RESEARCH PAPER

Sulphur flux through the sulphate assimilation pathway is differently controlled by adenosine 5’-phosphosulphate reductase under stress and in transgenic poplar plants overexpressing γ-ECS, SO, or APR

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Abstract

Sulphate assimilation provides reduced sulphur for the synthesis of cysteine, methionine, and numerous other essential metabolites and secondary compounds. The key step in the pathway is the reduction of activated sulphate, adenosine 5’-phosphosulphate (APS), to sulphite catalysed by APS reductase (APR). In the present study, [35S]sulphur flux from external sulphate into glutathione (GSH) and proteins was analysed to check whether APR controls the flux through the sulphate assimilation pathway in poplar roots under some stress conditions and in transgenic poplars. (i) O-Acetylserine (OAS) induced APR activity and the sulphur flux into GSH. (ii) The herbicide Acetochlor induced APR activity and results in a decline of GSH. Thereby the sulphur flux into GSH or protein remained unaffected. (iii) Cd treatment increased APR activity without any changes in sulphur flux but lowered sulphate uptake. Several transgenic poplar plants that were manipulated in sulphur metabolism were also analysed. (i) Transgenic poplar plants that overexpressed the γ-glutamylcysteine synthetase (γ-ECS) gene, the enzyme catalysing the key step in GSH formation, showed an increase in sulphur flux into GSH and sulphate uptake when γ-ECS was targeted to the cytosol, while no changes in sulphur flux were observed when γ-ECS was targeted to plastids. (ii) No effect on sulphur flux was observed when the sulphite oxidase (SO) gene from Arabidopsis thaliana, which catalyses the back reaction of APR, that is the reaction from sulphite to sulphate, was overexpressed. (iii) When Lemna minor APR was overexpressed in poplar, APR activity increased as expected, but no changes in sulphur flux were observed. For all of these experiments the flux control coefficient for APR was calculated. APR as a controlling step in sulphate assimilation seems obvious under OAS treatment, in γ-ECS and SO overexpressing poplars. A possible loss of control under certain conditions, that is Cd treatment, Acetochlor treatment, and in APR overexpressing poplar, is discussed.

Key words: APS synthetase, flux measurements, glutathione, poplar, roots, sulphate uptake, sulphite oxidase.

Introduction

Plants meet their demand for the essential nutrient sulphur by taking up inorganic sulphate from the soil, reducing it to sulphide, and incorporating it into cysteine (Cys) in the sulphate assimilation pathway (for reviews, see Leustek et al., 2000; Kopriva, 2006). Cys can be further incorporated into proteins or used as a sulphur donor for methionine and glutathione (GSH) synthesis and for a wide range of cofactors and secondary metabolites. Since sulphate is
chemically very stable, it has to be activated before reduction by adenylation to adenosine 5′-phosphosulphate (APS) in a reaction catalysed by ATP sulphurylase (ATPS). APS is reduced to sulphite by APS reductase (APR) and further to sulphide by sulphite reductase (Sir). Cys is synthesized by O-acetylserine(thiol)lyase from sulphide and O-acetylserine (OAS), which is formed in a reaction between serine and acetyl-CoA catalysed by serine acetyltransferase (SAT). A major sink for Cys is the tripeptide GSH, an essential component of the plant stress response and redox homeostasis (Foyer and Rennenberg, 2000; Foyer and Noctor, 2005; Meyer and Hell, 2005; Mullineaux and Rausch, 2005; Cairns et al., 2006; Meyer, 2008). GSH is synthesized in two consecutive enzymatic steps. The first step, catalysed by γ-glutamylcysteine synthetase (γ-ECs), joins Cys with glutamate. In the second step, glycine is added to γ-glutamylcysteine (γ-EC) by the glutathione synthetase (GSHS).

The synthesis of Cys represents a merging point of sulphate assimilation with nitrogen and carbon metabolism and it is thus not surprising that the pathway is strongly regulated by both nitrogen and carbohydrate availability (Reuveny et al., 1980; Brunold and Suter, 1984; Kopriva et al., 1999; Kopriva and Rennenberg, 2004). Sulphate assimilation can also respond to the availability of sulphur in a demand-driven manner (Lappartient and Touraine, 1996; Herschbach et al., 2000). Accordingly, the mRNA levels of most components of the sulphate assimilation pathway are increased in response to sulphur starvation (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003), feeding with OAS (Neuenschwander et al., 1991; Hirai et al., 2003), or after exposure to heavy metals, which induce synthesis of phytochelatins (PCs) and thus a rapid drain of reduced sulphur pools (Nussbaum et al., 1988). On the other hand, when plants are provided with reduced sulphur either by fumigation with hydrogen sulphide (Westerman et al., 2001) or by feeding with Cys or GSH (Vauclare et al., 2002), the pathway is repressed. Regulation of sulphate assimilation is well described and understood at the level of transcripts, enzyme activities, and metabolites, using system approaches as well as targeted analyses (Takahashi et al., 1997; Herschbach et al., 2000; Koprivova et al., 2000; Hopkins et al., 2004; Kawashima et al., 2005; Hirai et al., 2005). Analysis of metabolite composition and enzyme activities describes only a steady state. However, determination of incorporated labelled precursors into products (ap Rees and Hill, 1994) provides information about changes in the flux through a metabolic pathway that is important to understand its control (Fell, 1998). The controlling step and the regulation of this step, that is of the enzyme that controls the flux through a metabolic pathway, have to be distinguished (ap Rees and Hill, 1994). The controlling step means the enzymatic reaction by which the flux through the pathway is limited. APR seems to be the controlling step of assimilatory sulphate reduction in Arabidopsis roots (Vauclare et al., 2002; Kopriva and Rennenberg, 2004). Regulation of the controlling step indicates the mechanism(s) that influence(s) its reaction. Feedback inhibition, covalent modification of enzymes, as well as control of enzyme synthesis and degradation are mechanisms that can alter the flux through a metabolic pathway (Fell, 1992). For assimilatory sulphate reduction many studies revealed a regulation of APR at the transcriptional level by thiols (Bick et al., 2001; Vauclare et al., 2002; Hartmann et al., 2004), amino compounds (Brunold and Suter, 1984; Neuenschwander et al., 1991; Koprivova et al., 2000; Hesse et al., 2004; Hopkins et al., 2005), carbohydrates (Neuenschwander et al., 1991; Kopriva et al., 1999, 2002; Hesse et al., 2003), and hormones (Harada et al., 2000; Ohkama et al., 2002). More recently, APR was also found to be under post-transcriptional control (Bick et al., 2001; Koprivova et al., 2008).

Flux through sulphate assimilation can be estimated by incubation of plants with [35S]sulphate and measurement of the radioactivity in proteins and thiols (Neuenschwander et al., 1991; Kopriva et al., 1999, 2002; Koprivova et al., 2000; Vauclare et al., 2002).

In order to test whether APR controls the flux through sulphate assimilation under different environmental conditions, several treatments known to affect APR activity strongly by increasing the demand for reduced sulphur were selected. These treatments included (i) OAS exposure which greatly promotes Cys synthesis (Neuenschwander et al., 1991); (ii) Cd exposure that induces PC synthesis from GSH (Nussbaum et al., 1988); and (iii) exposure to Acetochlor, a pesticide which is detoxified via conjugation to GSH by glutathione S-transferase (GST; Jablonkai and Hatzios, 1991). Poplar was chosen because (i) this tree species is discussed as a plant that could be useful in phytoremediation of heavy metals and herbicides (Peuke and Rennenberg, 2005a, b); (ii) transgenic lines overexpressing γ-ECs that possess increased amounts of GSH are available; and (iii) sulphate assimilation is well understood in this species (Strohm et al., 1995; Arisi et al. 1997; Noctor et al., 1998; Hartmann et al., 2004; Kopriva et al., 2004; Rennenberg et al., 2007). In the present study, the 35S flux from sulphate taken up by excised roots of wild type (WT) poplar into GSH was determined under different environmental conditions. As a more precise calculation of APR flux control coefficients requires a wide range of changes in the
enzyme activity, *Lemna minor APR* was overexpressed in poplar. In addition, the effects on the $^{35}S$ flux of modulated levels of sulphite oxidase (SO), an enzyme which counter-acts sulphite production via APR by producing sulphate from sulphite (Eilers et al., 2001; Hänsch et al., 2006; 2007), were analysed. The effects of these treatments and metabolic modifications on the flux through sulphate assimilation in excised poplar roots and the consequences for the contribution of APR to flux control of this pathway are reported.

**Materials and methods**

**Plant material and growth conditions**

Cuttings of the poplar hybrid *Populus tremula* × *P. alba*, clone 717 1B4 (Institut National de la Recherche Agronomique, INRA) and all transgenic lines were micropropagated as described by Strohm et al. (1995) and Noctor et al. (1996). After 4 weeks, cuttings were transferred onto quartz sand (0.7–2 mm, Götz & Moritz, Freiburg, Germany) and were grown in a greenhouse (22 ± 5°C) under long day conditions. Seedlings were watered with a modified Hoagland solution consisting of 0.125 mM KNO$_3$, 0.25 mM Ca(NO$_3$)$_2$, 0.05 mM MgSO$_4$, 0.45 mM MgCl$_2$, 0.05 mM KH$_2$PO$_4$, 2 μM MnSO$_4$, 10 μM H$_2$BO$_3$, 0.2 μM CuSO$_4$, 0.2 μM ZnSO$_4$, 0.2 μM Na$_2$MoO$_4$, 0.04 μM CoSO$_4$, 0.1 mM FeSO$_4$, and 0.095 mM NaEDTA.

**Transgenic poplar lines**

Transgenic poplar lines that express the (35S) promoter, the coding region of the gene (cDNA) from *Escherichia coli* and target the protein either to the cytosol (line gg28) or to plastids (lines Lggs6, Lggs12, and Lggs20) were described previously (Arisi et al., 1997; Noctor et al., 1998). APR is considered the key enzyme of the sulphate assimilation pathway (reviewed in Kopriwa and Kopriwa, 2004). To analyse the impact of increased APR activity on S metabolism in poplar, APR cDNA from *Lemna minor* (Suter et al., 2000) was overexpressed in the 717 1B4 clone of *P. tremula* × *P. alba* under the control of the constitutive 35S promoter. Transgenic poplar lines overexpressing APR (lines 303, 304, 391, and 404) were constructed as follows: the cDNA encoding the complete APR open reading frame was amplified from total RNA of *Lemna minor* and cloned into the pCR2 plasmid carrying EcoRI and BamHI restriction sites at the 5’ and 3’ end, respectively (Suter et al., 2000). The restriction sites were used to prepare a translational fusion with the 35S promoter in the plasmid pCK/2 × 3SS. Transgenic lines that overexpressed the SO gene (cDNA) from *Arabidopsis thaliana* under the control of either the constitutive cauliflower mosaic virus (CaMV) 35S promoter (lines 158-2, 159, and 161) or the leaf-specific promoter from tobacco (ST-LS1 promoter; Stockhaus et al., 1987) (lines 93, 150-1, and 185) were created by PCR amplification of SO from pQE60-SO (Eilers et al., 2001). XhoI and BamHI restriction sites at the 5’ and 3’ end were used to clone the PCR fragment into the plasmids pCK/2 × 3SS; BamHI restriction sites at both ends are used for the creation of a translational fusion of the gene in sense orientation with the ST-LS1 promoter. HindIII fragments containing the chimeric genes consisting of the promoter, the coding region of the genes, and 3SS terminator were cloned into the binary vector pBin19 (Bevan, 1984).

WT poplars (clone 717 1B4) were transformed with these constructs using *Agrobacterium*-mediated transformation, according to published protocols (Leplé et al., 1992). Regenerated kanamycin-resistant plants were tested for the presence of the transgenes by genomic PCR, for the amount of protein by western blot, and for enzyme activity. Plants with the highest activity compared with WT poplars were selected, propagated, and analysed further.

**$^{35}S$Sulphate feeding**

Fine roots were sampled from 2- to 5-month-old poplar seedlings grown in sand. The roots were washed with water to remove sand particles. Fine roots up to a diameter of 1 mm were separated from the poplar root system and transferred into 10 ml of an incubation solution (Hoagland; see above) adjusted to 0.1 mM SO$_4^{2-}$. After 2 h pre-incubation, the incubation solution was exchanged and sulphate uptake measurement was started by adding 150 μCi (5.55 × 10$^7$Bq) of carrier-free $[^35]$SO$_4^{2-}$ (Hartmann Analytic, Braunschweig, Germany). After 4 h, sulphate uptake was stopped by washing roots three times with the incubation medium. Root samples were frozen in liquid nitrogen and stored until analyses at −80°C.

**Treatments**

OAS at 1 mM was applied to fine roots during the whole incubation, that is during the 2 h pre-incubation and 4 h incubation with $[^35]$S sulphate. Cd treatment was performed by adding 0.5 mM CdCl$_2$ to the watering solution for 4 weeks prior to the experiment, but was omitted in the incubation solutions. The herbicide Acetochlor was added to the watering solution for 2 d (66 μg ml$^{-1}$) before $[^35]$S sulphate uptake experiments were started.

**$[^35]$S analyses**

$[^35]$S sulphate uptake was determined by measurement of radioactivity in 20 mg of root tissue (powdered under liquid nitrogen) as reported by Herschbach and Rennenberg (1996). After solubilization with a tissue solubilizer (Soluene 350, Packard Instruments, Frankfurt, Germany) root samples were bleached with 200 μl of H$_2$O$_2$ (30%) overnight. After adding 5 ml of scintillation fluid (HiSafe 2, Packard Instruments, Frankfurt, Germany), radioactivity was determined by scintillation counting (Wallac System 1409, Wallac, Turku, Finland) and was corrected for quenching.

**$[^35]$S metabolite analyses**

Thiols were determined as described by Hartmann et al. (2000). A 30 μg aliquot of fine root tissue (powdered under liquid nitrogen) was homogenized in 750 μl of 0.1 M HCl that contained 50 mg of insoluble polyvinylpyrrolidone (PVPP). Root samples were centrifuged and 120 μl of the clear supernatant were added to 180 μl of CHES buffer (200 mM, pH 9.3). Reduction of thiols was performed with dithiothreitol (DTT; 30 μl, 15 mM) for 1 h at room temperature. Thiols were derivatized with monobromobimane (20 μl, 30 mM) and stabilized by adding 240 μl of acetic acid (10%, v/v) after 15 min of derivatization. Aliquots of 130 μl were taken to separate bimane conjugates by HPLC (SUPEROSILTM LC-18, 25 cm × 4.6 mm, 5 μm, Sigma-Aldrich) according to Schupp and Rennenberg (1988) using 10% (v/v) methanol, 0.25% (v/v) acetic acid (pH 3.9) as solvent A and 90% (v/v) methanol, 0.25% (v/v) acetic acid (pH 3.9) as solvent B. Bimane derivatives were detected by fluorescence detection (Schupp and Rennenberg, 1988). To determine the amount of $[^35]$S in thiols, 1 ml fractions of the eluate were collected with a fraction collector; after adding 4 ml of scintillation fluid (HiSafe 3, Packard Instruments, Frankfurt, Germany) the radioactivity was determined by liquid scintillation counting and classified as GSH by comparison with the fluorescent detector output. Acid-insoluble $[^35]$S was determined as reported by Herschbach and Rennenberg (1996). Sulphate was extracted and determined from 50 μg of root tissue (powdered under liquid nitrogen) by anion exchange chromatography (Herschbach et al., 2000). Radioactivity in sulphate was determined in 1.2 ml fractions collected after anion exchange chromatography.
APR measurements and protein determination

Fresh root material (~70–90 mg) was homogenized in 1 ml of extraction buffer (50 mM sodium/potassium phosphate buffer pH 8 with additions of 30 mM Na₂SO₃, 0.5 mM AMP, and 10 mM DTT) in a mortar. APR activity was measured using 20 μl of the extract by measuring the acid-volatile ³⁵S formed in the presence of [³⁵S]APS and DTT according to Brunold and Suter (1990).

The amount of protein was determined as described by Bradford (1976) using bovine serum albumin (BSA) as a standard. For determination of radioactivity in protein, 30 mg of frozen root powder of root tissue was homogenized in 1 ml of extraction buffer. Protein was precipitated in 10% (v/v) trichloroacetic acid (TCA) for 30 min on ice, washed, and re-dissolved in 0.2 M NaOH as described (Vauclare et al., 2002). The amount of ³⁵S incorporated into protein was determined in the re-dissolved protein by liquid scintillation counting after adding 5 ml of liquid scintillation fluid (HiSafe 2).

Calculation of flux control coefficients

In the present study, labelled [³⁵S]sulphate was used and its flow into thiols and proteins of excised poplar roots was analysed in relation to treatments or genetic modifications affecting APR activity. The ‘metabolic control analysis’ described by Vauclare et al. (2002) and Fell (1998) was used to calculate the flux control coefficient for this enzyme. For this purpose, the equation of Vauclare et al. (2002) was applied:

\[ C_{APR}^{flx} = \Delta \ln J / (\Delta \ln E_{APR} + \varepsilon \Delta \ln \text{APS}) \]

where \( C_{APR}^{flx} \) = the flux control coefficient, \( \Delta \ln J \) = changes in flux, \( \Delta \ln E_{APR} \) = changes in APR activity, and \( \Delta \ln \text{APS} \) = changes in APS. The minimum value of the control coefficient would be obtained when the elasticity (ε) of APR to a change in APS is 1. Changes in APS are assumed to be proportional to changes in internal SO₄²⁻ (the latter is used to calculate the flux control coefficient in the present study as described by Vauclare et al., 2002), when the first enzyme of the sulphate assimilation, the ATPS, was near equilibrium and exerted little control (Vauclare et al., 2002). This seems to be the case in poplar roots tested under nitrogen and sulphur deficiency (Kopriva et al., 2004). The interpretation is based on the summation theorem (Kacser and Burns, 1973), that is the sum of the flux control coefficients of the enzymes involved in a metabolic system is assumed to be 1. Thus for calculating the flux control coefficients in the present study, the calculation was started at internal sulphate and ended at the sulphate assimilation into GSH and protein (Vauclare et al., 2002). Significant differences between the control (WT) and the treatment or the transgenic lines were analysed according to the Student’s t-test for \( P < 0.05 \).

Results

Changes in sulphur metabolism due to different treatments

In order to address the dynamics of sulphate assimilation under different environmental conditions in poplar roots, the flux through the pathway was analysed. For this purpose, [³⁵S]sulphate was fed to excised fine roots, and sulphate uptake rates, total sulphate, GSH and protein contents were determined, as well as the ³⁵S flux into the sulphate, GSH, and protein pools. In addition, the activity of the key enzyme of the pathway, APR, was determined. After 4 h incubation with [³⁵S]sulphate, radioactivity was detected in all fractions, that is in sulphate, GSH, and proteins. The specific activity of the sulphate pool was always lower than the specific ³⁵S activity in the GSH fraction (Table 1).

OAS treatment: OAS treatment of excised fine roots resulted in a 1.8-fold increased APR activity, but it did not affect the sulphate uptake rate in the present experiment. Simultaneously, the metabolite contents, that is sulphate, GSH, and protein contents, did not change due to OAS exposure. Nevertheless, an ~2-fold higher flux of ³⁵S into the GSH pool and a reduced flux into the protein pool was found (Fig. 1). This was accompanied by a 3-fold increase in the ratio of soluble to insoluble [³⁵S]sulphur (Table 1) and by a higher specific ³⁵S activity of the GSH pool, although this increase was not significant at the \( P=0.05 \) level.

Table 1. Sulphate uptake rates, specific ³⁵S activities of the sulphate and GSH fraction, and the ratio between soluble and insoluble ³⁵S

<table>
<thead>
<tr>
<th></th>
<th>Sulphate uptake (nmol g⁻¹ FW h⁻¹)</th>
<th>Specific activity of sulphate (dpm nmol⁻¹)</th>
<th>Specific activity of glutathione (dpm nmol⁻¹)</th>
<th>³⁵S soluble/³⁵S insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAS control</td>
<td>25.5±17.2</td>
<td>793±332</td>
<td>3961±2495</td>
<td>2.6±0.9</td>
</tr>
<tr>
<td>OAS</td>
<td>24.2±8.8</td>
<td>1137±615</td>
<td>6331±3581</td>
<td>8.0±3.5*</td>
</tr>
<tr>
<td>Cd control</td>
<td>6.8±2.0</td>
<td>907±505</td>
<td>3388±972</td>
<td>4.8±1.8</td>
</tr>
<tr>
<td>Cd</td>
<td>4.6±1.1*</td>
<td>1063±277</td>
<td>2900±1486</td>
<td>3.3±1.0*</td>
</tr>
<tr>
<td>Acetochlor control</td>
<td>12.4±9.3</td>
<td>1329±1058</td>
<td>3065±958</td>
<td>8.7±2.7</td>
</tr>
<tr>
<td>Acetochlor</td>
<td>11.8±7.9</td>
<td>1018±576</td>
<td>4424±2192</td>
<td>7.3±2.3</td>
</tr>
<tr>
<td>Wild type</td>
<td>21.8±11.6</td>
<td>2167±596</td>
<td>4802±2091</td>
<td>2.3±0.7</td>
</tr>
<tr>
<td>APR sense</td>
<td>17.3±12.0</td>
<td>1224±913</td>
<td>3422±1462</td>
<td>2.3±0.8</td>
</tr>
<tr>
<td>Wild type</td>
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<td>5050±2487</td>
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<tr>
<td>ggs28</td>
<td>33.1±24.3</td>
<td>6285±4121</td>
<td>6170±386</td>
<td>4.4±1.0*</td>
</tr>
<tr>
<td>Lggs</td>
<td>12.3±6.3</td>
<td>1712±1018</td>
<td>3577±1453</td>
<td>1.5±0.5*</td>
</tr>
<tr>
<td>Wild type</td>
<td>22.5±10.6</td>
<td>1818±946</td>
<td>1816±761</td>
<td>6.5±2.4</td>
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<td>SO CaMV 35S</td>
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<td>1811±713</td>
<td>2582±1185</td>
<td>4.2±2.1</td>
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<tr>
<td>SO ST-LS</td>
<td>31.5±14.9</td>
<td>2434±1174</td>
<td>2662±1422</td>
<td>5.5±3.2</td>
</tr>
</tbody>
</table>

* Significant differences from the control treatment or from the wild type poplar trees at \( P < 0.05 \).
**Cd treatment**: Poplar trees were pre-treated with 0.5 mM CdCl₂ added to the Hoagland nutrient solution during watering for 4 weeks. This treatment resulted in an 18-fold increase in APR activity of the fine roots. Protein and GSH contents were not affected, while the sulphate content was reduced by ~50% (Fig. 2). Accordingly, sulphate uptake in Cd-treated plants was significantly reduced by 22% (Table 1). However, ³⁵S flux into internal sulphate, GSH, and protein was given as pmol ³⁵S g⁻¹ FW h⁻¹ (light blue squares). APR activity is indicated in a green square as nmol mg⁻¹ protein min⁻¹. Significant differences at P < 0.05 from control roots without Cd are indicated by asterisks.

**Acetochlor treatment**: Application of the herbicide Acetochlor to poplar plants 2 d prior the experiment did not affect sulphate uptake (Table 1) or the sulphate content in fine roots (Fig. 3). Also the ³⁵S flux into the internal sulphate pool was comparable in both Acetochlor-treated and control roots. However, a 42% reduction of GSH levels was observed although APR activity increased 5-fold. Surprisingly, ³⁵S flux into GSH and protein remained unaffected by the herbicide treatment.

**Changes in sulphur metabolism due to manipulation of gene expression**

**γ-ECS overexpression**: In the present study, GSH content was found to be 2-fold higher in the fine roots of line ggs28 (Figs 4C, 5B) and APR activity was also increased. As expected, overexpression of γ-ECS led to a significant increase in ³⁵S flux into the GSH pool while the flux to proteins was not different compared with the WT control (Fig. 5B). Sulphate uptake was enhanced in ggs28 plants (Table 1), thus leading to increased ³⁵S flux into the internal sulphate pool (Fig. 5). Consequently, the ratio between soluble and insoluble ³⁵S was significantly enhanced.

Targeting γ-ECS to the plastids in another set of transgenic lines (Lggs) increases GSH levels in leaves to the same extent as in the ggs lines where the enzyme is targeted to the cytosol (Noctor et al., 1998). However, flux analysis in fine roots of these lines did not reveal any differences from WT plants (Fig. 5C). Sulphate uptake (Table 1), ³⁵S fluxes, APR activity, and metabolite levels were not different in Lggs and WT plants. Nevertheless, a significant reduction in the ratio between soluble and insoluble ³⁵S was detected (Table 1).

**APR overexpression**: Fifty independent kanamycin-resistant plantlets that overexpressed *Lemma minor APR* were grown in tissue culture and were tested for APR activity in the leaves. Four lines (303, 304, 391, and 404) with 17- to 72-fold increased foliar APR activity (data not shown) were selected and further propagated. Initially, the plants did not show any obvious morphological differences compared with WT poplars when grown on sandy soil, although with prolonged growth on perlite, sand, and humus soil, some alterations in leaf shape and increased branching were

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**Fig. 1.** Sulphur metabolite contents and ³⁵S flux into different metabolite pools of OAS-treated poplar roots. Excised fine roots from poplar trees were pre-treated without (A, n=12) or with 1 mM OAS (B, n=12) for 2 h and subsequently exposed to [³⁵S]sulphate plus 1 mM OAS for 4 h for sulphate uptake and ³⁵S flux measurements. Sulphate (µmol g⁻¹ FW), GSH (nmol g⁻¹ FW), and protein (mg g⁻¹ FW) contents were determined (pink squares). ³⁵S flux into internal sulphate, GSH, and protein is given as pmol ³⁵S g⁻¹ FW h⁻¹ (light blue squares). APR activity is indicated in a green square as nmol mg⁻¹ protein min⁻¹. Significant differences at P <0.05 from control roots without OAS are indicated by asterisks.

**Fig. 2.** Sulphur metabolite contents and ³⁵S flux into different metabolite pools of fine roots excised from poplar plants pre-treated with Cd. Fine roots were excised from poplar trees watered without (A, n=10) or with 0.5 mM Cd (B, n=10) in the nutrient solution for 4 weeks. The excised fine roots were pre-incubated for 2 h and subsequently exposed to [³⁵S]sulphate for 4 h to measure sulphate uptake and ³⁵S flux into different metabolite pools. Sulphate (µmol g⁻¹ FW), GSH (nmol g⁻¹ FW), and protein (mg g⁻¹ FW) contents were determined (pink squares). ³⁵S flux into internal sulphate, GSH, and protein is given as pmol ³⁵S g⁻¹ FW h⁻¹ (light blue squares). APR activity is indicated in a green square as nmol mg⁻¹ protein min⁻¹. Significant differences at P <0.05 from control roots without Cd pre-treatment are indicated by asterisks.
Fig. 3. Sulphur metabolite contents and $^{35}$S flux into different metabolite pools of fine roots excised from poplar plants pretreated with Acetochlor. Fine roots were excised from poplar trees watered without (A, $n=9$) or with 66 $\mu$g ml$^{-1}$ Acetochlor (B, $n=9$) in the nutrient solution for 2 days. The excised fine roots were pre-incubated for 2 h and subsequently exposed to $[^{35}S]$sulphate for 4 h to measure sulphate uptake and $^{35}$S flux into different metabolite pools. Sulphate (nmol g$^{-1}$ FW), GSH (nmol g$^{-1}$ FW), and protein (mg g$^{-1}$ FW) contents were determined (pink squares). $^{35}$S flux into internal sulphate, GSH, and protein is given as pmol $^{35}$S g$^{-1}$ FW h$^{-1}$ (light blue squares). APR activity is indicated in a green square as nmol mg$^{-1}$ protein min$^{-1}$. Significant differences at $P<0.05$ from control roots without Acetochlor pretreatment are indicated by asterisks.

Other parameters such as sulphate (Fig. 4G) and protein content as well as APR activity (Fig. 4H) of the roots remained unchanged. However, the GSH content was lower than in WT plants or lines overexpressing the gene under the control of the 35S promoter when the roots of all transgenic lines overexpressing SO under the control of the leaf-specific promoter ST-LS1 were combined (Fig. 7).

Flux control coefficients

Flux control coefficients were calculated from the data of the presented experiments. Several conditions revealed that APR indeed is the main controller of sulphate assimilation. This was obvious from (i) OAS treatment ($C_{APR}^1=0.72$); (ii) from transgenic poplars overexpressing $\gamma$-ECS and targeting the protein either to the cytosol ($C_{APR}^1=0.41$) or to plastids ($C_{APR}^1=1.11$); and (iii) from transgenic poplars overexpressing SO, either under the CaMV 35S promoter ($C_{APR}^1=0.71$) or under the ST-LS1 promoter ($C_{APR}^1=0.49$). However, this was not evident for certain stress conditions. Cd ($C_{APR}^1=0.003$) and Acetochlor ($C_{APR}^1=-0.11$) treatments and also analyses of roots from APR overexpressing poplar plants ($C_{APR}^1=-0.29$) revealed that the flux control of sulphate assimilation via APR may be disturbed.

Discussion

There are four main ways by which the maximum catalytic activity of a given enzyme can be altered: inhibitors, induction/repression, mutation, and genetic manipulation (ap Rees and Hill, 1994). In the present S flux analysis study, APR activity was induced by OAS, Cd, and Acetochlor treatment, and several transgenic lines that overexpressed genes of the sulphur metabolism were used. The activity of the key enzyme of the sulphate assimilation pathway, APR, was determined to allow calculation of the flux control coefficient for the $^{35}$S flux into GSH and protein (Vauclare et al., 2002). After 4 h of incubation with $[^{35}S]$sulphate, radioactivity was detected in all metabolic fractions, that is in sulphate, GSH, and proteins. The specific activity of the sulphate pool was always lower than the specific $^{35}$S activity in the GSH pool (Table 1), since the cytosolic $[^{35}S]$sulphate pool was apparently diluted during extraction by sulphate from the vacuole. These results clearly show that vacuolar sulphate is not involved in GSH formation in poplar roots in the present study. The higher specific activity of the GSH pool can only be achieved when the $[^{35}S]$sulphate taken up into the cytosol is immediately transported into plastids for sulphate assimilation and further GSH synthesis. Thus, for the model to calculate the flux control coefficient used in the present study, consideration of compartmentation of metabolites is unnecessary. However, these results support the assumption of a defined pathway leading from external sulphate into Cys that legitimizes the calculation of flux control coefficients. Cys itself is the first product and a branching point from which methionine, proteins, GSH, and secondary sulphur-containing compounds are produced (Bergmann and Rennenberg,
Control of sulphate assimilation by APR

The flux control coefficient calculated in the present study from OAS treatment indicates that sulphate assimilation in poplar roots is controlled by APR. OAS stimulates sulphate assimilation in potato (Hopkins et al., 2005) and in Arabidopsis (Koprivova et al., 2000; Hesse et al., 2003), and the flux of $^{35}$S into both GSH and protein in Arabidopsis roots (Koprivova et al., 2000). Also in the present experiments with excised poplar roots, OAS enhanced the $^{35}$S flux into GSH and protein, which correlated with higher $^{35}$S in the acid-soluble fraction and increased APR activity. The flux control coefficient estimated a control of the sulphur flux through the sulphate reduction and assimilation pathway by APR of 72%. This strong control is consistent with findings of Koprivova et al. (2000) on the effect of feeding various nitrogen compounds to N-starved Arabidopsis plants. The closer the N source was related metabolically to OAS the higher was the incorporation of $^{35}$S into GSH and protein. However, OAS can also act as a positive regulator of sulphate transporter transcription and sulphate uptake (Smith et al., 1997; Hopkins et al., 2005; Hawkesford and De Kok, 2006). In the present study, sulphate uptake was not induced in OAS-treated poplar roots. This observation agrees with findings from Arabidopsis. There it was shown that the expression of AtSULTR1;1 and AtSULTR1;2 did not reveal a relationship to the internal OAS content (Rouached et al., 2008). Thus, sufficient sulphate supply to the roots can be assumed under the experimental conditions, that is despite low N supply.

GSH levels cannot only be modulated by external application of metabolites, but also by genetic manipulation of enzymes involved in sulphate assimilation and GSH synthesis (reviewed in Rennenberg et al., 2007). Increased GSH contents in poplar were observed in glutathione reductase (GR) (Foyer et al., 1995), $\gamma$-ECS (Strohm et al., 1995; Noctor et al., 1996, 1998; Arisi et al., 1997), and APR overexpressing poplar (Rennenberg et al., 2007). The best characterized example of transgenic plants with increased GSH levels are poplars expressing the bacterial GSH biosynthesis genes (Strohm et al., 1995; Arisi et al., 1997; Noctor et al., 1998). Overexpression of $\gamma$-ECS and targeting the protein to the cytosol was associated with a 2- to 4-fold increase in GSH levels in leaves and in roots (Arisi et al., 1997; Herschbach et al., 2000; Hartmann et al., 2004).

Overexpression of $\gamma$-ECS, the control step in GSH synthesis (Strohm et al., 1995; Noctor et al., 1996), and targeting the protein to the cytosol led to increased GSH contents in roots (Fig. 4C, line ggs28) as also found in previous studies for the line ggs28 (Herschbach et al., 2000; Hartmann et al., 2004). As Cys was shown to limit GSH synthesis in leaves (Noctor et al., 1996), one pre-requisite for flux analyses after ap Rees and Hill (1994).
for enhanced GSH synthesis is sufficient Cys formation. Hence, a higher sulphur flux through the sulphate assimilation pathway is required. This was indeed observed in the present experiments. 35S flux into GSH and proteins of transgenic poplar roots was significantly enhanced. Although poplar plants were grown at very low nitrogen nutrition, OAS seems not to be a limiting factor in this transgenic line. Thus APR activity increased and the flux control coefficient calculated for the APR activity revealed a control of only 40%. Vauclare et al. (2002) have already demonstrated the importance of sulphate uptake as a controlling step in sulphate assimilation. Sulphate uptake rates were not enhanced with the numbers of replicates in the present study. However, increased sulphate uptake rates have been demonstrated in an earlier experiment (Herschbach et al., 2000). The high variance of sulphate uptake rates by roots excised from poplar plants was observed across all experiments and results from a high standard deviation (Table 1). Nevertheless, the present results from Lggs transgenic poplar lines indicate a very strong control of the sulphur flux through the sulphate assimilation pathway by APR in the roots. Since neither the flux nor the APR activity was significantly different between transgenic and WT roots, it seems that the high flux control coefficient reflects a high control of the pathway already in the WT. The fact that, in
with such a barrier and allows increased GSH synthesis. Consequently, APR activity is increased when γ-ECS is targeted to the cytosol to provide the reduced sulphur necessary to sustain the increased GSH synthesis, but not when the γ-ECS is targeted to the plastids.

SO catalyses the back reaction from sulphite to sulphate, however, localized in peroxisomes. Thus, this enzyme is clearly separated spatially from the sulphate reduction pathway (Nowak et al., 2004; Hänsch and Mendel, 2005), in particular from APR which is located exclusively in plastids (Kopriva and Koprivova, 2004). The biological function of SO is still under debate (Hänsch et al., 2007). One established function is the detoxification of sulphite that is important especially under high atmospheric SO2 (Lang et al., 2007). However, SO is constitutively expressed in all tested tissues without any pronounced diurnal rhythm (Hänsch et al., 2007) and, thus, SO may be an essential housekeeping protein. Theoretically SO overexpression enforces the back reaction of APR (Nowak et al., 2004; Hänsch and Mendel, 2005). As SO may act against the normal flow of sulphate assimilation, overexpression of SO may diminish the availability of free sulphite for further reduction and Cys formation. Nevertheless, the flux control coefficients calculated from analyses of roots from transgenic poplar overexpressing SO revealed a control via APR of 72% and 49% for the sulphur flux through the sulphate assimilation pathway. Thus, the present results did not provide any indication that SO significantly disturbed or reduced the control of sulphate assimilation by APR. A further approach to test the capacity of SO keeping sulphite at non-toxic levels could be a simultaneous overexpression of both APR and SO.

Possible disturbance of the control of sulphate assimilation by APR

Despite numerous previous reports and the present results with γ-ECS or SO overexpressing plants, APR was found not to be important for the pathway control under several environmental conditions. When APR was overexpressed in poplar and when WT plants were treated with Cd or Acetochlor, negative flux control coefficients were calculated for APR. It seems that in these cases the control of sulphate assimilation via APR activity is disturbed. The common reason could be that under these conditions APR activity was at least 5-fold enhanced compared with the controls. Nevertheless, Cd treatment and APR overexpression did not enhance GSH levels in root tissues. In leaves of APR overexpressing poplars GSH levels enhanced to a similar extent were found when other enzymes of the sulphur assimilation pathway were overexpressed (reviewed in Rennenberg et al., 2007). Thus, the 35S flux through sulphate assimilation into GSH seems to be subject to an additional control. When Pseudomonas aeruginosa APR (PaAPR) was overexpressed in Arabidopsis, a strong deregulation of sulphate assimilation, evident from increased levels of sulphite, thiosulphate, Cys, γ-EC, and GSH, was observed and led to plant injury (Tsakraklides et al., 2002).
Increasing GSH contents were also found in *Zea mays* overexpressing the same gene (Martin *et al.*, 2005), but not in roots of transgenic poplar lines overexpressing *Lemna APR*, although leaf Cys and GSH contents increased (Ch Herschbach, unpublished results). As Cys formation in transgenic *Arabidopsis* overexpressing PaAPR might be limited by the availability of OAS (Tsakraklides *et al.*, 2002), nitrogen limitation in the present experiment may also limit OAS formation in poplar roots and, as a consequence, Cys and GSH formation. Therefore, at very high APR activity sulphate assimilation seems to be controlled at another metabolic step. From the results presented for the OAS treatment of poplar roots (increased flux into GSH, see above) the most likely step that may control Cys and GSH synthesis in APR overexpressing poplar roots seems to be the synthesis of OAS via serine acetyltransferase.

After long-term Cd exposure, APR activity increased and the flux of $^{35}$S through sulphate assimilation seems not to be controlled by APR, as indicated by the flux control coefficient. Similar to the findings of the present study, APR activity was also found to be increased upon Cd treatment in excised roots from *Brassica juncea* (Lee and Leustek, 1999). The increased APR activity indicates a high need for reduced sulphur. Surprisingly, the flux of $^{35}$S into GSH and protein of poplar roots was not affected. Moreover a diminished rate of sulphate uptake resulted in a reduction of $^{35}$S in the soluble fraction inside the roots. Cd can react with thiol groups of proteins and may inhibit sulphate uptake by a deactivation of sulphate transporter protein as a consequence of covalent binding. In contrast, increased uptake of sulphate and higher expression of SULTR genes have been observed in *Zea mays* upon Cd exposure (Nocito *et al.*, 2002, 2006). In the present study, $^{35}$S flux into GSH of Cd-treated poplar roots was not affected at reduced sulphate uptake. This indicates the preferential channelling of the sulphate that has been taken up into GSH synthesis. Under these conditions, the selected pathway with GSH and protein as an end-product does not take into consideration synthesis of PCs as additional end-products. Thus, under Cd exposure, flux analyses should be extended to PCs for the correct calculation of the flux control coefficient. This assumption is consistent with the observation that the GSH content was unchanged in poplar roots upon Cd exposure, as also found in previous studies (Koprivova *et al.*, 2002). In these studies, long-term Cd treatment left the GSH content unaffected, but increased the PC content in *Brassica juncea* (Zhu *et al.*, 1999a, b) and poplar (Koprivova *et al.*, 2002), whereas a decline in GSH after short-term Cd exposure corresponded to enhanced PC contents in maize roots (Nocito *et al.*, 2002, 2006). PCs are involved in heavy metal detoxification (Rauser, 1995, 1999; Cobbett, 2000a, b), and sulphur flux into PCs may be an additional sink of $^{35}$S taken up by the roots under Cd exposure. It may therefore be assumed that taking into consideration $^{35}$S flux into PCs can restore the control via APR, or that phytochelatin synthase constitutes a controlling step of sulphate assimilation under Cd exposure. In addition, the low nitrogen supply during plant growth may limit Cys synthesis via OAS availability. However, since all enzymes of the sulphate reduction pathway can be affected by Cd treatment (Ernst *et al.*, 2008), it cannot be excluded that the loss of control of sulphur flux through the sulphate assimilation pathway by APR is a consequence of the interaction of Cd with other enzymes.

Short-term application of Acetochlor resulted in a low contribution of APR to the control of sulphate assimilation. Although APR activity increased dramatically and the GSH content decreased, changes in the flux of $^{35}$S into GSH, sulphate, or proteins were not found. The slight increase in the specific $^{35}$S activity of the GSH pool may be an indication for a preferential channelling of the sulphate taken up into GSH for Acetochlor detoxification. Thus, processes involved in Acetochlor conjugation with GSH and the degradation of the conjugation product such as the reactions catalysed by GST (Edwards *et al.*, 2000) and/or phytochelatin synthase (Grzam *et al.*, 2006; Blum *et al.*, 2007) may execute flux control under these conditions. GST consumes GSH by its conjugation with xenobiotics and thereby contributes to herbicide detoxification. Thus, synthesis of GSH conjugates may have reduced the GSH content in poplar roots exposed to Acetochlor and may have removed GSH from the pools measured for flux determination. GSH conjugates, synthesized when Acetochlor is bound to GSH via GST (Jablonski and Hatzios, 1991; Mezzari *et al.*, 2005; Cho and Kong, 2008), are thought to be transported into the vacuole for further metabolism (Coleman *et al.*, 1997a, b). Recently phytochelatin synthase was shown to be involved in this metabolism (Grzam *et al.*, 2006; Blum *et al.*, 2007). Hence, both enzymes, GST and phytochelatin synthase, may take over the control of the flux through the sulphate assimilation pathway during xenobiotic exposure. On the other hand, as mentioned above, calculation of $^{35}$S flux rates into PCs and GSH conjugates may restore the control via APR. However, also during Acetochlor treatment OAS limitation due to the low nitrogen supply may be limiting Cys synthesis under high demand.

**Experimental constraints of flux analyses through the sulphate assimilation pathway**

For estimation of the flux control coefficient of APR, Vauclare *et al.* (2002) defined several assumptions: (i) for a given treatment there are no routes other than their effect on APR. This means that the change in APR activity must be specific for the treatments, that is for the present study for OAS, Cd, or Acetochlor exposure. This assumption was not confirmed in the current experiments with Cd and Acetochlor exposure. In these experiments, further metabolic steps that include PC synthesis and GSH–Acetochlor conjugation must be taken into account when the control via APR is discussed. (ii) The sulphate assimilation pathway starts at internal sulphate. Even Vauclare *et al.* (2002) calculated that sulphate uptake contributes significantly to the control of sulphur flux through the sulphate assimilation pathway. Hence under Cd treatment where sulphate uptake
was reduced and in the transgenic poplar line ggs28 that revealed a higher sulphate uptake, the sulphate uptake step has to be taken into account for metabolic control analysis. (iii) ATPS is near equilibrium and exerts little control, so that the internal sulphate reflects the APS concentration.

Other important assumptions concerning application of metabolic control analysis have been summarized by ap Rees and Hill (1994). The metabolites are distributed evenly throughout the tissue, cells, and compartments. The sulphate pool measured includes the sulphate from the cytosol and all organelles including the vacuole. As the vacuolar sulphate does not seem to contribute significantly to the flux through sulphate assimilation up to GSH, metabolic control analysis can be applied. Furthermore, caution is advisable when transgenic plants are analysed (ap Rees and Hill, 1994). It is crucial that only the enzyme in question is shown that other enzymes of sulphate assimilation are affected. In the case of γ-ECS overexpression it has not been shown that other enzymes of sulphate assimilation are affected. In the case of γ-ECS overexpression in the cytosol (ggs28) or in plastids (Lggs), and in the case of SO overexpression, APR activity was not affected. In conclusion, calculation of the flux control coefficient in the present study revealed some critical aspects, but nevertheless provided important information on the control of sulphur flux through the sulphate assimilation pathway.

Conclusion

The presented results show that the control of sulphur flux through the sulphate assimilation pathway in poplar roots was not apparent under certain conditions. This seems to be the case during Cd and Acetochlor treatment as well as in transgenic poplar overexpressing Lemma APR. Sulphur flux through the sulphate assimilation pathway may also be controlled by ATPS (Lappartient et al., 1999) or by the serine acetyltransferase/O-acetylserine(thiol)lyase complex (Wirtz and Hell, 2006). Furthermore, whether the primary site for Cys synthesis is in the mitochondria (Heeg et al., 2009) or in the cytosol (Krueger et al., 2009) is still under discussion. Therefore, differentiated analyses of the sulphur flux through the sulphate assimilation pathway that include separation of the sulphur pools of each compartment are required. A complete realistic view thus can only be obtained if all enzymatic steps of the sulphate assimilation pathway with their corresponding flux control coefficients are determined and if all metabolites that integrate 35S are analysed in further studies. This is of great importance because analyses of APR overexpression showed complete loss of control of sulphur flux via APR, whereas this was not the case when γ-ECS was overexpressed. Hence other component(s) of sulphate assimilation seem to take over the control in APR overexpressing poplar. On the other hand, APR overexpression and also γ-ECS overexpression did not affect sulphate assimilation only in roots but also in the shoot (Hartmann et al., 2004). GSH can be transported in the phloem to the roots (Hartmann et al., 2000). Thus, in addition, whole plant interactions, most importantly shoot to root signalling, may also influence the sulphur flux through the sulphate assimilation pathway in roots. This may also be important during Cd exposure when GSH and PCs increased in leaves (Koprivova et al., 2002). Furthermore, under conditions where GSH is increasingly used for other metabolic pathways such as PC synthesis and herbicide detoxification via GST, the sulphur flux into these compounds needs to be investigated.

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