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Modification of thiol contents in poplars (*Populus tremula* × *P. alba*) overexpressing enzymes involved in glutathione synthesis

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Abstract. The hybrid poplar (*Populus tremula* × *P. alba*) was transformed to express the *Escherichia coli* gene for γ-glutamylcysteine synthetase (EC 6.3.2.2: γ-ECS) in the cytosol. Four transformed lines of poplar were obtained. These were phenotypically indistinguishable from untransformed poplars. Three lines, ggs28 (Noctor et al. 1996, Plant Physiol 112: 1071–1078), ggs11 and ggs5 possessed high levels of bacterial gene transcripts. Line ggs17 had lower transcript levels. Antisera were prepared against bacterial γ-ECS and bacterial glutathione synthetase (EC 6.3.2.3: GS). Using the antiserum prepared against the purified His-tagged *E. coli* γ-ECS, lines ggs28, ggs11 and ggs5 were shown to possess abundant quantities of the bacterial protein, whereas ggs17 contained lower amounts. The antiserum prepared against the purified His-tagged *E. coli* GS was also effective in screening poplars transformed with the *E. coli* gene coding for this enzyme. Immunoblots of leaf extracts from poplars overexpressing GS using this antibody revealed two bands. The extractable foliar γ-ECS activities of the γ-ECS transformants were in quantitative agreement with the protein levels. Lines ggs28, ggs11 and ggs5 had approximately 30-fold higher γ-ECS activity than untransformed poplars, whereas in ggs17 this activity was only augmented about 3-fold. The lines strongly overexpressing γ-ECS, ggs28, ggs11 and ggs5, contained enhanced foliar levels of cysteine (up to 2-fold), γ-glutamylcysteine (5- to 20-fold) and glutathione (2- to 4-fold). Foliar thiol contents in ggs17 were no different to those of untransformed plants.

Key words: γ-Glutamylcysteine synthetase – Glutathione synthesis – Glutathione synthetase – Transgenic poplar – *Populus* (glutathione synthesis)

Abbreviations: γ-EC = γ-glutamylcysteine; γ-ECS = γ-glutamylcysteine synthetase; GSH = glutathione; GS = glutathione synthetase; GSSG = oxidised glutathione; WT = wildtype

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Introduction

Recent years have witnessed an upsurge of interest in the tripeptide thiol glutathione (GSH: γ-glu-cys-gly), as the multiplicity of its functions in plant metabolism has become evident. Glutathione plays several important roles in the defence of plants against environmental threat. Not only is it implicated in protecting the leaf against oxidative stress, particularly in its role as reductant of dehydroascorbate (Foyer and Halliwell 1976; Smith 1985; May and Leaver 1993; Foyer et al. 1995a), but GSH is also a precursor of the phytochelatins, which allow the plant to withstand supra-optimal concentrations of heavy metals (Grill et al. 1990; Rüegsegger and Brunold 1992; Chen and Goldsborough 1994). Moreover, as a substrate for the glutathione S-transferases, GSH enables neutralisation of potentially dangerous xenobiotics (for a review, see Marrs 1996). Since GSH often constitutes the major pool of non-protein reduced sulfur, it may considerably influence sulfur metabolism (Rennenberg 1982). Indeed, recent studies provide growing evidence for a co-ordinating role in this metabolism, owing to the inhibitory effect of GSH upon sulfur uptake at root level (Herschbach and Rennenberg 1991, 1994; Lappartient and Touraine 1996).

Despite the importance of the above roles, a clear understanding of the relative importance of the factors which regulate GSH synthesis is lacking. Glutathione synthesis takes place in two ATP-dependent steps (Fig. 1), through reactions catalysed by γ-glutamylcysteine synthetase (EC 6.3.2.2: γ-ECS) and glutathione synthetase (EC 6.3.2.3: GS). The foliar forms of these enzymes have been partially characterised and shown to exist in both chloroplastic and extra-chloroplastic compartments (Law and Halliwell 1986; MacNicol 1987; Hell and Bergmann 1988, 1990; Schneider and Bergmann 1995). Several reports of the cloning from plant sources of γ-ECS (May and Leaver 1994) and GS (Rawlins et al. 1995; Ullman et al. 1996) have recently appeared. Nevertheless, despite these advances,

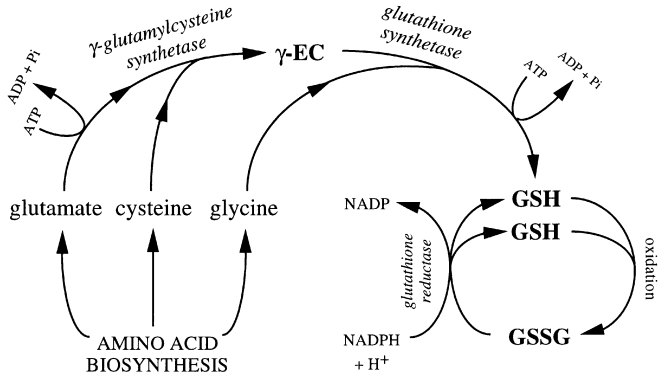


Fig. 1. Schematic illustration of the synthesis of glutathione from constituent amino-acids. The interconversion of the reduced form (GSH) and oxidised form (GSSG) of glutathione is also depicted. Italic characters denote enzymes

biochemical data relating to the kinetic characteristics of the purified plant γ-ECS and GS are as yet lacking.

Three factors considered likely to control the rate of GSH synthesis in leaves are the availability of cysteine (Buwalda et al. 1988; Strohm et al. 1995), feedback inhibition of γ-ECS by GSH (Hell and Bergmann 1990; Schneider and Bergmann 1995) and the amount of γ-ECS enzyme (Rüegsegger and Brunold 1992; Chen and Goldsborough 1994; Farago and Brunold 1994; Schneider and Bergmann 1995). Assessment of the relative contributions of these factors under given conditions is problematic. Firstly, although total foliar cysteine content is readily measured, the difficulties of subcellular fractionation and of accurately measuring compartment volumes complicate estimations of its concentration in different organelles. Secondly, although feedback inhibition of γ-ECS by GSH has been reported in vitro, its significance in vivo remains to be conclusively demonstrated. Thirdly, since neither γ-ECS nor GS has been purified from a plant source, relatively little is known about the kinetic properties of these enzymes. In particular, it is not known whether organelle-specific isoforms, with different kinetic characteristics, exist. Consequently, many studies on the control of GSH synthesis have been of a correlative nature, and have examined changes in GSH synthesis under stress conditions (e.g. Smith 1985; Rüegsegger and Brunold 1992; May and Leaver 1993; Chen and Goldsborough 1994).

An alternative approach is to attempt to influence the synthesis of GSH directly, through overexpression of the enzymes which catalyse GSH synthesis. Consequently, we have produced poplars which constitutively express *E. coli* genes encoding γ-ECS or GS. The aim of this project was twofold: firstly, to explore whether constitutively enhanced GSH concentrations could be engineered in leaves, and secondly, to investigate the contribution of the in vivo activities of these enzymes to the regulation of the pathway of GSH biosynthesis. Increasing an enzyme activity by overexpression will only influence substrate:product ratios of a reaction if the reaction is held out of thermodynamic equilibrium in the untransformed system. Transformation of poplar to overexpress GS (up to 200-fold increase in extractable activity) led to no increase in foliar GSH content (Foyer et al. 1995a; Strohm et al. 1995). A simple explanation of this lack of effect is that the ATP-dependent formation of GSH from γ-EC and glycine is not strongly regulated in untransformed poplars. In other words, the relative concentrations of the reactants are not far removed from the equilibrium position predicted thermodynamically, which would imply that the in vivo activity of GS does not play a major role in controlling the foliar GSH content in untransformed poplars. In contrast, the importance of γ-ECS activity was evidenced by the observation that the poplar line ggs28 (formerly gsh28), transformed to overexpress γ-ECS in the cytosol, possessed considerably augmented foliar contents of both γ-EC and GSH (Noctor et al. 1996).

In this work, we report the characterisation, at the level of gene transcripts, enzyme activities and foliar thiols, of ggs28 and three other independent lines of poplar overexpressing γ-ECS in the cytosol. In addition, we have produced antisera against the *E. coli* γ-ECS and GS. This has permitted rapid analysis of the respective bacterial protein contents, both in these poplar lines and in poplar lines overexpressing GS (Foyer et al. 1995a). Measurements of the foliar thiol contents of the three lines strongly overexpressing γ-ECS demonstrate that increasing the amount of this enzyme enables alleviation of a restriction existing in untransformed poplars over the synthesis of γ-EC, and, consequently, the production of GSH.

Materials and methods

Plant material. Untransformed and transformed hybrid poplars (*Populus tremula* × *P. alba*; Institut National de la Recherche Agronomique No. 717-1-B4, France) were micropropagated in vitro and then introduced into the greenhouse. For all analyses, unless stated otherwise, samples were taken from fully expanded leaves (seventh to tenth position from the apex) of plants which had grown three months in the greenhouse.

Gene cloning and transformation. Transformation of poplar to express the *E. coli* gene for glutathione synthetase (GS) has already been described in Foyer et al. (1995a). For transformation of poplars with the *E. coli* γ-glutamylcysteine synthetase gene, the original start codon TTG of the *gshI* cDNA (Watanabe et al. 1986) was changed to ATG by polymerase chain reaction. The *Hind*III/*Sma*I fragment containing the *gshI* coding sequence (1.7 kb) was inserted into the same sites of the plasmid pLBR19, which contains the cauliflower mosaic virus (CaMV) 35S promoter with double-enhancer sequence (p70) and the CaMV polyA sequence. The promoter-*gshI*-polyA cassette was cloned as an *Sst*I/*Xba*I fragment into the binary vector pBIN 19 (Bevan 1984) to create p70*gshI*. For the transformed line ggs28 (formerly gsh282d), p35S*gshI* was produced as described in Noctor et al. (1996). The binary vectors were introduced into the disarmed *Agrobacterium tumefaciens* strain C58 pMP90 (Koncz and Schell 1986) by triparental mating and transformation of poplar was carried out as described in Leplé et al. (1992).

Analysis of DNA and RNA gel blots . Genomic DNA was isolated from 3 g leaf material, using the procedure of Dellaporta et al. (1993), and purified on a CsCl-ethidium bromide gradient. Five micrograms of DNA was digested with *Sal*I and *Xba*I (unique sites

in the T-DNA). Total RNA was extracted from 1 g leaf material as described by Verwoerd et al. (1989). The RNA and DNA gel blots were performed according to standard protocols (Sambrook et al. 1989). The DNA probe was a 1.4-kb *Eco*RI internal fragment from the *gshI* cDNA, labelled for both blots by random priming with [^{32}P]dCTP (Pharmacia, St Quentin en Yvelines, France).

Isolation of fusion proteins for preparation of antibodies against the gshI and gshII gene products. The *E. coli* γ-ECS and GS proteins were expressed as N-terminally histidine-tagged proteins as follows. For γ-ECS, the entire *gshI* cDNA sequence was subcloned into the *Xho*I/*Bam*HI sites of the histidine-tag expression vector pET14b (Novagen, Abingdon, UK). For GS, a fragment of 0.9 kb from the *Msl*I sites of the *gshII E. coli* cDNA sequence (Gushima et al. 1984) was subcloned into the *Nde*I site of the pET14, blunt-ended after a Klenow treatment. The sequences were verified. The resulting plasmids pHis*gshI* and pHis*gshII* were transferred into *E. coli* BL21 (DE3) pLysS. The transformed bacteria were grown in the presence of 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h at 37 °C.

For native purification of the His-γ-ECS, the cell pellet from 100 ml culture was resuspended in 4 ml binding buffer [50 mM Na-phosphate (pH 8.0), 300 mM NaCl], sonicated, and centrifuged at 10 000 *g* for 20 min. The supernatant was mixed with 0.4 ml nickel affinity resin (Ni-NTA agarose; QIAGEN, Hilden, Germany), batched 1 h at 4 °C, and applied to the column. The column was washed with 20 ml binding buffer followed by 10 ml wash buffer [50 mM Na-phosphate (pH 6.0), 300 mM NaCl]. The His-γ-ECS was eluted with wash buffer containing 250 mM imidazole. The eluted fraction containing the highest concentration of protein (about 1 mg · ml^{-1}) was used for inoculation of rabbit.

Solubilization of the native form of the His-GS proved not to be possible, even following sonication and use of detergents. The His-GS fusion protein was therefore purified in denatured form as follows. The cell pellet from 100 ml culture was resuspended in 4 ml denaturing buffer [100 mM Na-phosphate (pH 8.0), 10 mM Tris-HCl, 6 M urea], incubated at room temperature, and centrifuged at 10 000 *g* for 20 min. The subsequent protocol was identical to that used for His-γ-ECS purification, with the following exceptions: the column was equilibrated with denaturing buffer rather than binding buffer, the wash buffer was 100 mM Na-phosphate (pH 6.0), 10 mM Tris-HCl, 6 M Urea, and the batch was carried out at room temperature. Fractions containing the highest concentrations of eluted protein were diluted to 0.5 mg · ml^{-1} prior to injection into rabbit.

Immunoblot analysis. Leaf discs (approx. 100 mg) were cut from the eighth leaf from the apex and frozen, then ground, in liquid N_2. Soluble protein was extracted into either 200 mM Tris-HCl (pH 8.0), 2% insoluble polyvinylpyrrolidone (PVP, w/v), 2 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 μM trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane or, to prevent possible proteolysis during the extraction, directly into denaturing buffer [50 mM Tris (pH 6.8), 1.6% SDS, 1 mM β-mercaptoethanol]. Following denaturing PAGE (12% or 15% acrylamide), proteins were transferred onto nitrocellulose (Sambrook et al. 1989). Blots were incubated 1 h at 37 °C with rabbit antiserum against His-γ-ECS or His-GS diluted 2000 times. Following incubation with the second antibody (goat anti-rabbit IgG alkaline phosphatase conjugate, the bands were visualised using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

Determination of leaf thiols. Total thiols were determined following labelling with monobromobimane and separation by reverse-phase HPLC (Newton et al. 1981; Kosower and Kosower 1987), by modification of a procedure described in Strohm et al. (1995). Leaf tissue (approx. 50 mg fresh weight, taken unless stated from leaves between the seventh and tenth position from the apex) was ground in 1 ml 0.1 N HCl, 1 mM EDTA with 80 mg insoluble PVP and centrifuged 30 min at 10 000 *g* and 4 °C. Sufficient quantities of 0.5 M 2-(N-cyclohexylamino)ethanesulfonic acid (Ches), pH 9.3 (about 90 μl) were added to 200-μl aliquots of the supernatant to reach a pH of between 8.0 and 8.5. Twenty microliters 10 mM dithiothreitol was added and the mixture incubated in the dark at room temperature. After 1 h, 20 μl 30 mM monobromobimane was added and the derivatization allowed to proceed for 15 min at room temperature in the dark. The reaction was stopped by addition of 800 μl 10% acetic acid. Samples were centrifuged 30 min at 10 000 *g*, filtered, and injected into a Waters (St Quentin en Yvelines, France) HPLC system. Separation of derivatized thiols was carried out on an RP18 column (100 mm long, 4.6 mm i.d; C18 Spheri 5; Applied Biosystems, Alltech France, Templeuve, France). Compounds were eluted using a linear gradient comprising buffer A (10% methanol, 0.25% acetic acid, pH 4.3) and buffer B (90% methanol, 0.25% acetic acid, pH 4.3). The gradient was from 100% A to 90% A:10% B within 8 min, at a flow rate of 1.5 ml · min^{-1}. Monobromobimane derivatives were detected fluorometrically (excitation 380 nm; emission 480 nm). By reference to mixed standards treated exactly as sample supernatants, three peaks at approx. 4, 5.4 and 7 min were identified as cysteine, γ-EC and GSH respectively. Within the range of the data shown, peak area was proportional to thiol concentration. Experiments in which known amounts of the three thiols were added at the leaf extraction stage gave recovery quotients of 88% (cysteine), 95% (γ-EC) and 94% (GSH).

Oxidised glutathione (GSSG) was determined in the same acid extract as total thiols, according to the following procedure, adapted from Griffith (1980). Sufficient amounts of 0.5 M Ches (pH 9.3) to reach pH 6.5–7.0 (approx. 70 μl) were added to 200 μl of the acid supernatant. Vinylpyridine (5 μl) was immediately added and the mixture left to stand at room temperature for 20 min. The mixture was then microfuged (5 min) and 100 μl supernatant taken for assay in the following medium: 100 mM NaH_2PO_4 (pH 7.5), 5 mM EDTA, 0.6 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.5 mM NADPH (final volume, 1 ml). The GSSG-dependent reduction of DTNB (25 °C) was initiated by addition of 5 units · ml^{-1} glutathione reductase (Boehringer, Meylan, France). The GSSG concentration was calculated by reference to GSSG standards treated in exactly the same way as the initial 200 μl supernatant (i.e. made up in extraction medium, neutralised with Ches, and incubated with vinylpyridine).

Measurement of enzyme activities. Glutathione synthetase (GS) and γ-glutamylcysteine synthetase (γ-ECS) were assayed from the same extract by methods adapted from those of Hell and Bergmann (1988, 1990). Leaf tissue (approx. 50 mg), taken unless stated from leaves between the seventh and tenth position from the apex, was ground in liquid N_2, extracted into 0.75 ml 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, and centrifuged 15 min at 10 000 *g* and 4 °C.

The activity of GS was assayed in 100 mM Tris (pH 8.4), 50 mM KCl, 20 mM $MgCl_2$, 1 mM γ-EC, 2 mM glycine, 5 mM ATP, 5 mM phosphoenolpyruvate, 5 mM dithioerythreitol, 10 units · ml^{-1} pyruvate kinase (30 °C). The reaction was started by addition of extract (130 μl, approx. 300 μg protein) to give a total volume of 500 μl. Standards contained 1 mM GSH instead of extract. For analysis, 25 μl of this mixture was taken and introduced into 200 μl 50 mM Ches (pH 8.4); 20 μl monobromobimane was immediately added and thiol derivatization allowed to proceed for 15 min at room temperature in the dark, before being stopped by addition of 800 μl 10% acetic acid. For extracts from plants transformed for GS, activities were calculated by subtraction of GSH formed within 5 min following the start of the reaction ('zero' sample) from GSH formed after 15 min. For untransformed plants and plants transformed for γ-ECS, the reaction times were 5 min ('zero' sample) and 125 min. Thiols were determined by HPLC, as described above for leaf thiol contents, and activities quantified by comparison of peaks with those given by aliquots taken from the standard solution.

For γ-ECS activity, the reaction was started by addition of extract (140 μl, taken from the Tris/$MgCl_2$/EDTA extract

described above) to give 500 µl assay mix containing 100 mM Hepes (pH 8.0), 50 mM $MgCl_2$, 20 mM glutamate, 1 mM cysteine, 5 mM ATP, 5 mM phosphoenolpyruvate, 5 mM dithioerythreitol, 10 units · ml^{-1} pyruvate kinase (30 °C). The standard solutions contained 1 mM γ-EC instead of extract. Derivatizations of thiols were carried out as described above for assay of GS. Assay times of γ-ECS were 20 min (γ-ECS transformants) or 60 min (untransformed plants and GS transformants). No loss of cysteine was observed within these times in the absence of extract; in the presence of extract, cysteine loss not atttributable to formation of γ-EC was approximately 10% (assay time 20 min), and up to 40% (assay time 60 min), of the initial concentration. In the latter case (i.e. assay of the endogenous poplar enzyme only), no γ-ECS activity was detectable. This was also the case for extracts subjected to ammonium sulphate precipitation, eluted from a Sephadex column, and assayed under anaerobic conditions in the absence of dithioerythreitol (Noctor et al. 1996). For extracts from the γ-ECS transformants, γ-ECS activities were comparable whether assayed as eluted precipitates under anaerobic conditions in the absence of dithioerythreitol or as crude supernatants of leaf extracts under aerobic conditions in its presence (data not shown).

Protein assay. Soluble leaf protein was measured in centrifuged extracts using the standard Biorad coomassie blue assay, after the method of Bradford (1976).

Results

Overexpression of γ-ECS. Hybrid poplar (*Populus tremula* × *P. alba*) was transformed with the bacterial gene encoding γ-ECS (*gshI*: Watanabe et al. 1986), using two different T-DNA constructs enabling constitutive expression in the cytosol (Fig. 2). In the first, p35S*gshI*, the *gshI* cDNA is under the control of the CaMV 35S promoter, whereas in the second, p70*gshI*, it is under the control of the CaMV 35S promoter with a double enhancer sequence (p70; Fig. 2). This second construct was used in an attempt to obtain the strongest possible expression of the *E. coli* γ-ECS in poplar. Following digestion of genomic DNA with *Sal*I and using an internal fragment of *gshI* as probe, DNA gel blot analysis revealed that one line transformed with the first construct, ggs28 (formerly gsh28; Noctor et al. 1996), contained one copy of the inserted gene (Fig. 2). Two lines produced using the second construct (ggs11 and ggs17) possess, respectively, two and at least four copies of the introduced gene (Fig. 2). For lines ggs11 and ggs17, DNA gel blot analysis following digestion of genomic DNA with *Xba*I confirmed the number of integration sites (data not shown). Data presented below show results of analyses of plants belonging to the above three lines (ggs28, ggs11, ggs17). It is important to note that, at all stages of development, these plants were phenotypically indistinguishable from untransformed control poplars (Foyer et al. 1995b).

The RNA gel blot analysis revealed that plants of lines ggs28 and ggs11 exhibited high levels of transcripts corresponding to the introduced *gshI* cDNA (Fig. 3, upper figure). Extracts of total RNA from plants of line ggs17 contained lower but, nevertheless, detectable transcript levels (Fig. 3, upper figure). All lanes were

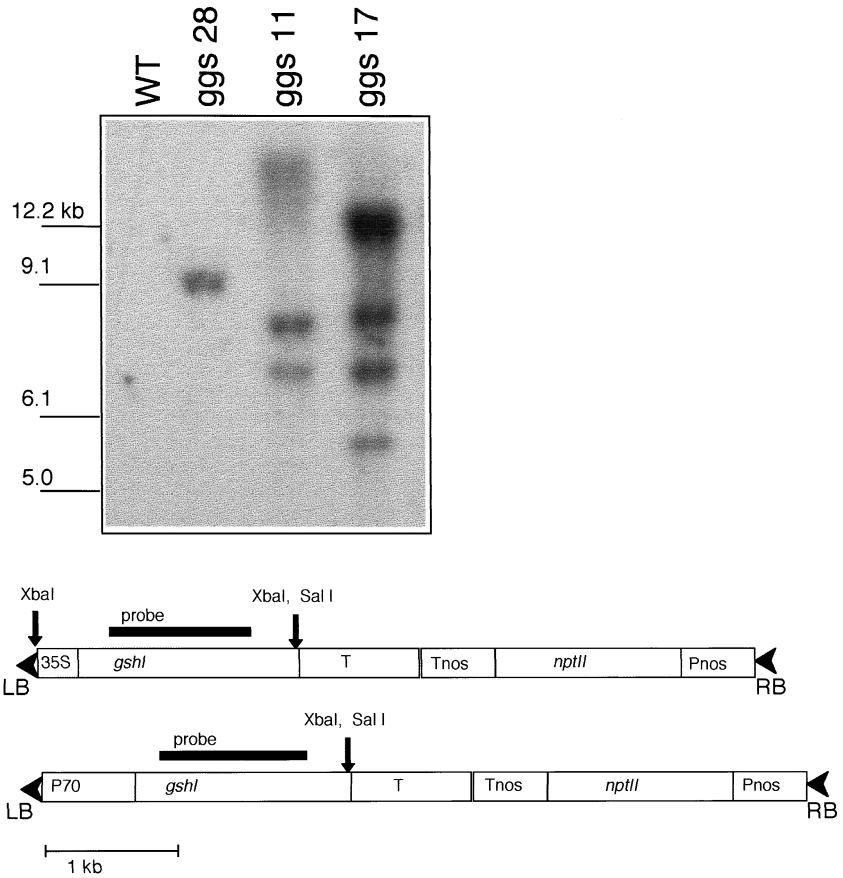


Fig. 2. DNA gel blot analysis and the T-DNA constructs used to transform poplars to express the *E. coli* gene for γ-ECS. DNA samples (5 µg) from untransformed (WT) and transformed (ggs28, ggs11, ggs17) poplars were digested with *Sal*I. The probe used corresponded to an internal 1.4-kb fragment of the *gshI* coding sequence

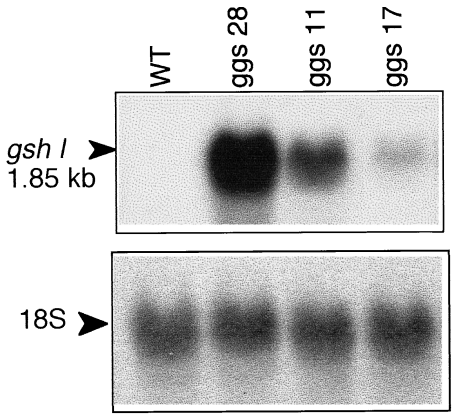


Fig. 3. RNA gel blot analysis of total leaf RNA from poplars transformed to express the *E. coli* gene for γ-ECS. Total RNA samples (30 μg) from untransformed (WT) and transformed (ggs28, ggs11, ggs17) poplars were loaded per lane. The probe used to detect γ-ECS mRNA corresponded to an internal 1.4-kb fragment of the *gshI* coding sequence (*upper figure*). The 18S probe used corresponded to a 0.5-kb fragment of a cDNA for the radish 18S rRNA (*lower figure*)

loaded with the same quantity of total RNA, as confirmed by the similar intensities of 18S rRNA bands on the blot (Fig. 3, lower figure).

Detection of E.coli γ-ECS and GS proteins in transformed poplars. To facilitate the screening of transformed plants, polyclonal antisera against *E. coli* γ-ECS and GS were produced. Histidine-tagged fusion γ-ECS or GS, produced by cloning of the γ-ECS and GS cDNAs into the vector pET14 and purification on a nickel affinity chromatography column, were injected into rabbit. The His-γ-ECS fusion protein was soluble and had high γ-ECS activity (data not shown). In contrast, the His-GS fusion protein was not soluble in native form, even following gradual dialysis. It was therefore purified in the denatured form.

Dilute solutions of both antisera were able to detect 30 ng of the respective purified fusion protein (Fig. 4A: His-γ-ECS; Fig. 4B: His-GS). No cross-reactions were observed between antisera raised against the *E. coli* γ-ECS and the purified *E. coli* GS (Fig. 4A: His-GS), or between antisera produced against the *E. coli* GS and the purified *E. coli* γ-ECS (Fig. 4B: His-γ-ECS). Immunoblotting of leaf extracts from untransformed poplars showed that these antibodies do not react with the poplar enzyme (Fig. 4A,B: WT). The absence of cross-reactivity is confirmed by the absence of reaction of either antibody with leaf extracts from poplar lines transformed for the other enzyme (Fig. 4A: gsh3; Fig. 4B: ggs28).

Leaf extracts from poplar lines which gave detectable levels of *E. coli gshI* transcripts (Fig. 3) showed a single defined band on the immunoblot, corresponding to the *E. coli* γ-ECS gene product (Fig. 4A). This band gives a calculated molecular weight for the protein of 58 kDa, in good agreement with the size predicted for the *E. coli* γ-ECS from the gene sequence (Watanabe et al. 1986). Lines ggs28 and ggs11 have considerably more γ-ECS

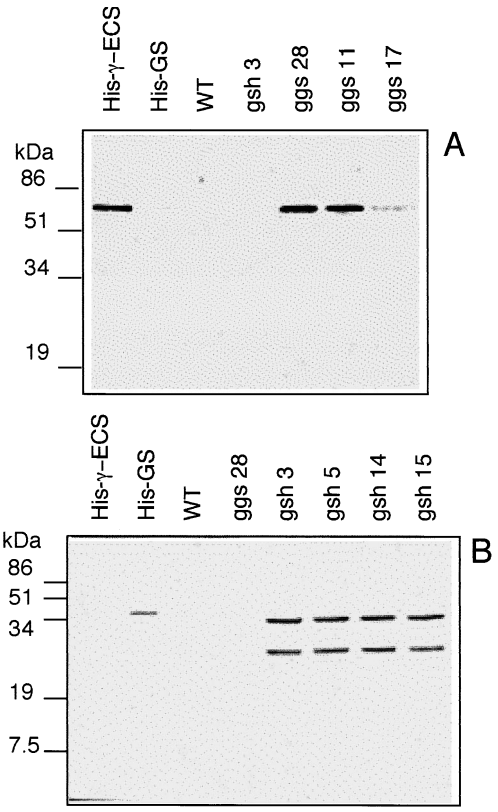


Fig. 4A,B. Immunoblot analyses of extracts of total soluble leaf protein from poplars transformed to overexpress the *E. coli* genes for γ-ECS and GS. Proteins were separated on gels of 12% (**A**) and 15% (**B**) acrylamide, transferred onto nitrocellulose membranes and incubated with antisera raised against the *E. coli* γ-ECS (**A**) or the *E. coli* GS (**B**). Lanes His-γ-*ECS* and His-GS: 30 ng purified fusion-proteins (the *E. coli* gene products fused with histidine tag sequences, allowing purification on a his-binding column). Other lanes correspond to poplars expressing the bacterial γ-ECS (ggs28, ggs11, ggs17) or the bacterial GS (gsh3, gsh5, gsh14, gsh15). For all plants, 20 μg total soluble leaf protein was loaded except ggs17, where 80 μg was loaded

protein than line ggs17, in agreement with their high transcript levels (Fig. 3). It should be noted that, in the blot shown in Fig. 4, four times as much soluble protein was loaded for ggs17 as for ggs28 and ggs11. Other blots, in which smaller amounts of protein were loaded for ggs11 and ggs28 extracts, showed that band intensity was proportional to the quantity of protein and that the band intensity resulting from 80 mg total soluble leaf protein from ggs17 was similar to that given by 6 mg protein from ggs11 (data not shown). It is noteworthy that the quantities of bacterial protein were comparable in leaves from lines ggs11 and ggs28 (Fig. 4), even though the latter possesses higher transcript levels (Fig. 3): this may reflect differences in transcript stability and/or turnover of the bacterial protein between these two plant types. Extractable enzyme activity was proportional to the amount of γ-ECS protein (see below).

Immunoblots of leaf extracts from poplars overexpressing GS (Foyer et al. 1995a) revealed two bands for all four lines analysed (Fig. 4B). The band of higher

Table 1. Activities of γ-ECS and GS in leaves from different poplar lines transformed to overexpress the bacterial genes coding for these enzymes. The data represent means ± SD of single determinations of three separate extractions of material taken from between the seventh and tenth leaf from the apex. Values followed by the same letter are significantly different at $P < 0.001$ (ANOVA, Tukey-Kramer test); n.d., not detected; n.D., not determined

Plant line	Enzyme overexpressed	Enzyme activity (nmol · (mg protein)$^{-1}$ · min^{-1})	
		γ-ECS	GS
Untransformed	none	n.d.	0.26 ± 0.02 a, b, c, d
gsh 3	GS	n.d.	75.0 ± 5.3 a, e, f, g
gsh 5	GS	n.D.	86.7 ± 7.7 b, h, i, j, k
gsh14	GS	n.D.	78.8 ± 1.9 c, l, m, n
gsh15	GS	n.D.	60.4 ± 8.5 d, h, o, p, q
ggs28	γ-ECS	7.37 ± 0.75 a	0.26 ± 0.05 e, l, o
ggs11	γ-ECS	8.42 ± 0.63 b	0.39 ± 0.03 f, j, m, p
ggs17	γ-ECS	0.47 ± 0.10 a, b	0.21 ± 0.01 g, i, k, n, q

molecular weight agrees well with the predicted molecular weight of the monomeric subunit of the *E. coli* GS (35.6 kDa: Gushima et al. 1984). The faster band corresponds to an apparent molecular weight of 26 kDa and was detected on the blot even for leaf samples extracted directly into denaturing buffer (data not shown). Immunoblots with diluted protein extracts demonstrated that the polypeptide of 26 kDa was present in lower quantities than that of 35.6 kDa (data not shown). Moreover, no bands of molecular weight greater than 5 kDa were detected on the blot (Fig. 4B). We conclude that the band at 26 kDa is a product of the introduced gene present in vivo and not a degradation product formed during the extraction procedure. It is noteworthy, nevertheless, that only one band was detectable on RNA gel blots of leaf extracts from these GS transformants (Foyer et al. 1995a).

Enzyme activities in the transformed lines. Table 1 shows that γ-ECS activity was detectable in leaf extracts of poplars transformed to overexpress γ-ECS (ggs28, ggs11, ggs17). Lines ggs28 and ggs11 exhibited extractable γ-ECS activities much higher than those detected in extracts from line ggs17. This is in agreement with the band intensities of the immunoblot of extracts from these lines (Fig. 4A). All ggs lines possessed GS activities similar to those of untransformed poplar (Table 1). The activity of γ-ECS in untransformed poplar is below the level of detection (Table 1). This prevents precise calculation of the extent of increase in extractable γ-ECS activity in the transformed poplars. Available data, where both γ-ECS and GS activities have been measured in the same tissue, show that these activities are often comparable (Hell and Bergmann 1990; Schneider and Bergmann 1995). Assuming this also to be the case for poplar leaves, and assuming the endogenous poplar activity to be absent from the γ-ECS assays of the ggs transformants, enhancements of γ-ECS activity due to transformation can be estimated as approximately 30-fold for lines ggs28 and ggs11 and about 3-fold for line ggs17 (Table 1: cf. γ-ECS and GS activities in the ggs transformants).

As previously reported (Foyer et al. 1995a; Strohm et al. 1995), all four lines of poplar overexpressing GS (gsh3, gsh5, gsh14, gsh15) exhibited very high extractable activities of this enzyme (Table 1). In these lines, the increase in extractable foliar GS activity relative to untransformed poplars was more than 200-fold (Table 1). As with extracts from untransformed poplars, no γ-ECS activity was detected in leaf extracts from these plants (Table 1; see γ-ECS activity for line gsh3). The high GS activities extractable from the lines overexpressing GS are in agreement with the intense bands on the immunoblot (Fig. 4B) and demonstrate that strong expression of this transgene has been maintained in these plants for over two years (cf. Foyer et al. 1995a).

Poplar lines overexpressing γ-ECS possessed total soluble leaf protein contents similar to those of untransformed plants [approx. 30 mg · (gFW)$^{-1}$; data not shown]. Activities of glutathione reductase were not significantly different between the different lines [50–70 nmol · (mg protein)$^{-1}$ · min^{-1}; data not shown].

Foliar thiol contents in poplars overexpressing γ-ECS. Figures 5 and 6 show the effect of overexpression of γ-ECS on foliar thiol contents in four different lines. It is important to note that all data shown in each figure were obtained using material sampled during the afternoon of the same day, from leaves between the seventh and tenth positions from the apex, from plants growing under identical conditions. Direct comparison of absolute thiol contents between Figs. 5 (measured in May) and 6 (measured in January) is precluded by seasonal variations in thiol contents. In both untransformed and transformed poplars, foliar contents of all thiols are up to twofold higher in summer than in winter. However, the relative increases brought about by γ-ECS overexpression remain the same throughout the year [cf. thiol contents in ggs28 and in untransformed poplars measured in May (Fig. 5) and in January (Fig. 6)]. As previously reported, leaves from line ggs28 were found to have 2–4 times more GSH and over 10 times more γ-EC than leaves from untransformed poplars (Noctor et al. 1996). Line ggs11, exhibiting similar amounts of γ-ECS protein (Fig. 4) and similar extractable γ-ECS activity (Table 1) to ggs28, also has markedly enhanced foliar γ-EC and GSH contents (Fig. 5). In contrast, foliar γ-EC and GSH contents in line ggs17, which has a γ-ECS activity enhanced only about threefold (Table 1), do not significantly differ from those of untransformed plants (Fig. 5). Figure 6 shows data for ggs5, a line recently obtained using the same construct as for ggs11 (35S promoter with double enhancer sequence). Line ggs5 has similar levels of bacterial transcripts and

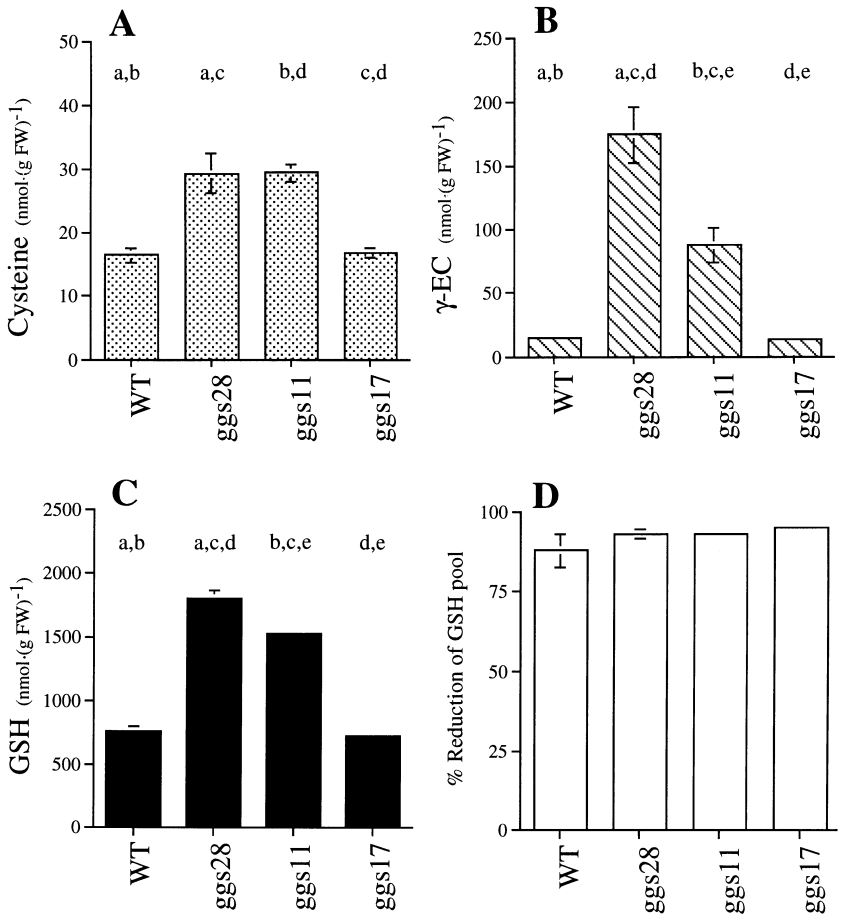


Fig. 5A–D. Foliar thiol contents of different plant lines overexpressing a bacterial gene coding for γ-ECS. The data represent means ± SD of single determinations of three separate extractions taken from leaves between the seventh and tenth position from the apex. Plants were growing in a greenhouse in full sunlight in May. Glutathione redox state is calculated assuming 1 GSSG = 2 GSH. The same letters above the columns indicate significant difference at $P < 0.001$ (ANOVA, Tukey-Kramer test). Similar results have been obtained in between 5 and 15 separate experiments

protein to ggs28 (data not shown). This is reflected in the similar extractable foliar γ-ECS activities and thiol contents (Fig. 6). The 18 separate extractions of foliar thiols in the three untransformed poplars shown in Fig. 6 gave values lying between 225 and 428 nmol · (g fresh weight)$^{-1}$. In ggs5 and ggs28, the lowest and highest values of 12 separate extractions were 890 and 1056 nmol · (g fresh weight)$^{-1}$. These increases in GSH content of between two- and four-fold in ggs28, ggs11 and ggs5 have been repeatedly verified over a period of 3 months (ggs5), 12 months (ggs11) and 30 months (ggs28). In the three lines strongly overexpressing γ-ECS, cysteine contents were up to 2 times greater than those of untransformed poplars (Fig. 5; Fig. 6, ggs5). This effect is, however, somewhat variable (see Fig. 6, ggs28 and Fig. 7b). Despite the enhanced foliar glutathione contents in ggs28, ggs11 and ggs5, no appreciable change in the reduction state of the glutathione pool was observed (Fig. 5; data not shown for ggs5). Equally, overexpression of γ-ECS had no effect on either the pool size or the redox state of foliar ascorbate in ggs28, ggs11 or ggs5 (data not shown).

Relationship between leaf age and foliar thiols/enzyme activities in the γ-ECS overexpressors. In order to characterise more fully the poplars overexpressing γ-ECS, and to explore the relationship between enzyme activities and foliar thiol contents, these were measured in leaves at differing positions. Figure 7A shows how the activities of γ-ECS and GS vary with leaf age in untransformed poplar and in poplar lines strongly (ggs28, ggs11) and weakly (ggs17) overexpressing γ-ECS. On a fresh-weight basis, the activity of the introduced γ-ECS enzyme in ggs28 and ggs11 was higher in upper leaves than in lower leaves (Fig. 7A). This difference is less marked if activity is calculated on a protein basis (data not shown), owing to the lower protein contents of the lower leaves (Fig. 7A). In the weak γ-ECS overexpressor ggs17, position-related changes in extractable γ-ECS activity were less apparent. In all plants, the endogenous GS activity was relatively constant with leaf position (Fig. 7A).

The thiol contents of the same leaves as those investigated for enzyme activities are shown in Fig. 7B. In all plants, both cysteine and GSH contents were highest in the upper leaves and dwindled in leaves towards the base of the stem. In untransformed plants and the weak γ-ECS overexpressor ggs17, γ-EC was very low or undetectable at all leaf positions (Fig. 7B). In contrast, γ-EC was present in substantial amounts in all leaves of poplars strongly overexpressing γ-ECS, the lowest quantities being detected in the uppermost and basal leaves (ggs28 and ggs11: Fig. 7B). It is worthy of note that, despite the position-related decrease in foliar GSH contents, the relative enhancement of GSH in the strong overexpressors ggs28 and ggs11 was constant with leaf age (2–3 times higher than in the corresponding leaf of untransformed poplar; Fig. 7B).

Poplar line	WT1	WT2	WT3	ggs5	ggs28
γ-ECS activity	n.d.	n.d.	n.d.	7.8 (1.0)	11.1 (1.6)
GS activity	0.39 (0.02)	0.45 (0.06)	0.48 (0.05)	0.28 (0.09)	0.51 (0.09)

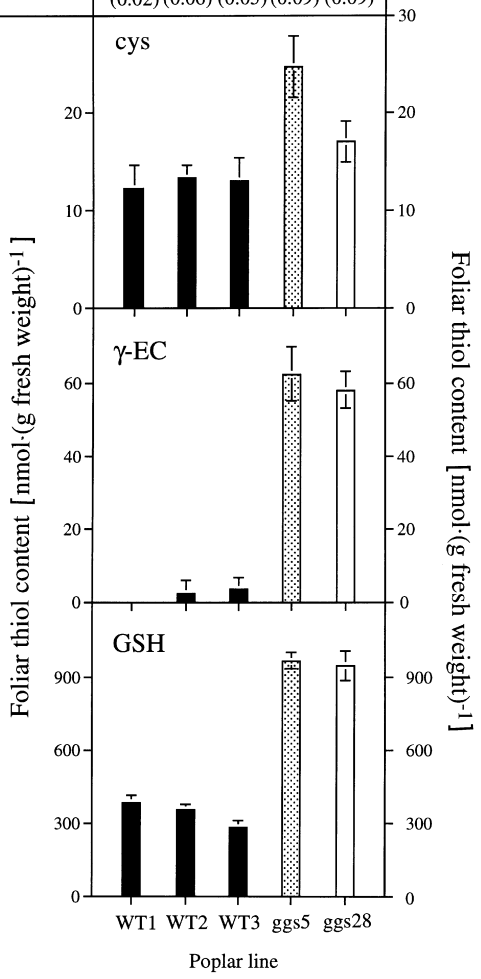
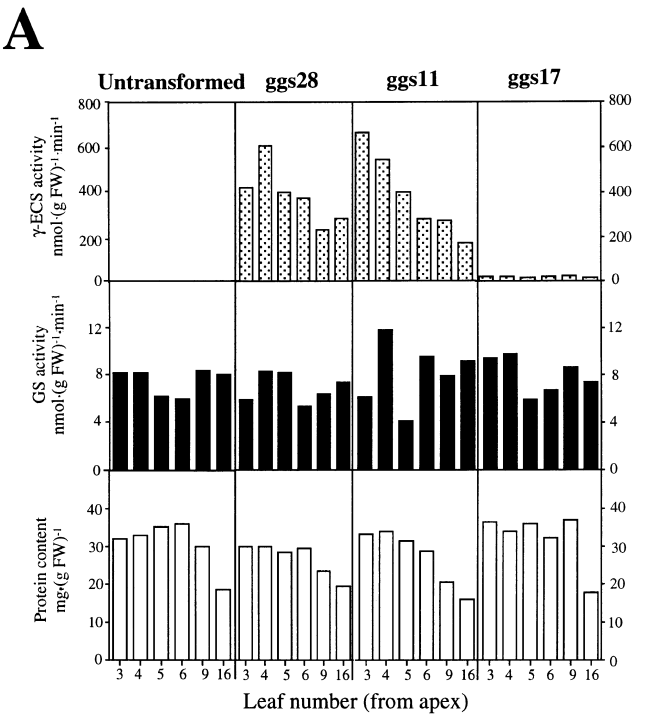


Fig. 6. Comparison of foliar thiol contents in three different untransformed poplar plants (WT1-3: *filled columns*) and a further poplar line expressing the bacterial γ-ECS in the poplar cytosol (ggs5: *dotted columns*). Extractable foliar activities of γ-ECS and GS [nmol · (mg protein)$^{-1}$ · min^{-1}] are shown above the graphs. Data for ggs28 (*open columns*) are shown for comparison. All leaf material was sampled between 2.00 pm and 3.00 pm on the same January day from plants growing in the greenhouse. All data for thiols are means ± SD of single analyses of six separate extractions. Data for enzyme activities are means of single analyses of three separate extractions; standard deviations are shown in parentheses beneath the means (*n.d.*, not detected)



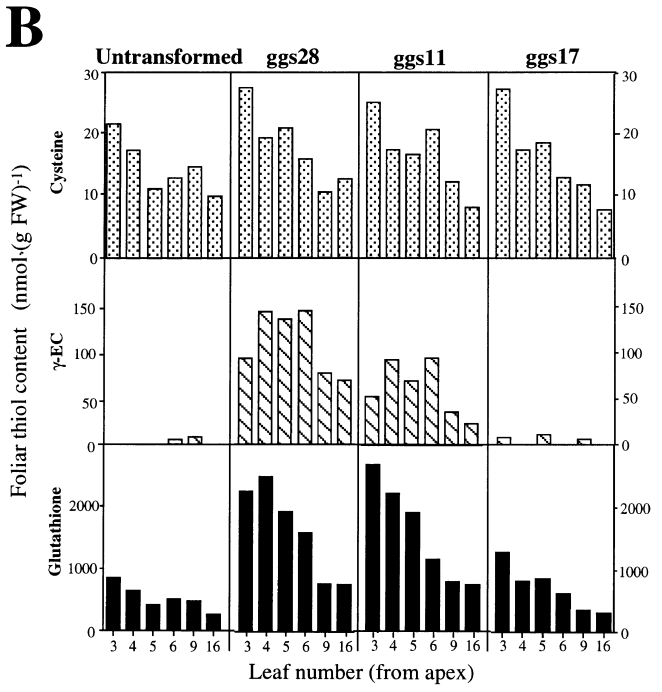


Fig. 7A,B. Vertical profiles of activities of γ-ECS and GS (**A**), protein contents (**A**) and shoot thiol contents (**B**) in poplars overexpressing γ-ECS. In each case, the leaf at a given position was divided lengthways. One half was used for determination of enzyme activities and protein contents while the second was used for analyses of thiols. Leaf numbers give the position from the youngest green leaf (position 1). Leaf 16 was the basal, or second basal, leaf on the shoot. Analytical procedures as described in *Materials and methods*

Discussion

Antisera produced against the *E. coli* γ-ECS and GS can be used to rapidly screen plants expressing the respective genes in poplar. Detected levels of bacterial γ-ECS protein are in good agreement with measured γ-ECS activities. Two lines produced from separate transformation procedures using similar but distinct constructs, ggs28 and ggs11 (Fig. 2), strongly express the introduced gene (Fig. 3), possess considerable amounts of bacterial protein (Fig. 4), and show markedly elevated extractable foliar activities of γ-ECS (Table 1). In consequence, the foliar γ-EC and GSH contents of these lines are significantly increased (Fig. 5). The similar increases in

extractable γ-ECS activity and foliar thiols in ggs5 (Fig. 6) confirm the importance of γ-ECS activity in determining foliar γ-EC and GSH contents. As observed previously, and in contrast to the increases in thiols provoked by overexpression of γ-ECS, poplars strongly overexpressing GS (Fig. 4, Table 1) possess foliar thiol contents no different from those of untransformed poplars (Foyer et al. 1995a; Strohm et al. 1995). Analogous results have been obtained by overexpression of the *E. coli* γ-ECS and GS genes in the tobacco chloroplast (Creissen et al. 1996). In these tobacco transformants, however, the increased GSH levels resulting from γ-ECS overexpression were accompanied by necrotic lesions and increased sensitivity to oxidative stress (Creissen et al. 1996). This is in contrast to the poplar γ-ECS transformants studied here, which show no evidence of deleterious effects (Foyer et al. 1995b). The difference between the poplar and tobacco transformants cannot be due to the intracellular localisation of the product of the introduced gene, since we have recently obtained poplar lines strongly overexpressing the *E. coli* γ-ECS in the poplar chloroplast. Like the lines overexpressing the enzyme in the cytosol, these chloroplastic lines show no negative effects of the transformation (not shown).

The data shown in Figs. 5–7 are measurements of thiol contents, rather than rates of synthesis. Hence, it is possible that the increased thiol contents in the poplars strongly overexpressing γ-ECS are caused not by an increased rate of synthesis, but rather by decreased rates of utilization, breakdown or export. Why this would be the case, however, is unclear. Alternatively, the increased γ-EC and GSH contents may reflect an increased rate of γ-EC synthesis in the transformants. In animal cells, the rate of synthesis of GSH is influenced by cysteine availability, the amount of γ-ECS enzyme and feedback control of γ-ECS by GSH (Sun et al. 1996). Several authors have considered that these factors are also likely to control the rate of GSH synthesis in leaves (Buwalda et al. 1988; Hell and Bergmann 1990; Rüegsegger and Brunold 1992; Farago and Brunold 1994; Schneider and Bergmann 1995). Since cysteine availability has previously been shown to limit GSH synthesis in leaf discs from untransformed poplar (Strohm et al. 1995), the increases in foliar cysteine content in lines ggs5, ggs11 and ggs28 (up to twofold; Figs. 5 and 6) may contribute to the increased synthesis of γ-EC and GSH. However, these increases in foliar cysteine are variable and sometimes absent (e.g. Fig. 7B), even though the augmentation of leaf γ-EC and GSH contents, relative to untransformed poplars, is constant. Since foliar glutamate contents are similar in transformed and untransformed poplars (Noctor et al. 1997b), and there exists, to our knowledge, scant literature data to support a limitation of GSH synthesis by ATP supply or by the phosphate potential, we conclude that the increase in γ-EC and GSH in the transformants is due to the presence of the bacterial γ-ECS.

Increased rates of γ-EC synthesis in the transformants may be due to differing kinetic characteristics of the introduced bacterial γ-ECS and the endogenous poplar enzyme. One possibility is that the former possesses higher affinities for cysteine and/or glutamate than the latter. Direct comparison of the two enzymes is precluded by the absence of detectable poplar γ-ECS activity. However, consideration of literature data shows that the affinities for cysteine (K_m) of γ-ECS from several sources are remarkably similar. These have been reported as 0.074 mM for the parsley enzyme (Schneider and Bergmann 1995), 0.19 mM for the tobacco enzyme (Hell and Bergmann 1990), 0.1 and 0.2 mM for the enzyme from two different strains of *E. coli* (Huang et al. 1988), and 0.3 mM for the enzyme from rat kidney (Huang et al. 1988). Assuming the K_m for cysteine of the poplar γ-ECS to be similar to those of the enzymes from tobacco and parsley, it seems unlikely that the increased synthesis of γ-EC and GSH in the transformed poplars results from a higher affinity of the bacterial enzyme for cysteine. Although measured K_m values of the *E. coli* γ-ECS for glutamate (0.7–1.7 mM; Huang et al. 1988) are somewhat lower than those reported for the enzyme from parsley (3.8 mM; Schneider and Bergmann 1995) and tobacco (10.4 mM; Hell and Bergmann 1990), the availability of glutamate has been shown not to be kinetically limiting for synthesis of GSH in poplar leaf discs (Noctor et al. 1996).

The increased γ-EC and GSH contents in the poplars strongly overexpressing γ-ECS might reflect a higher turnover number of the bacterial enzyme, which would increase the rate of cytosolic γ-EC synthesis, regardless of substrate concentrations. However, an increased rate of γ-EC synthesis would be expected even if the turnover rates of the two enzyme were identical, since strong expression of the bacterial enzyme leads to a marked increase of the number of catalytic sites. Both of these explanations invoke increased catalytic capacity for γ-EC synthesis in the poplars strongly expressing the bacterial enzyme. This augmented catalytic capacity leads to increased γ-EC (and thus increased GSH) in the cytosol of the transformants strongly overexpressing γ-ECS. Given that the substrate affinities of the bacterial enzyme are not markedly higher than those of the poplar enzyme (see above), two conclusions can be drawn. Firstly, the conversion of glutamate and cysteine to γ-EC is held out of equilibrium (i.e. regulated) in the cytosol of untransformed poplar. Secondly, one of the limiting factors responsible for this regulation is the activity of γ-ECS in this compartment. Hence, changes in the rate of de novo synthesis of endogenous γ-ECS would perhaps be one way in which foliar GSH content might be controlled. This last conclusion must, however, be drawn with some circumspection, since the regulatory properties of the poplar γ-ECS may differ from those of the bacterial enzyme. For instance, the latter may be less sensitive to feedback inhibition by GSH. Nevertheless, it is far than clear whether competitive feedback inhibition (Hell and Bergmann 1990; Schneider and Bergmann 1995) would be able to prevent an increase in reaction rate under conditions in which the number of catalytic sites is increased through augmentation of the amount of poplar γ-ECS (discussed in Noctor et al. 1997a). Indeed, several studies in other plant species have reported

elevated extractable activities of γ-ECS under circumstances where the rate of GSH synthesis is accelerated or the GSH content increased (Rüegsegger and Brunold 1992; Chen and Goldsborough 1994; Farago and Brunold 1994).

It is noteworthy that the leaf profiles of both untransformed poplars and those overexpressing γ-ECS showed a strong correlation between foliar contents of cysteine and GSH (Fig. 7B). In line ggs28, and particularly ggs11, leaf GSH content also correlated with the activity of the introduced γ-ECS (cf. Fig. 7A and Fig. 7B). Foliar GSH contents were shown to decline with leaf age in both pea (Bielawski and Joy 1986) and spinach (De Kok and Kuiper 1986). On a fresh-weight basis, the GSH and cysteine contents of young tobacco leaves were, respectively, almost fourfold and more than twofold higher than in old leaves (Herschbach and Rennenberg 1994). The lower cysteine contents of older, basal poplar leaves (Fig. 7B) may be linked to a decreased rate of transport of sulphate from the roots into leaves at these positions, as observed in older tobacco leaves (Herschbach and Rennenberg 1994). Declining foliar GSH levels would then reflect the decreased cysteine contents of older leaves, though in the case of ggs28 and ggs11, diminishing γ-ECS activities may also contribute.

The increases in cysteine in poplars strongly overexpressing γ-ECS are variable (cf. Figs. 5–7). Over a period of 14 months, we have carried out numerous measurements of foliar cysteine in lines ggs28 and ggs11: the cysteine content is either enhanced relative to untransformed poplars (by up twofold; Figs. 5, 6) or unchanged (Fig. 7B). Significantly, it is never decreased. This implies that the rate of cysteine synthesis may be accelerated in these poplars in order to meet the requirements of higher rates of γ-EC and GSH synthesis. Moreover, to support these higher rates, sufficient cysteine must be made available in the poplar cytosol, where the bacterial γ-ECS is located. Although the chloroplast is thought to be the major site of incorporation of inorganic sulfur into cysteine (Schwenn 1994), both serine acetyltransferase (Ruffet et al. 1995) and cysteine synthase (Lunn et al. 1990) have been detected in the leaf cytosol.

The production of poplar lines containing constitutively enhanced GSH levels will facilitate evaluation of the role of GSH in stress resistance. Preliminary data indicate that ggs28 poplars are no more resistant than untransformed poplars to either photoinhibition or methyl viologen exposure (data not shown). Perhaps this is due to overexpression of γ-ECS in the cytosol; as yet, it is not known whether the additional thiols synthesized in ggs28, ggs11 and ggs5 remain in the cytosol or are able to enter other subcellular compartments. Alternatively, it may reflect the importance of the glutathione redox state in protection against free radical damage. Despite the increase in the GSH content in lines ggs28, ggs11 and ggs5, neither the glutathione reductase activity (data not shown) nor the percentage reduction of the glutathione pool (Fig. 5) were appreciably different from untransformed poplars. In contrast, poplars overexpressing glutathione reductase possess a highly reduced glutathione pool (Foyer et al. 1995a). These plants exhibit greater resistance to photoinhibition than untransformed poplars and are able to maintain higher foliar ascorbate contents under conditions of oxidative stress (Foyer et al. 1995a). Nevertheless, absolute glutathione concentrations may be important in protection against other types of stress, such as exposure to heavy metals (Chen and Goldsborough 1994). Investigations of heavy-metal tolerance in these γ-ECS transformants, currently in progress, will allow this possibility to be examined.

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