defensive metabolism.

Key words: Oxidative stress, photorespiration, glutathione, ascorbate, poplar.

Introduction

Glutathione is found in the vast majority of prokaryotic and eukaryotic cells, where it often represents the major pool of non-protein reduced sulphur (Kunert and Foyer, 1993). The reduced form of glutathione (GSH) is a tripeptide thiol with the formula γ-glu-cys-gly. The pathway of GSH biosynthesis (Fig. 1) is well established: two sequential ATP-dependent reactions allow the synthesis of γ-glutamylcysteine (γ-EC) from L-glutamate and L-cysteine, followed by the formation of GSH by addition of glycine to the C-terminal end of γ-EC (Meister, 1988). These reactions are catalysed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS). Several plant species produce analogous tripeptide derivatives of γ-EC in addition to, or in place of, GSH (γ-EC-ala or homoglutathione: Klapheck, 1988; γ-EC-ser: Klapheck *et al*., 1992; γ-EC-glu: Meuwly *et al*., 1993). Since all functions thus far described for GSH in plants are related to the cysteine moiety of the tripeptide, these homologues may exercise similar biochemical roles to γ-EC-gly.

Numerous physiological functions have been attributed

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Abbreviations: AA, ascorbic acid; APS, adenosine 5′-phosphosulphate; AP, ascorbate peroxidase; CAM, crassulacean acid metabolism; CaMV, cauliflower mosaic virus; chl, chlorophyll; CH_2 THF, methylene tetrahydrofolate; C : O, ratio of the rates of carboxylation of RubP and oxygenation of RubP; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; γ-EC, γ-glutamylcysteine; γ-ECS, γ-glutamylcysteine synthetase; FW, fresh weight; GR, glutathione reductase, GS, glutathione synthetase; GSH, reduced glutathione; GSSG, glutathione disulphide; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; PC, phytochelatin; 3-PGA, 3-phosphoglycerate; prot., protein; rbcS, Rubisco small subunit; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; THF, tetrahydrofolate; triose P, triose phosphate.

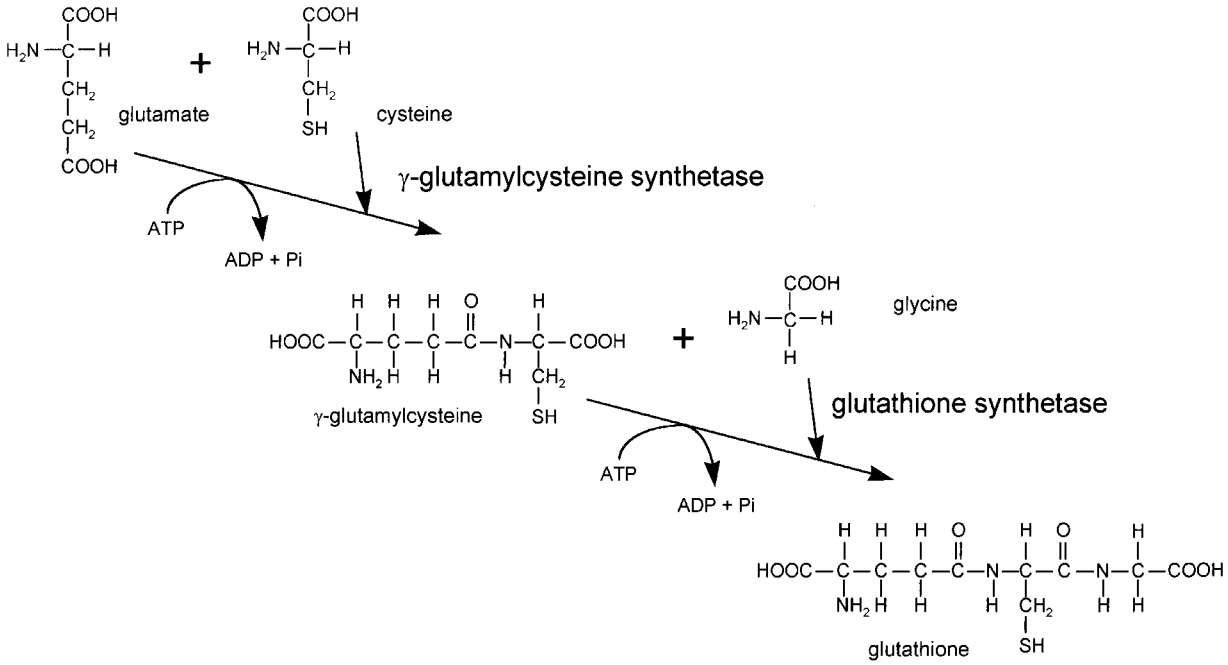

Fig. 1. Schematic representation depicting the pathway of glutathione biosynthesis from constituent amino acids.

to GSH in plants (Rennenberg, 1995; Foyer *et al.*, 1997). Important as a storage form of reduced sulphur, GSH also regulates inter-organ sulphur allocation (Herschbach and Rennenberg, 1991, 1994; Lappartient and Touraine, 1996). Glutathione has been shown to act as a regulator of gene expression (Wingate *et al.*, 1988; Baier and Dietz, 1997), is the precursor of phytochelatins, which bind supra-optimal concentrations of heavy metals (Grill *et al.*, 1987, 1989), and is a substrate for the GSH *S*-transferases, which catalyse the conjugation of GSH with potentially dangerous xenobiotics such as herbicides (Marrs, 1996). Glutathione may also be involved in the redox regulation of the cell cycle (Gyuris *et al.*, 1993; Russo *et al.*, 1995; Shaul *et al.*, 1996; Sanchez-Fernández *et al.*, 1997). Owing to its redox-active thiol group, GSH has often been considered to play an important role in defence of plants and other organisms against oxidative stress (Alscher, 1989; Grant *et al.*, 1996). In all cells where GSH is found, the reduced tripeptide form exists interchangeably with the oxidized form (glutathione disulphide: GSSG). While glutathione reductase (GR) uses NADPH to reduce GSSG to GSH, various free radicals and oxidants are able to oxidize GSH to GSSG (Fig. 2). The proportion of glutathione in the reduced form reflects the relative rates of reduction and oxidation and is always greater than 0.9 under non-stress conditions. Since the concentration of GSH in the chloroplast stroma is thought to be close to 5 mM, the reduced form of glutathione may act as an important redox buffer, preventing enzyme inactivation by protecting potentially susceptible protein thiol groups (Foyer and Halliwell, 1976; Halliwell and Foyer,

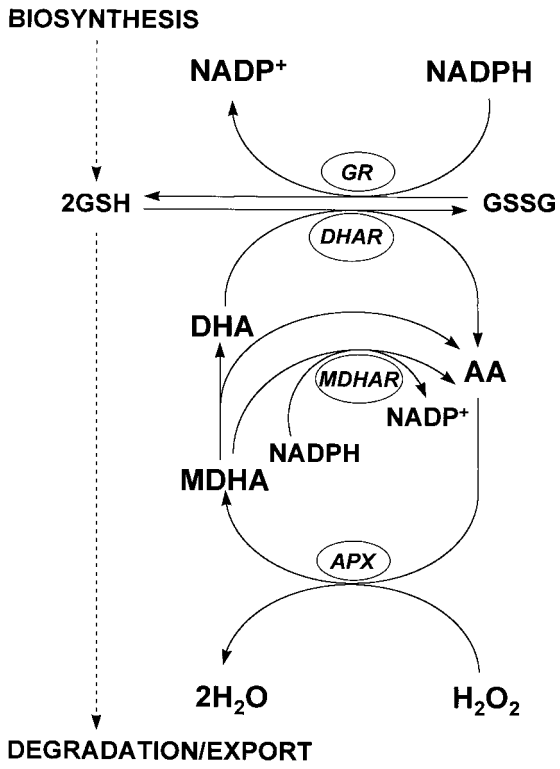

Fig. 2. Schematic representation of the relationships between glutathione biosynthesis and export together with interactions between the reduced and oxidized forms of glutathione and ascorbate in removal of H_2O_2.

1978; Alscher, 1989). A specific function in maintenance of the ascorbate pool in plant cells has been demonstrated. Dehydroascorbate (DHA) formed when ascorbate is oxidized must be re-reduced in order to prevent decreases in the total ascorbate pool. At alkaline pH values, GSH rapidly reduces DHA to ascorbate in a non-enzymic reaction (Foyer and Halliwell, 1976; Winkler, 1992). In plant tissues this reaction is catalysed by dehydroascorbate reductases (DHAR; Foyer and Halliwell, 1976, 1977). While specific DHARs catalysing this reaction have been purified from plants (Foyer and Halliwell, 1977; Kato *et al*., 1997), it is clear that the reaction between GSH and DHA is also catalysed by other enzymes such as glutaredoxins (thioltransferases), protein disulphide isomerases (Wells *et al*., 1990), and a Kunitz-type trypsin inhibitor (Trümper *et al*., 1994; Morell *et al*., 1997). The non-enzymic reduction of DHA by GSH is rapid at alkaline pH values, but the enzyme-catalysed reaction is significantly faster.

Recognition of the significance of GSH in plant metabolism has prompted efforts to identify the factors which regulate its synthesis and reduction state. Consequently, several reports of the cloning of γ-ECS (May and Leaver, 1994), GS (Rawlins *et al*., 1995; Ullman *et al*., 1996) and GR (Creissen *et al*., 1992, 1996) from plant sources have appeared in the last five years. Nevertheless, despite these advances, biochemical data relating to the kinetic characteristics of the purified plant γ-ECS and GS are lacking. This contrasts markedly with the bacterial and rat enzymes, which are well characterized (Huang *et al*., 1988, 1993, 1995). Moreover, although transformed tobacco plants have been produced which overexpress a GR cDNA from pea (Broadbent *et al*., 1995), no reports of plants transformed with the plant γ-ECS or GS clones have yet appeared.

During oxidative stress associated with catalase inhibition (Smith *et al*., 1984; Smith, 1985; May and Leaver, 1993), catalase deficiency (Chamnongpol *et al*., 1996; Willekens *et al*., 1997) or ozone exposure (Sen Gupta *et al*., 1991; Ranieri *et al*., 1993; Luwe, 1996), glutathione accumulates. High cellular glutathione levels are associated with resistance to heavy metals in tomato cells (Chen and Goldsborough, 1994), while heavy metal exposure has been shown to lead to accelerated GSH synthesis in roots and cultured cells (Rüegsegger and Brunold, 1992; Schneider and Bergmann, 1995). These correlative studies not only implicated glutathione in protection against various forms of stress but also drew attention to the regulatory factors mediating the metabolic signalling for modified rates of GSH synthesis and accumulation. In 1989, at the outset of our research programme, it was clear that many of the pertinent questions relating to glutathione metabolism in plants could not be answered by purely biochemical or physiological studies. A combined molecular and metabolic approach was therefore undertaken, involving the production of bacterial GR, GS or γ-ECS cDNA constructs. By inclusion or omission of sequences encoding a chloroplast transit peptide, the bacterial enzymes were targeted to either chloroplastic or cytosolic compartments. This specific metabolic targeting allowed the precision in manipulation required for incisive assessment of regulation. As shown for the pathways of lysine and threonine synthesis, the use of bacterial genes to produce plants with compartment-specific overexpression of enzymes can both lead to enrichment of a given end-product and provide invaluable insights into the regulation of metabolic pathways *in planta* (Shaul and Galili, 1992*a*, *b*). It was planned to explore glutathione metabolism in transformed and untransformed poplars with the following three principal questions in mind. (1) Can constitutively enhanced glutathione concentrations be engineered in leaves and other plant organs? (2) What can be learnt about the regulation of GSH biosynthesis and related metabolic pathways? (3) Does a constitutive increase in the glutathione content of leaves confer a physiological advantage in terms of increased stress tolerance? This review addresses these three questions in turn, describing to what extent transformed plants, with particular reference to our own work over the last eight years, have provided answers and to what extent they have clarified the problems which remain in understanding the role of glutathione in cell function.

Manipulating glutathione levels and redox state by plant transformation

Glutathione reductase

Glutathione reductase (NADPH: oxidized glutathione oxidoreductase; GR) catalyses the NADPH-dependent reduction of the disulphide bond of oxidized glutathione. Like other members of this family the *E. coli* GR is a homodimer in its active form (Arscott *et al*., 1989; Scrutton *et al*., 1988) with one FAD molecule per monomer. The catalytic mechanism involves reduction of the flavin molecule by NADPH followed by oxidation of the flavin by a redox-active cystine residue to produce a thiolate anion and cysteine. GSSG can then be reduced via reversible thiol-disulphide interchange reactions. If the reduced enzyme is not re-oxidized by GSSG reversible inactivation follows (Arscott *et al*., 1989). In chloroplasts the NADPH concentration can be several times higher than the K_m ($\sim 3\ \mu M$) for NADPH, but the importance of NADPH inhibition in the regulation of the activity of the enzyme when the GSSG pool is very low is uncertain. Evidence from the literature suggested that GRs from plant sources might be less sensitive to inhibition by NADPH than GR from *E.coli* and other sources (Connell and Mullet, 1986; Kalt-Torres *et al*., 1984). Our studies, described below, established that any such inhibition is

not sufficient to prevent the operation of *E.coli* GR in poplar leaves when it is present in large excess. Similar observations were obtained in transformed tobacco plants expressing a pea GR cDNA in the chloroplast (Broadbent *et al.*, 1995).

It was shown previously that GR is required to maintain the glutathione pool in *E.coli* (Kunert *et al.*, 1990) and that the presence of GR was essential for glutathione accumulation. GR expression in a glutathione-deficient *E. coli* mutant restored the glutathione pool to a level similar to that in the wild-type (Kunert *et al.*, 1990). However, overexpression of GR in tobacco leaves demonstrated that increases in GR activity in the cytosol of between two and ten times that measured in the untransformed controls did not result in any further increases in the foliar glutathione content (Foyer *et al.*, 1991). This result was confirmed in poplar where similar levels of overexpression of GR in the cytosol led to comparable results (Foyer *et al.*, 1995). Extractable foliar GR activities were between two and ten times higher than in untransformed poplars (Foyer *et al.*, 1995). Lines overexpressing GR in the chloroplast were also obtained, but in this case much higher levels of GR activity were achieved (Foyer *et al.*, 1995). Northern analysis of these lines showed that they contained transcripts of the bacterial gene which were approximately ten times more abundant than in poplars overexpressing the enzyme in the cytosol. The poplars overexpressing GR in the chloroplast had extractable GR activities which were up to 1000-fold higher than those of control poplars (Foyer *et al.*, 1995). While this effect was in part due to the action of the 35S promoter with double enhancer sequence, which has been shown to allow strong expression of genes introduced into poplar (Leplé *et al.*, 1992), differences between GR activities in the chloroplastic and cytosolic transformants (100- to 500-fold) did not primarily result from differences in the relative rates of expression in the chloroplast and cytosol. Rather, they are mainly due to greater stability of the bacterial enzyme in the poplar chloroplast than in the poplar cytosol.

While the transformants overexpressing GR in the cytosol had GSH contents similar to untransformed poplars, the chloroplastic transformants exhibited increased foliar GSH (Foyer *et al.*, 1995). Though this effect correlated only weakly with extractable GR activity, and was more pronounced in certain lines than others, some lines showed increases in GSH content of up to 2.5-fold (Foyer *et al.*, 1995). Moreover, chloroplastic overexpression of GR significantly increased the reduction state of the glutathione pool, an effect not observed in the cytosolic GR overexpressors (Foyer *et al.*, 1991, 1995). Mullineaux *et al.* (1994) also found that overexpression of GR in the tobacco chloroplast, but not the cytosol, increased both the reduction state and total pool of glutathione. Overexpression of a pea GR cDNA in the tobacco chloroplast, cytosol, or in both chloroplasts and mitochondria simultaneously, also led to increases in the total pool of glutathione, although redox state was unaffected (Broadbent *et al.*, 1995). The correlation of reduction state with total pool size, in poplar overexpressing GR in the chloroplast, may suggest that the oxidized form of glutathione, GSSG, is more readily degraded than GSH (Foyer *et al.*, 1995). This strongly implicates chloroplastic GR activity as a factor that influences glutathione levels through controlling the capacity for regeneration of GSH from GSSG. There may, however, be other mechanisms responsible for the increase in glutathione in the poplars overexpressing GR in the chloroplast: at least two lines possess significantly enhanced foliar cysteine contents (more than 2-fold greater than those of untransformed poplars; Noctor *et al.*, 1997*a*). Synthesis and availability of cysteine are factors which exert considerable influence over synthesis of GSH (see the section on Biosynthesis of cysteine and its interaction with glutathione synthesis). Whatever the mechanism, it is clear that GR of bacterial origin can function in the chloroplasts of plant cells to increase both the GSH/GSSG ratio and the total glutathione pool. NADPH-dependent inhibition of GR activity in the stroma does not negate the effects of increased GR activity.

Glutathione synthetase

Glutathione synthetase catalyses the ATP-dependent formation of a peptide bond between the α-carboxyl group of cysteine in γ-EC and the α-amino group of glycine to form GSH (Fig. 1). The catalytic mechanism involves an acylphosphate intermediate resulting from the transfer of the γ-phosphate of ATP to the cysteinyl carboxyl group. The α-amino group of glycine reacts with the acylphosphate group, forming a peptide bond and releasing inorganic phosphate. The bacterial GS is a tetramer of four identical subunits of 35.6 kDa molecular weight (Gushima *et al.*, 1984). Poplar was transformed to overexpress this enzyme in the cytosol (Foyer *et al.*, 1995). Immunoblotting of leaf extracts confirmed the presence of a polypeptide of approximately 35.6kDa (Arisi *et al.*, 1997). Extractable foliar GS activities were enhanced by up to 300-fold relative to untransformed poplars (Foyer *et al.*, 1995). Despite this huge increase in foliar GS activity, foliar thiol contents were not significantly affected (Strohm *et al.*, 1995; Foyer *et al.*, 1995). Leaf discs from these poplars were, however, capable of sustaining a higher rate of GSH synthesis when supplied with exogenous γ-EC (Strohm *et al.*, 1995). The biochemical significance of these findings is discussed later.

Five poplar lines, in which the bacterial GS is directed to the chloroplast, have recently been obtained. The presence of the bacterial protein in this compartment was

confirmed by chloroplast isolation (Noctor *et al*., 1998). Extractable foliar GS activities in these lines are enhanced by up to 500-fold; as in the transformants overexpressing GS in the cytosol, this markedly increased activity does not affect foliar thiol contents. These data are in agreement with results obtained for tobacco transformed to express the bacterial GS in the chloroplast (Creissen *et al*., 1996).

γ-Glutamylcysteine synthetase

γ-Glutamylcysteine synthetase (γ-ECS) catalyses the ATP-dependent ligation of cysteine and glutamate to form γ-EC in a reaction mechanism analogous to that catalysed by GS, except that it is the γ-carboxyl group of glutamate which condenses with the α-amino group of cysteine. Five transformed poplar lines overexpressing γ-ECS in the cytosol were obtained ('ggs'). Four of the five ggs lines strongly express the bacterial gene (Arisi *et al*., 1997). Use of an antibody raised against the *E. coli* γ-ECS (a monomeric protein of approximately 56 kDa: Watanabe *et al*., 1986) showed that the amount of bacterial protein in leaf extracts correlated with strength of expression of the transgene (Arisi *et al*., 1997). Crude leaf extracts from the four transformant lines strongly expressing the bacterial gene exhibit γ-ECS activities of 7–12 nmol mg^{-1} protein min^{-1} (Table 1). In both crude and partially purified leaf extracts, the endogenous poplar γ-ECS has proved so far undetectable, precluding precise calculation of the relative increase in activity due to transformation. In species where both GS and γ-ECS have both been measured, γ-ECS is most often the lower activity of the two (Hell and Bergmann, 1990; Chen and Goldsbrough, 1994; Schneider and Bergmann, 1995). Assuming this holds true for poplar, a minimum relative increase in γ-ECS activity in the poplar transformants can be calculated, by comparison of extractable activities of γ-ECS with those of GS. Since values for the latter in poplar are between 0.2 and 0.4 nmol mg^{-1} protein min^{-1} (Table 1), an approximate enhancement of γ-ECS activity in ggs lines of 18- and 60-fold can be calculated (Noctor *et al*., 1996; Arisi *et al*., 1997). Assuming the endogenous poplar activity also to be absent from assays of leaf extracts from the ggs transformants, the poplar line weakly overexpressing γ-ECS (ggs17) would possess a foliar γ-ECS activity about 2–3 times higher than that of untransformed poplars (Arisi *et al*., 1997). All lines overexpressing γ-ECS were shown to have similar foliar activities of GS and GR to untransformed poplars (Noctor *et al*., 1996; Arisi *et al*., 1997).

Determination of foliar thiols in ggs and untransformed poplars revealed that leaves from the four ggs lines strongly overexpressing γ-ECS have between two and four times more GSH than leaves from untransformed poplars (Table 1: Noctor *et al*., 1996; Arisi *et al*., 1997). These increases in GSH were accompanied by equivalent increases in GSSG. Thus, despite the marked increase in total glutathione, the redox state of the glutathione pool was not significantly different from that of untransformed poplars. This implies that the endogenous poplar GR activity is, at least under non-stressed conditions, in excess of that required to maintain the glutathione pool in its predominantly reduced state. In addition to the increases in glutathione, foliar γ-EC contents were also increased between 5-and 15-fold in these poplars (Noctor *et al*., 1996; Arisi *et al*., 1997). Elevated cysteine contents (up to 2-fold) were also observed, although these effects were more variable than the consistent and stable increases in γ-EC and GSH (Noctor *et al*., 1996; Arisi *et al*., 1997). Importantly, however, despite the pronounced increases in γ-EC and GSH contents in the poplars overexpressing γ-ECS, cysteine contents were never decreased: the significance of this observation is discussed later. Determinations of free amino acid pools in leaves from ggs and untransformed poplars showed that foliar glutamate and glycine contents were not significantly affected by strong overexpression of γ-ECS (Table 2; Noctor *et al*., 1997*a*). In the one poplar line weakly overexpressing γ-ECS (ggs17), foliar thiol contents were shown not to differ from those of untransformed poplars (Arisi *et al*., 1997). Possible explanations of this last observation are discussed in Noctor *et al*. (1997*c*).

Six independent lines, designated Lggs, in which the bacterial γ-ECS is targeted to the chloroplast, have recently been obtained (Noctor *et al*., unpublished

Table 1. *Extractable activities ± SD of γ-ECS and GS in untransformed poplars and three poplar lines strongly overexpressing γ-ECS in the cytosol*

Typical foliar glutathione contents (measured in plants growing in the greenhouse in January) are also shown. Values in parentheses give number of separate foliar extracts/number of independent experiments; n.d., not detected.

Plant line	Enzyme activity (nmol mg^{-1} protein min^{-1})		Glutathione content (nmol g^{-1} fresh weight)
	γ-ECS	GS	
Untransformed	n.d.	0.305 ± 0.113 (27/7)	277 ± 31 (6/2)
ggs28	9.67 ± 2.42 (26/7)	0.280 ± 0.107 (22/7)	1016 ± 115 (9/3)
ggs11	8.83 ± 2.10 (13/4)	0.390 ± 0.144 (13/4)	554 ± 20 (6/2)
ggs5	7.79 ± 0.95 (3/1)	0.281 ± 0.094 (3/1)	965 ± 32 (6/2)

Table 2. *Extractable foliar enzyme activities and total foliar concentrations of substrate and product for γ-EC and GSH synthesis in illuminated leaves from untransformed poplars and poplars overexpressing γ-ECS (ggs28)*

Values are means of three (all enzyme activities: all values for glu and gly) or four (values for cys, γ-EC, GSH) measurements under steady-state conditions (10 h light: 500 μmol m^{-2} s^{-1}). Standard deviations were between 5% and 15% of values shown; n.d. not detected.

	Enzyme activity (nmol mg^{-1} protein min^{-1})		Total foliar concentration (nmol g^{-1} fresh weight)					[γ-EC]/([cys]×[glu])	[GSH]/([γ-EC]×[gly])
	γ-ECS	GS	glu	cys	gly	γ-EC	GSH		
Untransformed	n.d.	0.24	5541	11.3	263	8.2	542	1.3×10^{-4}	0.25
ggs28	9.7	0.20	6399	19.7	340	138	2043	1.3×10^{-3}	0.044

results). Five of these lines have extremely high extractable foliar γ-ECS activities (between 25 and 40 nmol mg^{-1} protein min^{-1}), correlating with an intense band on western blots of leaf extracts. Isolation of chloroplasts from ggs and Lggs poplars confirmed that the transgene product is present in the chloroplast in Lggs and outside the chloroplast in ggs (Noctor *et al.*, unpublished results). The differences in enzyme activities between the two types of transformant (4- to 5-fold higher in Lggs) can only partly be explained by expression strength of the transgene, and perhaps suggest that, like the bacterial GR, the bacterial γ-ECS is more stable in the chloroplast than in the cytoplasm.

Like cytosolic overexpression, γ-ECS overexpression in the chloroplast also leads to marked increases in foliar thiols: the five Lggs lines strongly overexpressing the enzyme possess glutathione pools which are between two and four times higher than those of untransformed poplars (Noctor *et al.*, unpublished results). Foliar γ-EC is even more markedly enhanced in Lggs than in ggs (up to 50-fold higher than in untransformed poplars), although foliar contents of this thiol are markedly affected by light and dark in both types of transformant (see below). The increased thiols in ggs and Lggs demonstrate that GSH synthesis can be up-regulated by increased chloroplastic or cytosolic γ-ECS activity without detriment to plant growth (Fig. 3), foliar amino acid pools or photosynthetic performance (Noctor *et al.*, unpublished results). In contrast, transgenic tobacco containing a construct directing the bacterial γ-ECS to the chloroplast were found to show necrotic lesions and impaired growth (Creissen *et al.*, 1996). At present, it is difficult to assess the significance of the study with tobacco, since no detailed data has yet appeared relating to transgene expression, foliar enzyme activities or foliar thiol contents. The absence of general physiological perturbation in the poplars overexpressing γ-ECS underlines the flexibility of plant metabolism, especially when one considers the elevation of γ-EC from a low level intermediate to a metabolite whose foliar content may exceed those of major free amino acids such as aspartate, serine and glutamine (Noctor *et al.*, unpublished results). Halobacteria lack GSH but accumulate γ-EC instead, which (by virtue of a specific oxidoreductase) fulfils analogous physiological roles to GSH (Newton and Javor, 1985). Interestingly, a recent report provides evidence that γ-EC may be able to substitute for some of the antioxidant functions of GSH in yeast (Grant *et al.*, 1997).

Fig. 3. Photograph of untransformed poplars and poplars overexpressing γ-ECS in the cytosol or chloroplast.

Regulation of glutathione synthesis and its interaction with primary metabolism

Biosynthesis of cysteine and its interaction with glutathione synthesis

Foliar cysteine pools are often tightly controlled, depending on the availability of reduced sulphur (Buwalda *et al.*, 1988, 1990) and on the concentration of *O*-acetylserine (Rennenberg, 1983; Neuenschwander *et al.*, 1991; Saito *et al.*, 1994), whose synthesis from serine is restricted by the activity of serine acetyltransferase (Saito *et al.*, 1994; Ruffet *et al.*, 1995). Whereas activation of sulphate by the reaction catalysed by ATP sulphurylase is generally accepted as the initial step of sulphur assimilation in higher plants, the exact pathway of reduction of the adenosine 5′-phosphosulphate (APS) produced in this reaction is still a matter of debate (Brunold, 1990; Brunold and Rennenberg, 1997). Numerous biochemical and physiological studies led to

the conclusion that carrier-bound sulphite, produced by an APS-sulphotransferase activity, is an intermediate of sulphate reduction. Recent investigations indicate however that APS is reduced by an APS reductase to yield free sulphide (Brunold and Rennenberg, 1997). The *in vitro* assays frequently used to determine APS sulphotransferase activity cannot distinguish between APS reductase and APS sulphotransferase. Since the activity formerly considered to be APS sulphotransferase was frequently found to be regulated by nutritional and environmental factors (Brunold, 1990), it would appear that APS reductase is a regulatory enzyme exerting control over sulphate reduction and, hence, over cysteine availability for glutathione synthesis.

Two distinct but not exclusive interactions between cysteine and GSH synthesis may be distinguished. First, cysteine concentration may actively control the rate of GSH synthesis. Supplying excess exogenous cysteine to leaves or roots has been shown to increase tissue GSH contents, suggesting that one of the factors controlling GSH synthesis is cysteine concentration (Buwalda *et al*., 1990; Farago and Brunold, 1994; Strohm *et al*., 1995; Noctor *et al*., 1996, 1997*b*). This mechanism presumably operates through maintenance of cysteine concentrations within a range which is kinetically limiting for the reaction catalysed by γ-ECS. Control of GSH synthesis through availability of cysteine would allow integration of GSH synthesis with cysteine synthesis, consistent with the role of GSH as an important storage and transport form of reduced sulphur (Rennenberg, 1995). Second, the rate of cysteine synthesis may be tailored to the demand for cysteine in synthesis of GSH and other cysteine-requiring components. Although little work has been carried out on cysteine synthesis specifically, several studies have reported enhanced incorporation of sulphate under conditions of GSH accumulation (Smith *et al*., 1985; Kocsy *et al*., 1996). In contrast, work with canola roots has provided evidence that GSH accumulation can repress sulphate assimilation (Lappartient and Touraine, 1996).

Cysteine concentration limits glutathione biosynthesis whether γ-ECS activity is limiting or not: Studies in which cysteine was supplied to excised poplar leaf discs demonstrated that the availability of this amino acid influences synthesis of GSH (Strohm *et al*., 1995; Noctor *et al*., 1996, 1997*b*). In discs from both untransformed poplars and poplars overexpressing γ-ECS, foliar GSH contents were doubled by incubation with cysteine. Hence, is seems that cysteine concentration limits GSH synthesis whether γ-ECS activity is strongly limiting (untransformed poplars) or not (poplars overexpressing γ-ECS). This provides a dual control over γ-EC synthesis by enzyme activity and substrate supply. It is tempting to speculate that the relative significance of these controls under given conditions reflects different physiological functions. Thus, stringent control of γ-ECS activity by cysteine concentrations could serve to prevent excessive synthesis of GSH and drainage of the cysteine pool: GSH synthesis could then be integrated with sulphur metabolism by changes in cysteine supply (Buwalda *et al*., 1988). Modifications in γ-ECS activity, either through changed rates of enzyme synthesis or via modulation of the activity of existing enzyme, might act principally to tailor the rate of GSH production to the defensive needs of the plant (Smith *et al*., 1984; Smith, 1985; Ruegsegger and Brunold, 1992; May and Leaver, 1993; Chen and Goldsborough, 1994; Schneider and Bergmann, 1995). The increased requirement for cysteine could be met by up-regulation of cysteine synthesis, as observed in poplars overexpressing γ-ECS (see next section).

Up-regulation of GSH synthesis is accompanied by enhanced cysteine synthesis: The observation that cytosolic or chloroplastic overexpression of γ-ECS results in enhanced γ-EC and GSH without concomitant depletion of foliar cysteine pools shows that the necessary cysteine can be made available to support ongoing γ-EC and GSH synthesis (Noctor *et al*., 1996, 1998; Arisi *et al*., 1997). Some transformed lines contained enhanced foliar cysteine contents, suggesting marked up-regulation of cysteine synthesis when GSH synthesis is increased (Noctor *et al*., 1996; Arisi *et al*., 1997). This notion receives support from measurements of activities of the enzymes of assimilatory sulphate reduction in poplar leaves (Will and Rennenberg, unpublished results). Overexpression of γ-ECS in poplar leaves resulted in enhanced *in vitro* activities of APS reductase and serine acetyltransferase of between 2- and 4-fold, whereas the activities of ATP sulphurylase and *O*-acetylserine (thiol) lyase were not affected. Since APS reductase may limit sulphate reduction and serine acetyltransferase may limit the synthesis of the cysteine precursor, *O*-acetylserine, the observed changes in the *in vitro* enzyme activities may be responsible for improved cysteine supply for glutathione biosynthesis in leaves of poplars overexpressing γ-ECS. Apparently, enhanced γ-ECS activity affects the regulation of both APS reductase and serine acetyltransferase in poplar leaves.

Although the mechanism(s) mediating this regulation are as yet unclear, these data represent the first report that direct modulation of the capacity for GSH synthesis effects accompanying modulation of cysteine biosynthesis. Since increased cysteine availability was concomitant with GSH accumulation, the results would seem to be in conflict with those of Lappartient and Touraine (1996), which suggested a homeostatic role for GSH in controlling sulphate assimilation in canola. There are, however, two major differences between these studies. Firstly, poplar and canola have very different growth habits, and it is not clear that the phenomena which regulate whole-plant

nutrition will be identical in perennial species and in annual plants with very short life-cycles. Secondly, the interactions observed in poplar were the result of directly enhancing synthetic capacity in leaves on metabolically upstream events, whereas the study with canola involved modifying sulphate assimilation through changes in sulphate supply to roots (Lappartient and Touraine, 1996).

Synthesis of γ-glutamylcysteine and glutathione

Glutathione contents in plants are known to be affected by various environmental conditions, including sulphate supply (De Kok and Kuiper, 1986; Herschbach *et al*., 1995), fumigation with H_2S (Buwalda *et al*., 1988, 1990) or SO_2 (Herschbach *et al*., 1995), light (Bielawski and Joy, 1986; Koike and Patterson, 1988; Schupp and Rennenberg, 1990; Buwalda *et al*., 1990; Noctor *et al*., 1997*a*, *b*), oxidative stress (Smith *et al*., 1984, 1985; Smith, 1985; May and Leaver, 1993), exposure to heavy metals (Scheller *et al*., 1987; Rüegsegger and Brunold, 1992; Chen and Goldsborough, 1994), herbicide safeners (Rennenberg and Lamoureux, 1990) and atmospheric pollution (Sen Gupta *et al*., 1991; Ranieri *et al*., 1993; Luwe, 1996). The biochemical mechanisms through which these external factors modulate foliar contents of glutathione have not been unambiguously characterized. Candidates are altered rates of synthesis, degradation, import or export.

The rate of synthesis of any metabolic product may be influenced either by supply of necessary substrates, by modulation of the activities of enzymes involved in the pathway, or by both. Where a reaction is regulated by enzyme activity, changes in *in vivo* activity may be effected by changed rates of synthesis of the enzyme (changes in enzyme amounts), by post-translational modification (e.g. phosphorylation, reduction, glycosylation, methylation) or by direct metabolic control exerted by effectors and inhibitors which influence either maximum catalytic rates (V_{max} values) or substrate affinities (K_m values). Several studies have shown increases in maximum extractable activities of either γ-ECS or GS under conditions where GSH synthesis is accelerated. In maize roots exposed to a herbicide safener, Farago and Brunold (1994) reported a 2-fold increase in both GSH content and extractable γ-ECS activity. Accelerated GSH synthesis in maize roots exposed to Cd was correlated with increased extractable GS (Rüegsegger *et al*., 1990) or γ-ECS activity (Rüegsegger and Brunold, 1992). Increases in extractable activities of both γ-ECS and GS were observed in Cd-exposed tobacco cells (Schneider and Bergmann, 1995). Tomato cells resistant to Cd, and able to sustain high levels of GSH, had a 2-fold higher extractable γ-ECS activity than susceptible cells (Chen and Goldsborough, 1994). These increases in extractable activities presumably reflect increases in *de novo* synthesis of γ-ECS or GS, although an influence of post-translational modification (e.g. protein phosphorylation: Sun *et al*., 1996) cannot be discounted.

The question of whether such increases in extractable activities are able to account for the observed increases in synthetic rates *in vivo* is unresolved: in several of the above studies the inferred increases in the rate of γ-EC and GSH biosynthesis were markedly higher than those in extractable γ-ECS or GS activities (Rüegsegger and Brunold, 1992; Schneider and Bergmann, 1995). Schneider and Bergmann (1995) suggested that the increase in maximum extractable activity of γ-ECS in cells treated with Cd could not account for calculated increases in rates of γ-EC and GSH synthesis. They therefore attributed the major influence to alleviation of feedback inhibition by GSH, due to consumption of the latter for phytochelatin synthesis. Feedback inhibition of γ-ECS by GSH is well-characterized in animal systems and is competitive with respect to glutamate (Meister, 1995). Although *in vitro* studies with γ-ECS from tobacco and parsley have shown that GSH acts as an inhibitor that is competitive with glutamate in plant cells (Hell and Bergmann, 1990; Schneider and Bergmann, 1995), the significance of the operation of this control mechanism *in vivo* is not yet clear. It is evident that end-product inhibition is a homeostatic mechanism and that it cannot, in itself, allow up-regulation of GSH synthesis if this is accompanied by GSH accumulation in the compartment where γ-ECS is located. In this respect, studies where cells or plants are treated with Cd may be a very poor model for control of GSH synthesis under other conditions, since Cd exposure is a condition where accelerated GSH synthesis and decreased GSH contents coincide (Scheller *et al*., 1987; Rauser *et al*., 1991; Rüegsegger and Brunold, 1992; Klapheck *et al*., 1995; Schneider and Bergmann, 1995). Feedback inhibition may, however, operate in concert with other control mechanisms, e.g. isoforms with differing GSH sensitivity or modulation of GSH sensitivity by post-translational modifications analogous to those which operate in animal systems (for a more detailed discussion, see May *et al*., 1998).

Effective control of foliar GSH synthesis could also be mediated by subcellular compartmentation. The dearth of reliable information concerning the intracellular location of the synthesis of cysteine and GSH is probably the single biggest obstacle to detailed and unequivocal knowledge of regulation of these pathways. Highest rates of cysteine synthesis probably occur in the chloroplast (Schwenn, 1994), although activities of both serine acetyltransferase and *O*-acetylserine(thiol)lyase have also been shown to be associated with cytosolic and mitochondrial compartments (Lunn *et al*., 1990; Rolland *et al*., 1992; Ruffet *et al*., 1995; Takahashi and Saito, 1996). Glutathione is found in both cytosol and chloroplast

(Foyer and Halliwell, 1976; Bielawski and Joy, 1986; Klapheck *et al*., 1987), but the precise determination of compartment concentrations is confounded by the possible exchange between the different compartments during subcellular fractionation (Klapheck *et al*., 1987). Little is known concerning the likelihood of movement of cysteine, γ-EC or GSH between compartments by specific amino acid or peptide transporters. Possible GSH transporters have been described (Frommer *et al*., 1994; Steiner *et al*., 1994), but specific kinetic studies of GSH transport have been confined to the plasmalemma (Schneider *et al*., 1992; Jamai *et al*., 1996). Likewise, although GS and γ-ECS activities have been shown to exist in the chloroplast and cytosol (Klapheck *et al*., 1987; Hell and Bergmann, 1988, 1990), no information concerning the possibility of isoforms, with distinct kinetic characteristics, has been reported. The recent cloning of these enzymes from plant sources (May and Leaver, 1994; Rawlins *et al*., 1995; Ullman *et al*., 1996) may contribute to the solution of this problem.

In addition to factors influencing synthesis or transport, GSH concentrations may be affected by degradation. The pathway of GSH degradation in animals is well established, occurring as part of the 'γ-glutamyl cycle' (Meister, 1988). This sequence involves the removal of the glutamate moiety by a γ-glutamyltranspeptidase, followed by metabolism of the glutamyl moiety of the resulting peptide to oxo-proline by γ-glutamylcyclotransferase. Oxo-proline is hydrolysed to glutamate by 5-oxo-prolinase and cysteinylglycine is cleaved by a dipeptidase (Meister, 1988). Work with green tobacco cells indicates that a different pathway may operate in plants, in which removal of the glycine moiety, catalysed by a carboxypeptidase, occurs first (Steinkamp and Rennenberg, 1985); γ-EC is then cleaved to glutamate and cysteine by the sequential actions of γ-glutamylcyclotransferase and 5-oxo-prolinase (Rennenberg *et al*., 1981; Steinkamp *et al*., 1987). This putative pathway would have significance for the control of GSH metabolism in plant tissues because it would mean that GSH could be degraded either partially (to γ-EC and glycine) or completely (to glutamate, cysteine and glycine). Participation of a γ-glutamyltranspeptidase cannot, however, be ruled out, since (1) the enzyme is frequently found in plant tissues (Steinkamp and Rennenberg, 1984), (2) the product of the hydrolytic reaction catalysed by the enzyme, cysteinylglycine, has been detected in several plant species (Bergmann and Rennenberg, 1993), (3) dipeptidase activity required to process cysteinylglycine has also been demonstrated in plant cells (Bergmann and Rennenberg, 1993), and (4) a gene encoding a plant transpeptidase has recently been cloned (Kushnir *et al*., 1995). The enzymes of GSH breakdown appear to be localized either partially or exclusively in the cytosolic compartment (Rennenberg *et al*., 1981; Steinkamp *et al*., 1987; Steinkamp and Rennenberg, 1984). An alternative pathway leading to effective breakdown of GSH may be the vacuolar metabolism of GSH S-conjugates (Marrs, 1996).

Glutathione synthesis is limited by γ-ECS activity but not GS activity: Unlike antisense expression (Stitt and Sonnewald, 1995), overexpression of an enzyme cannot be used to quantify control exerted by a given enzyme activity on flux through a given pathway (see Kacser and Porteus, 1987, for review). It can, however, indicate whether or not a given enzyme limits metabolite concentrations under a given set of physiological conditions. This is because supplementary enzyme is expected to mitigate control exercised by kinetically limiting endogenous enzyme activity. Thus, the simplest explanation of the finding that strong overexpression of GS does not affect the foliar concentrations of the reactants (γ-EC, glycine, GSH), is that the *in vivo* capacity of the poplar enzyme does not kinetically limit this reaction. The absence of effect cannot be explained by compartmentation of substrates, since overexpression of GS in the cytosol (Foyer *et al*., 1995; Strohm *et al*., 1995) or chloroplast (Noctor *et al*., unpublished results) has no effect on leaf GSH contents. Rather, it may indicate that the condensation of γ-EC and glycine to form GSH is not appreciably removed from thermodynamic equilibrium in untransformed poplar. This conclusion must, however, be drawn with caution, since it is possible that the bacterial GS possesses much lower affinities for its substrates than the poplar enzyme. Alternatively, the absence of effect of GS overexpression on foliar GSH levels could reflect induction of a corresponding and compensatory increase in the rate of GSH breakdown in the transformants. Although there is no evidence for such an effect, it is not possible to discount it completely. Appreciably increased rates of export of GSH from the transformant leaves is another possibility, which would allow accelerated foliar GSH synthesis without accompanying accumulation in the leaf. This eventuality can, however, be discounted: measurements of thiol contents in phloem samples collected along the poplar tree axis showed no difference in phloem cysteine and GSH concentration between poplars overexpressing GS and untransformed poplars (Herschbach *et al*., 1998).

The huge increase in maximum GS activity observed in leaf extracts may overestimate the actual enhancement of enzyme capacity *in vivo*. Feeding of γ-EC to poplar leaf discs was shown to lead to faster rates of GSH accumulation for poplars overexpressing GS in the cytosol than for untransformed poplars (Strohm *et al*., 1995). The extractable foliar GS activity of the transformant was about 50-fold higher than in the untransformed control, but the rate of γ-EC-induced GSH accumulation was only about three times faster. This disparity may indicate that the maximum activity of the introduced enzyme is down-regulated in the poplar cytosol.

The finding that supplying exogenous γ-EC was able to increase foliar GSH levels in untransformed poplars suggests that the low availability of this intermediate restricts the production of GSH. Control of GSH synthesis by the rate of γ-EC production has been considered by several authors (Hell and Bergmann, 1990; Ruegsegger and Brunold, 1992; Chen and Goldsbrough, 1994; Schneider and Bergmann, 1995; Rennenberg, 1995; Noctor *et al.*, 1997*c*) and would be consistent with the widespread view that the predominant control over a biochemical sequence resides in the first dedicated reaction of the pathway. The observation that overexpression of γ-ECS brings about marked increases in foliar GSH (Noctor *et al.*, 1996, 1998; Arisi *et al.*, 1997) demonstrates that elevation of this activity removes a major limitation over the rate of GSH synthesis. This implies that the synthesis of γ-EC from glutamate and cysteine is held far from equilibrium in untransformed poplar leaves. To illustrate this point, Table 2 shows a comparison of the foliar contents of GSH and precursors measured in untransformed poplars and in line ggs28, overexpressing γ-ECS in the cytosol, together with derived product:substrate ratios for the two reactions (synthesis of γ-EC and synthesis of GSH). Although foliar contents of ATP, ADP and phosphate have not been measured, it is unlikely that these values vary markedly between the two plant types, given their comparable physiology and photosynthetic rates. The data show that the product:substrate ratio for the synthesis of γ-EC is very low in untransformed poplars, but is increased 10-fold by overexpression of γ-EC (Table 2). For the second reaction, the product:-substrate ratio in untransformed poplars is much higher than that of the first reaction, but falls about 5-fold as a result of overexpression of γ-ECS. The simplest explanation of these effects is that overexpression of γ-ECS brings the first reaction closer to equilibrium and pushes the second reaction away from equilibrium. Similar results were obtained by chloroplastic overexpression of γ-ECS (Noctor *et al.*, unpublished results). Thus, in poplars overexpressing γ-ECS, the second reaction becomes much more limiting than in untransformed poplars. One corollary of this hypothesis is that overexpression of both γ-ECS and GS in the same plant line should lead to even greater increases in foliar GSH than those obtained by overexpression of γ-ECS alone.

It should be stressed that the increases in foliar contents of γ-EC and GSH in poplars overexpressing γ-ECS only partially reflect the true increase in reaction rate. Analysis of phloem exudates showed that phloem GSH concentration is an order of magnitude greater in the poplars overexpressing γ-ECS than in untransformed poplars (Herschbach *et al.*, 1998). In addition, unlike untransformed poplars, the transformants phloem exudates contain detectable levels of γ-EC (Herschbach *et al.*, 1998). Since growth was not affected by the transformation, it appears that transport of reduced sulphur in the phloem is controlled by source activity rather than by sink strength.

Interaction of glutathione synthesis with carbon and nitrogen metabolism

Most studies concerning GSH metabolism have focused on the interactions of GSH with sulphur metabolism (for review, see Rennenberg, 1995). This reflects the quantitative importance of the GSH pool compared to those of other non-protein thiols. However, since total foliar GSH contents are typically in the region of 0.5 μmol g^{-1} FW, GSH represents a considerable non-protein pool of both glutamate and glycine. This is particularly true for glycine, whose total free foliar content, on a molar basis, may be considerably lower than that of GSH (Noctor *et al.*, 1997*a*, unpublished results).

In contrast to cysteine, there exist few data which support a kinetic limitation of GSH synthesis by glutamate availability. Similarly, at least in illuminated leaves under non-stress conditions, glycine concentration would seem to be sufficiently high to support maximal rates of GSH synthesis. Glycine availability has, however, been shown to become limiting in darkened leaves (Buwalda *et al.*, 1990; Noctor *et al.*, 1997*b*).

In non-photosynthetic tissues, glycine is synthesized from serine, which is in turn produced from 3-PGA by either the 'phosphorylated' or 'non-phosphorylated' pathway (Fig. 4; reviewed by Kleczkowski and Givan, 1988). Although glycine production via these pathways may also occur in photosynthetic tissues, the major pathway of glycine synthesis in illuminated leaves from plants with C_3 metabolism is undoubtedly from glycollate produced as a result of photorespiration (Fig. 4; Keys, 1980).

Buwalda *et al.* (1988) showed that fumigation with H_2S brought about marked increases in foliar cysteine levels in spinach. Under these conditions, maximum rates of GSH formation from γ-EC were light-dependent: in the dark, GSH contents decreased and γ-EC accumulated to unusually high levels. Supplying glycine through the petiole prevented the dark accumulation of γ-EC and allowed GSH contents to remain at similar values to those in the light (Buwalda *et al.*, 1990).

Upregulation of GSH synthesis in the chloroplast or cytosol requires photorespiratory glycine: Phenomena similar to those observed in H_2S-fumigated spinach have been observed in poplars overexpressing γ-ECS in the cytosol (Noctor *et al.*, 1997*a*, *b*) or chloroplast (Noctor *et al.*, unpublished results). Foliar cysteine contents in these poplars were not appreciably different from untransformed poplars and were not markedly affected by illumination. In contrast, γ-EC contents, already enhanced in the light, relative to untransformed poplars, increased in the dark to attain extremely high levels (as high as

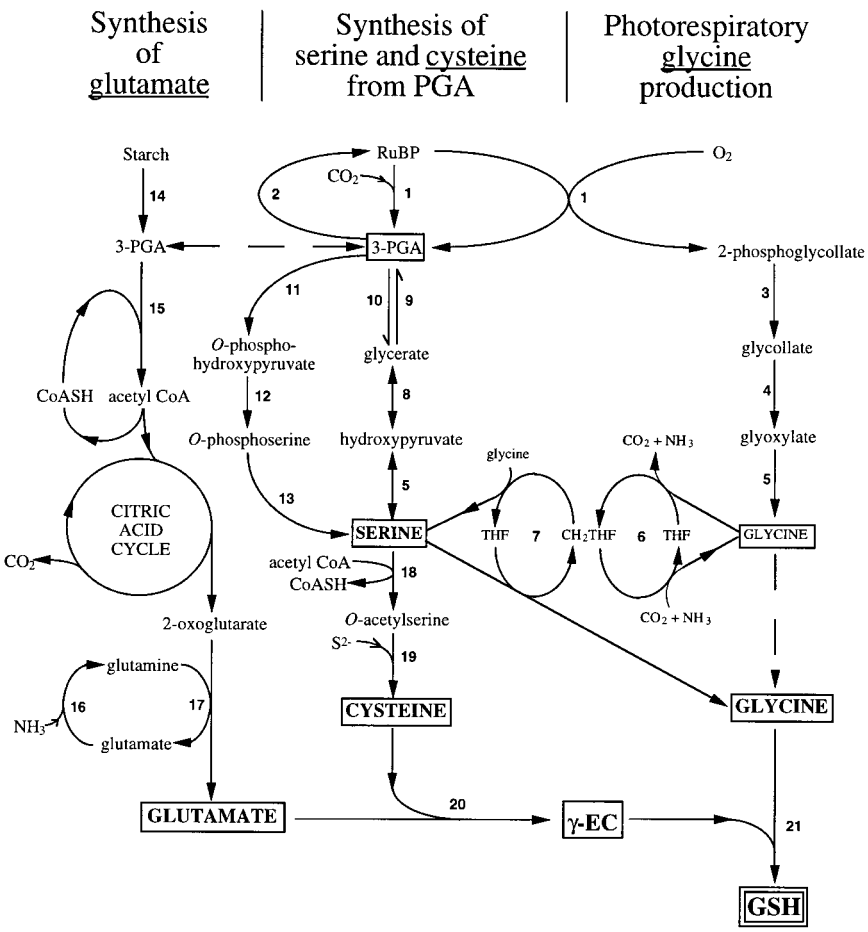

Fig. 4. Schematic non-stoichiometric depiction of the interaction of glutathione synthesis with assimilation of carbon and nitrogen. Numbers denote the following enzymes or sequences of enzymes: (1) Rubisco, (2) other Calvin cycle enzymes, (3) 2-phosphoglycollate phosphatase, (4) glycollate oxidase, (5) serine:glyoxylate aminotransferase (other amino acids may also act as amino donors for glyoxylate or hydroxypyruvate, while other oxo acids may accept the serine amino group), (6) glycine decarboxylase complex, (7) serine hydroxymethyl transferase, (8) hydroxypyruvate reductase, (9) D-glycerate kinase, (10) 3-PGA phosphatase, (11) 3-PGA dehydrogenase, (12) glutamate:*O*-phosphohydroxypyruvate aminotransferase, (13) *O*-phosphoserine phosphatase, (14) enzymes of starch metabolism and glycolysis, (15) other glycolytic enzymes, (16) glutamine synthetase, (17) glutamate synthase (glutamate:2-oxoglutarate aminotransferase), (18) serine acetyltransferase, (19) *O*-acetylserine (thiol) lyase, (20) γ-glutamylcysteine synthetase, (21) glutathione synthetase.

1 μmol g^{-1} FW). These values were up to 2-fold higher than the foliar GSH contents of untransformed poplars. Concomitant with this increase in γ-EC contents, GSH levels fell to approximately half those in illuminated leaves. Measurements of free amino acid contents showed that foliar glycine contents were highly light-dependent (Noctor *et al.*, 1997*a*, unpublished results). Supplying glycine through the petiole, or incubation of leaf discs on glycine, prevented dark build-up of γ-EC and allowed GSH synthesis to continue (Noctor *et al.*, 1997*b*, unpublished results). The importance of photorespiration in supplying the glycine for GSH synthesis was demonstrated by experiments in which pre-darkened leaves, with high γ-EC and relatively low GSH, were illuminated at 0.4% CO_2 or 0.2% O_2. Under these conditions, which prevent photorespiration, glycine contents remained at the comparatively low dark level and the light-induced conversion of γ-EC to GSH was not observed (Noctor *et al.*, 1997*a*, unpublished results). These data suggest that, when the capacity for γ-EC synthesis is increased by high γ-ECS activity, photorespiratory glycine becomes necessary to allow efficient conversion of γ-EC to GSH. This effect may result from a kinetic limitation of low glycine availability on the rate of the reaction catalysed by GS or by thermodynamic adjustment of the GSH:γ-EC ratio in response to changing glycine concentrations.

These observations demonstrate that photorespiration, whose adaptive significance has been much discussed, allows the production of intermediates for at least one biosynthetic pathway. It has been argued that such production is unlikely on theoretical grounds, since it would hinder maximal recycling of carbon diverted from the Calvin cycle by oxygenation of RuBP (see Conclusions

and perspectives). This argument would seem difficult to sustain, however, since if glycine produced from glycollate is not used for biosyntheses, then it must originate from serine produced from 3-PGA by other pathways (Kleczkowski and Givan, 1988).

Although illumination, or supplying glycine in the dark, was shown to prevent dark accumulation of γ-EC in untransformed poplars, these factors only brought about a marked increase in GSH contents when the capacity for γ-EC synthesis was elevated by feeding cysteine (Noctor *et al.*, 1997*b*). It therefore seems that photorespiratory glycine becomes increasingly necessary for GSH synthesis as the capacity of γ-EC production is increased. This capacity can be increased either by an increased γ-ECS activity (Noctor *et al.*, 1997*a*, unpublished results) or by an enhanced cysteine pool (Buwalda *et al.*, 1988, 1990; Noctor *et al.*, 1997*b*). These conclusions point to an important role for photorespiration in supporting enhanced production of GSH under stress conditions, where increased rates of GSH synthesis are thought to result largely from accelerated synthesis of γ-EC (Smith, 1985; Rüegsegger and Brunold, 1992; May and Leaver, 1993; Schneider and Bergmann, 1995). Under these conditions, photorespiratory glycine will become crucial in allowing the efficient conversion of γ-EC to GSH.

The importance of photorespiration in GSH metabolism begs the question of the origin of glycine for GSH synthesis in C_4 plants and CAM plants. Although there is some synthesis of glycine from glycollate in C_4 plants, rates are much reduced relative to plants of C_3-type metabolism (Morot-Gaudry *et al.*, 1980). In maize, Rubisco and the enzymes which catalyse the formation of glycine and serine from glycollate are located in the bundle sheath cells (Edwards and Walker, 1983), whereas the intercellular location of the enzymes of GSH biosynthesis is not known. Intriguingly, it is thought that the ratio of oxygenation:carboxylation of RuBP, very low in maize leaves under optimal conditions, may increase significantly under stress conditions (Edwards and Walker, 1983). Another question of interest is whether light–dark changes in GSH synthesis also occur in plants which synthesize GSH analogues rather than, or in addition to, γ-EC-gly. For instance, does γ-EC also accumulate in darkness in plants producing γ-EC-ala, γ-EC-ser or γ-EC-glu? Since the metabolism of serine is tightly linked to that of glycine, photorespiration may also be important in providing serine for γ-EC-ser production. The role of photorespiration in plants producing γ-EC-ala or γ-EC-glu is less evident. Nevertheless, it is noteworthy that foliar alanine contents in poplar are also increased by illumination (Noctor *et al.*, 1997*a*, unpublished results).

In addition to the observed up-regulation of cysteine synthesis in poplars overexpressing γ-ECS, specific enhancement of certain other amino acids were observed in the poplars in which the enzyme was targeted to the chloroplast (Noctor *et al.*, unpublished results). The most notable increases were in valine, leucine and isoleucine, which constitute the 'branched chain' family of amino acids (Singh and Shaner, 1995). Other significant increases were observed in tyrosine and lysine contents (Noctor *et al.*, unpublished results). Since all these amino acids are thought to be synthesized exclusively or predominantly in the chloroplast (Hermann, 1995; Singh and Shaner, 1995; Shaul and Galili, 1992*a*, *b*), it is interesting that these effects should be confined to plants with enhanced γ-ECS in the chloroplast.

Glutathione and stress tolerance

Glutathione is an attractive target for engineering stress tolerance in plants, because of its multiple roles in plant defences against both biotic and abiotic stresses (Foyer *et al.*, 1997). Stimulation of GSH biosynthesis is frequently observed in stress conditions. Similarly, GSH accumulation is found to compensate for decreases in the capacity of other antioxidants; for example, in catalase-deficient mutants and in plants where catalase activity has been reduced by antisense technology (Smith *et al.*, 1984; Smith, 1985; Chamnongpol *et al.*, 1996; Willekens *et al.*, 1997). Furthermore, when GSH is depleted, increases in sensitivity to oxidative stress have frequently been found (Hibberd *et al.*, 1978; Kunert *et al.*, 1990; Kushnir *et al.*, 1995; Grant *et al.*, 1996). In instances where GSH depletion has not increased sensitivity to oxidative stress (Greenberg and Demple, 1986; May *et al.*, 1996) it is probable that other antioxidant molecules are increased to compensate for decreases in glutathione.

GSH is a major water-soluble antioxidant in plant cells. It directly reduces most active oxygen species. It reacts rather slowly with hydrogen peroxide and GSH-dependent reduction of hydrogen peroxide is not a major route of hydrogen peroxide destruction in plants. Glutathione peroxidases are induced in plants in response to stress (Eshdat *et al.*, 1997). These enzymes are involved in the detoxification of lipid peroxides rather than hydrogen peroxide *per se*. In plants, the major substrate for reductive detoxification of H_2O_2 is ascorbate, which must therefore be continuously regenerated from its oxidized forms (Fig. 2). A major function of glutathione in protection against oxidative stress is the re-reduction of ascorbate in the ascorbate-glutathione cycle (Foyer and Halliwell, 1976; Groden and Beck, 1979; Nakano and Asada, 1980). In this pathway, glutathione acts as a recycled intermediate in the reduction of H_2O_2 using electrons derived, ultimately, from H_2O (Foyer, 1997). Efficient recycling of glutathione is ensured by GR activity. The components of this cycle exist in both chloroplast and cytosol (Foyer, 1993), and evidence for their presence in mitochondria,

glyoxysomes and peroxisomes has been recently reported (Jiménez *et al*., 1997).

In peroxisomes hydrogen peroxide can be destroyed by either catalase or ascorbate peroxidase. Catalase produces molecular oxygen and water from two molecules of hydrogen peroxide. Since these two molecules must impinge simultaneously at the active site, catalase has a very high maximum velocity, but a very poor affinity for its substrate. In contrast, the ascorbate peroxidases have a high affinity for hydrogen peroxide, but they require a reducing substrate, ascorbate. In the ascorbate peroxidase reaction hydrogen peroxide is reduced to water and ascorbate is oxidized to monodehydroascorbate (MDHA), the univalent product of ascorbate oxidation. In the chloroplasts MDHA is rapidly reduced to ascorbate by reduced ferredoxin. Other membrane-associated electron transport systems such as those on the plasmalemma may also be instrumental in re-reducing MDHA non-enzymically. In addition, MDHA reductases (MDHAR) rapidly reduce MDHA to ascorbate using NAD(P)H (Fig. 2). The presence of these mechanisms for recycling MDHA has cast doubt on the requirement for GSH-dependent reduction of DHA in ensuring ascorbate regeneration. When MDHA is not reduced it rapidly disproportionates to ascorbate (AA) and DHA, the divalently oxidized product (Fig. 2). Although DHA is reduced to ascorbate by DHAR, DHA is always detectable in plant tissues (Foyer *et al*., 1983) and AA/DHA ratios are relatively low compared to GSH/GSSG ratios, particularly under field conditions. It has recently been argued that DHA detected in extracts is artefactual and that *in vivo* levels are much lower or negligible (Morell *et al*., 1997). This contention was principally supported by the observation that inclusion of DHA in enzyme assays, at concentrations thought to exist in the chloroplast *in vivo*, led to oxidative inactivation of two enzymes known to be regulated by the thioredoxin system (Morell *et al*., 1997). The authors therefore concluded that significant formation of DHA *in vivo* must be avoided (Morell *et al*., 1997). This conclusion is erroneous. It is well established that the soluble stromal enzymes regulated by the thioredoxin system require ongoing reduction to remain active (Leegood and Walker, 1982; Leegood *et al*., 1985; Noctor and Mills, 1988). Data obtained by addition of oxidants to enzymes removed from the light- and membrane-dependent thioredoxin reduction system lack any relevance whatsoever to *in vivo* conditions, where the activation state of thiol-regulated enzymes will reflect the differences between reductive and oxidative fluxes. This means that it is important that DHA is reduced back to ascorbate, not that DHA cannot exist *in vivo*. It is therefore crucial to establish the significance of GSH as a reductant in this process, particularly in relation to stress tolerance. Some indication of the requirement for DHAR in ascorbate regeneration and associated stress tolerance has been obtained from studies on a tropical fig mutant that is devoid of DHAR activity and is sensitive to high light (Yamasaki *et al*., 1995). In the chloroplast the direct reduction of both molecular oxygen and MDHA by the photosynthetic electron transport chain generates a high transthylakoid pH gradient which facilitates the regulated decrease in the quantum efficiency of photosystem II (Foyer *et al*., 1990; Schreiber and Neubauer, 1990; Neubauer and Yamamoto, 1992). Similarly, GSH-dependent regeneration of ascorbate enhances the transthylakoid pH gradient through utilization of NADPH by GR in the re-reduction of GSH from GSSG oxidized by DHA (Foyer *et al*., 1990; Foyer, 1997).

Key questions, which require answers if a full understanding of the contribution made by glutathione to stress resistance is to be reached, are: how important is glutathione concentration in determining the plant cell's capacity to control the concentrations of harmful oxygen species? How crucial is the rate of regeneration of GSH from GSSG? How important is the subcellular localization of glutathione? Transformed plants, with compartment-specific enhancement of capacities for either glutathione synthesis or glutathione reduction, offer a model system in which to explore these questions. The significance of glutathione under three principal stress conditions will be considered here: (1) light-associated stress (photoinhibition and exposure to paraquat); (2) ozone stress; (3) heavy metal stress.

Photoinhibition and paraquat exposure

In the combined conditions of high light and low temperature, where metabolism is slowed relative to photochemistry, the Mehler reaction may occur at increased rates. Hence, providing that the resulting superoxide and peroxide can be efficiently metabolized, O_2 reduction could play an important role in allowing the ongoing utilization of light energy absorbed by the photosynthetic apparatus. Enhanced operation of the Mehler reaction may thus diminish the extent of photoinhibition, the slowly-reversible reduction in photosynthetic efficiency and capacity which occurs when light energy is in excess. In addition, high AA/DHA and GSH/GSSG ratios may protect the thiol-modulated enzymes of the Benson–Calvin cycle from oxidation by hydrogen peroxide and allow photosynthesis to proceed at relatively high rates even during oxidative stress. At room temperature, very high rates of O_2 reduction at Photosystem I can be induced in photosynthetic systems by exposure to the herbicidal Mehler reagent paraquat, leading to markedly augmented rates of formation of active oxygen species. Both glutathione concentration and GR activity have been correlated with increased resistance to paraquat exposure (Madamanchi *et al*., 1994).

Enhanced chloroplastic GR protects against oxidative stress: Despite the elaborate mechanisms of ascorbate and glutathione regeneration already in place in the chloroplast, overproduction of GR has been shown to provide additional protection against oxidative stress and photoinhibition. Poplars overexpressing GR in the chloroplast, containing highly reduced, enhanced, foliar glutathione pools, were more resistant to photoinhibition than untranformed plants (Foyer *et al*., 1995). By comparing visible injury, Aono *et al*. (1993) adjudged tobacco plants overexpressing the *E. coli* GR in the chloroplast more resistant to damage brought about by exposure to paraquat. Resistance was also obtained in tobacco simultaneously overexpressing GR and superoxide dismutase in the cytosol (Aono *et al*., 1995*a*). Some lines of tobacco expressing a pea cDNA encoding GR also showed decreased sensitivity to paraquat (Broadbent *et al*., 1995). Conversely, increased sensitivity was reported in tobacco antisensed for GR (Aono *et al*., 1995*b*).

Unlike poplars in which GR was targeted to the chloroplast, poplars overexpressing the enzyme in the cytosol did not exhibit protection against paraquat or photoinhibition (Foyer *et al*., 1991, 1995). This may have been due to the absence of enhanced glutathione accumulation or increases in the GSH/GSSG ratio in the leaves of the cytosolic overexpressors (Foyer *et al*., 1991, 1995). Since cytosolic overexpression led to much less marked increases in extractable GR activity than chloroplastic overexpression, these data may suggest that there is a minimum increase in GR activity that has to be reached before any real physiological advantage is conferred, possibly due to threshold effects on glutathione content and redox state (see the section on Glutathione reductase). Alternatively, the failure of cytosolic overexpression of GR to affect resistance to paraquat or photoinhibition in poplar may be due to the chloroplastic origin of these stresses. Again, it could reflect limitations by other antioxidant components in the cytosol, implying that the rate-limiting reactions operative in these conditions involve components other than glutathione. In the case of paraquat, for example, it is possible that the rate of the superoxide dismutase reaction limits hydrogen peroxide production since overexpression of this enzyme increases protection of photosynthesis in the presence of this herbicide (Foyer *et al*., 1994).

Increased GR activity protects foliar ascorbate: Confirmation of the critical role of GSH-dependent DHA-reduction in maintaining the ascorbate pool has come from studies on transformed plants over-expressing GR. Poplars overexpressing GR in the chloroplast had higher levels of foliar ascorbate than untransformed poplars (Foyer *et al*., 1995). Moreover, both tobacco and poplar transformants overexpressing GR were better able to maintain their foliar glutathione and ascorbate pools during exposure to paraquat (Foyer *et al*., 1991, 1995). These observations show that the glutathione pool and GR play a crucial role in maintaining the ascorbate pool in both optimal and stress conditions.

Increased total glutathione pools per se *do not ameliorate stress responses:* Despite 3-fold higher glutathione contents than untransformed poplars, transformed lines overexpressing γ-ECS in the chloroplast or cytosol did not contain significantly enhanced pools of foliar ascorbate (Fig. 5). Likewise, the GSH/GSSG ratio was similar to that in untransformed poplars.

In experiments performed in Versailles, foliar ascorbate, glutathione, and chlorophyll contents were measured during a period of illumination following overnight incubation on 10 μM paraquat (Fig. 6). This concentration led to some loss of ascorbate and glutathione in the dark, which was more marked for the untransformed poplars than for the transformants. Similarly, the paraquat-induced decreases in glutathione in the light were slower in the transformants (Fig. 6). Interpretation of these effects is complicated, however, due to the higher absolute glutathione contents of the transformants and because of the pronounced light-induced accumulation observed in these plants under non-stress conditions (Fig. 6; discs illuminated on H_2O: for an explanation of this effect see the section on the Interaction of glutathione synthesis with carbon and nitrogen metabolism). Therefore, glutathione contents in the presence of paraquat were plotted as a fraction of contents at the same timepoint in its absence (data not shown). These derived data showed no differences between the disc types in the sensitivity of the glutathione pool to paraquat in the light. However, the increased resistance of the glutathione pool to paraquat exposure in the dark was still apparent in the transformants, particularly in the chloroplastic overexpressors (after 16 h incubation on paraquat in the dark, the foliar glutathione contents of the control, ggs and Lggs poplars were 47%, 64% and 94%, respectively, of those in the corresponding discs incubated on H_2O; Fig. 6, time 0). Significant loss of chlorophyll was only observed after 7 h illumination and did not differ between the disc types (Fig. 6). Higher concentrations of paraquat (50 μM) brought about more extensive degradation of chlorophyll (Fig. 7). Unlike ascorbate and glutathione loss, chlorophyll degradation was totally dependent on the light-induced action of paraquat: even at concentrations as high as 1 mM, overnight incubation in the dark did not significantly affect chlorophyll contents. Figure 7 shows a marginally lower sensitivity to paraquat in the discs from poplars overexpressing γ-ECS in the cytosol. In contrast, chlorophyll contents of poplars overexpressing GR in the chloroplast were no more resistant than those of untransformed poplars (Fig. 7). The difference between these data and those of Aono *et al*. (1993, 1995*a*)

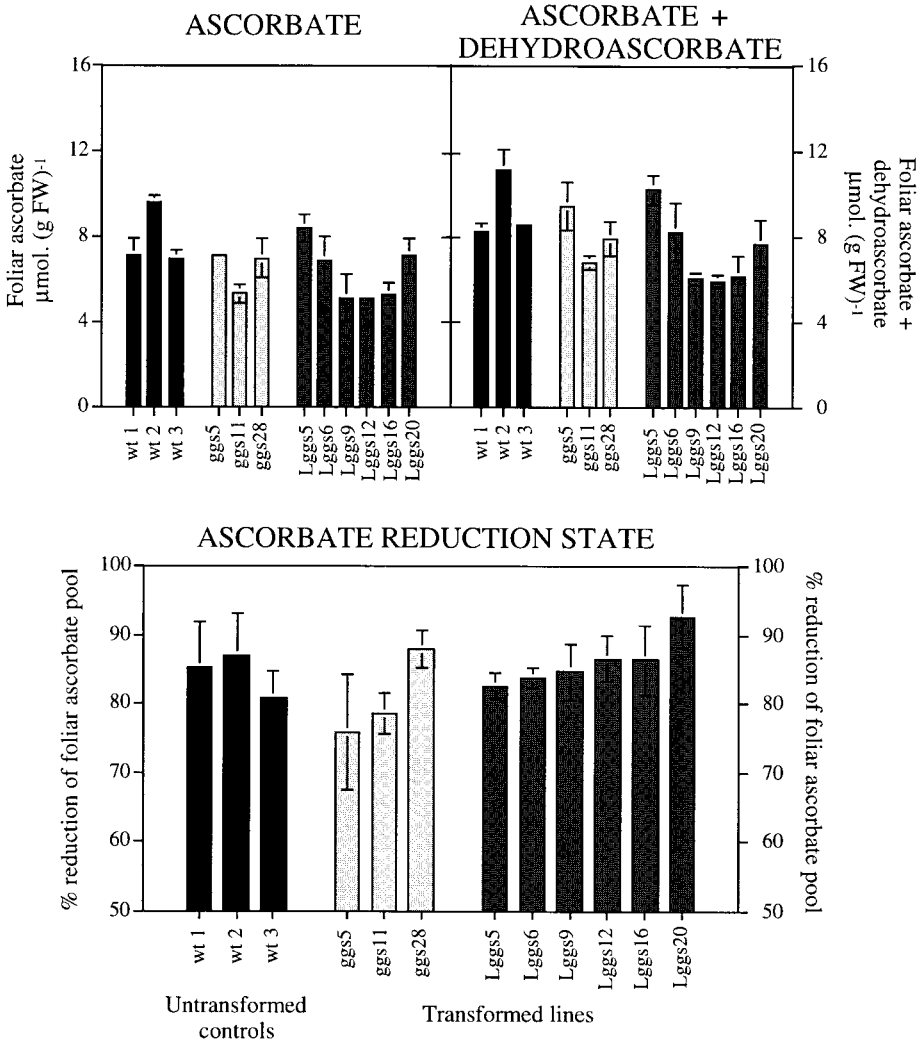

Fig. 5. Foliar ascorbate contents in three different untransformed poplars (filled columns) and poplars overexpressing γ-ECS in the cytosol (three different ggs lines: dotted columns) or the chloroplast (six different Lggs lines: hatched columns). Data are the means $\pm$ SD of three separate extractions of laminar leaf material taken during the afternoon from poplars growing in the greenhouse.

may be due to the higher paraquat concentrations used in the experiment shown in Fig. 7.

Results obtained in Freiburg also showed that a significant loss of chlorophyll was observed when leaf discs of untransformed poplar were exposed to paraquat in the light. Addition of GSH to the leaf discs was able to prevent this loss. However, differences in the susceptibility to paraquat were not observed between leaf discs from untransformed poplars and transformants overexpressing γ-ECS in the cytosol. This result may be a consequence of much higher GSH accumulation induced by supplying exogenous GSH to leaf discs from untransformed poplars, as compared to the endogenous GSH levels of leaf discs from plants overexpressing γ-ECS.

Conclusions: The foliar response to oxidative attack and associated stresses can be manipulated through overexpression of enzymes involved in glutathione metabolism. The contrast between the absence of effects on redox processes brought about by increasing the rate of GSH synthesis and the marked changes incurred by GR overexpression clearly demonstrates the rather unique effect of enhanced GR activity on the ascorbate-glutathione cycle. The capacity for regeneration of glutathione (overexpression of GR) seems to be a more important determinant of resistance to oxidative stress than increases in the total pool size (brought about by overexpression of γ-ECS). It remains to be seen whether simultaneous overexpression of these enzymes would be more effective than overexpression of either alone.

Ozone exposure

The air pollutant, ozone, causes severe tissue damage resulting in chlorosis, water-logging, stipple, premature loss of chlorophyll, and leaf abscission in a variety of

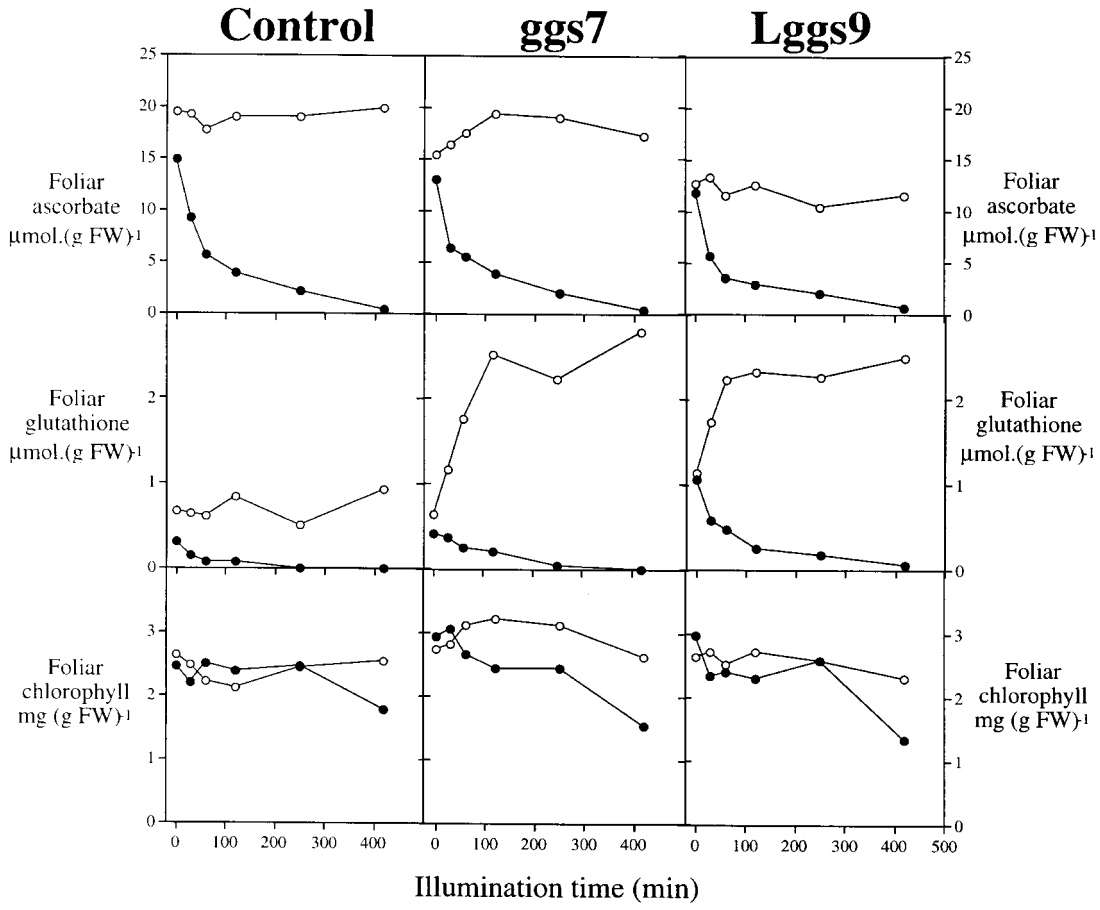

Fig. 6. Comparison of the responses of total foliar ascorbate, glutathione and chlorophyll to paraquat in untransformed poplar (control) and in poplars overexpressing γ-ECS in the cytosol (ggs7) or chloroplast (Lggs9). Leaf discs were floated overnight in the dark (16 h) on deionized water (empty symbols) or 10 μM paraquat (filled symbols). The following day, they were illuminated at a light intensity of 400 μmol m^{-2} s^{-1} and samples taken at the times shown for determination of ascorbate, glutathione and chlorophyll.

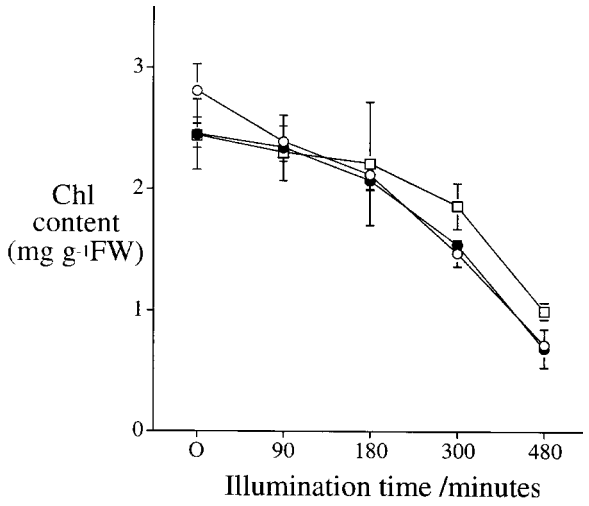

Fig. 7. Paraquat-dependent chlorophyll destruction in untransformed poplar (filled circles), 70gor (overexpressing GR in the chloroplast: empty circles) and ggs28 (overexpressing γ-ECS in the cytosol: squares). Leaf discs were floated overnight (16 h) on 50 μM paraquat in the dark. The following day, they were illuminated at a light intensity of 400 μmol m^{-2} s^{-1}. Data are the means $\pm$SD of three separate extractions.

plant species (Heath, 1994; Heggestad, 1991). Ozone-induced decreases in photosynthesis and accelerated senescence have been observed even in asymptomatic leaves (Reich and Amundson, 1985; Krupa and Manning, 1988). Accelerated senescence following exposure to ozone is associated with a rapid decline in the quantity and activity of Rubisco (Lehnherr *et al.*, 1987; Dann and Pell, 1989; Matyssek *et al.*, 1991; Landry and Pell, 1993; Nie *et al.*, 1993). Numerous investigations indicate that poplar species are highly sensitive to acute ozone stress (Jensen and Dochinger, 1974; Karnovsky, 1976; Harkov and Brennan, 1982; Berang *et al.*, 1986). In studies on ozone tolerance in tobacco, Tanaka *et al.* (1990) found that GR activity limited turnover of the ascorbate-glutathione cycle. Furthermore, the reduction in the rate of photosynthesis observed in poplars exposed to ozone (Sen Gupta *et al.*, 1991; Pell *et al.*, 1992; Matyssek *et al.*, 1993) is preceded by an increase in foliar glutathione content (Sen Gupta *et al.*, 1991).

Ozone sensitivity studied in poplars overexpressing GR and GS: Untransformed poplar and lines overexpressing GS in the cytosol by 200- to 300-fold, GR in the cytosol by

5-fold, or GR in the chloroplast by 150- to 250-fold, were exposed to acute ozone stress (Strohm, 1996; Strohm *et al*., unpublished results). This treatment led to visible foliar injury consisting of dark-brown lesions on the leaves of all lines. A band of red-violet discoloured cells separated these lesions from green tissues. This band of cells showed yellow autofluorescence in blue light and blue autofluorescence in UV light, suggesting ozone-induced accumulation of phenolic compounds in this single cell layer. Visible injury was mainly restricted to mature leaves whereas young leaves were much less affected. These differences in susceptibility to acute ozone exposure between young and mature poplar leaves cannot be attributed to differences in pollutant influx caused by differences in leaf conductance. Prior to ozone exposure, leaf conductance was similar in young and mature leaves. Following ozone exposure, conductance was significantly reduced, but to a similar extent in both young and mature leaves. No differences in response were observed between untransformed and transformed poplar lines (Strohm, 1996; Strohm *et al*., unpublished results). It was concluded that (1) enhanced foliar activities of GS or GR do not increase tolerance to acute ozone exposure, and (2) ozone sensitivity is controlled by unknown factors that change with leaf development and are different from leaf conductance, GS or GR activity.

Ozone-induced increases in glutathione cannot be explained by alleviated feedback inhibition of GSH synthesis: Acute ozone exposure enhanced foliar GSH contents in all poplar lines irrespective of leaf age, but did not affect the glutathione redox state (Strohm, 1996). The increase in total glutathione without changes in reduction state does not support the hypothesis that ozone-induced increases in glutathione are due to release of redox control over GSH synthesis (Sen Gupta *et al*., 1991).

Resistance to ozone does not correlate with glutathione content or reduction state: Neither foliar glutathione accumulation nor the redox state of the pool correlated with ozone sensitivity (Strohm, 1996; Strohm *et al*., unpublished results). The absence of an effect of increased GSH on ozone tolerance may be due to the compartmentalization of this antioxidant. Ozone enters the leaf through open stomata and produces active oxygen species in the apoplastic fluid which therefore represents the first line of defence against ozone damage. In many plant species, however, the apoplast contains little or no glutathione (Polle *et al*., 1990; Vanacker *et al*., 1998). Hence increases in total foliar glutathione contents would be of little avail in improving defence against ozone if all the glutathione was sequestered within the cytoplasm. Acute ozone exposure led to increases in GR activity in both untransformed poplar and in lines overexpressing GR in the cytosol. GR activity was increased more in the leaves of the GR transformants than in those of the untransformed controls. The CaMV 35s promoter used for GR overexpression in these studies contains a well-characterized enhancer sequence, the *ocs* element (also called the *as-1* site (Lam *et al*., 1989)) which is induced by auxin, salicylic acid and hydrogen peroxide in stress conditions. It is therefore possible that increases in GR activity induced by ozone in the GR transformants are due to activation of this element as well as induction of the native forms of the enzyme. In transformed poplars overexpressing GR in the chloroplasts, GR activity already far exceeds that of the untransformed controls. Ozone exposure increased GR activity even further but this conferred no benefit in terms of ozone tolerance.

Increased GR activity protects the ascorbate pool from oxidation during ozone exposure: In contrast to the foliar glutathione pool, the foliar ascorbate pool was significantly oxidized upon acute ozone exposure. Ascorbate oxidation was observed in leaves of untransformed and transformed poplar overexpressing GR in the cytosol, irrespective of leaf age (Strohm, 1996). In poplar lines overexpressing GR in the chloroplast, ozone-mediated oxidation of the foliar ascorbate pool was prevented. Again, this illustrates the importance of the capacity for reduction of the glutathione pool in maintaining the ascorbate pool. However, increased GR activity did not overcome ozone-induced damage and the redox state of the ascorbate pool in poplar leaves did not correlate with ozone sensitivity. Total ascorbate contents, ascorbate peroxidase, MDHAR and DHAR activities were not affected by acute ozone exposure (Strohm *et al*., unpublished results).

Conclusions: Ozone exposure increases total glutathione but not total ascorbate. It leads to oxidation of the ascorbate pool but not the glutathione pool. The increases in glutathione are therefore difficult to explain in terms of alleviated feedback inhibition brought about by conversion of GSH to GSSG (Smith *et al*., 1984; Smith, 1985; Hell and Bergmann, 1990; Sen-Gupta *et al*., 1991). As observed on paraquat exposure, enhanced GR activities protect the foliar ascorbate pool from oxidation during exposure to ozone. None of the following are responsible for the developmentally-determined tolerance of poplar leaves to acute ozone exposure: increased glutathione content, enhanced GS activity, or enhanced GR activity. Likewise, decreased oxidation of the ascorbate pool was not correlated with ozone tolerance. This result might be taken to be in conflict with the isolation of ascorbate-deficient *Arabidopsis* mutants by their sensitivity to ozone (Conklin *et al*., 1996). However, it should be noted that cytoplasmic and apoplastic ascorbate pools appear not to be related (Polle *et al*., 1990; Vanacker *et al*., 1998). The absence of a correlation between glutathione metabolism and ozone tolerance may be explained by the extremely low levels of glutathione in the apoplast (Polle

et al., 1990; Vanacker *et al.*, 1998). Other antioxidants such as polyamines, which are rapidly induced by a variety of stress conditions and which have a high affinity for biological membranes, may be involved in protecting young leaves from ozone-damage (Barna *et al.*, 1993; Tiburcio *et al.*, 1997).

Heavy metal tolerance

The principal pathway by which plants sequester heavy metals involves the formation of complexes with cysteine-rich peptides, called phytochelatins (PCs). Phytochelatins contain between 2 and 11 repeated γ-EC units, usually followed by a C-terminal amino acid which may be glycine, alanine, serine or glutamate (Rauser, 1995; Zenk, 1996). In higher plants, they are induced by heavy metals (Grill *et al.*, 1987). Cadmium, a well-known inducer of phytochelatins, is thought to be chelated in complexes in the cytosol and transported to the vacuole (Rauser, 1995). The biosynthesis of phytochelatins from the precursor glutathione is catalysed by the enzyme γ-EC-dipeptidyl-transpeptidase, which transfers a γ-EC unit from glutathione or PC to an acceptor GSH or PC molecule (Grill *et al.*, 1989). Glutathione depletion following Cd exposure has been observed in cultured cells and in roots (Scheller *et al.*, 1987; Rauser *et al.*, 1991; Rüegsegger and Brunold, 1992; Klapheck *et al.*, 1995; Schneider and Bergmann, 1995). In maize, decreased GSH was accompanied by increases in γ-ECS activity and γ-EC contents (Rüegsegger and Brunold, 1992). Inhibition of PC accumulation by buthionine sulphoximine, an inhibitor of γ-ECS, demonstrated the importance of γ-ECS activity in PC synthesis (Grill *et al.*, 1987). Elevated γ-ECS activity was shown to correlate with Cd resistance in cultured tomato cells (Chen and Goldsborough, 1994) while inhibition of γ-ECS markedly enhanced the negative effects of Cd on growth in birch (Gussarsson *et al.*, 1996).

Heavy metal tolerance studied in plants overexpressing γ-ECS: Poplar overexpressing γ-ECS presents an excellent model system in which to investigate the roles of this enzyme and GSH synthesis in phytochelatin-mediated resistance to heavy metals. In preliminary experiments, transformants overexpressing γ-ECS in the cytosol, with enhanced GSH contents, were exposed for 14 d to different Cd concentrations (0–1000 μg g^{-1} soil). In transformed and untransformed poplars, Cd accumulated to a similar extent. Cadmium-dependent increases in foliar GSH content (up to 2-fold) were observed in both untransformed and transformed poplars, suggesting that the enhanced GSH content in the γ-ECS overexpressors did not repress further increases in foliar contents of this thiol. Changes in enzyme activities related to carbohydrate utilization were much less evident in γ-ECS overexpressors exposed to Cd than in untransformed poplars (Arisi *et al.*, unpublished results). Although no extensive study has yet been undertaken, the study of heavy metal tolerance will probably be one of the most important applications of plants overexpressing γ-ECS.

Conclusions and perspectives

The application of transgene technology to the study of glutathione biosynthesis, metabolism and function has proved remarkably successful. Within the remit of the project with transformed poplars, it has been possible to provide answers to the questions posed at the outset. In addition, the demonstrated feasibility of genetic manipulation of glutathione metabolism opens up a promising vista of prospective industrial and environmental applications.

Engineering plants with constitutively increased glutathione

An important factor in the protection of metabolism against stress is the rapidity with which a plant can activate or ameliorate its protective system. One way to circumvent the damage which might result from insufficiently rapid defence activation is through constitutive enhancement of defence components. The study with poplar has demonstrated that constitutive increases in glutathione levels can be achieved through the introduction of genes encoding either the enzymes of glutathione biosynthesis or GR. The increases in glutathione contents are systemic, notably owing to export of glutathione from the leaves of plants with a transgenically-enhanced capacity for GSH synthesis. Hence, the enrichment of glutathione will influence not only foliar processes but will potentially be of benefit to the whole plant.

Internal homeostatic mechanisms, such as end-product inhibition of γ-ECS by GSH or NADPH-dependent inhibition of GR activity in the chloroplast stroma, can be overcome by strong overexpression of these enzymes. The increases obtained by overexpression of either GR or γ-ECS are of the order of 2- to 4-fold. It is not unrealistic to surmise that overexpression of these enzymes together, or concerted overexpression of both γ-ECS and GS, would allow production of plants possessing glutathione contents as much as 10-fold greater than untransformed plants. Whether such huge increases would be accompanied by negative physiological effects is unclear. The constitutive increases in glutathione already obtained do not appear to give rise to negative effects on growth and morphology. Indeed, it is interesting to note that the presence of elevated GSH contents does not prevent induction of an even greater capacity for GSH biosynthesis in stress situations, as evidenced by the results of our studies with heavy metals and with ozone. Production of even greater constitutive increases in glutathione will clearly depend upon substrate availability, in particular cysteine since, in leaf discs from all types of

poplars, cysteine incubation induced significant increases in foliar glutathione. However, the results already obtained show that, at least in the light, substrates can be made available to match the increased demand imposed by increased enzyme activities. The results present no evidence of exhaustion of the cysteine pool when γ-ECS activity is augmented. On the contrary, the inclusion of a powerful sink for cysteine confirms that GSH formation strongly influences the pathway of cysteine synthesis. This may reflect an elasticity of sulphate uptake and reduction in poplar, with the rates of these processes being set by the downstream demand. Specific up-regulation of sulphate reduction (APS reductase) and cysteine synthesis (serine acetyltransferase) appears to be involved.

Control of glutathione biosynthesis

The increases in foliar glutathione brought about by enhanced γ-ECS activities demonstrate that the endogenous activity of this enzyme limits glutathione accumulation. This effect contrasts with the unchanged glutathione contents in poplars strongly overexpressing GS, suggesting that the potential activity of the second enzyme is in relative excess in untransformed poplars. These results offer no data in favour of significant control of GSH synthesis by feedback inhibition of γ-ECS. However, increased GSH synthesis induced by overexpression of γ-ECS does not mean that feedback inhibition by GSH does not operate *in vivo*, merely that it can be overcome. It should be noted that increased GSH synthesis has been repeatedly correlated with increased γ-ECS transcript level, protein and extractable activity in animals (Godwin *et al.*, 1992; Shi *et al.*, 1994; Rahman *et al.*, 1996) and that overexpression of the endogenous γ-ECS in human cells leads to increases in cellular GSH contents similar to those in poplars overexpressing γ-ECS (Mulcahy *et al.*, 1995). This demonstrates that competitive feedback inhibition of γ-ECS by GSH can be overcome simply by up-regulating *de novo* synthesis of the endogenous enzyme, as theoretical considerations predict (Noctor *et al.*, 1997*c*). The requirement for these two control mechanisms, feedback inhibition and *de novo* synthesis of γ-ECS, is explained by the potentially conflicting demands of GSH homeostasis and increased GSH synthesis. Obviously, modulated feedback inhibtion will only permit increased synthesis of GSH under conditions where this increased synthesis does not result in GSH accumulation in the intracellular compartment(s) where γ-ECS is located. One example is up-regulation of GSH synthesis to provide substrates for phytochelatins. The accumulation of GSH observed under certain conditions will require increased synthesis of γ-ECS. If one considers the possible regulatory effect of increasing intracellular cysteine concentrations, it is apparent that no one, simple, global mechanism operates to regulate GSH synthesis: rather, there exist multiple controls whose relative influence may vary according to the conditions. Despite their increased GSH contents, the ggs lines showed even greater proportional increases in γ-EC levels. This effect cannot be explained in terms of limitation by glycine, since the foliar [GSH]/[γ-EC][gly] ratio, and not simply the [GSH]/[γ-EC] ratio, was decreased by γ-EC overexpression. This implies that GS activity exerts a significant limitation over GSH synthesis, once the severe restriction due to low γ-ECS activity is removed. It therefore seems that, even though GS appears not to limit GSH synthesis in untransformed plants, activity of this enzyme is not in great excess and could perhaps become limiting under certain stress conditions.

Glutathione and amino acid metabolism

The synthesis of glutathione is, to our knowledge, the only biosynthetic pathway thus far demonstrated to utilise photorespiratory intermediates. It is clear that photorespiration is able to influence glutathione synthesis: it may be asked whether the converse is true. It is often averred that photorespiratory glycine production occurs as part of an integrated and strict recycling of carbon from phosphoglycollate to PGA and that utilization of intermediates would deplete this pathway and drain the Calvin cycle of the sugar phosphate levels required to maintain ongoing fixation of CO_2 (see, for instance, Ogren, 1984). Table 3 shows an attempt to evaluate the effect of glutathione synthesis on regeneration of RuBP by the Calvin cycle. The drainage of intermediates to glutathione is calculated for various ratios of carboxylation to oxygenation of RubP (C:O), using a rate of RuBP utilization expected from measured rates of CO_2 fixation in poplar. The rate of glutathione synthesis is set at a constant 180 nmol mg^{-1} chl h^{-1}: this rate is derived from measured rates of GS activity in crude poplar extracts. Although such rates are measured under saturating conditions, it should be noted that they are probably well below the maximum capacity of the enzyme (Hell and Bergmann, 1988). Assuming C:N and N:S net assimilation ratios of 10 and 20 (Cram, 1990), the net ratio of C:S incorporation would be 200. From Table 4, the ratio between the rates of gross CO_2 assimilation (90 μmol mg^{-1} chl h^{-1} at the lowest rate of oxygenation) and GSH synthesis is 500. At this ratio, the percentage of potentially recyclable carbon which is used, as glycine, for GSH synthesis is relatively small and decreases as the rate of oxygenation increases (Table 4: % loss of glycollate carbon to synthesis of GSH). At the same time, however, the % loss of total assimilated carbon due to use of glycine for GSH synthesis increases (Table 4: far right column). This reflects the increasing importance of the recycling of glycollate as oxygenation rate increases. For C_3 plants in typical atmospheric conditions, a likely C:O ratio is about 2.6

Table 3. *Effect of the utilization of glycine for GSH biosynthesis on maximum rates of RuBP regeneration at different ratios of carboxylation/oxygenation (C:O) of RuBP*

Absolute rates of RuBP utilisation are set at 100 μmol mg^{-1} chl h^{-1}. The rate of glutathione biosynthesis is set at 180 nmol mg^{-1} chl h^{-1}. The calculations assume (1) no RuBP is consumed for reactions other than RuBP regeneration and GSH synthesis; (2) all phosphoglycollate produced is converted to glycine; (3) recycling of phosphoglycollate to RuBP occurs with the conversion stoichiometry of 2 phosphoglycollate : 1 serine : 0.6 RuBP; (4) there is no refixation of CO_2 released from glycine decarboxylation; (5) all glycine for GSH synthesis originates from phosphoglycollate; (6) the rates of RuBP utilization and GSH synthesis are independent of the C : O ratio. All values other than % values are in units of μmol mg^{-1} chl h^{-1}.

C : O	Triose P produced (no recycling of glycollate)	RuBP produced (no recycling of glycollate)	RuBP produced with recycling of glycollate	Pentose phosphates from glycollate recycling		% loss of glycollate carbon to synthesis of GSH	Total net production of pentose phosphates		% loss of assimilate to synthesis of GSH
				−GSH synthesis	+GSH synthesis		−GSH synthesis	+GSH synthesis	
9	190	114	117	3	2.946	1.87	17	16.946	0.32
7	187.5	112.5	116.25	3.75	3.696	1.49	16.25	16.196	0.33
4	180	108	114	6	5.946	0.90	14	13.946	0.39
3	175	105	112.5	7.5	7.446	0.75	12.5	12.446	0.43
2	166.7	100	110	10	9.946	0.56	10	9.946	0.54
1	150	90	105	15	14.946	0.36	5	4.946	1.08
0.67	140	84	102	18	17.946	0.31	2	1.946	2.80
0.5	133.3	80	100	20	19.946	0.27	0	−0.054	–

(Sharkey, 1988), which would mean that the use of photorespiratory glycine for GSH synthesis involves a loss of approximately 0.5% of net assimilate. Hence, it appears that GSH synthesis alone is unlikely to entail unsustainable drainage of the Calvin cycle. Nevertheless, it should be noted that under stress conditions, the rate of photosynthesis may decline and γ-EC synthesis may accelerate, so that increases in glycine demand and decreases in glycine supply may be concurrent. Such effects may result, for instance, from cold stress, where a decrease in the rate of photosynthetic metabolism would be further aggravated, as far as the supply of glycine is concerned, by a high C : O ratio (Edwards and Walker, 1983). On the other hand, drought stress may favour a low C : O ratio. Finally, a more general consideration of the demands made by glutathione synthesis upon carbon and nitrogen assimilation must take into account not only glycine, but also glutamate and cysteine: though glutathione may be a relatively concentrated store of reduced sulphur, it nevertheless has C : S and N : S ratios of 10 and 3, respectively.

Producing plants with ameliorated reactions to stress

Results obtained with the poplars overexpressing GR suggest that high activity of this enzyme endows an increased capacity to cope with oxidative stress.This conclusion is in agreement with other work using transgenic plants (Aono *et al.*, 1993, 1995*a*, *b*; Broadbent *et al.*, 1995). For poplars overexpressing γ-ECS, with substantial increases in total foliar glutathione, little evidence of increased resistance has been obtained. On the other hand, these poplars, in particular those overexpressing γ-ECS in the chloroplast, appear at least as able as untransformed poplars to maintain their glutathione pools under oxidizing conditions. This has important implications for subsequent attempts to engineer more substantial increases in resistance through multifactorial changes: it appears that the enhanced glutathione pools in the γ-ECS transformants, far from being redox-fragile, are at least as robust in the face of oxidative attack as the smaller glutathione pools of untransformed poplars.

Industrial and environmental applications

In addition to the scientific information derived from these studies, overexpression of the enzymes of glutathione metabolism has opened the door to novel possibilities for industrial exploitation. As a manipulatable product, glutathione has dual interest. First, it has intrinsic value as a flavour precursor in food and as an anti-carcinogen (Ho *et al.*, 1992; Jones *et al.*, 1992). Due to the latter property, glutathione has appreciable potential value as a medicinal product and as an additive for pharmaceutical, food or cosmetic applications. Secondly, GSH is a simple tripeptide and can therefore be used as a model for the biotechnological production of heterologous peptides in plant cell cultures. The major barrier at present to large-scale industrial production of GSH is cost. The current method of GSH production for industrial purposes is through expensive chemical synthesis. Alternative biological sources are therefore required. Plants enriched in GSH may provide a useful source of GSH for industrial purposes, particularly if transformed plant cells with enhanced GSH biosynthesis can be grown in culture. Plant cell cultures can readily be prepared from transformed poplar calli and constitute an excellent alternative means of GSH production. The productivity of cell cultures is potentially higher than that of genetically modified whole plants in the field for several reasons. Firstly, production may be optimized in cultures cells by convenient supply of substrates. Secondly, the culture medium may be considered as an 'extracellular vacuole', allowing excretion of the product from the cell and

enabling continuous extraction. Thirdly, because of the ongoing excretion of the product, overproduction is unlikely to be restricted by internal regulatory factors such as end-product inhibition.

There is also considerable industrial interest in the use of non-conventional breeding strategies for conferring resistance to fungal disease and improving environmental stress tolerance in plant species which are multiplied by micropropagation (e.g. fruit trees). In plants that are traditionally multiplied by cuttings, a relatively small number of transformed mother-stock plants can provide the starting material required to produce the final product by micropropagation.

Poplars (and other forest trees) with increased capacity for GSH synthesis may prove useful for eco-detoxification, through the 'trapping' of xenobiotic pollutants permitted by an increased capacity for glutathione-mediated conjugation. Similarly, plants with enhanced capacities for phytochelatin synthesis, due to increased rates of GSH formation, could be applied to bioremediation of poor soils through the removal of heavy metals.

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