plant biology

Plant Biology ISSN 1435-8603

RESEARCH PAPER

Over-expression of *gsh1* in the cytosol affects the photosynthetic apparatus and improves the performance of transgenic poplars on heavy metal-contaminated soil

L. A. Ivanova[1], D. A. Ronzhina[1], L. A. Ivanov[1], L. V. Stroukova[2], A. D. Peuke[3] & H. Rennenberg[3]

1 Botanical Garden of Ural Division, Russian Academy of Sciences, Yekaterinburg, Russia

2 Chemical-Technological Faculty, Ural Federal University, K-2, Yekaterinburg, Russia

3 Institut für Forstbotanik und Baumphysiologie, Albert-Ludwigs-Universität Freiburg, Freiburg, Germany

Keywords

Cell number; cell size; chloroplast; γ-glutamylcysteine synthetase; glutathione; mesophyll structure.

Correspondence

L. A. Ivanova, Botanical Garden of Ural Division, Russian Academy of Sciences, 8 Marta 202a, 620144 Yekaterinburg, Russia.
E-mail: Larisa.Ivanova@botgard.uran.ru

Editor

R. Mendel

Received: 25 August 2010; Accepted: 25 October 2010

doi:10.1111/j.1438-8677.2010.00422.x

ABSTRACT

Recent studies of transgenic poplars over-expressing the genes *gsh1* and *gsh2* encoding γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase, respectively, provided detailed information on regulation of GSH synthesis, enzymes activities and mRNA expression. In this experiment, we studied quantitative parameters of leaves, assimilating tissues, cells and chloroplasts, mesophyll resistance for CO_2 diffusion, chlorophyll and carbohydrate content in wild-type poplar and transgenic plants over-expressing *gsh1* in the cytosol after 3 years of growth in relatively clean (control) or heavy metal-contaminated soil in the field. Over-expression of *gsh1* in the cytosol led to a twofold increase of intrafoliar GSH concentration and influenced the photosynthetic apparatus at different levels of organisation, *i.e.*, leaves, photosynthetic cells and chloroplasts. At the control site, transgenic poplars had a twofold smaller total leaf area per plant and a 1.6-fold leaf area per leaf compared to wild-type controls. Annual aboveground biomass gain was reduced by 50% in the transgenic plants. The reduction of leaf area of the transformants was accompanied by a significant decline in total cell number per leaf, indicating suppression of cell division. Over-expression of γ-ECS in the cytosol also caused changes in mesophyll structure, *i.e.*, a 20% decrease in cell and chloroplast number per leaf area, but also an enhanced volume share of chloroplasts and intercellular airspaces in the leaves. Transgenic and wild poplars did not exhibit differences in chlorophyll and carotenoid content of leaves, but transformants had 1.3-fold fewer soluble carbohydrates. Cultivation on contaminated soil caused a reduction of palisade cell volume and chloroplast number, both per cell and leaf area, in wild-type plants but not in transformants. Biomass accumulation of wild-type poplars decreased in contaminated soil by more than 30-fold, whereas transformants showed a twofold decrease compared to the control site. Thus, poplars over-expressing γ-ECS in the cytosol were more tolerant to heavy metal stress under field conditions than wild-type plants according to the parameters analysed. Correlation analysis revealed strong dependence of cell number per leaf area unit, chloroplast parameters and mesophyll resistance with the GSH level in poplar leaves.

INTRODUCTION

The sulphur-containing tripeptide glutathione (GSH) is the major low molecular mass peptide in plants and is present at millimolar concentrations within cells. It is involved in many cellular processes through its influence on intracellular redox state (Noctor & Foyer 1998), its function as a transport form and reservoir of reduced sulphur (Rennenberg 2001) and its significance in the cross-talk between sulphur, nitrogen and carbon metabolism (Kopriva & Rennenberg 2004). In addition, GSH plays an important role in the defence of plant cells against reactive oxygen species (Noctor & Foyer 1998), xenobiotics (Edwards & Dixon 2005) and heavy metals (Cobbett & Goldsbrough 2002).

Lines of the poplar hybrid, *Populus tremula* × *P. alba*, over-expressing the bacterial genes *gsh1* or *gsh2* encoding γ-glutamylcysteine synthetase (γ-ECS) or glutathione synthetase, respectively, are widely known as good models for studies of the effects of enhanced glutathione (GSH) biosynthesis in plants (Noctor *et al.* 1998; Kopriva & Rennenberg 2004; Rennenberg & Peuke 2005). Therefore, these poplar transformants are among the best-characterised transgenic plants (Noctor *et al.* 1998). In laboratory experiments, transgenic poplars over-expressing γ-ECS in the cytosol did not differ from wild-type lines morphologically during initial growth and development (Noctor *et al.* 1996; Gullner *et al.* 2001) but had distinct functional features. Transformants were more tolerant towards chloroacetanilide herbicides than

wild-type plants (Gullner *et al.* 2001) and showed elevated heavy metal uptake (Bittsánszky *et al.* 2005). Still the over-expression of γ-ECS in transgenic poplar did not increase the general tolerance to heavy metals or ozone (Arisi *et al.* 2000; Rennenberg & Will 2000; Koprivova *et al.* 2002).

In tobacco, over-expression of γ-ECS in chloroplasts resulted in reduced rates of photosynthesis and strongly modified morphology of the plants (Creissen *et al.* 1999). Changes in photosynthesis and growth were also observed in poplars over-expressing γ-ECS in the chloroplasts upon prolonged growth during one growing season in the greenhouse. These changes were attributed to down-regulation of photosystem II reaction centres and correlated with accumulation of γ-glutamylcysteine and GSH in leaves, roots and phloem exudate (Herschbach *et al.* 2010). Apparently, the effects of GSH are complex and it is very difficult to identify its precise roles in individual cells or to predict the consequences of altered glutathione biosynthesis during the life cycle of plants (North & Kopriva 2007; Herschbach *et al.* 2010).

Sulphate assimilation and glutathione synthesis are closely connected with N and C metabolism. Sugars induce the activity of sulphur assimilation enzymes and, after introduction to and transformation in nitrogen metabolism, provide the acceptor of sulphide for biosynthesis of cysteine and other metabolites for sulphate assimilation (Kopriva & Rennenberg 2004; Kopriva 2006). It has previously been reported that leaves of transgenic poplars over-expressing *gsh1* in the cytosol did not significantly differ from wild-type lines in chlorophyll, carotenoid, soluble protein levels (Noctor *et al.* 1996) and the rate of photosynthesis (Herschbach *et al.* 2000) when grown under greenhouse conditions. It seems that functional parameters of individual chloroplasts were not affected by manipulating GSH synthesis in the cytosol, at least upon short-term cultivation of poplars. In contrast, field experiments showed marked changes in chloroplast size and shape of the same transformants in comparison with wild-type poplars (Ivanova *et al.* 2009). These results support the idea that structural reorganisation in photosynthetic tissues is affected by increased thiol content. It is well known that the defined arrangement of chlorophyll-containing cells and chloroplasts inside the leaf is a decisive factor for optimum photosynthesis and productivity of plants (Nobel & Walker 1985; Terashima *et al.* 2001; Oguchi *et al.* 2005).

Unlike the detailed knowledge on biochemical features such as metabolite composition, enzyme activities and gene expression, little is known about precise parameters of photosynthetic tissues and cells in transformed poplars. The aim of this study was to test, under prolonged growth for several seasons and in field conditions, whether the photosynthetic apparatus in poplars is influenced by over-expression of γ-ECS in the cytosol. For this purpose, we compared quantitative parameters of assimilating tissues, cells and chloroplasts, diffusion resistance of mesophyll, as well as chlorophyll and carbohydrate content in transgenic and wild-type poplars after 3 years of growth in a field experiment.

MATERIAL AND METHODS

Plant material and experimental sites

Wild-type poplars (*Populus tremula* × *P. alba*) of the INRA female clone 717 1-B4 and a transgenic line of this clone (line *ggs11*: Noctor *et al.* 1996; Arisi *et al.* 1997) over-expressing the bacterial gene *gshI* encoding γ-ECS in the cytosol. The transgenic line chosen shares common biochemical features with a number of other transgenic lines studied intensively under laboratory conditions (Noctor *et al.* 1998).

Plants were grown for 3 years at a relatively clean (control) field site and a field site contaminated with heavy metals near Yekaterinburg (Sverdlovskaja oblast, Russia: E59°59′ E, 58°00′ N). The soil heavy metal content at the control site was 170 ppm, including 49 ppm Cr, 48 ppm Ni, 32 ppm Zn, 17 ppm Cu, 15 ppm Co, 8 ppm Pb and 0.3 ppm Cd. The sum of heavy metals at the contaminated site was 1185 ppm, including 573 ppm Zn, 391 ppm Cu, 149 ppm Pb, 35 ppm Cr, 22 ppm Ni, 12 ppm Co and 3.4 ppm Cd. The experimental sites were located in the southern taiga. Intensive plant growth in this region begins in May and continues until late August. The average period with air temperatures above +10 °C is 127 days (from May to September). The average temperature in July is +17.6 °C; the annual mean precipitation is 435 mm, annual mean air temperature is 1 °C (Nauchno-prikladnoi spravochnik po klimatu SSSR 1990). The absolute maximum air temperature during the experiment was 38 °C.

Plant parameters

Shoot fresh and dry weights were measured for 10 individuals of each plant type at the end of August of each year of the experiment. Annual biomass gain was determined as difference between shoot biomass of the third and the second year of cultivation, divided by the shoot biomass of the second year. Total leaf area per plant was determined by dividing leaf dry weight by leaf mass area (LMA, dry weight per leaf area unit). LMA was calculated on fully expanded leaves of the middle part of the tree crown exposed to the south, using leaf dry weight and area in 10 replicates for each variant.

Mesostructure of photosynthetic apparatus

Quantitative parameters of photosynthetic tissues and cells, named 'mesostructure of the photosynthetic apparatus', were determined according to the method of Mokronosov (1981) described in detail in Pyankov *et al.* (1998) and Ivanova & Pyankov (2002). Fully expanded leaves from the middle of the crown, exposed to the south, were collected from 10 individuals of each plant type. Measurements of leaf, tissue and cell parameters were carried out with the image analysis system SIAMS Mesoplant™ (SIAMS, Yekaterinburg, Russia; see Appendix). Leaf area was measured on fresh leaves of 10 individuals of each plant type. For quantitative anatomical analysis, leaf disks from the middle part of the leaf blade without big veins were fixed in 3.5% glutaraldehyde in 0.15 M phosphate buffer, pH 7.4.

Leaf thickness, average diameter of veins and covering tissue area were obtained by measuring 20–30-μm thick leaf cross-sections produced with a freezing microtome (Technom, Yekaterinburg, Russia). The portion of covering tissue was calculated as the percentage of the area occupied by the upper and lower epidermis in a total cross-section area. Conductive tissue volume per leaf area unit was determined by multiplying the total vein projection area per leaf area unit

and the average vein diameter. Vein projection area (total area of small vines of the second and third order) per leaf surface area unit was measured by microscopic analysis of leaf disks (Axiostar plus; Zeiss, Jena, Germany). The volume of air spaces was calculated by subtracting the share of covering tissues, conductive tissues, and palisade and spongy mesophyll cells (described below) from the whole leaf volume.

The number of cells per leaf area unit was determined in a haemocytometer after maceration of fixed leaf disks in 20% KOH (described in detail by Ivanova & Pyankov 2002). Cell number per whole leaf was calculated by multiplying the cell number per leaf area unit and average leaf area. The chloroplast number per leaf area was calculated by multiplying the chloroplast number per cell and cell number per leaf area for palisade and spongy tissue, accordingly. Cell sizes and chloroplast numbers per cell were determined after maceration of leaf disks in 1 N HCl and heating to 40–50 °C. The mesophyll cell volume (V_{cell}) and surface area (S_{cell}) were calculated according to the projection method (Ivanova & Pyankov 2002) using average cell projection area (A_{cell}) and perimeter (P_{cell}):

$$S_{cell} = b \times A_{cell}, V_{cell} = \frac{A_{cell}^2}{P_{cell}} \sqrt{b^3 \times K_p}$$

with the geometric coefficients b and K_p depending on cell shape. The total cell surface area per leaf area unit (A_{mes}/A) was determined separately for palisade and spongy tissue by multiplying the average cell surface area by cell number per leaf area.

Chloroplast parameters, such as chloroplast number per cell, chloroplast volume and surface area, were taken from Ivanova *et al.* (2009). Chloroplast projection area and perimeter were measured by visualising the leaf cross-sections with a light microscope on a computer display and were analysed with the image analysis system SIAMS Mesoplant™. The chloroplast volume (V_{chl}) and surface area (S_{chl}) were determined using the projection method described in detail by Ivanova & Pyankov (2002):

$$S_{chl} = b \times A_{chl}, V_{chl} = \frac{A_{chl}^2}{P_{chl}} \sqrt{b^3 \times K_p}$$

with the geometric coefficients $b = 4$ and $K_p = 0.11$. The total chloroplast surface area per leaf area unit (A_{chl}/A) was determined by multiplying the average chloroplast surface area by chloroplasts number per leaf area.

Mesophyll resistance to CO_2 diffusion ($s \cdot cm^{-1}$) was determined as a theoretical value according to Laisk *et al.* (1970):

$$r_m = \frac{L}{D \times (A_{mes}/A) \times k \times b}$$

where $L = 1$ μm is average distance from the cell surface to the chloroplasts, $D = 8 \times 10^{-6}$ $cm^2 \cdot s^{-1}$ is the constant for CO_2 diffusion in the liquid phase of cells; $b = 0.68$ is the solubility of CO_2 in water at 30 °C; and A_{mes}/A is the total surface of mesophyll cells per unit leaf area. k is the coefficient (<1) describing the portion of the cell surface contacting the chloroplasts:

$$k = \frac{S_{chl} \times Chlp}{2 \times S_{cell}}$$

where *Chlp* is the chloroplast number per cell. Diffusion resistance of the intercellular spaces ($s \cdot cm^{-1}$) was calculated according to the formula:

$$r_{is} = \frac{T_{mes}}{D_c \times V_{is} \times 100}$$

where T_{mes} is thickness of the leaf mesophyll (μm); $D_c = 0.16$ $cm^2 \cdot s^{-1}$ is a constant for CO_2 diffusion in air; and V_{is} is relative volume of intercellular spaces in the mesophyll (%; Laisk 1977).

Biochemical analyses of leaves

Chlorophyll and thiol content of wild-type and transgenic poplars was taken from Ivanova *et al.* (2009). Pigments were extracted with 80% acetone from leaf disks of known area and measured using an SF-46 spectrophotometer (LOMO; Saint-Petersburg, Russia). The pigment content was calculated according to the equations described in Lichtenthaler & Wellburn (1983). Thiol content was determined as monobromobimane derivatives after reverse-phase HPLC separation with fluorimetric detection, as previously described (Strohm *et al.* 1995).

For determination of carbohydrate content, leaves from five individuals of each plant type were fixed at 125 °C and than dried in an air thermostat at 75 °C. Soluble sugars were double extracted from plant material using 80% ethanol at 40 °C. After centrifugation, the supernatant was used to determine the total amount of soluble sugars (sucrose, glucose and fructose), and the pellet was boiled for 3 h in 3% HCl to hydrolyse nonstructural polysaccharides (starch and fructanes) (Poorter & Bergkotte 1992). The amount of soluble sugars and nonstructural polysaccharides after hydrolysis was measured using a colorimetric method with anthrone (Sigma, St. Louis, MO, USA) (Fales 1951) in a spectrophotometer (Odyssey DR/2500; HACH, Loveland, CO, USA).

The content of chlorophylls and nonstructural polysaccharides per one chloroplast was determined by dividing chlorophyll and polysaccharide content per leaf area by chloroplast number per leaf area. GSH concentrations in leaves were calculated by dividing the GSH content per leaf area by leaf volume (multiplying leaf area unit and leaf thickness). GSH content per cell of palisade or spongy tissues was estimated from the GSH concentration in the leaf and the corresponding cell volume. The size of the GSH pool in chloroplasts of wild-type and transgenic poplars was taken as 50% of total GSH per fresh weight (Hartmann *et al.* 2003). GSH concentrations in chloroplasts were calculated using the chloroplast volume data from Ivanova *et al.* (2009).

Statistical analysis

For each of the four studied variants (plant type*place), 10 individuals were used for biometric (shoot weight, leaf area

and thickness, LMA) and thiol analysis. Leaves from these individuals were taken for quantitative anatomical measurements, *i.e.*, 20 measurements for cell number counting and 30 replicates for chloroplast numbers, cell and chloroplast sizes. For biochemical analysis (pigments, carbohydrates), five individuals were studied. Significance of differences between variants was evaluated using a paired *t*-test. Differences were considered to be significant at $P \leq 0.05$. In figures and tables, means ± SE are presented. Different letters on figures and tables indicate significant differences. Data were subjected to two-way analysis of variance (ANOVA, variable: type, place and interaction type*place).

RESULTS

Effect of enhanced GSH synthesis on biometric parameters of poplar leaves

The leaves of transgenic and wild-type poplars did not exhibit any visible morphological differences. However, biometric analysis of leaves showed significant differences in leaf size at the control field site. After 3 years of growth, the transformants had a twofold smaller total leaf area per plant than the wild type (Fig. 1A). The development of individual leaves was significantly retarded in transgenic plants, which had a 1.6-fold decreased leaf area per leaf (Fig. 1C). Annual aboveground biomass gain in the poplar mutants was half the biomass gain of the wild type (Fig. 1B). Heavy metal contamination caused a decrease of leaf area and biomass parameters in the wild type, but did not influence leaf parameters of transgenic poplars (Fig. 1A and C). At the contaminated site, wild-type plants showed a decrease in total plant leaf area by more than a factor of three and of individual leaf area by a factor of two (Fig. 1A and C). The biomass gain of wild-type poplars and transformants decreased more than 30-fold and twofold, respectively, when controls and contaminated sites were compared. As a consequence, the annual biomass gain of the mutants was 12-fold higher than the biomass gain of wild-type poplars under heavy metal contamination (Fig. 1B).

Leaf thickness and LMA did not differ between wild-type and transformed plants in controls, but increased in wild-type plants under heavy metal stress (Fig. 1D and E). Over-expression of γ-ECS led to a twofold decrease in total cell number per leaf in transformants at the control site (Fig. 1F). At the same time, heavy metal contamination caused a twofold reduction of cell amount per leaf in wild-type but not in transgenic poplars.

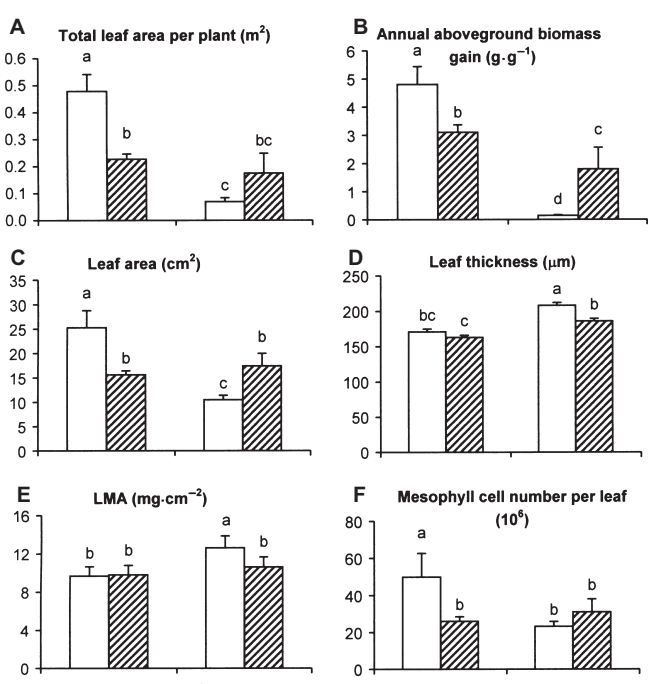

Fig. 1. Biometric parameters of wild-type (light columns) and transgenic (stroked columns) poplar leaves at the control and contaminated sites.

Effects of enhanced GSH synthesis on photosynthetic tissue architecture

The general view of leaf cross-sections, tissues arrangement and number of cell layers was indistinguishable between transgenic and wild-type poplars, as previously described (Ivanova *et al.* 2009). However, transgenic and wild-type poplars showed different quantitative parameters for photosynthetic tissues. Figure 2 presents the volume fractions of tissue components in the leaf of wild-type and transgenic poplars at the two experimental sites. At the control site, transgenic poplars had an enhanced amount of air spaces in the leaf in comparison with the wild type due to a decreased share of palisade tissue. Heavy metal contamination of the soil caused a significant increase in intercellular spaces in both wild-type and transgenic poplars. Expansion of air spaces in both poplar types was at the expense of a reduction in palisade and epidermal portions, although in the transformants this was smaller. The spongy and conductive tissue relative volume was not affected by either poplar type or site.

Over-expression of γ-ECS led to a decrease in cell number per leaf area to about 80%, which was significant, both at the control and at the contaminated site (Fig. 3A). As chloroplast number per cell in transgenic poplars was unchanged (Ivanova *et al.* 2009), the chloroplast number per

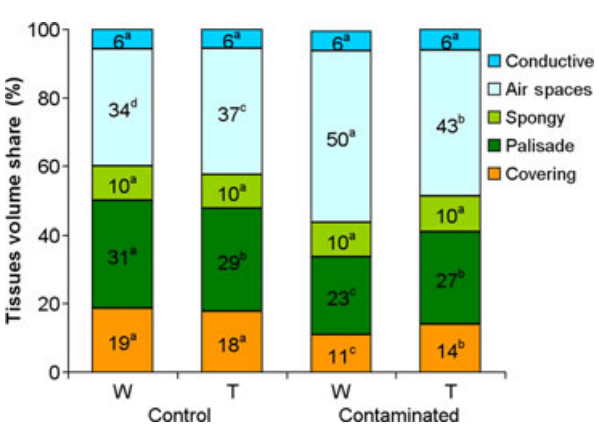

Fig. 2. Volume fractions of leaf tissue components in wild-type and transgenic poplar from the control and contaminated sites. W, wild type, T, transformant.

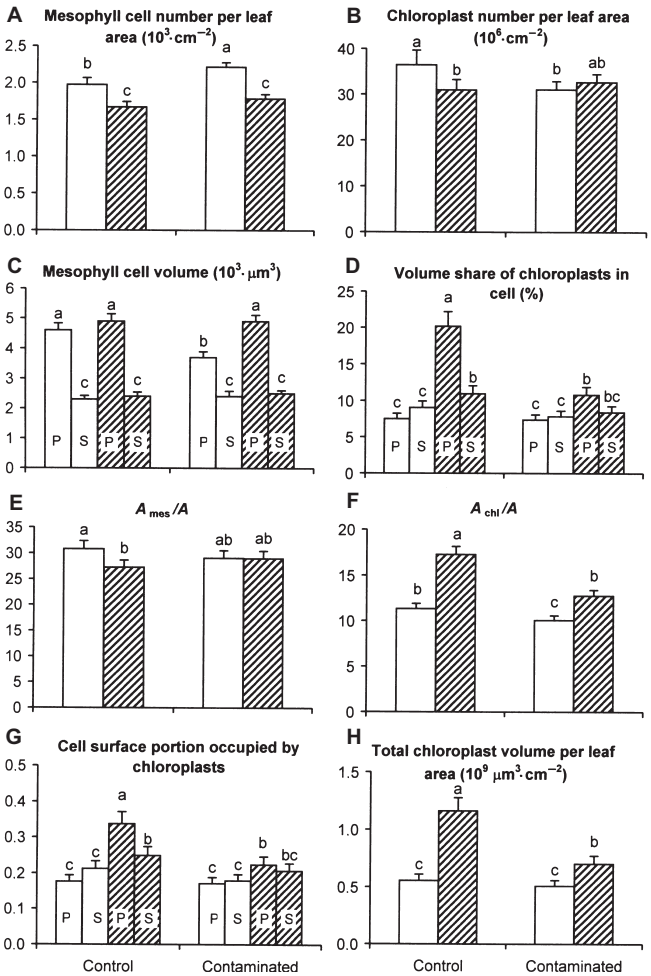

Fig. 3. Quantitative parameters of photosynthetic tissues of wild-type (light columns) and transgenic (stroked columns) poplar at the control and contaminated sites. P, palisade, S, spongy. A_{mes}/A – total cell surface area per leaf area unit, A_{chl}/A – total chloroplast surface area per leaf area unit.

leaf area was reduced to 80% in the transformants (Fig. 3B). At the control site, both types of poplar had the same mesophyll cell sizes (Fig. 3C). Cultivation on soil contaminated with heavy metals caused a reduction of palisade cell volume, chloroplast number both per cell and leaf area and enhanced the cell number per leaf area in wild-type poplars (Fig. 3).

Due to marked chloroplast swelling, especially in palisade cells of transgenic poplars (Ivanova *et al.* 2009), the relative volume of chloroplasts per palisade cell was much larger than in wild-type plants (Fig. 3D). Reduced cell numbers in the mutants led to a slight decrease of A_{mes}/A, but the increase in chloroplast size enhanced A_{chl}/A 1.5-fold at the control site (Fig. 3E and F). Heavy metal contamination affected these cell and chloroplast parameters in wild-type plants but not in transformants, except for the cell surface portion occupied by chloroplasts and the total chloroplast volume per leaf (Fig. 3G and H). At the control site, both types of poplar had similar palisade/spongy cells number ratios, which decreased under heavy metal contamination.

Photosynthetic pigment and nonstructural carbohydrate content

Photosynthetic pigment content in poplar leaves depended on the site of growth, but not on the poplar type (Table 1, Table S1). Transgenic and wild-type poplars had no significant differences in chlorophyll and carotenoid content in the leaves. Contamination with heavy metals caused a *ca.* 30% reduction in pigment content in both poplar lines (Table 1). By contrast, the content of nonstructural carbohydrates per leaf dry weight depended on both poplar type and site of growth (Table 1, Table S1). Transformed poplars had *ca.* 1.3-fold lower soluble carbohydrate content in leaves, both at control and contaminated sites. Contamination of soil by heavy metals reduced the amount of nonstructural carbohydrates in both poplars types.

Transgenic and wild-type leaves had the same amount of chlorophyll *a* and *b* per one chloroplast, which decreased to 70% under heavy metals stress (Table 1). The amount of nonstructural carbohydrates per chloroplast was similar in both wild-type and transgenic poplar at both study sites. However, our calculations of the concentration of chlorophyll and carbohydrate per chloroplast volume showed a marked decrease in the concentration of these substances in transgenic poplars (Table 1).

Relationships between structural parameters of poplar leaves and GSH content

Glutathione concentration per leaf fresh weight was closely related to plant height and total leaf area at the control site, both in wild-type and transgenic poplars (Fig. 4A and C). A similar dependence was not observed within the two groups of plants of each poplar type (control soil/contaminated soil). At the contaminated site, no significant correlation between GSH content in leaves and plant parameters was found (Fig. 4B and D). Close relationships were observed between leaf GSH content and quantitative parameters of photosynthetic tissues (Fig. 5). Chloroplast volume and surface area related to leaf volume and area, respectively, increased with increasing GSH content of the leaves (Fig. 5B and C). The slope of regression for palisade parenchyma was much steeper than for spongy tissue. In contrast, the cell number declined with increasing GSH content with a similar slope in both palisade and spongy tissue (Fig. 5D). Mesophyll resistance to CO_2 diffusion decreased with increasing GSH content in the leaves (Fig. 6A). Diffusion resistance of intercellular air spaces was stable, but its share in relation to total mesophyll resistance increased with increasing GSH content (Fig. 6B).

DISCUSSION

Transgenic poplars over-expressing γ-ECS in the cytosol possess enhanced activity of γ-ECS and have a two to fourfold elevated GSH content in leaves, as reported in numerous laboratory studies (Strohm *et al.* 1995; Noctor *et al.* 1996; Kopriva & Rennenberg 2004). The transformants also synthesised twice as much GSH in the leaves compared to the wild type in a 3-year field experiment (Ivanova *et al.* 2009). In the cytosol of leaves of transgenic plants, GSH concentrations increased twofold in both, photosynthetic and non-pho-

Table 1. Photosynthetic pigment and nonstructural carbohydrate content in wild-type and transgenic poplar over-expressing the bacterial gene *gsh1* after 3 years of cultivation at a control and a contaminated site.

	control		contaminated	
parameters	wild type	transformant	wild type	transformant
mg·g^{-1} DW				
total chlorophyll	3.5 ± 0.6^{a}	3.1 ± 0.3^{ab}	2.8 ± 0.4^{bc}	2.5 ± 0.6^{c}
chlorophyll *a*	2.5 ± 0.4^{a}	2.3 ± 0.2^{ab}	2.2 ± 0.4^{ab}	1.9 ± 0.4^{b}
chlorophyll *b*	1.0 ± 0.2^{a}	0.7 ± 0.1^{ab}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}
soluble carbohydrates	72 ± 5^{ab}	54 ± 6^{c}	69 ± 5^{b}	48 ± 4^{c}
nonstructural polysaccharides	124 ± 21^{a}	94 ± 11^{ab}	81 ± 13^{b}	83 ± 23^{b}
total nonstructural carbohydrates	195 ± 26^{a}	149 ± 17^{b}	150 ± 17^{b}	131 ± 25^{b}
GSH/Chlorophyll, nmol·μg^{-1}	0.29 ± 0.04^{c}	0.68 ± 0.09^{a}	0.19 ± 0.02^{d}	0.49 ± 0.06^{b}
mg 10^{-9} chloroplasts				
chlorophyll *a*	0.71 ± 0.06^{a}	0.77 ± 0.07^{a}	0.56 ± 0.05^{b}	0.54 ± 0.05^{b}
chlorophyll *b*	0.28 ± 0.03^{a}	0.24 ± 0.03^{a}	0.15 ± 0.02^{b}	0.18 ± 0.02^{b}
nonstructural polysaccharides	32.8 ± 7.8^{a}	29.9 ± 6.0^{a}	32.9 ± 7.0^{a}	26.9 ± 6.2^{a}
μg 10^{-7} μm^{-3} chloroplasts				
total chlorophyll	0.64 ± 0.10^{a}	0.31 ± 0.08^{b}	0.43 ± 0.08^{ab}	0.36 ± 0.07^{b}
nonstructural polysaccharides	21 ± 3^{a}	9 ± 1^{b}	20 ± 2^{a}	13 ± 1^{b}

Mean ± standard deviation of five plants is given. Different superscript letters in rows indicate significant differences at $P \leq 0.05$. DW = dry weight.

tosynthetic cell types (Hartmann *et al.* 2003). GSH concentrations also doubled per leaf volume unit in transformants (Table 2). As GSH is involved in many cellular processes, the increased GSH concentration can have numerous effects on the structure and function of leaf tissues. The present results show that over-expression of *gsh1* in the cytosol of poplars not only leads to enhanced intercellular concentrations of GSH, but also influences the photosynthetic apparatus at different levels of organisation, *i.e.*, chloroplasts, photosynthetic tissues, leaves (Fig. 7).

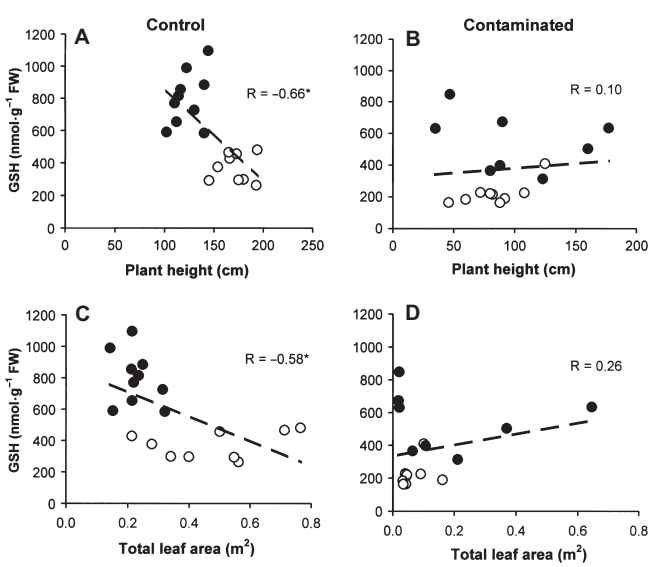

Fig. 4. Relationships between biometric parameters and GSH content in poplar leaves studied at the control site (A, C) and the site contaminated with heavy metals (B, D). Glutathione concentration per fresh weight (FW) of leaves from individual plants was plotted against corresponding stem height and total leaf area per plant. Light circles – wild type, filled circles – transgenic plants. Correlation coefficients (R) significant at $P < 0.01$ are marked with an asterisk.

Effects on chloroplasts of γ-ECS over-expression in the cytosol

Enhanced GSH synthesis in transformants demands increased sulphate reduction and cysteine synthesis, which take place in plastids (Leustek *et al.* 2000; Kopriva & Rennenberg 2004). It has previously been reported that the size of the chloroplastic GSH pool is similar in wild and transgenic poplar (Hartmann *et al.* 2003). Here, we show that transformants had a marked decrease in chloroplast number per leaf fresh weight (Table 2). This means that at the same total chloroplastic GSH level and a 20% decreased chloroplast number per fresh weight, the transgenic poplars should have *ca*. 20% more GSH in a single chloroplast compared to the wild type. The increased chloroplast volume found in transformants (Ivanova *et al.* 2009; Figs. 3 and 5) is probably linked to the elevated amount of GSH per one chloroplast. An influence of GSH synthesis on chloroplast size was also observed for *Arabidopsis thaliana* (Wojcik *et al.* 2009). In these studies, decreased GSH biosynthesis in plants treated with buthionine sulphoximine (BSO) led to a decreased chloroplast sectional area, while enhancing GSH synthesis by growing plants with Cu resulted in enhanced chloroplast size. Thus, over-expression of γ-ECS in the cytosol not only causes elevated cytosolic and chloroplastic GSH content, but also increased chloroplast volume (Fig. 7).

The increase in chloroplast volume mediated by enhanced GSH biosynthesis took place to a greater degree in palisade compared to spongy tissue (Figs. 3 and 5). GSH concentrations were similar for palisade and spongy cells in leaves of transgenic poplars (Hartmann *et al.* 2003). At the same time, palisade cells of poplars were twice as big as spongy cells (Fig. 3). Apparently, the total GSH amount per cell is about twofold higher in palisade tissue (Table 2). This is consistent

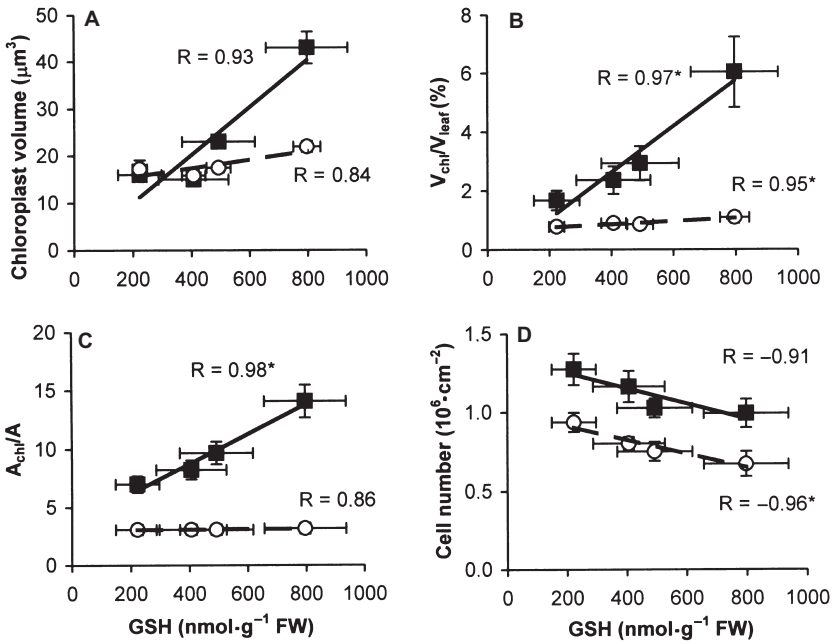

Fig. 5. Correlation of structural parameters of palisade (■) and spongy (○) tissues with GSH content in poplar leaves. Each plot corresponds to the average value of parameters for one studied variant (type*place). A: volume of one chloroplast, B: share of total chloroplast volume in leaf volume, C: total surface area of chloroplasts per leaf area, D: mesophyll cell number per leaf area. FW, fresh weight. Correlation coefficients (R) significant at $P < 0.01$ are marked with an asterisk.

with the finding that GSH biosynthesis is stimulated by light (Kopriva & Rennenberg 2004). As most sunlight is absorbed in the upper layers of the mesophyll, *i.e.*, palisade tissue, the highest GSH synthesis can be expected in this tissue.

The increased chloroplast volume in the transgenic plants was not linked to changes in pigment or starch content (Table 1), indicating that the chloroplast volume increase is most likely caused by a change in stroma volume, where processes of cysteine synthesis take place. The mechanisms adjusting cysteine biosynthesis to a higher demand for GSH synthesis in γ-ECS transgenic trees are still unknown (Kopriva 2006). To date, it is not clear why γ-ECS transformants react differently to increased GSH level mediated by enhanced endogenous synthesis compared to feeding exogenous GSH to wild-type plants (Hartmann *et al.* 2004).

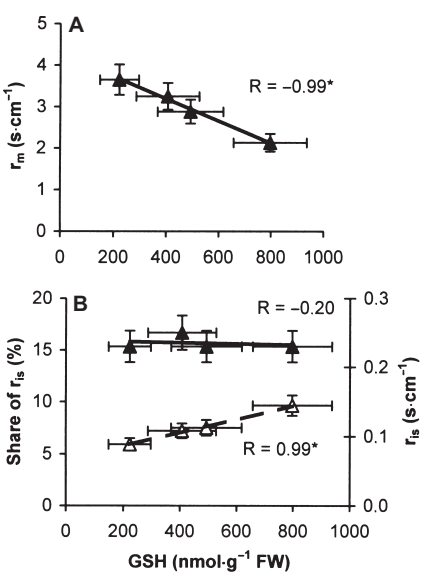

Fig. 6. Relationships between inner leaf resistance to CO_2 diffusion and GSH content in poplar leaves. A: mesophyll resistance, B: resistance of intercellular air spaces (▲) and its proportion of total mesophyll resistance (Δ). FW, fresh weight. Correlation coefficients (R) significant at $P < 0.01$ are marked with an asterisk.

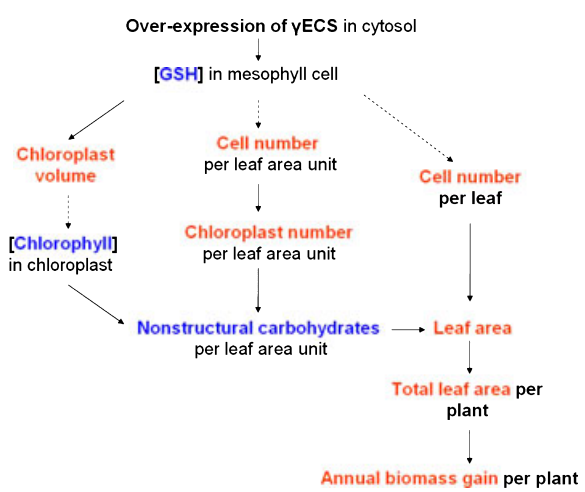

Fig. 7. Schematic representation of the effects of over-expression of *gsh1* in the cytosol on the photosynthetic apparatus of transgenic poplar. Black arrows represent positive correlation; dashed lines represent negative effects. [GSH] – GSH concentration, [Chlorophyll] – chlorophyll concentration.

Table 2. Glutathione content (GSH) and concentration ([GSH]) in leaf photosynthetic tissues of wild-type and transgenic poplar at the control site. Data are estimated from GSH content in leaves and cells, as well as chloroplast number and size (see Material and Methods).

parameters	wild type	transformant
GSH per leaf area, nmol·dm^{-2} leaf area	828 ± 92^{b}	1562 ± 116^{a}
[GSH] in leaf, mM	0.5	1.0
GSH per palisade cell, nmol 10^{-6} cells	2.4	5.0
GSH per spongy cell, nmol 10^{-6} cells	1.1	2.5
GSH per leaf weight, nmol·g^{-1} FW	407 ± 45^{b}	797 ± 56^{a}
GSH in chloroplasts, mg·g^{-1} FW	204	204
Chloroplast number per leaf weight, 10^{9} g^{-1} FW	1.51 ± 0.03^{a}	1.21 ± 0.02^{b}
GSH per chloroplast, nmol 10^{-9} chloroplasts	135	170
[GSH] in chloroplast, mM	8.8	5.2

FW = fresh weight. Different letters indicate significant differences at $P \leq 0.05$.

Despite a three to fourfold increase in GSH level in the mutants, the activities of enzymes of sulphate assimilation were not affected, while feeding exogenous GSH caused activity of adenosine 5′ phosphosulphate reductase (APR) and mRNA accumulation to decrease (Hartmann *et al.* 2004). We hypothesise that chloroplast volume regulation may be a mechanism of adaptation to the increased GSH level and may occur in the course of growth and development of transgenic poplar plants. The increased chloroplast volume in mutants probably constitutes a mechanism to maintain or even reduce the GSH concentration inside chloroplasts (Table 2), thereby preventing the inhibitory effect of excess glutathione on enzymes of sulphate assimilation. This might also explain the down-regulation of ATP sulphurylase (ATPS) and adenosine 5′ phosphosulphate reductase (APR) by exogenous GSH, because a change in chloroplast volume cannot occur after a definite stage of leaf development. During leaf development, the chloroplast volume increases quickly in the first days (*e.g.*, in potato, 6–7 days) and then remains constant, despite subsequent intense growth of the leaf blade (Mokronosov 1981); therefore, exogenous GSH could not change the plastid size of *Arabidopsis thaliana* leaves (Wojcik *et al.* 2009). As a consequence, feeding exogenous GSH to plants with developed leaves will considerably increase the GSH concentration of the chloroplast and will inhibit the activities of sulphate assimilation enzymes, given that active uptake of external glutathione by chloroplasts takes place (Noctor *et al.* 2002). Thus, adaptation to increased GSH levels in poplar transformants can take place only at the first stages of leaf growth, as far as the chloroplast structure is concerned.

Effects of γ-ECS over-expression on mesophyll cell and photosynthetic tissue organisation

Our results demonstrate that over-expression of γ-ECS in the cytosol is accompanied in poplars by structural changes in photosynthetic tissues and cells. Unlike chloroplasts, elevated GSH in the transformants did not affect cell size, but decreased cell number per leaf area unit, as indicated by the negative correlation between cell density and GSH content of leaves (Fig. 5D). As a result of the reduced cell number per leaf area, mesophyll porosity of the transgenic poplars, *i.e.*, volume portion of intracellular airspaces in mesophyll, increased as compared to wild-type plants at the control site (Fig. 2). Mesophyll porosity is proportional to the intercellular conductance for CO_2 diffusion (Parkhurst 1994), a parameter that is important for leaf gas exchange and response to environmental stress (Flexas *et al.* 2008). Under heavy metal stress, the proportion of airspaces strongly increased in both types of poplar, but in wild-type plants to a higher degree compared to the transformants. In general, mesophyll structure of the transformants was less sensitive to heay metal contamination of the soil. This is consistent with the observation that the contribution of airspaces to mesophyll resistance was strongly proportional to GSH content (Fig. 6B).

Nevertheless, the volume of airspaces is not a major determinant of total internal resistance of the leaf to CO_2 diffusion (Evans *et al.* 1994; Hanba *et al.* 1999), as diffusion in the gaseous phase is 10^{4}-times faster than in the liquid phase of cells (Nobel 1991). Therefore, a negative correlation was obtained between mesophyll porosity and leaf internal conductance (Hanba *et al.* 1999). Mesophyll resistance to CO_2 diffusion depends to a greater degree on total surface area of mesophyll cells and chloroplasts (A_{mes}/A and A_{chl}/A accordingly) (Evans *et al.* 1994; Hanba *et al.* 1999). At the control site, A_{mes}/A was reduced in leaves of transgenic plants as a consequence of a reduced cell number, which could increase mesophyll resistance. However, cell surface area occupied by chloroplasts increased twofold due to chloroplast swelling thereby decreasing mesophyll resistance to CO_2 diffusion in the transformants (Fig. 5C). Thus, the alterations in cell number per leaf area, volume portion of chloroplasts and mesophyll diffusion resistance depend on plant type and heavy metal contamination, and these factors are linked to foliar GSH level.

Another consequence of the decreased cell density of the transformants was a reduced number of chloroplasts per leaf area unit. Over-expression did not effect chloroplast replication since the chloroplast number per cell was unchanged (Ivanova *et al.* 2009); the chloroplast number in leaves declined proportional to cell number and may by related to lower photosynthetic capacity (Mokronosov 1981). Transformed poplars have a twofold increased chloroplast volume fraction per cell, as well as per leaf volume, due to the increase in chloroplast volume (Fig. 3D and H). At the same time, most plants have an inverse relation between chloroplast volume and number per cell (Ellis & Leech 1985). Thus, the total volume of chloroplasts in cells usually remains almost constant (Tselniker 1978). For instance, in *Arabidopsis* mutants with strongly reduced chloroplast number per cell, their sizes were several times larger that required to support the chlorophyll content of the leaf (Austin & Webber 2005). In our case, the increase in volume fraction of chloroplasts was not accompanied by chlorophyll content changes (Table 1). As a result, the chlorophyll concentration in chloroplasts decreased twofold with increasing plastid volume. The chloroplast volume increase reduced the surface/volume

ratio, which can reduce the diffusion of CO_2 from the cytosol to the photosynthetic centres inside chloroplasts. Therefore, chloroplast volume regulation is necessary for efficient photosynthesis (McCain & Markley 1992). In *Arabidopsis*, volume increase and quantity decrease of chloroplasts reduced photosynthetic activity, even with an unchanged chlorophyll amount (Austin & Webber 2005). However, seedlings of transgenic poplar over-expressing γ-ECS in the cytosol did not significantly differ from the wild type in terms of net photosynthesis under laboratory conditions (Herschbach *et al.* 2000).

In summary, structural changes in photosynthetic tissues of the transformants, such as a reduction of A_{mes}/A, decrease in chloroplast number per leaf area and decrease in chloroplast surface/volume ratio, should negatively influence the photosynthetic capacity of leaves. The increase in airspaces and in the share of cell surface occupied by chloroplasts could partly compensate these changes by facilitating CO_2 diffusion inside leaves.

Effects of enhanced GSH level on productivity and tolerance to heavy metals

Investigations of poplar under laboratory conditions did not show differences between γ-ECS transgenic and wild-type plants in terms of plant height, or shoot and root fresh weight (Gullner *et al.* 2001). Our field experiment with γ-ECS transgenic poplars revealed, after 3 years of growth, a marked decrease in total foliage area per plant that was caused to a greater degree by a reduction in single leaf area than by a decrease in leaf number (Fig. 1). The reduction of leaf area resulted from a decline in the total cell number per leaf, indicating inhibition of cells replication, *i.e.*, mitotic activity of cells in the transformants. In previous studies, a strong correlation was observed between GSH content and proliferation capacity of cells in the root apical meristem (May *et al.* 1998). Feeding GSH to roots of spruce seedlings decreased the rate of cell division, induced mitotic abnormalities and affected the ultrastructure of cells (Zellnig *et al.* 2000). The present results imply similar effects of GSH in leaves. Also, other experiments revealed toxicity of exogenous GSH and a negative influence on plant growth (Lee *et al.* 2003; Wojcik *et al.* 2009). The inverse relation between plant height, foliage area and GSH level in poplars (Fig. 4) found in the present study supports the hypothesis of a negative influence of excess GSH on plant productivity (Fig. 7).

Prolonged growth at the contaminated site revealed a higher tolerance of the transformants to heavy metals as compared to the wild type. Heavy metal contamination strongly influenced leaf parameters of wild-type plants: total leaf area per plant decreased sevenfold and single leaf area 2.5-fold, while these leaf features remained unchanged in the transformants. As a result of heavy metal contamination, the biomass gain declined 30-fold in the wild type, but only twofold in the transformants (Fig. 1). An increase in tolerance to heavy metals was not observed in transgenic poplars under laboratory conditions (Arisi *et al.* 2000; Rennenberg & Will 2000; Koprivova *et al.* 2002), although fresh weight and growth rate of transgenic poplars was less reduced by herbicides than that of wild-type plants (Gullner *et al.* 2001). However, enhanced concentrations of phytochelatins due to over-expression of genes for GSH synthesis increased Cd tolerance in *Brassica juncea* (Zhu *et al.* 1999). In our investigation under field conditions, poplars over-expressing γ-ECS in the cytosol were more tolerant to heavy metal stress than wild-type plants, according to a set of parameters including lower reduction in annual biomass gain, stability of leaf expansion, stability of cell and chloroplast parameters and maintenance of replication level of cells and chloroplasts.

Thus, prolonged field exposure of transgenic poplars revealed that over-expression of *gsh1* in the cytosol linked to enhanced GSH levels led to an increase of tolerance to heavy metal contamination. However, the over-expression of *gsh1* also caused changes in the photosynthetic apparatus at different levels of organisation that resulted in decreased productivity as compared to the wild type in the absence of soil contamination with heavy metals. We assume that the transformants are adapted to enhanced GSH synthesis through an increased chloroplast volume that reduces the GSH concentration inside chloroplasts and prevents the inhibitory effect of excess glutathione on enzymes of sulphate assimilation. Reasons for the productivity loss of the transformants at the control site were reduced leaf area due to suppression of cell replication and photosynthetic tissue reorganisation, such as a decrease in cell and chloroplast density.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Bundesministerium für Bildung und Forschung (BMBF), Germany (FKZ 0312638F). We are grateful to Seregey Chrebtikov ('Green Movement' Association, Russia) for excellent technical help.

SUPPORTING INFORMATION

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

Table S1. Summary of two-way analysis of variance (ANOVA).

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

REFERENCES

Arisi A.C., Noctor G., Foyer C.H., Jouanin L. (1997) Modification of thiol contents in poplars (*Populus tremula x P. alba*) over-expressing enzymes involved in glutathione synthesis. *Planta*, **203**, 362–372.

Arisi A-C.M., Mocquot B., Lagriffoul A., Mench M., Foyer C.H., Jouanin L. (2000) Responses to cadmium in leaves of transformed poplars over-expressing γ-glutamylcysteine synthetase. *Physiologia Plantarum*, **109**, 143–149.

Austin J., Webber N. (2005) Photosynthesis in *Arabidopsis thaliana* mutants with reduced chloroplast number. *Photosynthesis Research*, **85**, 373–384.

Bittsánszky A., Kömives T., Gullner G., Gyulai G., Kiss J., Heszky L., Radimszky L., Rennenberg H. (2005) Ability of transgenic poplars with elevated glutathione content to tolerate zinc^{2+} stress. *Environment International*, **31**, 251–254.

Cobbett C., Goldsbrough P. (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology*, **53**, 159–182.

Creissen G., Firmin J., Fryer M., Kular B., Leyland N., Reynolds H., Pastori G., Wellburn F., Baker N., Wellburn A., Mullineaux P. (1999) Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. *Plant Cell*, **11**, 1277–1291.

Edwards R., Dixon D.P. (2005) Plant glutathione transferases. *Methods in Enzymology*, **401**, 169–186.

Ellis J.R., Leech R.M. (1985) Cell size and chloroplast size in relation to chloroplast replication in light-grown wheat leaves. *Planta*, **165**, 120–125.

Evans J.R., von Caemmerer S., Setchell B.A., Hudson G.S. (1994) The relationship between CO_2 transfer, conductance and leaf anatomy in transgenic tobacco with reduced content of Rubisco. *Australian Journal of Plant Physiology*, **21**, 475–495.

Fales F.W. (1951) The assimilation and degradation of carbohydrates by yeast cells. *The Journal of Biological Chemistry*, **193**, 113–124.

Flexas J, Ribas-Carbó M, Diaz-Espejo A, Galmés J, Medrano H. (2008) Mesophyll conductance to CO_2: current knowledge and future prospects. *Plant, Cell and Environment*, **31**, 602–621.

Gullner G., Kömives T., Rennenberg H. (2001) Enhanced tolerance of transgenic poplar plants over-expressing γ-glutamylcysteine synthetase towards chloroacetonilide herbicides. *Journal of Experimental Botany*, **52**, 971–979.

Hanba Y.T., Miyazawa S., Terashima I. (1999) The influence of leaf thickness on the CO_2 transfer conductance and leaf stable carbon isotope ratio for some evergreen tree species in Japanese warm-temperate forests. *Functional Ecology*, **13**, 632–639.

Hartmann T.M., Fricker M.D., Rennenberg H., Meyer A.J. (2003) Cell-specific measurement of cytosolic glutathione in poplar leaves. *Plant, Cell and Environment*, **26**, 965–975.

Hartmann T., Hoenicke P., Wirtz M., Hell R., Rennenberg H., Kopriva S. (2004) Regulation of sulphate assimilation by glutathione in poplars (*Populus tremula x P. alba*) of wild type and over-expressing γ-glutamylcysteine synthetase in the cytosol. *Journal of Experimental Botany*, **55**, 837–845.

Herschbach C., van der Zalm E., Schneider A., Jouanin L., De Kok L., Rennenberg H. (2000) Regulation of sulphur nutrition in wild-type and transgenic poplar over-expressing γ-glutamylcysteine synthetase in the cytosol as affected by atmospheric H_2S. *Plant Physiology*, **124**, 461–473.

Herschbach C., Rizzini L., Mult S., Hartmann T., Busch F., Peuke A.D., Kopriva S., Ensminger I. (2010) Over-expression of bacterial γ-glutamylcysteine synthetase (*GSH1*) in plastids affects photosynthesis, growth and sulphur metabolism in poplar (*Populus tremula x Populus alba*) dependent on the resulting γ-glutamylcysteine and glutathione levels. *Plant, Cell and Environment*, **33**, 1138–1151.

Ivanova L.A., Pyankov V.I. (2002) Structural adaptation of the leaf mesophyll to shading. *Russian Journal of Plant Physiology*, **49**, 419–431.

Ivanova L.A., Ronzhina D.A., Ivanov L.A., Stroukova L.V., Peuke A.D., Rennenberg H. (2009) Chloroplast parameters differ in wild type and transgenic poplars over-expressing *gsh1* in the cytosol. *Plant Biology*, **2009**, 11.

Kopriva S. (2006) Regulation of sulfate assimilation in *Arabidopsis* and beyond. *Annals of Botany*, **97**, 479–495.

Kopriva S., Rennenberg H. (2004) Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism. *Journal of Experimental Botany*, **55**, 1831–1842.

Koprivova A., Kopriva S., Jäger D., Will B., Jouanin L., Rennenberg H. (2002) Evaluation of transgenic poplars over-expressing enzymes of glutathione synthesis for phytoremediation of cadmium. *Plant Biology*, **4**, 664–670.

Laisk A.Kh. (1977) *Kinetika fotosinteza i fotodykhaniya C3-rastenii (Kinetics of photosynthesis and photorespiration in C3 plants)*. Nauka, Moscow, Russia, 196 pp.

Laisk A., Oya V., Rakhi M. (1970) Anatomy-dependent diffusion resistance in leaves. *Fiziologia Rastenij* (*Soviet Plant Physiology*, English translation), **17**, 40–48.

Lee S., Moon J.S., Ko T.-S., Petros D., Goldsbrough P.B., Korban S.S. (2003) Over-expression of *Arabidopsis* phytochelatin synthase paradoxically leads to hypersensitivity to cadmium. *Stress Plant Physiology*, **131**, 656–663.

Leustek T., Martin M.N., Bick J.A., Davies J.P. (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. *Annual Review of Plant Physiology and Plant Molecular Biology*, **51**, 141–165.

Lichtenthaler H.K., Wellburn A.R. (1983) Determination of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochemical Society Transactions*, **603**, 591–592.

May M.J., Vernoux T., Leaver C., Van Montagu M., Inzé D. (1998) Glutathione homeostasis in plants: implications for environmental sensing and plant development. *Journal of Experimental Botany*, **49**, 649–667.

McCain D.C., Markley J.L. (1992) In vivo study of chloroplast volume regulation. *Biophysical Journal*, **61**, 1207–1212.

Mokronosov A.T. (1981) *Ontogeneticheskii aspekt fotosinteza (developmental aspects of photosynthesis)*. Nauka, Moscow, Russia, 196 pp.

Nauchno-prikladnoi spravochnik po klimatu SSSR Permskaya, Sverdlovskaya, Chelyabinskaya, Kurganskaya oblasti, Bashkirskaya ASSR (1990) (A Handbook on the Climate of the Soviet Union: Perm, Sverdlovsk, Chelyabinsk, and Kurgan Oblasts, the Bashkir Autonomous Republic). Gidrometeoizdat, Leningrad, Russia, Ser. 3, issue 9.

Nobel P.S. (1991) *Physicochemical and environmental plant physiology*. Academic Press, San Diego, CA, USA.

Nobel P.S., Walker D.B. (1985) Structure of leaf photosynthetic tissue. In: Barber J., Baker N.R. (Eds), *Photosynthetic mechanisms and the environment*. Elsevier Science, Amsterdam, the Netherlands, pp 501–536.

Noctor G., Foyer C.H. (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology*, **49**, 249–279.

Noctor G., Strohm M., Jouanin L., Kunert K.J., Foyer C.H., Rennenberg H. (1996) Synthesis of glutathione in leaves of transgenic poplar over-expressing γ-glutamylcysteine synthetase. *Plant Physiology*, **112**, 1071–1078.

Noctor G., Arisi A.-C.M., Jouanin L., Kunert K.J., Rennenberg H., Foyer C.H. (1998) Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. *Journal of Experimental Botany*, **49**, 623–647.

Noctor G., Gomez L., Vanacker H., Foyer C. (2002) Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. *Journal of Experimental Botany*, **53**, 1283–1304.

North K.A., Kopriva S. (2007) Sulfur in resistance to environmental stress. In: Hawkesford M.J., De Kok L.J. (Eds), *Sulfur in plants – an ecological perspective*. Springer, Heidelberg, Germany, pp 143–168.

Oguchi R., Hikosaka K., Hirose T. (2005) Leaf anatomy as a constraint for photosynthetic acclimation: differential responses in leaf anatomy to increasing growth irradiance among three deciduous trees. *Plant, Cell and Environment*, **28**, 916–927.

Parkhurst D.F. (1994) Diffusion of CO_2 and other gases inside leaves. *New Phytologist*, **126**, 449–479.

Poorter H., Bergkotte M. (1992) Chemical composition of 24 wild species differing in relative growth rate. *Plant, Cell and Environment*, **15**, 221–229.

Pyankov V.I., Ivanova L.A., Lambers H. (1998) Quantitative anatomy of photosynthetic tissues of plant species of different functional types in a boreal vegetation. In: Lambers H., Poorter H., van Vuuren M.M.I. (Eds), *Inherent variation in plant growth: physiological mechanisms and ecological consequences*. Backhuys, Leiden, the Netherlands, pp 71–87.

Rennenberg H. (2001) Glutathione – an ancient metabolite with modern tasks. In: Grill D., Tausz M., De Kok L.J. (Eds), *Significance of glutathione in plant adaptation to the environment*. Kluwer Academic, Dordrecht, the Netherlands, pp 1–11.

Rennenberg H., Peuke A.D. (2005) Improved phytoremediation of contaminated soils by changes in sulfur metabolism. In: Saito K., de Kok L.J., Stulen I., Hawkesford M., Schnug E., Sirko A., Rennenberg H. (Eds), *Sulfur transport and assimilation in plants in the postgenomic era*. Backhuys, Leiden, the Netherlands, pp 201–208.

Rennenberg H., Will B. (2000) Phytochelatin production and cadmium accumulation in transgenic poplar (*Populus tremula×P. alba*). In: Brunold C., Rennenberg H., De Kok L.J., Stulen I., Davidian J.-C. (Eds), *Sulfur nutrition and sulfur assimilation in higher plants*. Paul Haupt, Berne, Switzerland, pp 393–398.

Strohm M., Jouanin L., Kunert K.J., Pruvost C., Polle A., Foyer C.H., Rennenberg H. (1995) Regulation of glutathione synthesis in leaves of transgenic poplar (*Populus tremula x P. alba*) over-expressing glutathione synthetase. *The Plant Journal*, **7**, 141–145.

Terashima I., Shin-Ichi M., Hanba Y.I (2001) Why are sun leaves thicker than shade leaves? Consideration based on analyses of CO_2 diffusion in the leaf. *Journal of Plant Research*, **114**, 93–105.

Tselniker U.L. (1978) Chloroplast replication, its regulation and significance for photosynthesis. In: Mokronosov A.T. (Ed.), *Mesostructure and functional activity of photosynthetic apparatus*. Ural State University Publ, Sverdlovsk (Yekaterinburg), Russia, pp 31–46.

Wojcik M., Pawlikowska-Pawlega B., Tukiendorf A. (2009) Physiological and ultrastructural changes in *Arabidopsis thaliana* as affected by changed GSH level and Cu excess. *Russian Journal of Plant Physiology*, **56**, 820–829.

Zellnig G., Tausz M., Pesec B., Grill D., Miiller M. (2000) Effects of glutathione on thiol redox systems, chromosomal aberrations, and the ultrastructure of meristematic root cells of *Picea abies* (L.) Karst. *Protoplasma*, **212**, 227–235.

Zhu Y.L., Pilon-Smiths E.A.H., Tarun A.S., Jouanin L., Terry N. (1999) Over-expression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. *Plant Physiology*, **119**, 73–79.

APPENDIX

Description of SIAMS MesoPlant™

SIAMS MesoPlant™ is an automated complex for image analysis of plant photosynthetic apparatus. It consists of three parts – a microscope with video output, image input tools (digital camera and scanner) and SIAMS MesoPlant™ special software. The complex is designed for both laboratory and field use. The software implements algorithms for image improvement, transformation and morphometry, and stereological reconstruction of mesophyll cells. SIAMS MesoPlant™ is an innovative and robust spreadsheet-based image analysis software that delivers 'Natural Automation™' for repetitive image analysis tasks. SIAMS® allows visualising of every step taken in the process while delivering accurate and easily replicable results.

SIAMS MesoPlant™ software includes three modules: 'MACRO' – for leaf lamella analysis; 'MESO' – for analysis of leaf tissues; and 'MICRO' – for analysis of mesophyll cells of plant leaves. The 'MACRO' module allows determination of leaf biometric parameters, including leaf area, leaf perimeter, form coefficients and leaf venation parameters. The leaf image is captured using a scanner or digital camera and displayed on a computer. The 'MESO' module processes images of leaf cross-sections in order to determine partial volumes of leaf tissues. The 'MICRO' module allows measurement of cell area, perimeter, form coefficients and mesophyll cell classification by type (palisade or spongy) based on shape. The images for modules 'MESO' and 'MICRO' are obtained using a light microscope with a digital camera.