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Over-expression of bacterial γ-glutamylcysteine synthetase (*GSH1*) in plastids affects photosynthesis, growth and sulphur metabolism in poplar (*Populus tremula* × *Populus alba*) dependent on the resulting γ-glutamylcysteine and glutathione levels

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ABSTRACT

We compared three transgenic poplar lines over-expressing the bacterial γ-glutamylcysteine synthetase (*GSH1*) targeted to plastids. Lines Lggs6 and Lggs12 have two copies, while line Lggs20 has three copies of the transgene. The three lines differ in their expression levels of the transgene and in the accumulation of γ-glutamylcysteine (γ-EC) and glutathione (GSH) in leaves, roots and phloem exudates. The lowest transgene expression level was observed in line Lggs6 which showed an increased growth, an enhanced rate of photosynthesis and a decreased excitation pressure (1-qP). The latter typically represents a lower reduction state of the plastoquinone pool, and thereby facilitates electron flow along the electron transport chain. Line Lggs12 showed the highest transgene expression level, highest γ-EC accumulation in leaves and highest GSH enrichment in phloem exudates and roots. This line also exhibited a reduced growth, and after a prolonged growth of 4.5 months, symptoms of leaf injury. Decreased maximum quantum yield (F_v/F_m) indicated down-regulation of photosystem II reaction centre (PSII RC), which correlates with decreased PSII RC protein D1 (PsbA) and diminished light-harvesting complex (Lhcb1). Potential effects of changes in chloroplastic and cytosolic GSH contents on photosynthesis, growth and the whole-plant sulphur nutrition are discussed for each line.

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Key-words: APS reductase; ATP sulphurylase; chlorophyll fluorescence; glutathione; long-distance transport; maximum quantum yield; phloem; photosystem II; sulphate assimilation; transgenic poplar.

Abbreviations: γ-EC, γ-glutamylcysteine; γ-ECS, γ-glutamylcysteine synthetase; GSH, glutathione (reduced state); *GSH1*, γ-glutamylcysteine synthetase gene; *GSH2*, glutathione synthetase gene; GSSG, glutathione (oxidized state).

INTRODUCTION

Glutathione (GSH) is an important component of the primary metabolism of plants. GSH is involved in various processes including storage and transport of reduced sulphur (Rennenberg, Schmitz & Bergmann 1979; Rennenberg 1984; Herschbach 2003), stress response to reactive oxygen (Polle & Rennenberg 1993; Foyer & Noctor 2005a,b) and heavy metals (Rauser 1995, 1999; Cobbett 2000a,b; Peuke & Rennenberg 2005a,b), as well as detoxification of xenobiotics (Rennenberg & Lamoureux 1990; Edwards & Dixon 2005). In addition, GSH is a key component to maintain the cellular redox state (Meyer & Hell 2005; Mullineaux & Rausch 2005). For example, under oxidative stress (Rouhier, Lemaire & Jacquot 2008) or during flower development (Xing, Lauri & Zachgo 2006), synthesis of various cellular proteins is under GSH-mediated redox control. The GSH-mediated redox control can occur via glutathionylation of proteins, and thus at the transcriptional and/or post-transcriptional level (Dietz 2008; Meyer 2008). GSH is synthesized in two ATP-dependent steps catalysed by consecutive action of γ-glutamylcysteine synthetase (γ-ECS), which forms γ-glutamylcysteine (γ-EC) from cysteine (Cys) and glutamate, and glutathione synthetase (GSHS), which adds glycine to the γ-EC.

Various transgenic lines with modifications in the sulphur metabolism have been generated, for example in the dicots *Arabidopsis thaliana*, *Nicotiana tabacum* and *Populus* sp., resulting in increased GSH contents in different tissues of various plants (Rennenberg *et al*. 2007). Depending on the over-expressed gene, GSH contents increased two- to sixfold, and two studies also reported a phenotype (Creissen *et al*. 1999; Matityahu *et al*. 2006). *Met25* encodes *O*-acetylhomoserine–*O*-acetylserine sulphydrylase, and catalyses the synthesis of Cys from the precursor *O*-acetylserine. Over-expression of *Met25* from *Saccharomyces cerevisiae* in tobacco targeted either to the cytosol or to plastids, revealed higher Cys and GSH contents in both sets of transgenic plants. These plants were taller and greener than wild-type (WT) tobacco (Matityahu *et al*. 2006). By contrast, over-expression of *O*-acetylserine (thiol) lyase from spinach (Saito *et al*. 1994), rice (Harada *et al*. 2001) or wheat (Youssefian *et al*. 2001) in tobacco did not result in such a phenotype. Because the increase in GSH was in the same range in all these transgenic tobacco lines, total GSH accumulation seems not responsible, causing the observed phenotype. Because *Met25* in yeast functions as *O*-acetylhomoserine sulphydrylase, this may cause a disturbance of Met synthesis in the transgenic tobacco plants, and consequently the observed phenotype.

In the other study Creissen *et al*. (1999) describe a phenotype resulting from over-expression of bacterial *γ-ECS* (*GSH1*) targeted to plastids. The transgenic tobacco plants showed chlorosis and necrosis in response to high light intensity, which paradoxically resulted from increased oxidative stress. The transgenic tobacco exhibited a shift in the redox state of the GSH and γ-EC pool to a more oxidized state, which was accompanied by enhanced H_2O_2 levels that either occurred from increased production or defect reactive oxygen species (ROS) scavenging (Creissen *et al*. 1999). The authors concluded that the low redox state disturbed redox-sensing processes in the chloroplasts. Interestingly, this phenotype has not been observed in young poplar over-expressing the same bacterial *GSH1* gene targeted to chloroplasts (Noctor *et al*. 1998), even though the increase in GSH was within the same range. Creissen *et al*. (2000) concluded that these differences could be caused by different growth habits and ecological niches of these species. As symptoms of leaf injury like in tobacco (Creissen *et al*. 1999) were not visible in 4.5-month-old poplar plants that over-expressed the *GSH1* gene targeted to the cytosol (Herschbach, Jouanin & Rennenberg 1998), it is assumed that the subcellular targeting of the GSH1 protein is of importance. The GSH pool size in chloroplasts seemed to be unaffected in leaves of transgenic poplar that over-expressed *GSH1* in the cytosol, but was larger when the GSH1 protein was targeted to plastids (Hartmann 2002). The latter may be the consequence of the low GSH transport capacity from the chloroplast into the cytosol (Hartmann *et al*. 2003).

Transgenic tobacco that targeted the over-expressed bacterial *GSH1* to plastids showed a decreased rate of photosynthetic CO_2 uptake, a pattern that did not occur when bacterial GSHS (in bacteria encoded by the *GSH2* gene) was over-expressed. This decrease in CO_2 assimilation was prevented when *GSH2* was co-expressed with *GSH1* (Creissen *et al*. 1999). By contrast, no changes in net CO_2 assimilation were observed in transgenic poplar over-expressing *GSH2* targeted to the cytosol (Foyer *et al*. 1995).

Therefore, we hypothesized that in the perennial poplar GSH levels in the chloroplast rather than bulk leaf GSH concentrations, affect photosynthesis, growth and sulphur metabolism, and that the effects can be detected prior to any visible symptom of leaf injury. We chose three different lines of transgenic grey poplar, Lggs6, Lggs12 and Lggs20 (Noctor *et al*. 1998), to test our hypothesis. All three lines over-express bacterial *γ-ECS* (*GSH1*) targeted to plastids and have multiple copies of the transgene (two copies in the lines Lggs6 and Lggs12, and three copies in line Lggs20; Arisi 1997). The insertion site of the *GSH1* copies is not known. Using these three lines, we were able to assess the effects of *GSH1* over-expression in plastids: (1) on growth parameters; (2) on parameters of photosynthesis; (3) on foliar sulphur metabolism; and (4) on the sulphur metabolism of the whole plant.

MATERIALS AND METHODS

Plant material

The hybrid poplar trees (*Populus tremula* × *Populus alba*) expressing the *Escherichia coli* gene for *γ-ECS* (*GSH1*) under the control of CaMV 35S promoter, and targeting the protein to the plastids because of the rbcS transit peptide used in this study were described and characterized by Noctor *et al*. (1998; lines Lggs6, Lggs20, Lggs12). All transformed lines and WT poplar were micropropagated *in vitro* (Strohm *et al*. 1995). After 4 weeks, cuttings were transferred into pots (10 cm height, length and width) containing a soil mixture of one part silica, sand particle size 0.06–0.2 mm, one part sterile commercial soil and one part perlite, and fertilized as described previously (Herschbach *et al*. 1998). The plants were grown in a greenhouse under natural light conditions. Metal halide lamps were used as additional light source in the morning and in the evening to provide an extended photoperiod of 16 h (long-day conditions). Light intensity thus typically varied from 60 to 600 μmol m^{-2} s^{-1} depending on weather conditions. The plants were harvested and analysed after 8–10 weeks of growth. Two subsets of poplar plants were transferred into larger pots with 19 cm diameter and 16 cm height after 10–12 weeks, and were grown further in the greenhouse for approx. 4.5 months.

Collection of phloem exudates, leaves and roots

Phloem exudates were collected from stem sections of the apex, the basipetal stem and lateral roots of 8-week-old plants, as well as from five trunk sections and lateral roots of 4.5-month-old trees. Phloem exudation was performed as described by Herschbach *et al*. (1998, 2000). Young mature

leaves of 8-week-old plants were collected at a developmental stage of *c*. 70% expansion (i.e. the 9th–11th leaf from the apex). The entire trunk of 4.5-month-old poplars was dissected into five equally long sections. The lowest trunk section (section 5) represented the oldest leaves that sometimes carried symptoms of senescence. The following two sections (sections 4 & 3) contained old mature leaves, and section 2 contained mature leaves. The highest trunk section (section 1; up to the shoot apex) contained young mature (*c*. 70% expanded) and developing leaves.

The root systems of both 8-week- and 4.5-month-old trees were washed in water to remove sand, soil and perlite particles. The root system was divided into lateral roots and fine roots (*c*. 0.5 mm diameter). All samples were frozen in liquid nitrogen and stored at –80 °C until analysis, except for *in vitro* assays of APS reductase, ATP sulphurylase, *O*-acetylserine (thiol) lyase and sulphite reductase, for which the activities were measured immediately in freshly sampled material.

Analysis of sulphur compounds and carbohydrates in phloem exudates

Thiols were analysed as previously described by Herschbach *et al*. (1998). Sulphate was analysed by ion chromatography as reported by Herschbach *et al*. (2000). Total sucrose and other sugars were determined as anthrone-positive compounds (Schneider *et al*. 1996). For this procedure, 50 μL aliquots of phloem exudates were diluted 1:4 with Millipore (Billerica, MA, USA) water, and then 1 mL anthrone reagent [50 mg anthrone and 1 g thiourea in 100 mL 68.4% (v/v) H_2SO_4] was added. This mixture was boiled for 15 min. The samples were cooled to room temperature, and the extinction at 620 nm was determined. Carbohydrates were quantified with standard solutions containing 100–500 μM sucrose.

Analysis of sulphur compounds, carbohydrates and chlorophyll in tissues

Frozen leaves and roots were homogenized in liquid nitrogen, and thiols were extracted, derivatized and quantified as described by Strohm *et al*. (1995) and Herschbach *et al*. (2000). Sulphate in leaves and roots was determined as reported in Herschbach *et al*. (2000). Soluble carbohydrates were determined in young mature leaves and roots either with a commercial standard kit (Boehringer Mannheim, Mannheim, Germany) in 8-week-old poplar or as anthrone-positive compounds in 4.5-month-old poplar. For the determination of anthrone-positive compounds, soluble carbohydrates were extracted 1:10 (w/v) in Millipore water. An aliquot of 50 μL was subjected to the procedure described above. For determination with commercial standard kits after manufacturer's instruction, soluble sugars were extracted from approximately 300 mg powdered leaf or 500 mg powdered root material in 9.5 mL Millipore water. Proteins were precipitated by adding 500 μL 85 mM $K_4[Fe(CN)_6]$/250 mM $ZnSO_4$, and the pH was subsequently adjusted to 7.5–8.5 with NaOH. After filtration and centrifugation, sucrose, glucose and fructose were detected in the supernatant and quantified with a standard test kit according to the manufacturer's instructions.

Determination of enzyme activities

For measurements of ATP sulphurylase, sulphite reductase and *O*-acetylserine (thiol) lyase, the leaves and roots were extracted 1:10 (w/v) in ice-cooled extraction buffer as described by Hartmann *et al*. (2000). For SAT activity measurements, extracts were passed through a Sephadex G-25 M column (PD-10; Pharmacia, Freiburg, Germany), and subsequently concentrated by ultrafiltration with Vivispin 20 concentrators (Vivascience, Lincoln, UK). *In vitro* ATP sulphurylase (ATPSase; EC 2.7.7.4) activity was determined luminometrically, by measuring the ATP production from APS and inorganic PP_i (reverse reaction) according to Schmutz & Brunold (1982). A 5 μL aliquot of the leaf extract or 10 μL of the root extract was used to determine the activity of this enzyme according to Hartmann *et al*. (2000).

In vitro sulphite reductase (SiRase; EC 1.8.7.1), serine acetyltransferase (SATase, EC 2.3.1.30) and *O*-acetylserine (thiol) lyase (OASase; EC 4.2.99.8) activities were determined as described by Hartmann *et al*. (2000). *In vitro* APS reductase (APR; EC 1.8.99.-) activity was determined from the formation of [^{35}S]sulphite from [^{35}S]APS in the presence of DTE according to Brunold & Suter (1990). Leaves and roots were extracted 1:50 (w/v) in a Tris/HCl buffer (Hartmann *et al*. 2000) either in the presence of 0.5 mM AMP and 1% (w/v) Tween 80 (Hartmann *et al*). The enzyme assay contained 50 or 40 μL 1:2 diluted leaf or root extract, respectively. Protein contents of leaf and root extracts were measured in enzyme extracts according to Bradford (1976).

Isolation of total RNA and Northern blotting

Frozen leaves and roots were homogenized in liquid nitrogen, and total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Electrophoresis of 5 μg RNA was performed on 1% formaldehyde agarose gels at 120 V. RNA was transferred onto Hybond-N nylon membranes (Amersham, Freiburg, Germany), and hybridized with ^{32}P-labelled cDNA probes for ATP sulphurylase from *Arabidopsis thaliana* (GenBank Accession Number U05218), APS reductase (AY353039) and tubulin (AY353093) from poplar, and for *GSH1* from *E. coli*. The membranes were washed three times at different concentrations of SSC in 0.1% SDS for 20 min, the final washing step being 1 × SSC, 0.1% SDS at 65 °C, and exposed to an X-ray film (Kodak BioMax MS, Integra Biosciences, Fernwald, Germany) at –80 °C for 3 d. The autoradiograms were quantified with a densitometer GS-670 (Bio-Rad, Munich, Germany) using the software 'Molecular Analyst' and normalized to tubulin expression. The APS reductase and tubulin cDNA probes were obtained by RT-PCR

amplification of total RNA from poplar leaves with oligonucleotide primers derived from conserved domains. The *GSH1* from *E. coli* was amplified from genomic DNA with primers derived from known sequence of the *GSH1* gene (Watanabe *et al*. 1986).

Chlorophyll fluorescence and CO_2 assimilation measurements

Five plants were selected from each transgenic line and from WT poplar. One leaf (11th or 12th leaf from shoot apex) was used for chlorophyll fluorescence measurements at pre-dawn, midday and at the beginning of the dark period (07:00, 13:00 and 19:00 h, respectively). Chlorophyll fluorescence was measured with a pulse-amplitude-modulated fluorometer (Mini-PAM, Walz, Effeltrich, Germany) with attached leaf clip holder. Photosynthetic photon flux density during the measurements was obtained from the built-in quantum sensor of the leaf clip holder which was calibrated against a Li-Cor Li-185B quantum sensor (Li-Cor, Lincoln, NE, USA). Maximum quantum yield of PSII (F_v/F_m) was measured at pre-dawn and during the day after 30 min of dark adaptation. F_m' and F_t (the maximum and the transient flourescence of a light-adapted sample, respectively) were measured at noon at 850–900 μmol photons m^{-2} s^{-1}, using the built-in actinic light source of the Mini-Pam. F_m', F_t and pre-dawn F_m were used to calculate the fraction of absorbed light that was utilized in photochemistry ($\Phi_{PSII} = 1 - F_t/F_m'$) or that was dissipated thermally via regulated non-photochemical quenching ($\Phi_{NPQ} = F_s/F_m' - F_s/F_m$) and via non-regulated fluorescence and constitutive thermal processes ($\Phi_{f,D} = F_t/F_m$) according to Hendrickson, Furbank & Chow (2004). F_0' was calculated according to Oxborough & Baker (1997). The excitation pressure on PSII, 1-qP, as a measure of the redox state of the Q_A pool was calculated according to Huner, Öquist & Sarhan (1998) as $1 - [(F_m' - F_t)/(F_m' - F_0')]$.

The rate of CO_2 assimilation was determined using an LCA-4 gas exchange measurement system attached with the 'broadleaf' cuvette (ADC, Hoddesdon, UK). In parallel to the chlorophyll fluorescence measurements, the rate of photosynthetic gas exchange was determined at 10:00 and 16:00 h on 2 d for the transgenic lines Lggs6, Lggs12, Lggs20 and WT poplar. Leaves were allowed to equilibrate in the cuvette until steady-state photosynthesis was attained, indicated by a stable signal of the rate of CO_2 exchange. During measurements, the 'broadleaf' cuvette was kept at constant temperature which was comparable to the growth temperature in the greenhouse (19 ± 2 °C during the day).

Protein extraction, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

Frozen leaves were ground to a fine powder in liquid nitrogen. The proteins were extracted and separated as described by Busch, Hüner & Ensminger (2007). Following separation, proteins were transferred to a nitrocellulose membrane (0.2 mm pore size, Bio-Rad, Hercules, CA, USA) and probed with antibodies against PsbA, Lhcb1 and RbcL (Agrisera, Vännäs, Sweden), as well as against PsaA/B. The proteins were then detected and quantified according to Busch *et al*. (2007). Statistical analyses were done with the program SPSS (SPSS for Windows, Release 10, Chicago, IL, USA).

Data analysis

Differences between the accessions (WT and the three transformed lines) in chlorophyll fluorescence, photosynthetic gas exchange, transpiration and stomatal conductance were assessed by using five individual plants per accession per day and at each measuring time-point. Significant differences were estimated by analysis of variance (ANOVA) multifactorial analyses using the statistics package of Origin 6.1 (Northampton, MA, USA).

Differences in metabolite levels and enzyme activities between the WT and transgenic lines were assessed using 4–12 poplar trees per line. Significant differences were estimated by Student's *t*-test or Duncan's multifactorial test using the statistics program SPSS (SPSS for Windows, Release 10).

RESULTS

Transgene expression

In order to correlate growth and morphological changes with the expression of the transgene (bacterial *GSH1* coding for γ-ECS), we prepared RNA from mature leaves and fine roots of 8-week-old poplars, as well as from leaves of section 2 (mature leaves), section 5 (oldest leaves) and from fine roots of 4.5-month-old trees (Fig. 1). Northern blot analysis revealed the absence of any bacterial *GSH1* transcript in leaves and roots of WT plants (Fig. 1). Expression of bacterial *GSH1* in leaves of 8-week-old poplar increased from line Lggs6, to Lggs20 and line Lggs12. The same pattern was found in 4.5-month-old poplar trees. The *GSH1* mRNA levels were similar in leaves from sections 2 and 5 (i.e. mature and oldest leaves). This demonstrates that over-expression of *GSH1* was stable in leaves at least over a period of 4.5 months of growth.

The detected levels of the bacterial *GSH1* transcript were also different in fine roots of the investigated transgenic lines. Whereas line Lggs6 revealed no bacterial *GSH1* transcript in roots of 8-week-old plants, it was detected in line Lggs20 and at a higher level in line Lggs12. In fine roots of 4.5-month-old poplar, this pattern was reversed with the bacterial *GSH1* transcript decreasing from line Lggs6 to Lggs20 to line Lggs12 (Fig. 1).

Growth characteristics

After 8 weeks of growth in the greenhouse, there were no discernable symptoms of any insufficiencies in the

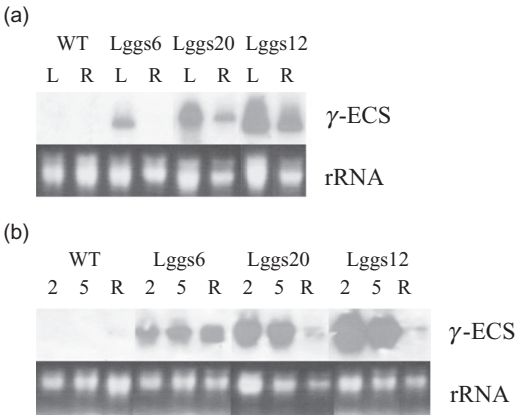

Figure 1. *GSH1* transcript levels of 8-week- and 4.5-month-old wild-type (WT) and transgenic poplar trees. Total RNA was isolated from the 10th leaf (L) from the apex and from fine roots (R) of 8-week-old poplar (a), as well as from leaves of section 2 (2) and 5 (5), and from fine roots (R) of 4.5-month-old poplars (b). The RNA (5 μg) was separated on 1% agarose in the presence of formaldehyde, blotted onto Hybond-N nylon membrane and hybridized with ^{32}P-labelled *GSH1* DNA fragment. Ethidium bromide-stained gel is shown as control of RNA loading.

transgenic lines over-expressing bacterial *GSH1* targeted to the plastid (Fig. 2). However, growth performance in a large number of individual replicates of the transgenic lines was altered compared to the WT. This was indicated by changes in biometric characteristics (Fig. 3). Tree height and the number of leaves of lines Lggs6 and Lggs20 were significantly increased compared to WT poplar (Fig. 3 and Supporting Information Table S1). In contrast, the transgenic line Lggs12, with the highest expression level of the bacterial *GSH1* (Fig. 1), revealed a decreasing number of leaves (Supporting Information Table S1). In addition, shoot fresh weight was lower in Lggs12 than in WT poplar (Fig. 3), and the shoot-to-root ratio was decreased in Lggs12 and Lggs20 (Supporting Information Table S1).

The phenotype we observed in line Lggs12 after 8 weeks was even more distinctive after a period of 4.5 months (Fig. 3). All biometric parameters (i.e. shoot height, number of leaves, shoot fresh weight and root fresh weight) were diminished, and the shoot-to-root ratio was increased in the transgenic line Lggs12 compared to WT plants (Supporting Information Table S2). More importantly, old mature leaves of line Lggs12 showed symptoms of leaf necrosis, premature leaf senescence and leaf fall (Fig. 2). These results were confirmed in a second experiment in which old mature leaves from line Lggs20 also revealed symptoms of leaf senescence. The increased growth of line Lggs20 observed after 8 weeks was no longer apparent after 4.5 months of growth (Fig. 2, Supporting Information Table S2). Moreover, after 4.5 months of growth, tree height, stem diameter and root fresh weight of line Lggs20 were decreased, whereas shoot fresh weight remained unaffected. Phenotypic and biometric parameters of line Lggs6 (which had the lowest expression level of the transgene) were not different from the WT poplar after 4.5 months of growth (Supporting Information Table S2).

Sulphur compounds in leaves and roots

Over-expression of bacterial *GSH1* targeted to the plastid resulted in higher Cys, γ-EC and GSH contents in leaves of 8-week-old transgenic poplar plants of lines Lggs6 and

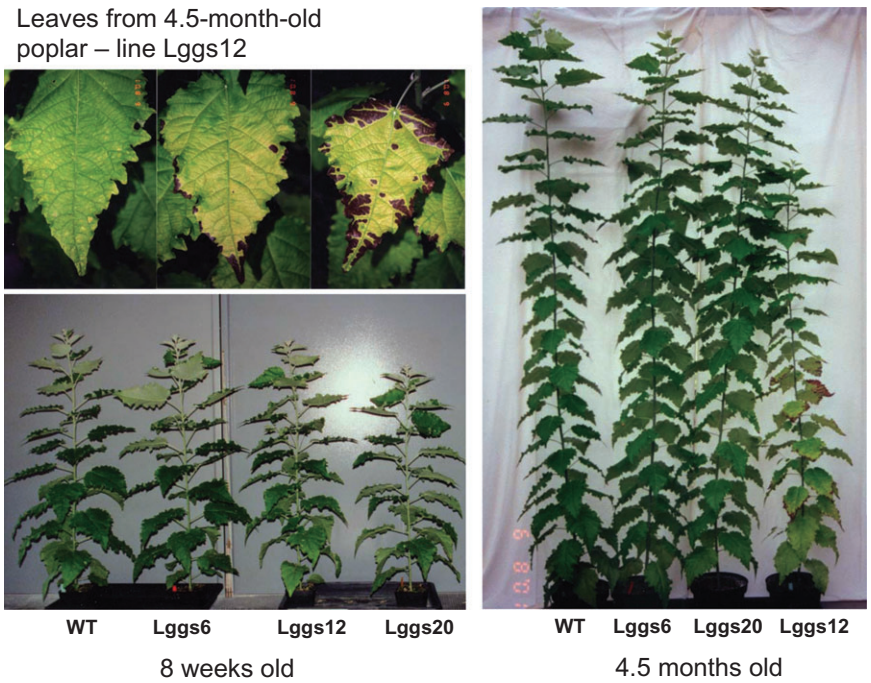

Figure 2. Phenotypes of 8-week- and 4.5-month-old poplar trees. Poplar lines: wild type (WT), transgenic poplar lines Lggs6, Lggs12 and Lggs20 as indicated.

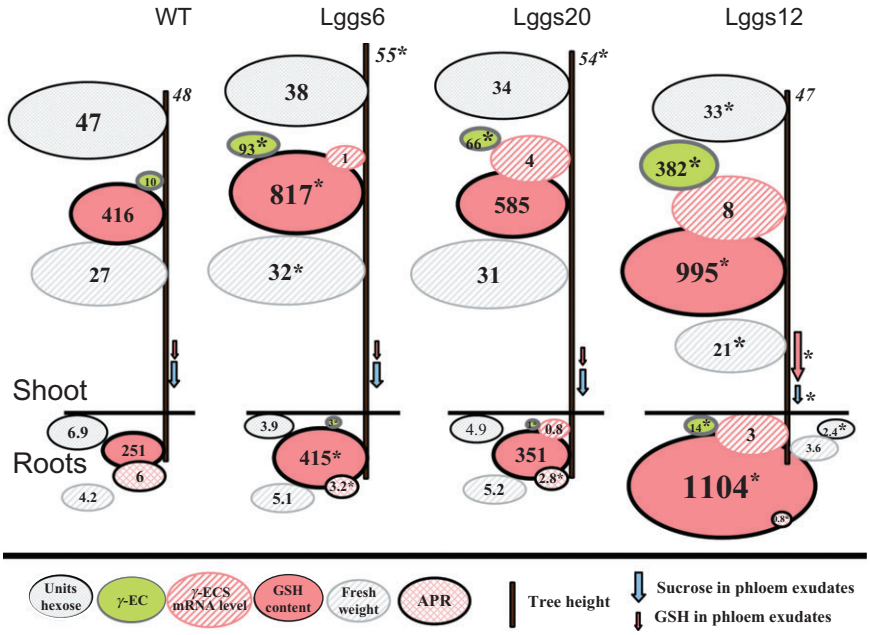

Figure 3. Overview of growth, metabolite contents, APS reductase activity and bacterial *GSH1* expression of 8-week-old wild-type (WT) and transgenic poplar plants over-expressing *GSH1* targeted to plastid. WT and transgenic poplar lines, Lggs6, Lggs12 and Lggs20 were grown under long-day conditions in a greenhouse for 8 weeks. Mean values of tree height (cm), as well as shoot and root fresh weight (g), are calculated from at least 44 plants. Thiols ($n = 6$, nmol g^{-1} tissue FW), and hexose units ($n = 12$; nmol g^{-1} tissue FW) contents, APS reductase activity ($n = 6$; nmol $^{35}SO_2$ g^{-1} tissue FW min^{-1}) and *GSH1* expression (relative units) were determined in *c*. 70% expanded leaves (the 10th leaf from the apex, young mature) and fine roots. Phloem exudates were sampled from bark slices of apical stem sections, basipetal stem sections and lateral roots. Therein, sucrose (nmol g^{-1} bark FW) and thiols (nmol μmol^{-1} sucrose) were determined. Data given are mean values without the calculated standard deviation. The latter is given in Table 1 as supplemental material. Asterisk (*) denotes significant differences to WT poplar at $P < 0.05$, according to Student's *t*-test.

Lggs12 (Fig. 3; Supporting Information Table S1). Leaves of line Lggs12, which had the highest expression level of the transgene, showed the highest amount of thiols, with a 36-fold increase in γ-EC (Fig. 3; Supporting Information Table S1) and the highest foliar γ-ECS activity (Noctor *et al.* 1998). Cys and GSH in leaves of line Lggs20 were slightly increased, although this increase was statistically not significant. There was also a considerable increase in γ-EC (sixfold), while sulphate concentrations in leaves of 8-week-old plants remained unaffected in all transgenic lines (Supporting Information Table S1). A similar pattern was revealed by 4.5-month-old poplars. Highest γ-EC and GSH contents were found in leaves of line Lggs12. However, after prolonged growth, concentrations of both thiols were also increased in leaves of line Lggs20 (for details, see Supporting Information Fig. S1).

Over-expression of *GSH1* targeted to plastids effected thiol levels not only in leaves, but also in fine roots (Fig. 3; Supporting Information Table S1). For example, γ-EC was increased in fine roots of 8-week-old poplars of each transgenic line. In line Lggs12, the γ-EC content was almost 460-fold higher than in WT poplar roots. GSH contents increased in fine roots of lines Lggs12 and Lggs6 with the highest increase in GSH compared to the WT (a 4.4-fold increase) in Lggs12. The GSH content of roots was about 50% lower compared to leaves of WT poplar and of lines Lggs6 and Lggs20. In contrast, similar GSH concentrations were observed in leaves and roots of line Lggs12 (Fig. 3; Supporting Information Table S1). In fine roots of 4.5-month-old Lggs12, neither γ-EC nor GSH was enhanced compared to the WT (Supporting Information Fig. S1).

Sugar compounds in leaves and roots

Soluble sugars were analysed to get an idea about the nutritional state of the plant regarding photosynthesis and plant growth. A small, but not significant, decrease in soluble carbohydrates (glucose, fructose and sucrose) was observed in leaves from 8-week-old transgenic poplar lines Lggs12 and Lggs20 (Supporting Information Table S1). When the pool of glucose, fructose and sucrose was converted to hexose equivalents, this amount was significantly reduced in line Lggs12 (Fig. 3). The effect was even more noticeable after 4.5 months of growth for lines Lggs12 and Lggs20. In leaves of both lines, soluble carbohydrate contents were significantly decreased (Supporting Information Fig. S1). However, in line Lggs20, this decrease of soluble carbohydrates occurred only in mature leaves, but not in young developing and young mature leaves. In fine roots, glucose and sucrose contents decreased in all transgenic poplar lines after 8 weeks of growth, although this was significant only in the transgenic line Lggs12 (Supporting Information

Table S1). There was no decrease in sugars in fine roots of 4.5-month-old transgenic poplars (Supporting Information Fig. S1).

Sulphur compounds in phloem exudates

In 8-week-old plants, phloem exudates were collected from the apex, the basal trunk section and from lateral roots. Compared to WT poplar, Cys, γ-EC (data not shown) and GSH in phloem exudates increased only in line Lggs12 (Fig. 3; Supporting Information Table S1). Anion analysis also revealed an enhanced sulphate concentration (Fig. 3; Supporting Information Table S1). GSH concentration in phloem exudates of 8-week-old poplars increased from apical stem sections to the roots (Fig. 3; Supporting Information Table S1). The opposite pattern was observed in 4.5-month-old plants (for details, see Supporting Information Fig. S2), where γ-EC and GSH contents decreased from apical stem sections towards lower sections in lines Lggs12 and Lggs20. However, in phloem exudates of lateral roots of lines Lggs12 and Lggs20, GSH contents were increased again.

Carbohydrates in phloem exudates

Sucrose contents in phloem exudates of 8-week-old transgenic poplars were not significantly different from WT. A slight increase was observed in phloem exudates of the transgenic poplar line Lggs6 and the contrary pattern in line Lggs12 (Supporting Information Table S1). In 4.5-month-old plants of line Lggs12, the concentration of sucrose in phloem exudates was significantly decreased (Supporting Information Fig. S2).

In vitro activities of enzymes involved in sulphate assimilation

In order to assess whether the increased demand for reduced sulphur for the enhanced GSH synthesis affects sulphate assimilation, enzymes involved in this pathway were analysed in young mature leaves (approximately 70% expanded) of 8-week-old trees. Neither the activities of the key enzymes ATP sulphurylase or APS reductase nor any other enzyme of the sulphate assimilation pathway [i.e. sulphite reductase, *O*-acetylserine (thiol) lyase and serine acetyltransferase] were affected by over-expression of *GSH1* in the plastid in the three transgenic lines (data not shown). This was paralleled by mRNA levels of ATP sulphurylase and APS reductase mRNA, which also remained unaffected (data not shown).

Compared to leaves, in fine roots of the WT, ATP sulphurylase and APS reductase activities were only 6 and 9%, respectively. In transgenic poplar lines, ATP sulphurylase and APS reductase activities in fine roots were also decreased compared to leaf activities. However, this down-regulation in enzymatic activities occurred to a larger degree than in the WT (Fig. 4, Supporting Information Table S1). All other enzymes (i.e. sulphite reductase, *O*-acetylserine (thiol) lyase and serine acetyltransferase) remained unaffected (data not shown). Line Lggs12 with the highest level of *GSH1* expression (Figs 1 & 3), the highest foliar γ-ECS activity (Noctor *et al.* 1998) and the highest thiol content in leaves (Fig. 3) and roots (Fig. 3) revealed the lowest APS reductase activity (only 14% of the WT level), and also significantly diminished ATP sulphurylase activity (only 75% of WT plants). In the transgenic lines Lggs6 and Lggs20, APS reductase activity was reduced to approximately 54 and 47%, but ATP sulphurylase activity remained unaffected. Northern blot analysis revealed that the mRNA level of ATP sulphurylase in fine roots was not affected in transgenic poplars, but the steady-state level of the APS reductase mRNA was decreased to approximately 80% in all transformants compared to the WT (Fig. 4).

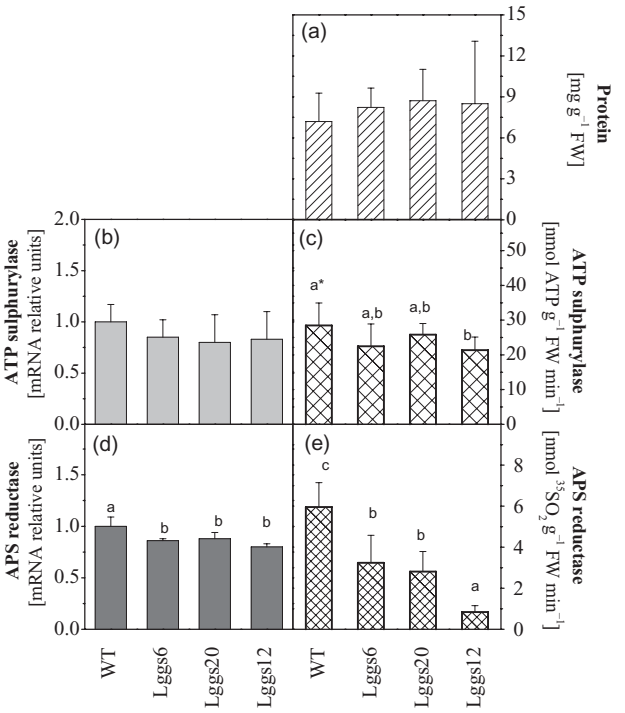

Figure 4. (a) Soluble protein content, (b) mRNA levels of ATP sulphurylase, (c) *in vitro* activity of ATP sulphurylase, (d) mRNA levels of APS reductase, (e) *in vitro* activity of APS reductase in fine roots of 8-week-old poplar plants. Proteins were extracted from fine roots ($n = 6$), and the enzyme activities were determined as described in Materials and methods. Total RNA was extracted from fine roots (b,d; $n = 4$), resolved on an agarose gel in the presence of formaldehyde, transferred onto Hybond-N nylon membrane and hybridized with ^{32}P-labelled cDNA fragments of ATP sulphurylase and APS reductase. The autoradiograms were quantified with a densitometer, and normalized to tubulin expression. Data given are mean values ± SD at the number of replicates indicated. Significant differences at $P < 0.05$ between transgenic lines and wild-type (WT) poplar are indicated by asterisks.

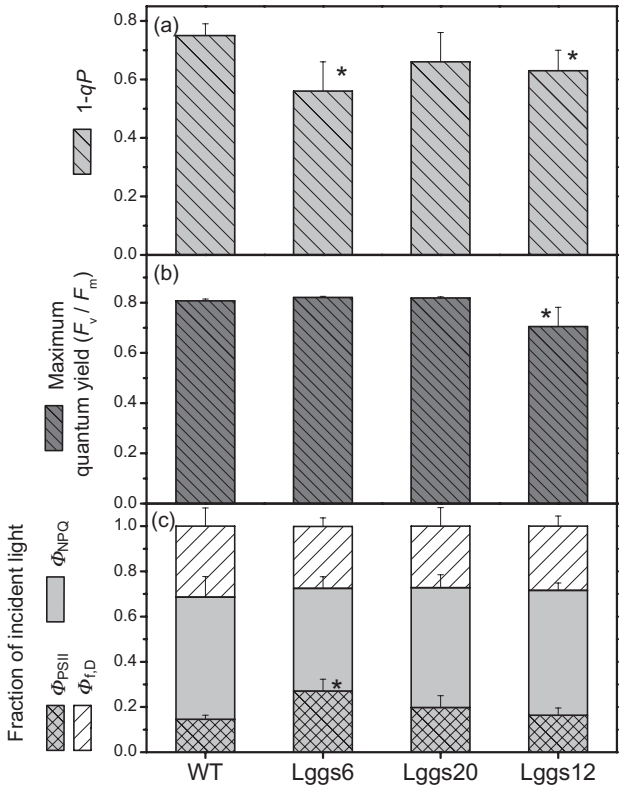

Figure 5. Maximum quantum yield at pre-dawn (b), 1-*qP* (a) and fraction of incident light used for photochemistry and heat dissipation (c) of wild-type (WT) and transgenic poplar lines over-expressing *GSH1* targeted to plastids during midday. Maximum quantum yield (F_v/F_m) was measured at pre-dawn. 1-*qP* was calculated according to the following equation $1\text{-}qP = (F_m' - F_t)/(F_m' - F_0')$ from measurements in light-adapted leaves at 13:00 h during light saturation (850–900 μmol photons m^{-2} s^{-1}). From the same data observed at 13:00 h, the fractionation of absorbed light used for Φ_{PSII}, Φ_{NPQ} and $\Phi_{f,D}$ was calculated after Hendrickson *et al.* (2004). Data given are mean values ± SD from five poplar trees for the WT, and each of the transgenic lines was measured on three consecutive days. (*) Asterisk indices indicate significant differences between transgenic lines and WT poplar at $P \leq 0.05$.

Chlorophyll fluorescence and CO_2 assimilation

The average maximum pre-dawn quantum yield of PSII (F_v/F_m) of young mature leaves was 0.81 in WT poplar and in lines Lggs6 and Lggs20, but was decreased to 0.70 in line Lggs12 (Fig. 5b). The reduction state of the pool of the primary electron acceptor Q_A of the photosynthetic electron transport chain was assessed by using the parameter 1-*qP*. An increase in 1-*qP* typically represents an increase in reduced Q_A and thereby limits the flow of electrons along the electron transport chain. In line Lggs6, 1-*qP* was significantly lower as compared to the WT and the other transgenic lines (Fig. 5a), thus indicating a more oxidized state of Q_A. Simultaneously, line Lggs6 revealed an increased fraction of incident light that was quenched photochemically (Φ_{PSII}) during midday, whereas the fraction of incident light that was utilized for non-photochemical quenching (Φ_{NPQ}) was slightly decreased (Fig. 5c). However, this effect was not statistically significant. In lines Lggs20 and Lggs12, the energy partitioning of absorbed light being utilized in Φ_{PSII} or being dissipated thermally via Φ_{NPQ} or via non-regulated fluorescence and constitutive thermal processes ($\Phi_{f,D}$) was not affected compared to WT poplar (Fig. 5c).

Compared to WT poplar, the rate of CO_2 assimilation and the rate of transpiration were increased in line Lggs6 (Fig. 6), but not in line Lggs20. In line Lggs12, CO_2 assimilation was not affected, but the rate of transpiration was increased and accompanied by an increase in stomatal conductance (Fig. 6).

Changes in proteins involved in photosynthesis and carbon fixation

Over-expression of *GSH1* targeted to plastids caused decreased levels of the reaction centre proteins PsbA of (D1) photosystem (PS) II and PsaA/B of PSI in Lggs6 and Lggs12, but not in Lggs20 poplar lines (Fig. 7). The effect of over-expression was most obvious on the detected levels of the Lhcb1 protein, the major light-harvesting complex of PSII. Compared to the WT, there was a 21% decrease in Lggs6 and a 27% decrease in Lggs12, but only a 5% decrease in the Lggs20 poplar line (Fig. 7). A comparison of RbcL protein levels, which represents the large subunit of ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco), showed only little effects of *GSH1* over-expression (Fig. 7). However, the overall pattern with generally decreased protein abundances and the Lggs12 line being most affected was consistent with the pattern observed for the above proteins (Fig. 7).

DISCUSSION

Consequences of high *GSH1* over-expression levels in line Lggs12

The present study showed that poplars over-expressing bacterial *GSH1* and targeted the protein to plastids developed a phenotype similar as described for tobacco (i.e. earlier leaf senescence and necrotic patches; Creissen *et al.* 1999); however, only after an extended growth period (4.5 months) in line Lggs12 which had the highest expression level of the transgene (Fig. 1), the highest foliar γ-ECS activity (Noctor *et al.* 1998), the highest γ-EC and GSH accumulation in leaves (Fig. 3; Noctor *et al.* 1998) and roots (Fig. 3), but the lowest level of hexose units in leaves and roots (Fig. 3). Photosynthetic parameters, like the rate of CO_2 assimilation, 1-*qP* and energy partitioning remained unaffected. The only exception was the decrease in maximum quantum yield (F_v/F_m), which may indicate constitutive impairment or down-regulation of PSII (Fig. 5b). The reaction centre of PSI was also affected because PsaA/B levels were decreased. Surprisingly, although the rate of CO_2 assimilation was not affected (Fig. 6), soluble sugar contents were decreased (Fig. 3). This may indicate an impaired formation of sugars. The

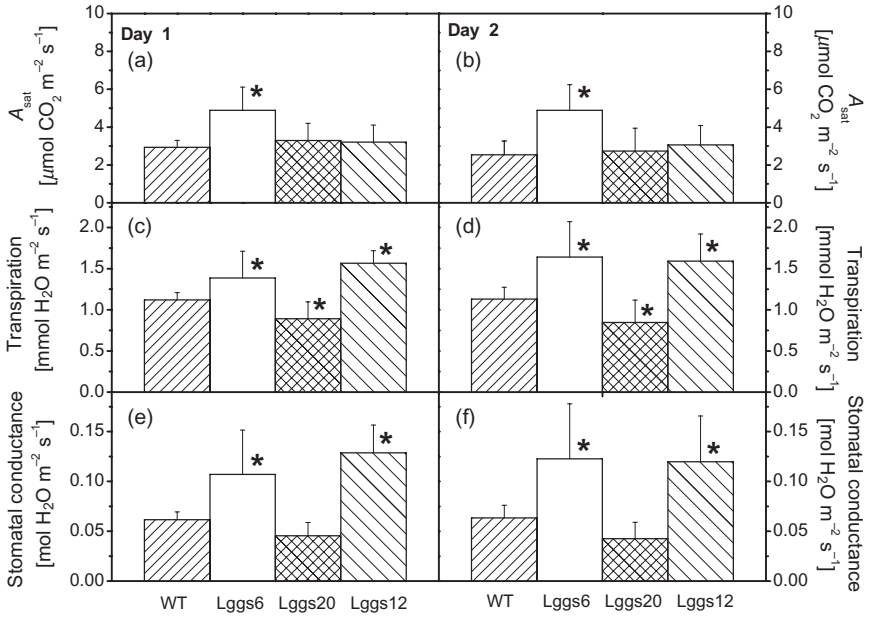

Figure 6. Rate of CO_2 assimilation (a,b), transpiration rate (c,d) and stomatal conductance (e,f) measured on 2 d at 16:00 h. Leaves from the five poplar plants of each line which were chosen for chlorophyll fluorescence measurements were also used for gas exchange measurements at 16:00 h at days 1 and 2. Data given are means ± SD Significant differences between the transgenic lines and wild-type (WT) poplar are given at $P \leq 0.05$.

amount of Rubisco did not vary between transgenic lines and WT plants (Fig. 7), and therefore post-translational control of enzymes involved in carbon metabolism may be more important. Thioredoxin f glutathionylation can affect enzymes of the Calvin cycle (Fig. 8; Schürmann & Jacquot 2000; Buchanan & Balmer 2005; Michelet *et al.* 2005; Lemaire *et al.* 2007), and glutathionylation via glutaredoxin (GRX) (Dietz 2008; Meyer 2008) may affect enzymes of the carbon metabolism (Ito, Iwabuchi & Ogawa 2003; Rouhier *et al.* 2008). Increased GSH concentration in the chloroplast may thus cause down-regulation of hexose synthesis (Lemaire *et al.* 2007; Rouhier *et al.* 2008) in leaves of line Lggs12.

On the other hand, alternative metabolic pathways (i.e. increased respiration and defence reactions) could use an enhanced part of carbon. Increased GSH levels in combination with the induction of genes involved in defence reactions were shown in various studies (Wingate, Lawton & Lamb 1988; Wingsle & Karpinski 1996; Karpinski *et al.* 1997; Mou, Fan & Dong 2003). Therefore, an enhanced GSH synthesis in plastids of line Lggs12 could result in an altered expression of genes involved in defence reactions (Fey *et al.* 2005; Mullineaux & Rausch 2005; Nott *et al.* 2006).

A higher availability of γ-EC and GSH in the cytosol as a result from an expanded γ-EC and/or GSH synthesis in chloroplasts needs enhanced rates of export into the cytosol, which can be paralleled by higher rates of phloem loading. Indeed, increased GSH contents were found in phloem exudates of line Lggs12 (Fig. 3). The exchange of GSH from chloroplasts to the cytosol must be considered as very low (Hartmann *et al.* 2003). Transport of reduced sulphur out of the chloroplasts can effectively occur as γ-EC in *Arabidopsis* (Mullineaux & Rausch 2005; Wachter *et al.* 2005; Pasternak *et al.* 2008; Krueger *et al.* 2009). Sulphide may constitute another important transport form to export reduced sulphur out of chloroplasts (Heeg *et al.* 2008; Krueger *et al.* 2009). For line Lggs12, it can be assumed that a high rate of γ-EC synthesized in the chloroplast promotes γ-EC efflux into the cytosol, where GSH is synthesized by

Protein	WT Lggs6 Lggs12 Lggs20	WT	Lggs 6	Lggs 12	Lggs 20
PsbA		1.00 ± 0.08	0.86 ± 0.10	0.84 ± 0.03	1.02 ± 0.05
PsaA/B		1.00 ± 0.05	0.95 ± 0.06	0.88 ± 0.09	1.02 ± 0.11
Lhcb1		1.00 ± 0.10	0.79 ± 0.10	0.73 ± 0.05 *	0.95 ± 0.04
RbcL		1.00 ± 0.10	0.95 ± 0.08	0.88 ± 0.08	0.94 ± 0.09

Figure 7. The effect of γ-EC over-expression targeted to plastids on expression levels of key proteins of photosynthesis in leaves compared to the wild type (WT). The average optical density of the WT was arbitrarily scaled to 1. Typical bands from the original Western blots are shown next to the values, with each lane loaded on an equal protein basis. Each value represents the average of $n = 4$ ± SE biological replicates. An asterisk (*) denotes significant differences to WT poplar at $P < 0.05$, according to Student's *t*-test.

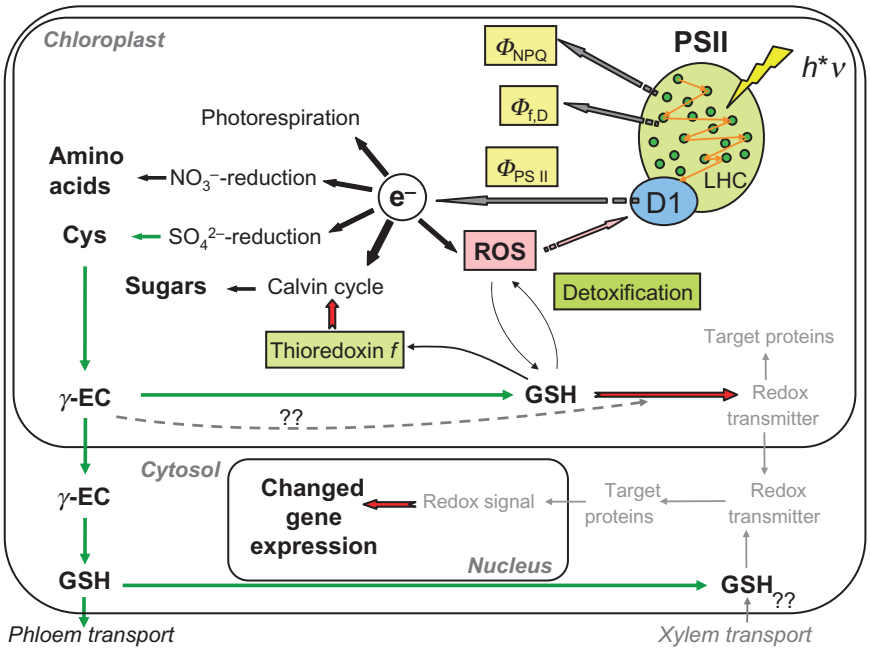

Figure 8. Hypothetical model of enhanced γ-EC synthesis in plastids and its interaction with glutathione (GSH) synthesis, its role in long distance transport and its influence on regulatory processes. After absorption of light energy by the light-harvesting complex (LHC), the energy is transferred to the reaction centre (*psbA*). From there, the electrons are consumed in: (1) the dark reactions for sugar synthesis; (2) nitrate reduction to form amino acids; (3) sulphate reduction to Cys synthesis; (4) photorespiration; or (5) in the transfer to oxygen by which reactive oxygen species (ROS) are formed that can damage the psbA protein. The potential for electron formation can be measured through the quantum yield of PSII photochemistry ($\Phi_{PS\ II}$). During the energy transfer within the LHC, the absorbed energy can be dissipated by non-photochemical quenching Φ_{NPQ} or by regulated thermal processes as fluorescence and constitutive thermal processes ($\Phi_{f,D}$). Within the chloroplast, γ-EC is synthesized and transported to the cytosol (Pasternak *et al.* 2008) where GSH is formed and can then be transported into the phloem. GSH is also synthesized in the chloroplast, where it can participate in different regulatory processes: (I) GSH can react with thioredoxin *f* by glutathionylation, and through this pathway enzymes of the Calvin cycle are decreased (Lemaire *et al.* 2007); (II) GSH is used during ROS detoxification via the GSH–ascorbate cycle (Polle & Rennenberg 1993); (III) ROS can increase the redox potential in the chloroplast, resulting in an increased γ-EC pool. This can lead to an increased GSH pool in the cytosol, which may trigger the induction of stress-related genes like NPR1 (Foyer & Noctor 2005a); (IV) GSH can be involved in changing redox signals via glutathionylation by glutaredoxin (GRX) (Meyer 2008), which changes the activities of several enzymes (Rouhier *et al.* 2008). Black arrows indicate the possible flow of electrons. Grey arrows indicate energy dissipation. Green arrows indicate the flow of reduced sulphur at high *GSH1* expression of line Lggs12. Red arrows indicate regulatory processes.

the GSHS. The resulting high GSH content in the cytosol may cause enhanced phloem loading and also the induction of genes involved in defence reactions (Fig. 8).

It seems unlikely that GSH itself is the signal, because transgenic poplars over-expressing *GSH1* targeted to the cytosol revealed comparable GSH contents in leaves and phloem exudates without showing any visible symptoms of leaf injury, even after 5 months of growth (Herschbach *et al.* 1998). It would also be possible that as found by Creissen *et al.* (1999), an enhanced γ-EC content in plastids of line Lggs12 is responsible for the observed effects on maximum quantum yield (F_v/F_m), on PSII and PSI reaction centre proteins and on induction of defence reactions that lead to early leaf senescence. These assumptions and whether H_2O_2 or other ROS (Creissen *et al.* 1999) or a yet unidentified redox transmitter may be involved in signal transduction between chloroplasts and cytosol in line Lggs12 (Fig. 8) could only be answered in further studies.

Consequence of low *GSH1* over-expression levels in line Lggs6

The low *GSH1* expression in line Lggs6 (present study) combined with slightly increased γ-ECS activity (Noctor *et al.* 1998) and foliar γ-EC content correlates with an enhanced tree height and weight after 8 weeks of growth (Fig. 3). This suggests an increased investment of carbon skeletons into biomass production, which is supported by an increase in CO_2 assimilation (Fig. 6). Chlorophyll fluorescence measurements provide further evidence for this assumption: (1) the fraction of incident light utilized in photochemical processes (Φ_{PSII}) at 13:00 h was significantly increased (Fig. 5c), whereas non-photochemical quenching (Φ_{NPQ}) was decreased; and (2) 1-*qP*, an indicator of the excitation pressure and a relative measure of the redox state of the primary electron acceptor Q_A and of the plastoquinone pool (PQ) (Pfannschmidt 2003), was lower at 13:00 h (Fig. 5a). This clearly shows that Lggs6 has a higher

capacity than WT to keep the electron transport chain oxidized at the same light intensity. Elevated γ-EC and GSH levels in leaves of line Lggs6 might protect PSII from damages by ROS (Fig. 8, Foyer & Noctor 2000).

Although foliar GSH contents of 8-week-old Lggs6 and Lggs12 poplars were about the same, biomass accumulation was reduced in line Lggs12, but enhanced in line Lggs6. GSH levels in phloem exudates of line Lggs6 remained unchanged, while it increased in line Lggs12. This might indicate an unaffected GSH levels in the cytosol of line Lggs6. It can be assumed that the enrichment of γ-EC in chloroplasts is too low to enforce increased γ-EC transport into the cytosol for GSH synthesis. Consequently, sufficient GSH enrichment in the cytosol that might induce expression of defence genes does not occur. This is supported by metabolite analyses within 4.5-month-old plants that did not show any differences to WT plants (Supporting Information Figs S1 & S2). Consequently, leaf necrosis and chlorosis were not even observed after a prolonged growth in line Lggs6 (Fig. 2).

Consequences of moderate *GSH1* over-expression levels in line Lggs20

The third transgenic poplar line, Lggs20, expressed an intermediate level of the transgene (Noctor *et al*. 1998), and has enhanced height and weight (Fig. 1). GSH contents, the rate of CO_2 fixation, the fraction of incident light utilized in photochemistry, the fraction of reduced Q_A, indicated by 1-*qP* and the levels of the reaction centre proteins of PSII and PSI remain unaffected (Figs 5–7). These findings suggest that line Lggs20 did not develop any additional protection against ROS, which might use additional carbon. Rather, the lower concentrations of soluble sugar suggest increased investment of carbon into growth of 8-week-old poplars of line Lggs20 that is comparable with line Lggs6. After 4.5 months of growth, this enhanced increment in tree height and biomass of line Lggs20 disappeared. Moreover, both parameters were even decreased. After the prolonged growth, several other parameters were also comparably affected as found in line Lggs12, including increased GSH levels in leaves and phloem exudates, and decreased levels of soluble sugars in leaves (Supporting Information Figs S1 & S2). Thus, only at the age of several months, γ-EC synthesis in plastids appears to suffice increased GSH synthesis in the cytosol in a similar way as discussed above for line Lggs12.

Effects of *GSH1* over-expression on whole-plant sulphur nutrition

From the present study, it is evident that chloroplastic γ-ECS enhancement affects whole-plant sulphur nutrition; a pattern consistent with previous observations in lines with γ-ECS targeted to the cytosol (Herschbach *et al*. 1998, 2000; Hartmann *et al*. 2004). In the transgenic lines targeting γ-ECS to plastids, a concomitant increase of GSH and decreased activities of key enzymes of the sulphate assimilation pathway (i.e. APS reductase and ATP sulphurylase) in roots were found. GSH content increased in roots of line Lggs6 and Lggs12, although no transcript of *γ-ECS* was detected in roots of line Lggs6. This suggests that the increased GSH content in roots of line Lggs12 may be caused by increased GSH synthesis in the roots, but increased transport from the shoot in line Lggs6. GSH was only increased in phloem exudates of line Lggs12 (Fig. 3; Supporting Information Table S1). Thus, a major contribution of thiols translocated via the phloem to the GSH pool of the roots of 8-week-old poplar is unlikely.

APS reductase activity and mRNA levels in roots of lines Lggs6, Lggs20 and in particular in line Lggs12 decreased (Fig. 4) when the GSH content increased (Fig. 3). As this enzyme is subjected to a feedback inhibition by GSH (Vauclare *et al*. 2002), this finding is coherent. The regulation in *Arabidopsis* occurred predominantly on the transcriptional level, as APR mRNA levels significantly decreased in response to GSH treatment (Vauclare *et al*. 2002). However, in poplar, another component seems to be involved in this regulation, because the corresponding mRNA levels of APR in roots were less affected than enzymatic activities (Fig. 4). Indeed, post-translational regulation of APR by reducing compounds was found by Bick *et al*. (2001) and Koprivova, North & Kopriva (2008). Poplar plants that over-expressed *GSH1* in the cytosol and had a similar increase of GSH in roots showed APR activity and mRNA levels that were not affected (Hartmann *et al*. 2004). This alludes to different effects of plastidic and cytosolic GSH pools on APR activity, and is consistent with a direct inactivation of the enzyme by GSH (Kopriva 2006). APS reductase activity and mRNA transcript accumulation are also diminished by decreasing sugar contents (Kopriva & Rennenberg 2004; Kopriva 2006). As both, increasing GSH and decreasing sucrose levels (probably because of decreasing sugar formation as discussed above) were observed in roots, it cannot be concluded yet whether GSH or sugar triggers down-regulation of APS activity and mRNA in the roots of poplars over-expressing γ-ECS in plastids.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Thiol, sulphate and sucrose contents of leaves and roots in dependence of the trunk height of 4.5-month-old poplar. Mean values ± SD of the apex, the leaves and roots from wild-type (WT) poplar (light grey columns, $n = 4$) and transgenic poplar lines Lggs6 (white columns), Lggs20 (hatched columns**)** and Lggs12 (dark grey columns, each $n = 4$) are given. Significant differences at $P < 0.05$ between each transgenic poplar line and WT poplar are indicated by asterisks.

Figure S2. Sucrose, thiol and sulphate contents in phloem exudates of 4.5-month-old poplars. Phloem exudates were sampled from bark slices from five trunk sections and from lateral roots of wild-type (WT) poplars (solid squares) and

transgenic poplar lines Lggs6 (open circle), Lggs20 (open diamonds) and Lggs12 (open triangles). Data given are mean values ± SD, and significant differences at $P < 0.05$ between each transgenic poplar line and the WT poplar are indicated by asterisks ($n = 4$). One of two independent experiments with similar results is shown.

Table S1. Biometric data, thiol, sugar and anion contents, as well as enzyme activities in young mature leaves, fine roots and phloem exudates of 8-week-old wild-type (WT) and transgenic poplar over-expressing GSH1 targeted to plastids. WT and transgenic poplar lines, Lggs6, Lggs12 and Lggs20 were grown under long-day conditions in a greenhouse for 8 weeks. Biometric data given are mean values ± SD from 44 plants. Thiols ($n = 6$), anions ($n = 6$) and soluble carbohydrate ($n = 12$) contents and enzyme activities ($n = 6$) were determined in ca. 70% expanded leaves (the 10th leaf from the apex, young mature) and fine roots. Phloem exudates were sampled from bark slices of apical stem sections, basipetal stem sections and lateral roots. Data given are mean values ± SD. Asterisk (*) denotes significant differences to WT poplar at $P < 0.05$, according to Student's *t*-test. n.d., not determined.

Table S2. Growth characteristics of 4.5-month-old poplars. Wild-type (WT) and transgenic poplar lines Lggs6, Lggs12 and Lggs20 were grown under long-day conditions in a greenhouse for 4.5 months ($n = 4$ WT and $n = 5$ transgenic poplars; one of two independent experiments with similar results is presented).

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