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# Synthesis of Glutathione in Leaves of Transgenic Poplar Overexpressing γ-Glutamylcysteine Synthetase[1]

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**Internode stem fragments of the poplar hybrid *Populus tremula* × *Populus alba* were transformed with a bacterial gene (*gshI*) for γ-glutamylcysteine synthetase (γ-ECS) targeted to the cytosol. Lines overexpressing γ-ECS were identified by northern analysis, and the transformant with the highest enzyme activity was used to investigate the control of glutathione synthesis. Whereas foliar γ-ECS activity was below the limit of detection in untransformed plants, activities of up to 8.7 nmol $mg^{-1}$ protein $min^{-1}$ were found in the transformant, in which the foliar contents of γ-glutamylcysteine (γ-EC) and glutathione were increased approximately 10- and 3-fold, respectively, without affecting either the reduction state of the glutathione pool or the foliar cysteine content. A supply of exogenous cysteine to leaf discs increased the glutathione content from both transformed and untransformed poplars, and caused the γ-EC content of the transformant discs to increase still further. The following conclusions are drawn: (a) the native γ-ECS of untransformed poplars exists in quantities that are limiting for foliar glutathione synthesis; (b) foliar glutathione synthesis in untransformed poplars is limited by cysteine availability; (c) in the transformant interactions between glutathione synthesis and cysteine synthesis operate to sustain the increased formation of γ-EC and glutathione; and (d) the foliar glutathione content of the transformant is restricted by cysteine availability and by the activity of glutathione synthetase.**

In plants the tripeptide thiol glutathione (γ-Glu-Cys-Gly) is central to sulfur metabolism and plays an important role in the defense against active oxygen species and xenobiotics (Alscher, 1989; Rennenberg, 1995). Tissue concentrations are highly dependent on both internal regulation and environmental influences. Glutathione levels in leaves undergo diurnal and seasonal fluctuations (Polle and Rennenberg, 1994; Rennenberg, 1995; Rennenberg and Herschbach, 1995) and are modulated by various forms of stress (Alscher, 1989; Smith et al., 1990; Rennenberg and Brunold, 1994). Processes that are capable of influencing the cellular glutathione content include synthesis and degradation, membrane and long-distance transport, as well as the use of glutathione as a substrate in the synthesis of phytochelatins, glutathione conjugates, and mixed disulfides (Rennenberg, 1995). Currently, the mechanisms controlling these processes have not been sufficiently studied to allow an evaluation of their physiological significance, and in particular, the factors regulating the synthesis of glutathione have not been unequivocally established.

Glutathione is synthesized in plants as well as in other organisms from its constituent amino acids in a two-step reaction sequence, as shown in Figure 1 (Meister and Anderson, 1983; Bergmann and Rennenberg, 1993; Rennenberg, 1995). In the first reaction the synthesis of γ-EC from glutamate and Cys is catalyzed by γ-ECS (EC 6.3.2.2). GluS (EC 6.3.2.3) then catalyzes the addition of Gly to the C-terminal carboxy group of γ-EC (Meister and Anderson, 1983; Bergmann and Rennenberg, 1993; Rennenberg, 1995). This pathway is well established, and the foliar forms of these enzymes have been characterized (Law and Halliwell, 1986; Hell and Bergmann, 1988, 1990; Schneider and Bergmann, 1995). However, whether the activity of either exerts significant control over the rate of glutathione synthesis, or whether the availability of amino acid substrates plays a more decisive role remains to be demonstrated.

Two factors that are considered likely to influence the rate of glutathione synthesis are end-product inhibition of γ-ECS by GSH (Fig. 1) (Bergmann and Rennenberg, 1993; Schneider and Bergmann, 1995) and Cys concentration (Rüegsegger and Brunold, 1992; Strohm et al., 1995). Feedback inhibition of γ-ECS by physiological concentrations of GSH has been demonstrated in vitro (Hell and Bergmann, 1990; Schneider and Bergmann, 1995) and is competitive with respect to glutamate (Schneider and Bergmann, 1995). Nevertheless, the operation of this mechanism in vivo is

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Abbreviations: γ-EC, γ-glutamylcysteine; γ-ECS, γ-glutamylcysteine synthetase; GluS, glutathione synthetase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione.

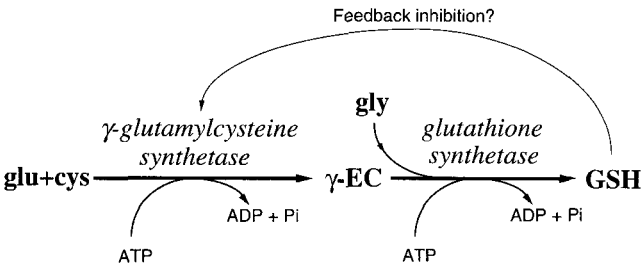


**Figure 1.** Schematic diagram showing the two-step synthesis of glutathione from constituent amino acids. Hypothetical feedback inhibition of γ-ECS by the end product is also depicted.

uncertain, since cellular glutathione concentrations are frequently found to be considerably greater than the $K_i$ values of glutathione for γ-ECS (Hell and Bergmann, 1990; Smith et al., 1990; Polle and Rennenberg, 1994; Rennenberg and Brunold, 1994; Schneider and Bergmann, 1995). Moreover, feedback inhibition alone may not always be an adequate or appropriate means of control, especially in cases in which the activity of an enzyme is high compared with the flux through the pathway in which it operates (Giovanelli, 1990). Recent experiments using suspension cultures may provide evidence for feedback control of γ-ECS by glutathione in vivo (Bergmann and Rennenberg, 1993; Schneider and Bergmann, 1995).

Alternatively, the rate of glutathione synthesis may be controlled by the amount of γ-ECS and/or GluS present. We have previously shown in poplar (*Populus tremula* × *Populus alba*) that strong overexpression of bacterial GluS (up to 100-fold increased activity in poplar leaves) did not affect the foliar glutathione content (Rennenberg and Polle, 1994; Foyer et al., 1995b; Strohm et al., 1995), suggesting that the quantity of GluS alone does not exert significant control over the steady-state level of glutathione in the leaves. The enhancement of glutathione levels observed on feeding Cys indicates that Cys availability plays a more important role (Strohm et al., 1995). Nevertheless, these studies show that foliar glutathione content never exceeded a ceiling value of approximately 1200 nmol $g^{-1}$ fresh weight, even after a prolonged feeding of Cys. This apparent maximum was greatly surpassed, however, on incubating leaf discs in γ-EC, rather than Cys, an effect presumably attributable to the circumvention of a limitation at the level of γ-ECS (Strohm et al., 1995). From these observations, we concluded that in the presence of sufficient quantities of Cys, the rate of glutathione synthesis from constituent amino acids is limited either by the amount of γ-ECS or by feedback inhibition of this enzyme.

To assess the significance of the role played by the quantity of γ-ECS in controlling glutathione synthesis, and to explore the possibility of obtaining plants with constitutively enhanced foliar levels of glutathione, we have transformed poplars with the *Escherichia coli* gene for γ-ECS. Since considerable importance has been attributed to the activity of γ-ECS in the regulation of γ-EC and glutathione synthesis (Hell and Bergmann, 1990; Rüegsegger and Brunold, 1992; Chen and Goldsborough, 1994), these transformants represent an excellent model system in which to investigate the relationships between substrate availability, enzyme activity, and glutathione content.

## MATERIALS AND METHODS

### Vector Construction, Transformation, and Molecular Characterization

All DNA recombinant techniques were performed according to standard protocols (Sambrook et al., 1989). The original start codon (TTG) of the *Escherichia coli* γ-ECS (*gshI*; Watanabe et al., 1986) was changed to ATG using PCR. The coding sequence was cloned into pJIT62 (Guerineau et al., 1989) as a *Hin*dIII/*Bam*HI fragment; an *Xba*I fragment from this plasmid, comprising the 35S cauliflower mosaic virus promoter/*gshI* coding sequence/cauliflower mosaic virus terminator, was cloned into pBIN19 (Bevan, 1984) to create *pgsh*28. This binary vector contained a neomycin phosphotransferase gene (*nptII*) under control of the nopaline synthase promoter. This gene confers kanamycin resistance to the transformed cells. The binary vectors were introduced into the *Agrobacterium* strain C58C1/pMP90 (Koncz and Schell, 1986) by triparental mating. The construct was introduced into the hybrid poplar *Populus tremula* × *Populus alba* INRA 717 1B4 via *Agrobacterium* according to Leplé et al. (1992). Before transfer to the greenhouse, all lines were amplified by micropropagation in vitro. Extraction of total RNA and northern-type analyses were performed as described by Tourneur et al. (1993).

### Plant Material Used for Biochemical Analysis

For determination of thiol contents, protein contents, pigment composition, and enzyme activities, poplar trees were grown to the age of 3 months in an environmental growth chamber. Control plants (untransformed poplar) underwent the same tissue-culturing process as the transformant, and both were transferred to the greenhouse at the same time. Day length was 16 h at a light intensity of 150 μE $m^{-2}$ $s^{-1}$. Temperature and RH were 20/15°C and 60/80% (light/dark). For feeding experiments with glutathione precursors, leaf discs (10 mm in diameter) were cut from the seventh leaf from the top and floated on 10 mM glutamate, 10 mM Cys, both, or on distilled water (all at pH 6.0). The discs were incubated in the growth chamber in the light under the same environmental conditions in which the plants were grown. For each sample, four leaf discs were taken for extraction at the times indicated. The pH of the feeding solutions did not change during the course of the experiment. The data shown are the means of three independent experiments.

### Analytical Techniques

GluS activity in poplar leaves was determined as described previously (Strohm et al., 1995). For determination of γ-ECS activity leaf extracts prepared by the same procedure were analyzed by a modified version of the method of Hell and Bergmann (1990). Three-hundred-microliter aliquots of leaf extract were incubated in Fernbach tubes (Kontes, Vineland, NJ) with an assay mixture containing 20

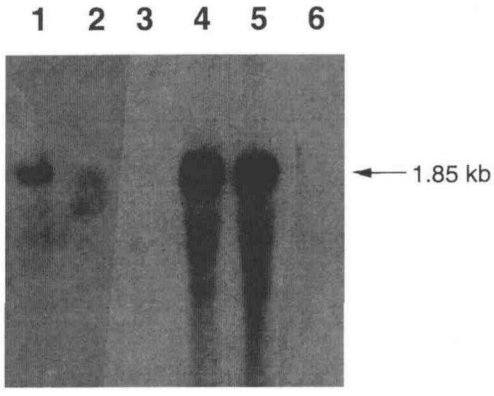


**Figure 2.** Northern blot of total RNA from leaves of poplar transformed with the *E. coli* gene for γ-ECS. Lane 1, gsh283b; lane 2, gsh2817a; lane 3, gsh281c; lanes 4 and 5, gsh282d; and lane 6, untransformed control. All lanes were loaded with 30 μg of RNA.

mM glutamate, 50 mM $MgCl_2$, 10 mM NADH, 4 mM ATP, 4 mM phosphocreatine, 100 mM Hepes (pH 8.0), 10 units $mL^{-1}$ phosphocreatine kinase, 240 units $mL^{-1}$ catalase, and 1 unit $mL^{-1}$ Glc oxidase, in a total volume of 0.5 mL. To prevent the oxidation of thiols during incubation, oxygen was removed from the assay mixtures by degassing and by washing the test tubes with argon three times for 2 min each time, as described by Papen et al. (1986). Following preincubation for 30 min at 30°C the reaction was started by the addition of 25 μL of degassed and argon-washed Cys (20 mM) and Glc (500 mM). After incubation for 20 to 60 min at 30°C, aliquots of the assay mixture were withdrawn and subjected to thiol analysis. For determination of GR activity, leaves were harvested and immediately ground in liquid $N_2$. An aliquot of 200 mg of the frozen powder was extracted with 7 mL of $K11_2PO_1/K_2HPO_4$, 0.5% Triton X-100 (pH 7.8), and 400 mg of insoluble PVP and centrifuged at 48,000*g* and 4°C for 30 min. Supernatant GR activity was determined by spectrophotometric analysis at 340 nm as described by Polle et al. (1990), based on the method of Foyer and Halliwell (1976).

Thiols in leaf extracts and in enzyme assay mixtures were separated by reverse-phase HPLC and quantified as their monobromobimane derivatives by fluorometric analysis, as described previously (Strohm et al., 1995). Determination of oxidized glutathione was based on the reduction of GSSG after irreversible alkylation by *N*-ethylmaleimide of the free thiol group for the GSH present in the extracts (Strohm et al., 1995). GSH that was formed from GSSG was then measured by HPLC as above. Pigment contents were determined spectrophotometrically, following the addition of 5 mL of 80% (v/v) acetone to 30 mg of frozen leaf powder, and calculated according to Lichtenthaler and Wellburn (1983). Soluble protein contents were measured in centrifuged crude extracts by spectrophotometric analysis, using the bicinchoninic acid assay (Pierce) and applying the manufacturer's instructions. For calculation of relative water contents, dry weights of leaf material were determined after drying for 72 h at 80°C.

## RESULTS

A construct containing the *E. coli* gene for γ-ECS (*gshI*), flanked with the regulatory signals required for its expression in the cytosol, was used to transform internode stem fragments of the poplar hybrid *P. tremula* × *P. alba*. Of the four regenerated lines propagated and grown in the greenhouse, two showed high levels of transcripts of the bacterial gene (Fig. 2). Our aim in this study was not to investigate the consequences of transformation itself, nor to examine the effects of γ-ECS overexpression upon physiological characteristics such as stress resistance. Consequently, the line with the highest transcript levels (line gsh282d) was chosen as a model in which to explore the regulation of glutathione synthesis under conditions in which the potential capacity for production of γ-EC was expected to be augmented. Nevertheless, it should be noted that plants of this transgenic line were phenotypically indistinguishable from untransformed poplars (Foyer et al., 1995a) and did not differ from the latter with respect to composition of major pigments, protein content, or dry weight (Table I).

Whereas foliar γ-ECS activity was below the limit of detection in leaves of untransformed plants, high activities were found in leaves of the transformant gsh282d (Table II). In contrast, foliar GluS and GR activities did not differ between the two plant types (Table II). Although we were unable to detect γ-ECS activity in untransformed poplar, we estimated the relative increase in the transformed plants in two ways. First, the limit of detection of the method used allowed us to calculate an activity at least 80 times inferior to that found in the transformant. Second, data from the literature show that in most plant species investigated, extractable activities of γ-ECS and GluS are approximately equal (Hell and Bergmann, 1990; Schneider and Bergmann, 1995). If this also holds true for poplar, then we may calculate that the transformant possesses an extractable foliar γ-ECS activity that is 24-fold that of untransformed plants (Table II).

Table III shows that this strongly augmented γ-ECS activity leads to an increase in both γ-EC and glutathione in gsh282d plants. Although both transformed and untransformed poplars showed considerable variation in thiol contents between experiments, on a given day the relative increases in γ-EC and glutathione contents in the transfor-

**Table I.** *Comparison of pigment contents, protein contents, and relative water contents in leaves of untransformed and transformed poplars*

Data are means of three independent analyses of the seventh leaf of three different plants ($n = 9$).

| Parameter | Untransformed | gsh282d |
|---|---|---|
| | *mg $g^{-1}$ fresh wt* | |
| Total chlorophyll | 2.37 ± 0.28 | 2.11 ± 0.41 |
| Chlorophyll *a* | 1.88 ± 0.19 | 1.68 ± 0.32 |
| Chlorophyll *b* | 0.49 ± 0.10 | 0.43 ± 0.09 |
| Carotenoids | 0.45 ± 0.05 | 0.42 ± 0.06 |
| Soluble protein | 26.8 ± 7.2 | 25.5 ± 5.6 |
| Relative water content (%) | 74.8 ± 2.1 | 75.0 ± 3.2 |

**Table II.** *Activities of glutathione-related enzymes in leaves of untransformed and transformed poplars*

Data are means of six (γ-ECS) and three (GluS and GR) independent extractions of the seventh leaf.

| Enzyme | Untransformed | gsh282d |
|---|---|---|
| | *nmol mg$^{-1}$ protein min$^{-1}$* | |
| γ-ECS | n.d.[a] | 8.67 ± 1.56 |
| GluS | 0.33 ± 0.02 | 0.36 ± 0.08 |
| GR | 19.8 ± 4.8 | 17.8 ± 2.8 |

[a] n.d., Not detected (the limit of detection was approximately 0.1 nmol mg$^{-1}$ protein min$^{-1}$).

mant were reproducible (3–4 and 10–15 times greater for glutathione and γ-EC, respectively [Table III]). This suggests that the fluctuations in thiol contents from experiment to experiment were related to the physiological or environmental changes to which both types of plants were subjected. The increased glutathione content in the transformant involved no change in the redox state of the glutathione pool, with GSSG remaining at 4 to 5% of the total pool (Table III). This implies that the GR activity of untransformed poplar is in excess of that required to maintain the foliar glutathione pool in a predominantly reduced state. The increased capacity of the transformant for synthesis of γ-EC is confirmed by the strikingly high accumulation of this thiol (Table III). Such enhanced accumulation of γ-EC and glutathione in the transformant is expected to entail a marked increase in Cys demand. Nevertheless, no diminution of foliar Cys concentration was observed in the transformants; the content of this thiol was unchanged or slightly elevated (Table III).

Previous experiments in which glutathione precursors were supplied to excised poplar leaf discs suggested that in the presence of sufficient amounts of Cys, the rate of formation of glutathione is limited by the activity of γ-ECS (Strohm et al., 1995). Consequently, similar studies were conducted using plants of line gsh282d, in which this activity is greatly enhanced. Leaf discs were incubated in glutamate, Cys, both, or neither for a period of 7 h (Fig. 3). Even in the absence of exogenous substrates (Fig. 3, $H_2O$), there was some accumulation of glutathione over the course of the experiment in discs from both untransformed and gsh282d poplars (open and closed circles, respectively), demonstrating that the excised discs are fully competent in glutathione synthesis. Because in whole poplar plants glutathione is transported from the leaves to the roots (data not shown), the basal level of accumulation observed on incubation of discs in water may be a consequence of interrupted long-distance transport. Supplying glutamate led to no increase above this basal level in leaf discs from either type of plant, indicating that the availability of glutamate does not limit glutathione synthesis under these conditions (Fig. 3, glu). In contrast, supplying Cys to the discs resulted in an approximately 3.5-fold increase in both types of plants; this increase was linear and was not accelerated by the addition of glutamate in concert with Cys (Fig. 3, cf. cys and cys+glu). This observation indicates that even during the high accumulation of glutathione produced by supplying Cys to leaf discs of transformed poplar, endogenous glutamate does not restrict the

**Table III.** *Foliar thiol contents in untransformed and transformed poplars*

Thiols were determined by HPLC in three identical experiments, carried out on different days. Each data point represents the mean of four extractions from the seventh mature leaf.

| Thiol | Experiment | Untransformed | gsh282d | Fold Increase in Transformants |
|---|---|---|---|---|
| | | *nmol g$^{-1}$ fresh wt* | | |
| GSH+GSSG | 1 | 255 ± 26 | 751 ± 4 | 2.95 |
| | 2 | 173 ± 12 | 482 ± 51 | 2.79 |
| | 3 | 258 ± 13 | 1071 ± 25 | 4.15 |
| GSSG | 1 | 10.8 ± 0.4 | 30.0 ± 3.1 | 2.78 |
| | 2 | 11.6 ± 0.8 | 45.7 ± 1.2 | 3.94 |
| | 3 | 12.2 ± 0.8 | 41.1 ± 4.6 | 3.37 |
| γ-EC | 1 | 9.2 ± 1.1 | 99.7 ± 12.8 | 10.8 |
| | 2 | 22.5 ± 3.1 | 265.4 ± 8.0 | 11.8 |
| | 3 | 7.6 ± 0.6 | 115.0 ± 4.6 | 15.1 |
| Cys | 1 | 12.6 ± 0.3 | 18.9 ± 2.1 | 1.50 |
| | 2 | 6.8 ± 0.3 | 17.5 ± 2.6 | 2.57 |
| | 3 | 7.4 ± 0.7 | 9.6 ± 1.0 | 1.30 |

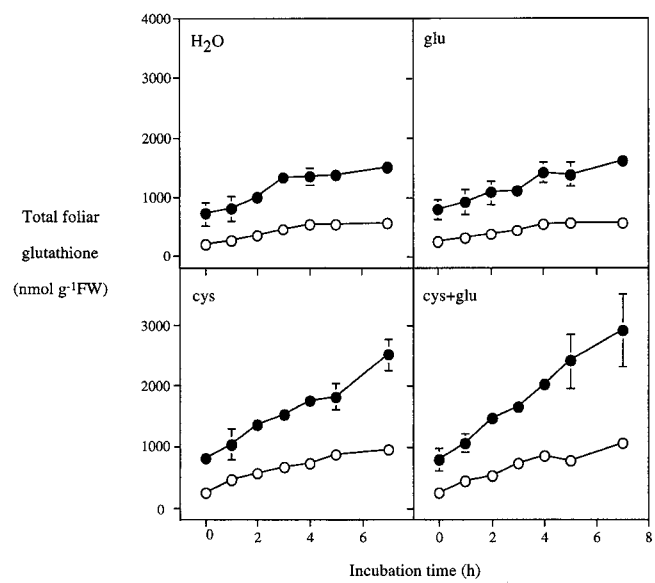


**Figure 3.** Effect of glutathione precursors on the glutathione content in leaf discs of untransformed and transformed poplar. Leaf discs (0.83 cm$^2$ each) were cut from the seventh mature leaf of poplars and floated on distilled water containing the indicated amino acids at a concentration of 10 mM water, with the control containing neither glutamate nor Cys. The pH of all feeding solutions was 6.0. Open circles, Untransformed control; closed circles, gsh282d. At the times indicated, four leaf discs were collected for each treatment, washed with distilled water, and homogenized. Total glutathione was determined fluorometrically following reduction by DTT, derivatization with monobromobimane, and separation by reverse-phase HPLC. Values are expressed as the means of three independent experiments. Where error bars are not apparent, SE values are smaller than the symbol diameter. FW, Fresh weight.

rate of glutathione synthesis. For discs from untransformed plants, the glutathione content that was attained after 7 h of incubation was similar to that previously observed in analogous experiments (about 1000 nmol $g^{-1}$ fresh weight; Strohm et al., 1995), whereas for discs from gsh282d poplar, the final glutathione content was approximately 3-fold greater (Fig. 3).

The enhanced accumulation of glutathione in the gsh282d leaf discs is not unexpected, given the greatly increased capacity for γ-EC synthesis in these plants. Nevertheless, the magnitude of enhancement (approximately 3-fold) is not commensurate with the increased γ-ECS activity (24–80 times higher), pointing to a restriction of glutathione synthesis by additional factors. Therefore, it was interesting to compare the accumulation of glutathione with the formation of γ-EC in these experiments. Figure 4 shows that increasing the supply of Cys brought a further enhancement of the γ-EC content in leaf discs of the transformant, suggesting that glutathione synthesis is limited in these circumstances by the utilization of γ-EC (Fig. 4, cys, closed circles). In comparison with the linear accumulation of glutathione under the same conditions (Fig. 3, cys, closed circles), γ-EC content increased rapidly to reach a new steady state within 1 h of incubation (Fig. 4). Further contrast between the response of γ-EC and glutathione to the supply of Cys to leaf discs of the transformant was observed on the simultaneous addition of glutamate and Cys; whereas the inclusion of glutamate had little effect on the rate of glutathione synthesis (Fig. 3), the final content of γ-EC was almost doubled (Fig. 4, cf. cys and cys+glu). Although not evident from Figure 4, the γ-EC pool in discs from untransformed poplar increased because of the incubation in Cys (Fig. 4, cys, open circles). This response, however, was less marked due to the low γ-EC content in these leaves and, moreover, was uninfluenced by glutamate (γ-EC contents after 7 h of incubation were 64.1 and 62.3 for discs from untransformed plants supplied with Cys and Cys plus glutamate, respectively).

One possible explanation for the saturation kinetics exhibited by the γ-EC pool in gsh282d leaf discs in response to Cys might be related to the feedback inhibition of γ-ECS by the increased glutathione contents under these conditions. However, the product of the *E. coli* gene used in this study reportedly lacks the domain that confers sensitivity to inhibition by GSH (Watanabe et al., 1986). Clearly, if this is true, effective restriction of the rate of γ-EC synthesis by glutathione is unlikely in our transformed poplars, where the overwhelmingly major part of the extractable activity is presumably attributable to the bacterial enzyme. Yet, the data in Table IV show that inclusion of glutathione in the assay medium did inhibit the γ-ECS activity extractable from gsh282d poplars. The concentrations of glutathione required, however, were substantially higher than those reported for the enzymes from tobacco (Hell and Bergmann, 1990) and parsley (Schneider and Bergmann, 1995).

## DISCUSSION

Poplar overexpressing γ-ECS in the cytosol contain elevated foliar γ-ECS activity and, as a result, enhanced quantities of foliar γ-EC and glutathione (Tables II and III). This finding contrasts with the unchanged foliar content of these thiols in poplar overexpressing GluS (Strohm et al., 1995) and implies that of the two enzymes, it is the activity of γ-ECS that most strongly affects the glutathione content in untransformed poplar. Increases in the extractable activity of γ-ECS have been reported for various plant species under conditions in which glutathione synthesis is accelerated (Rüegsegger and Brunold, 1992; Bergmann and Rennenberg, 1993; Chen and Goldsborough, 1994; Schneider and Bergmann, 1995). However, these increases are often modest and disproportionate to the measured or inferred rates of glutathione synthesis, indicating that feedback inhibition of γ-ECS activity by glutathione may be physiologically more important in controlling the glutathione content than the quantity of enzyme that is present (Bergmann and Rennenberg, 1993; Schneider and Bergmann, 1995). Although the data presented here do not conflict with this view, they do suggest that, at least in poplar leaves, endogenous γ-ECS is present in sufficiently low amounts to impose a limitation on the rate of glutathione synthesis. Therefore, it follows that augmented de novo synthesis of this enzyme is an effective means by which increases in the rate of glutathione synthesis might occur, for example, in response to stress.

The 3-fold increase in the foliar glutathione content in the transformed poplars demonstrates that glutathione is unable to control its own synthesis effectively when the quantity of γ-ECS is markedly increased. This is not surprising; although we did not determine the in vivo γ-ECS activity in the transformant, we estimate that the extractable activity is between 24- and 80-fold greater than the activity of the native poplar enzyme. Hence, elevated in vivo activity of γ-ECS in the transformant could represent an instance in which the activity of an enzyme is in excess of that required by the flux through the reaction it catalyzes. In these circumstances feedback inhibition is unlikely to function as an effective means of restraint (Giovanelli, 1990). However, feedback control by the enhanced levels of glutathione in the transformant may be considered responsible for preventing an even greater enhancement of foliar glutathione concentrations. This explanation can be discounted, however, since feeding Cys to transformant leaf discs caused the glutathione pool to increase in a linear fashion over 7 h to attain values close to 10-fold greater (around 3000 nol $g^{-1}$ fresh weight) than those found in untransformed poplar in the absence of added Cys (Fig. 3). Thus, even these high glutathione concentrations proved incapable of effectively inhibiting the activity of the bacterial enzyme, a failure that may have been partly due to the comparative insensitivity of this enzyme to glutathione (Table IV).

Despite the increased glutathione accumulation in the transformant overexpressing γ-ECS, there are clearly other factors that play determinant roles in the control of glutathione synthesis in untransformed poplars; chief among these is Cys. In leaf discs from untransformed plants, addition of Cys increased the content of both γ-EC (Fig. 4) and glutathione (Fig. 3; Strohm et al., 1995). These data demonstrate that Cys availability is limiting for the synthe-

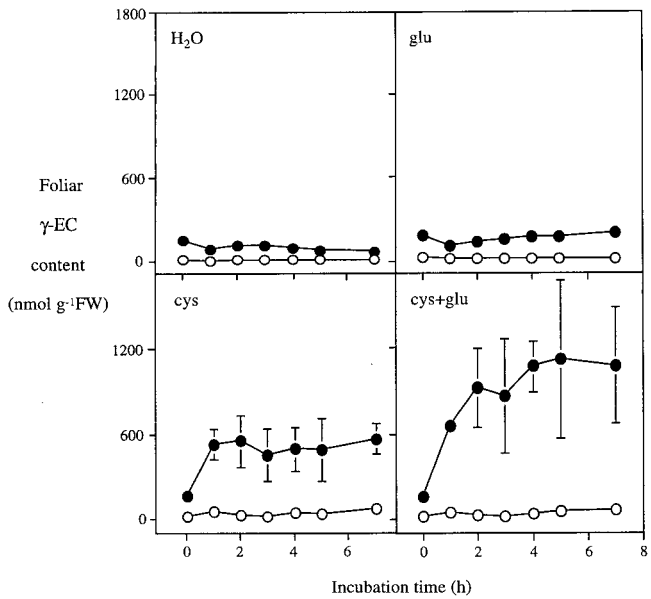


**Figure 4.** Effect of glutathione precursors upon γ-ECS in leaf discs of untransformed and transformed poplar. Experimental details are as for Figure 3. Open circles, Untransformed control; closed circles, gsh282d. All data represent the means of three independent experiments. FW, Fresh weight.

sis of both thiols, implying that overexpression of γ-ECS elicits increased rates of Cys synthesis to sustain the excess γ-EC and glutathione produced in transformed poplars. This suggestion is supported by measured values of foliar Cys contents in the transformants, which were no lower than those of untransformed plants (Table III). The possibility of regulatory interactions between γ-ECS activity and Cys concentration is an intriguing one, since Cys is synthesized in the chloroplast (Giovanelli, 1990), whereas the transformed poplar studied here overexpresses γ-ECS in the cytosol. This compartmentation may, to some extent, explain the limitation exercised by Cys availability in the transformant. However, in tobacco, pea, and spinach, γ-ECS and GluS activities have been demonstrated in both the chloroplastic and cytoplasmic fraction (Hell and Bergmann, 1988, 1990). Presumably, therefore, Cys synthesized in the chloroplast is able to cross the chloroplast envelope to support glutathione synthesis in the cytoplasm. It nevertheless remains possible that, in the transformant possessing enhanced capacity for γ-EC synthesis, the rate of Cys transport through the envelope contributes to a restriction over the rate of this synthesis. Whether the additional γ-EC and glutathione synthesized in the transformant are localized exclusively in the cytoplasm or not is a question currently under investigation.

Since Cys content was not markedly increased in the transformant (Table IV), it was expected that its availability would restrict the formation of γ-EC and glutathione. The increased synthesis of these thiols on feeding Cys to transformant leaf discs (Figs. 3 and 4) demonstrates that this is the case. Furthermore, data presented in Figure 4 suggest that when Cys is plentiful and γ-ECS activity is no longer limiting, i.e. in transformant leaf discs supplied with Cys, the availability of glutamate may begin to impose a restriction on the rate of formation of γ-EC (Fig. 4, cf. cys and cys+glu). Alternatively, this effect of glutamate might be considered evidence in favor of the operation of feedback inhibition of γ-ECS under these conditions. Since glutathione acts as a competitive inhibitor with respect to glutamate (Schneider and Bergmann, 1995), high concentrations of the latter were expected to mitigate any inhibitory effect of glutathione observed when Cys is supplied alone. However, there was little evidence for stimulation of glutathione synthesis by glutamate in these circumstances (Fig. 3, cf. cys and cys+glu), an observation that, in tandem with the synergistic effect of glutamate and Cys on γ-EC formation, argues for restricted conversion of γ-EC to glutathione in the transformant. Two additional pieces of evidence support this contention. First, overexpression of γ-ECS led to a proportionally greater increase of foliar γ-EC content than of foliar glutathione content (Table III). Second, the kinetics of the increase in synthesis of these two thiols on the supply of Cys to transformant leaf discs were markedly different. Whereas glutathione increased in a gradual, linear fashion over 7 h, the increase in γ-EC occurred much more rapidly; after 1 h of feeding Cys, γ-EC content was 3.25-fold greater than in the absence of exogenous Cys (Fig. 4). At this point, under the same conditions glutathione content had increased only 1.22-fold (Fig. 3).

Two factors that limit the conversion of γ-EC to glutathione in the transformants are the availability of Gly and the activity of GluS. The former has been shown to restrict the rate of glutathione formation under conditions in which γ-EC is high and Gly is low (Buwalda et al., 1990). In the present study, however, all thiol measurements were performed using either illuminated plants or illuminated leaf discs. Under these conditions Gly content is likely to be relatively high, due to its synthesis during photorespiration (Ogren, 1984). It is important that, upon supply of Cys to leaf discs from the γ-ECS overexpressor, the kinetics and extent of glutathione accumulation (Fig. 3) were strikingly similar to those observed on incubating leaf discs from untransformed poplars with γ-EC and Gly (Strohm et al., 1995). This observation is most easily explained by the hypothesis that the same factor limits the rate of glutathione synthesis in both cases, i.e. the activity of GluS. If this is correct, then concerted overexpression of γ-ECS and

**Table IV.** *Inhibition of the extractable foliar γ-ECS activity of transformed poplars by glutathione*

γ-ECS activity was measured in extracts from the seventh mature leaf of poplars of line gsh282d. GSH was included in the assay mix at the concentrations indicated. Data are the mean activities of three extractions. Figures in parentheses show percentage of activity in the absence of glutathione.

| Glutathione Concentration | γ-ECS Activity |
|---|---|
| *mM* | *nmol $g^{-1}$ fresh wt $min^{-1}$* |
| 0 | 235 ± 51 (100) |
| 10 | 190 ± 29 (82) |
| 20 | 82 ± 20 (35) |
| 30 | 26 ± 8 (11) |

GluS may offer a means of obtaining plants with even greater constitutive enhancement of foliar glutathione than that obtained by γ-ECS overexpression alone, even if the present data suggest that further increases may depend on sufficient Cys availability.

We conclude that the availability of Cys imposes a restriction on the rate of glutathione synthesis in poplar leaves, whether the activity of γ-ECS is limiting or not. This conclusion is in no way paradoxical, but simply reflects a simultaneous operation of a kinetic restraint exercised by the concentration of an essential substrate and one imposed by the effective activity of a limiting enzyme, i.e. the activity of γ-ECS in untransformed poplars and that of GluS in the transformant. In the latter, for instance, overexpression of γ-ECS leads to augmented foliar glutathione, but brings about a decrease in the glutathione-to-γ-EC ratio from a mean value of 17.4 to 4.8 (calculated from the data shown in Table III). Given that feedback inhibition appears not to be significant in the transformed poplars, this new ratio probably reflects a balance between the limitation of γ-EC formation by Cys availability and the restriction of γ-EC utilization by GluS activity. Assuming that this is the case, elevated cellular Cys concentrations, produced on feeding exogenous Cys, are expected to lead rapidly to increased γ-EC formation (Fig. 4 shows a 3-fold increase within 1 h). This would decrease the glutathione-to-γ-EC ratio in the leaf discs still further (in our experiments, to 1.95 after 1 h of feeding Cys; cf. Figs. 3 and 4). This extremely low glutathione-to-γ-ECS ratio would in turn be expected to favor glutathione formation, albeit at a slower rate than γ-EC synthesis (Fig. 3), due to the kinetic restriction imposed on the transformant by the activity of GluS.

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