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# Sulphur limitation and early sulphur deficiency responses in poplar: significance of gene expression, metabolites, and plant hormones

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## Abstract

The influence of sulphur (S) depletion on the expression of genes related to S metabolism, and on metabolite and plant hormone contents was analysed in young and mature leaves, fine roots, xylem sap, and phloem exudates of poplar (*Populus tremula*×*Populus alba*) with special focus on early consequences. S depletion was applied by a gradual decrease of sulphate availability. The observed changes were correlated with sulphate contents. Based on the decrease in sulphate contents, two phases of S depletion could be distinguished that were denominated as 'S limitation' and 'early S deficiency'. S limitation was characterized by improved sulphate uptake (enhanced root-specific sulphate transporter Pta*SULTR1;2* expression) and reduction capacities (enhanced adenosine 5′-phosphosulphate (APS) reductase expression) and by enhanced remobilization of sulphate from the vacuole (enhanced putative vacuolar sulphate transporter Pta*SULTR4;2* expression). During early S deficiency, whole plant distribution of S was impacted, as indicated by increasing expression of the phloem-localized sulphate transporter Pta*SULTR1;1* and by decreasing glutathione contents in fine roots, young leaves, mature leaves, and phloem exudates. Furthermore, at 'early S deficiency', expression of microRNA395 (miR395), which targets transcripts of Pta*ATPS3/4* (ATP sulphurylase) for cleavage, increased. Changes in plant hormone contents were observed at 'early S deficiency' only. Thus, S depletion affects S and plant hormone metabolism of poplar during 'S limitation' and 'early S deficiency' in a time series of events. Despite these consequences, the impact of S depletion on growth of poplar plants appears to be less severe than in Brassicaceae such as *Arabidopsis thaliana* or *Brassica* sp.

**Key words:** APS reductase, ATP sulphurylase, miR395, plant hormones, poplar, sulphate transporter (SULTR), sulphur deficiency.

## Introduction

Sulphur (S) is one of six macroelements which are essential for proper plant growth and development. It forms part of amino acids and proteins and plays an important role in redox control of cellular processes (reviewed in Foyer and Noctor, 2009) and in plant defence mechanisms (reviewed in Rausch and Wachter, 2005; Noctor, 2006). Plants take up S mainly in the form of sulphate from the soil. Within the plant, sulphate either remains in the roots or is transported via the xylem to the leaves, the main sites of sulphate reduction and assimilation (reviewed in Rennenberg, 1984; Brunold, 1990). Part of the sulphate from the leaves is redistributed back to the roots via the phloem (Hartmann

*et al.*, 2000; Herschbach and Rennenberg, 2001). Furthermore, sulphate is largely stored in the vacuoles (Leustek and Saito, 1999) and can be mobilized from these storage compartments if required (Bell *et al.*, 1994, 1995). All these sulphate distribution processes within the plant are controlled by sulphate transporters (SULTRs) (reviewed in Buchner *et al.*, 2004*b*; Hawkesford and De Kok, 2006). These processes require a complex regulation at the whole plant level and have to be coordinated with sulphate reduction and assimilation (reviewed in Kopriva and Rennenberg, 2004; Takahashi, 2010; Takahashi *et al.*, 2011). Sulphate uptake and its distribution within the plant are thought to be driven by the demand for reduced S compounds (reviewed in Davidian and Kopriva, 2010). Sulphate itself, but also cysteine and/or glutathione (GSH), are supposed to serve as signals in this demand-driven regulation (Rennenberg *et al.*, 1988, 1989; Herschbach and Rennenberg, 1991; Lappartient *et al.*, 1999; Herschbach *et al.*, 2000; Hartmann *et al.*, 2004; van der Zalm *et al.*, 2005). Beside these S compounds, other compounds such as *O*-acetylserine, the precursor of cysteine, and sugars also influence sulphate uptake and assimilation, emphasizing the strong regulatory connection with nitrogen and carbon metabolism (reviewed in Kopriva and Rennenberg, 2004). Moreover, plant hormones such as cytokinins and auxins, but also stress-related hormones such as abscisic acid (ABA) or jasmonate, are believed to be involved in the regulation of S metabolism (reviewed in Kopriva, 2006; Takahashi *et al.*, 2011).

S depletion has been shown to induce enhanced sulphate uptake and assimilation capacity, which are strongly regulated at the transcriptional level (Bolchi *et al.*, 1999; Lee and Leustek, 1999; Vidmar *et al.*, 1999; Takahashi *et al.*, 2000; Nikiforova *et al.*, 2003; Buchner *et al.*, 2004*a*, *b*; Nocito *et al.*, 2006; Parmar *et al.*, 2007). This response includes increased expression of several sulphate transporters and of enzymes involved in sulphate assimilation such as ATP sulphurylase (ATPS) and adenosine 5′-phosphosulphate (APS) reductase (APR) (Bolchi *et al.*, 1999; Vidmar *et al.*, 1999; Takahashi *et al.*, 2000; Hirai *et al.*, 2003; Nikiforova *et al.*, 2003; Buchner *et al.*, 2004*a*; Kataoka *et al.*, 2004; Maruyama-Nakashita *et al.*, 2004*a*, 2006; Parmar *et al.*, 2007). Still considerable differences were observed between genes and species in their responsiveness to S depletion. In *Arabidopsis* and *Brassica*, the expression of *SULTR1;1* and *SULTR4;2* seems to be more susceptible to S depletion than the expression of *SULTR1;2* and *SULTR4;1* (Yoshimoto *et al.*, 2002; Buchner *et al.*, 2004*a*; Kataoka *et al.*, 2004; Parmar *et al.*, 2007; Barberon *et al.*, 2008). Also the expression of different ATPS isoforms was regulated differently upon S depletion. Expression of the *Arabidopsis* ATPS At*APS3* increased during S depletion (Liang *et al.*, 2010; Kawashima *et al.*, 2011), while results for At*APS2*, the putatively cytosolic isoform (Hatzfeld *et al.*, 2000; Rotte and Leustek, 2000), were inconsistent between different studies (Logan *et al.*, 1996; Takahashi *et al.*, 1997; Kawashima *et al.*, 2011). At*APS1*, At*APS3*, and At*APS4* were shown to be post-transcriptionally regulated by microRNA395 (miR395), and the expression of miR395 was shown to be induced upon

S depletion (Jones-Rhoades and Bartel, 2004; Kawashima *et al.*, 2009, 2011; Liang *et al.*, 2010). However, a different cellular or subcellular localization of miR395 and its targets can prevent the post-transcriptional regulation and might result in a discrepancy between gene expression and enzyme activity of AtAPS (Kawashima *et al.*, 2009, 2011).

Beside these changes in gene expression, S depletion is also accompanied by changes in metabolite concentrations such as decreasing levels of glutathione which were shown to repress sulphate uptake and assimilation (Rennenberg *et al.*, 1988, 1989; Herschbach and Rennenberg, 1991, 1994; Bolchi *et al.*, 1999; Lappartient *et al.*, 1999; Vidmar *et al.*, 1999, 2000; Vauclare *et al.*, 2002; Hartmann *et al.*, 2004). Phenotypically, the first visible S depletion symptoms appear on young leaves which constitute the main sink organ for newly acquired sulphate (Burke *et al.*, 1986; Anderson, 2005). Nutrient deficiency in general induces root growth (Lopez-Bucio *et al.*, 2003) which might result in a rising sink capacity for sulphate in the roots. This enhanced sink capacity of the roots may decrease the transport of newly acquired sulphate via the xylem to the leaves and presumably causes the strong impact of S depletion on young leaves (Clarkson and Hawkesford, 1993; Anderson, 2005). In *Brassica napus*, the overall decreasing sulphate content within the plant during sulphate depletion is accompanied by a shift in the transport direction of remobilized sulphate from mature leaves in favour of the roots instead of young leaves (Abdallah *et al.*, 2010). This is consistent with an enhanced sink capacity for sulphate of the roots and implies changes in activity and abundance of SULTRs involved in the plant-internal distribution of sulphate.

Plant hormones are also involved in the regulation of sulphate uptake and assimilation. The participation of cytokinins in regulation of S metabolism was demonstrated for At*SULTR1;1* and At*SULTR1;2*. Expression of both genes was negatively affected by cytokinins in roots of *Arabidopsis thaliana* (Maruyama-Nakashita *et al.*, 2004*b*; Werner *et al.*, 2010). In contrast, Ohkama *et al.* (2002) showed a positive transcriptional regulation of At*APR1* and At*SULTR2;2* by cytokinins in leaves of *A. thaliana*. The involvement of auxin in regulation of S metabolism was demonstrated by increasing expression and activity of nitrilase 3 (NIT3) upon S depletion in *Arabidopsis* (Kutz *et al.*, 2002). This indicates an enhanced production of auxin upon degradation of glucosinolates, resulting in support of root growth and branching (Kutz *et al.*, 2002). However, this auxin-dependent regulation relates to the glucosinolate pathway which exists almost exclusively in the plant family order of Brassicales (Halkier and Gershenzon, 2006).

Although the involvement of plant hormones such as cytokinins and auxins in regulation of S metabolism has been demonstrated, investigations revealing changes in the endogenous plant hormone contents during S depletion are extremely rare. The present study was aimed to characterize the consequences of S depletion in poplar, including endogenous changes of plant hormones parallel to the analyses of the expression of a broad spectrum of genes involved in S uptake and metabolism and of thiol and sugar contents.

Poplar was chosen as the model species for trees (Cronk, 2005; Jansson and Douglas, 2007) which are assumed to possess a large S storage capacity (Rennenberg *et al.*, 2007), and because of its commercial relevance in short rotation forestry for biomass/bioenergy production (Ceulemans and Deraedt, 1999; Mitchell *et al.*, 1999). Such plantations might be the first 'forests' to be affected by the decreasing atmospheric S inputs (Schnug, 1991; McGrath and Zhao, 1996; Zhao *et al.*, 1999). S depletion was induced by a gradual decrease of sulphate availability that allowed classification of S depletion responses into different phases in a time-resolved manner. To address the question of whole plant regulation in a comprehensive way, differences in the composition of S metabolites, sugars, and plant hormones were analysed not only in fine roots, young leaves, and mature leaves, but also in xylem sap and phloem exudates.

## Materials and methods

*Plant material*

The experiment was carried out with the poplar hybrid *Populus tremula*×*Populus alba*, clone 717 1B4 (Institute National de la Recherche Agronomique, INRA, France). Plants were micropropagated by cuttings according to Strohm *et al.* (1995) and Noctor *et al.* (1996). Thirty-day-old poplar cuttings were transferred to pots of 1.0 l in size containing quartz sand (grain size 0.7–1.2 mm). The sand in each pot was pre-treated with 200 ml of 25% modified Hoagland solution (Hoagland and Arnon, 1950) [0.6 mM $KNO_3$, 1.3 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.3 mM $MgSO_4 \cdot 7H_2O$, 1.5 mM $MgCl_2 \cdot 6H_2O$, 0.25 mM $KH_2PO_4$, 2.3 µM $MnCl_2 \cdot 4H_2O$, 10 µM $H_3BO_3$, 0.08 µM $CuCl_2 \cdot 4H_2O$, 0.2 µM $ZnCl_2$, 0.2 µM $Na_2MoO_4 \cdot 4H_2O$, 0.04 µM $CoCl_2 \cdot 6H_2O$, 22.5 µM $Na_2$-EDTA, 22.5 µM $FeCl_2$, pH 5.5] including 0.15% fungicide solution (Proplant, Dr. Stählern GmbH, Stade, Germany). Plants were cultivated in a greenhouse (26±5 °C) under long day conditions (described by Hartmann *et al.*, 2000) and were watered with 25% modified Hoagland solution.

*S depletion treatment*

S depletion was started after 9 weeks of plant growth on sand by changing 25% modified Hoagland solution to S-free medium (25% modified Hoagland solution without $MgSO_4$, but with 1.8 mM $MgCl_2 \cdot 6H_2O$). Prior to the beginning of the depletion treatment, the sand in each pot was flushed three times with 200 ml of 1:10 diluted S-free medium to reduce the sulphate content in the sand. Controls were treated in the same way but were watered afterwards with full nutrient solution. S depletion was started time-shifted according to a time series of 0, 2, 5, 9, 14, and 21 d so that all plants were harvested on the same day. During the whole treatment, medium passing through the sand and flowing out of the pot was collected and analysed for sulphate content by anion exchange chromatography (described below). In each case the outflow from five plants standing together in one tray was mixed (one data point in Fig. 1). These measurements demonstrated that sulphate availability to the plants was reduced already by flushing the sand and further decreased with prolonged treatment with S-free medium (see Fig. 1).

Five replicates, each a pool of two plants, were harvested per every point of the time series (six different time points). For harvest, poplar plants were separated into (i) young leaves (third to sixth leaf counted from the first leaf with a clear petiole); (ii) mature leaves (ninth and 10th leaf); and (iii) fine roots with a diameter up to 1 mm. Fine roots were rinsed with tap water and dried with paper tissue. Additionally, xylem sap and phloem exudates were collected at each time point.
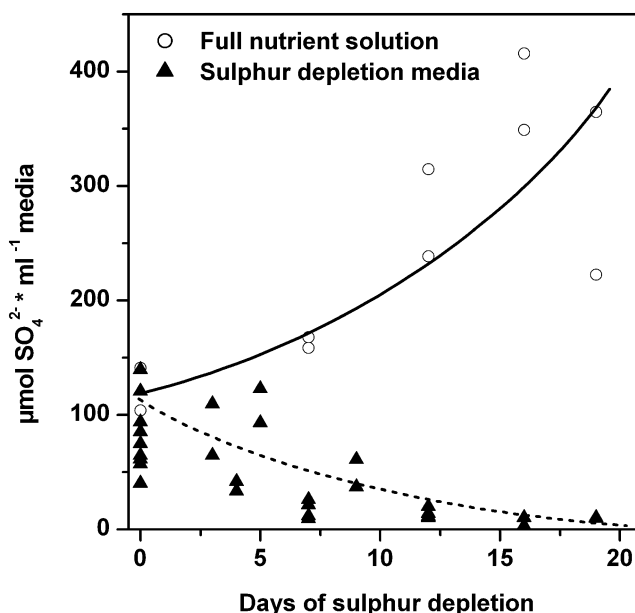


**Fig. 1.** Sulphate content in the flow-through solution during sulphur depletion treatment. Before starting sulphur depletion treatment, each plant pot was flushed three times with 1/10 diluted sulphur depletion medium (0 d). Solid (control) and dashed (sulphur depletion treatment) lines indicate approximated sulphate contents in the flow-through solution after watering the pots with full nutrient solution (filled triangle) or sulphur-free media (white circles), respectively. Each symbol represents one measurement which was collected as a mixture from the flow-through solution of five plants standing together in one tray.

Xylem sap was taken from the basal stem section of the poplar plants using the modification of the gas pressure technique of Scholander *et al.* (1965) described by Rennenberg *et al.* (1996). Phloem exudates were collected from bark pieces taken from the region around mature leaves (Rennenberg *et al.*, 1996). For thiol measurements, the phloem exudation method described by Herschbach *et al.* (1998) was applied. Ion and carbohydrate analyses were performed with exudates collected as reported by Gessler *et al.* (2004). All samples were frozen in liquid nitrogen and stored at –80 °C until analysis.

*Extraction of total RNA and cDNA preparation*

All tissue samples were homogenized in liquid nitrogen. Total RNA was extracted from 90 mg of leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol or from 100 mg of ground fine roots using the method described by Kolosova *et al.* (2004). The purity and the amount of RNA were determined using a nano-spectrophotometer (Nano-Drop® ND-1000, Peqlab, Erlangen, Germany) and the quality of the RNA was checked on a 1% agarose gel. To prevent contamination with genomic DNA, total RNA was treated with DNase I (Fermentas, GmbH, St.Leon-Rot, Germany) prior to cDNA synthesis. The reverse transcription reaction was performed with 1 µg of total RNA using a final concentration of 500 nM random primer R12 [5′-NNN NNN NNN NNN-3′] and Superscript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Samples without reverse transcriptase were prepared for each sample and used as a control.

*Isolation and expression analyses of poplar gene sequences*

The gene-specific primers used for isolation of partial gene sequences of Pta*ATPS1/2*, Pta*ATPS3/4*, Pta*ANT* (*Aintegumenta*),

**Table 1.** Sequence comparison between predicted protein ATP sulphurylase sequences of *Populus trichocarpa* and protein sequences of *Arabidopsis thaliana*

Similarities were calculated by CLUSTALW (SDSC Biology Work-Bench 3.2, http://workbench.sdsc.edu/, Thompson *et al.*, 1994) using pairwise alignment with the BLOSUM matrix and an open gap penality of 11 and a gap extension of 1.

| | AtAPS1 (At3g22890) | AtAPS2 (At1g19920) | AtAPS3 (At4g14680) | AtAPS4 (At5g43780) |
|---|---|---|---|---|
| PtcATPS1 (protein ID: 754047) | 70% | 82% | 72% | 73% |
| PtcATPS2 (protein ID: 818792) | 71% | 83% | 73% | 74% |
| PtcATPS3 (protein ID: 720943) | 78% | 67% | 75% | 72% |
| PtcATPS4 (protein ID: 658460) | 77% | 66% | 76% | 73% |

and Pta*bTub* (*β-tubulin*) were prepared according to sequence information in the *Populus trichocarpa* genome database (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html, Tuskan *et al.*, 2006) and are listed in Supplementary Table S1 available at *JXB* online. Four gene models were described in *P. trichocarpa* for Ptc*ATPS* (Table 1). By comparing the predicted amino acid sequence of these models with the protein sequences of *A. thaliana*, Ptc*ATPS1* and Ptc*ATPS2* were more similar to AtAPS2 while Ptc*ATPS3* and Ptc*ATPS4* were more closely related to AtAPS1, 3, and 4 (Table 1). Similarities were calculated by CLUSTALW (SDSC Biology Work-Bench 3.2, http://workbench.sdsc.edu/, Thompson *et al.*, 1994) using pairwise alignment with the BLOSUM matrix and an open gap penalty of 11 with a gap extension of 1. Because each two of the four Ptc*ATPS* gene sequences were highly similar (93% identity score for Ptc*ATPS1* and Ptc*ATPS2* and 92% between Ptc*ATPS3* and Ptc*ATPS4*), primers were designed so that they fit to both of the closely related sequences. The corresponding gene sequences were named accordingly Pta*ATPS1/2* and Pta*ATPS3/4*.

The PCRs to isolate the gene sequences of *P. tremula*×*P. alba* were performed using a standard Taq-DNA Polymerase (Fermentas GmbH, St.Leon-Rot, Germany) according to the manufacturer's instructions with cDNA from fine roots and leaves of *P. tremula*×*P. alba*. The amplified DNA segment was separated on a 0.8% agarose gel and subsequently ligated into the pCR2.1 vector (Invitrogen). The vector was transformed into *Escherichia coli* cells (INVF′α, Invitrogen) and the cloned gene segment was sequenced (Eurofins MWG Operon, Ebersberg, Germany) and verified by alignment with the corresponding gene sequences of *P. trichocarpa* (ALIGN function of SCSC Biology WorkBench, http://workbench.sdsc.edu/, Thompson *et al.*, 1994). Sequences for sulphate transporter (*SULTR*) genes were taken from Dürr *et al.* (2010), the sequence for *APR* from Kopriva *et al.* (2004), and the sequence for elongation factor 1β (*Ef1b*) from Wildhagen *et al.* (2010).

Expression analyses were performed using quantitative real-time PCR (qRT-PCR). The set-up was performed as described by Wildhagen *et al.* (2010) with 2.5 μl (25 ng) of cDNA per reaction. Sequences of the oligonucleotide primers used for gene expression analysis with qRT-PCR and the resulting length of the PCR fragment are listed in Supplementary Table S2 at *JXB* online. The specificity of the primer pairs was tested by qRT-PCR, subsequent agarose gel electrophoresis of the amplified segments, and by sequencing of the amplification products.

### Extraction of total RNA including small RNAs

Extraction of total RNA including the small RNA fraction was performed either using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions (leaf tissues) or using a modified method of Kolosova *et al.* (2004) and Chang *et al.* (1993) (fine roots). For the latter, 100 mg of ground plant tissue were mixed with 1 ml of extraction buffer [2% cetyltrimethylammonium bromide (CTAB), 2% (w/v) PVP K40, 100 mM TRIS-HCl, pH 8.0, 25 mM $Na_2EDTA$, 2 M NaCl, 0.5 g $l^{-1}$ spermidine, supplemented with 2% (v/v) 2-mercaptoethanol before use and pre-warmed at 65 °C] by vigorous shaking. After 10 min incubation at 65 °C in

a water bath with shaking every 2 min, RNA was extracted twice with 1 ml of chloroform/isoamylalcohol (24:1, v/v) followed by centrifugation at 15 000 *g* for 15 min at 4 °C. RNA in the supernatant was precipitated by adding 1 ml of 100% ethanol containing 1/20 vol. of 1 M acetic acid and by incubation at –20 °C overnight. After centrifugation (15 000 *g* for 15 min at 4 °C), the pellet was washed three times with 75% ethanol and dissolved in nuclease-free water (Fermentas). RNA concentrations were determined using a nano-spectrophotometer (NanoDrop® ND-1000, Peqlab) and by comparison with control RNA of known concentration separated on an agarose gel. Separation on agarose gel was also used to check the quality of the RNA. Isolated RNA samples were stored at –80 °C.

### Northern blot analysis for microRNAs

Aliquots of 15 μg (fine roots and young leaves) or 70 μg (young and mature leaves) of total RNA were separated in a 13% polyacrylamide gel containing 8.3 M urea in 1×TBE buffer and electroblotted onto nylon membranes (Hybond-NX for 15 μg of total RNA and Hybond-N+ for 70 μg of total RNA; Amersham, Freiburg, Germany) for 1 h at 400 mA. Oligonucleotides complementary to miRNA sequences were radiolabelled with [γ-$^{32}$P]ATP using T4 polynucleotide kinase (Fermentas). The oligonucleotide probes used were 5′-GAGTTCCTCCAAACCCTTCAG-3′ (miR395) and 5′-ACGGGATTCTGCAATTCACAC-3′ (5S rRNA). The oligonucleotide sequence for detecting miR395 corresponds to the mature sequence of miR395a of *P. trichocarpa* (accession no. MIMAT0002031; taken from the microRNAs database 'miRbase' (http://www.mirbase.org). Blot hybridization was carried out in a buffer containing 0.05 M sodium phosphate (pH 7.2), 1 mM EDTA, 6× SSC, 1× Denhardt's, and 5% SDS. Blots were washed 2–3 times with 2× SSC, 0.2% SDS, and once with 1× SSC, 0.1% SDS. Subsequently, blots were hybridized and washed at temperatures 10 °C below the $T_m$ of the oligonucleotide.

### Detection of RNA cleavage products by RNA ligase-mediated 5′ random amplification of cDNA ends (RLM 5′ RACE)

RLM 5′ RACE-PCR was performed with a modified GeneRacer Kit (Invitrogen) protocol (Llave *et al.*, 2002). PCRs were performed using the GeneRacer forward primer 5′-CGACTGGAGCACGAG-GACACTGA-3′ and the gene-specific reverse primer 5′-TTCTAT-CACCTCCAAGTCCCCTCCAATC-3′ followed by a PCR with the nested GeneRacer forward primer 5′-GGACACTGACATG-GACTGAAGGAGTA-3′ and the nested gene-specific reverse primer 5′-CTTCAACGTAAGGTAAACCAGGGGCAGT-3′. Amplification products corresponding to the size of expected cleavage products were excised from the gel, cloned, and sequenced.

### Determination of anions

Sulphate and phosphate contents were analysed using anion exchange chromatography as described by Herschbach *et al.* (2000). Aliquots of 50 mg of powdered tissue were added to 1 ml

of deionized $H_2O$ containing 100 mg of polyvinylpolypyrrolidone (PVPP). The mixtures were shaken for 1 h at 4 °C, boiled for 10 min, and then centrifuged for 10 min at 15 000 $g$ at 4 °C. The supernatants were diluted (1:1 for fine roots, 1:4 for leaves) before they were injected into the chromatography system DX-120 (Dionex, Idstein, Germany). A 300 μl aliquot of xylem sap or 1 ml of phloem exudates was mixed with 20 mg of PVPP and treated in the same way as tissue samples except for the boiling step. Both supernatants were subjected undiluted to anion exchange chromatography. The flow-through media collected from the pots after watering the plants were diluted at a ratio of 1:4 with deionized $H_2O$ and analysed directly. In each case, 50 μl were injected into the ion chromatography system.

### Measurement of soluble carbohydrates

For analysis of soluble carbohydrates, tissue samples were prepared in the same way as described for anion analyses. Samples of xylem sap and phloem exudates were diluted prior to extraction (1:100 xylem sap, 1:20 phloem exudates). Soluble carbohydrates were separated, detected, and quantified by HPLC as described by Jaeger *et al.* (2009). Tissue samples were analysed (Dionex DX 500 HPLC system; Dionex, Sunnyvally, USA) after dilution (1:10 for fine roots, 1:40 for leaves), whereas xylem sap and phloem exudates were measured without further dilution. In each case, 50 μl of sample were injected into the HPLC system.

### Determination of thiol contents

Thiol contents in tissue samples were measured as described by Strohm *et al.* (1995) and Herschbach *et al.* (2000) using 30 mg of powdered leaf tissue or 100 mg of powdered root tissue. Phloem exudates were analysed according to Herschbach *et al.* (2000), and thiol contents in xylem sap were analysed as described by Schupp *et al.* (1991) and Herschbach *et al.* (2000).

### Plant hormone measurements

For plant hormone measurements, powdered tissue samples were lyophilized for 5 d (Freeze dryer: Alpha 2-4 with an LDC-1M modul for system control; Martin Christ, Germany). Freeze-dried tissue samples (each 10–15 mg) were analysed for plant hormone contents as described by Kojima *et al.* (2009). A 300 μl aliquot of xylem sap and 2 ml of phloem exudates were directly lyophilized and were subjected completely for plant hormone analyses.

### Statistical analyses

All statistical analyses were performed using the software R version 2.8.1 for windows 2000 (R Development Core Team). For testing statistical differences between groups, analysis of variance (ANOVA; R function 'aov()') was performed combined with the Tukey's honest significant differences test (R function 'TukeyHSD()') as post-hoc test. Not normally distributed (tested either by Kolmogorov–Smirnov-test (R function 'lillie.test()') or by Shapiro–Wilk test (R function 'shapiro.test()')) or non-homogeneous data {tested using the Bartlett test (R function 'bartlett.tes()') for homogeneity of variances} were either transformed using an appropriate transformation or subjected to the non-parametrical Kruskal–Wallis rank sum test including the multiple comparison test after Kruskal–Wallis.

Bivariate Pearson correlation (for normally distributed data) or Spearman's rank correlation analysis (for non-normally distributed data) were used to analyse correlations of transcript levels, thiol, sugar, or individual plant hormone contents with sulphate contents. The correlation coefficient ρ (Pearson) or $ρ_s$ (Spearman) and the corresponding *P*-values were calculated by using the function 'cor.test()'. Quantile–quantile plots (R function 'qqnorm') and appropriate statistical tests (Kolmogorov–Smirnov-test (R function 'lillie.test()') or Shapiro–Wilk test (R function 'shapiro.test()') were applied to test for normal distribution of the residuals. Further-more, data were analysed for homogeneity of variance and linearity of the residuals (residuals plotted over fitted values) as well as for heteroscedasty (Goldfeld–Quandt test; R function 'gqtest()').

### Accession numbers

Sequences can be found in the NCBI database (http://www.ncbi.nlm.nih.gov) under the following accession numbers for *P. tremula*×*P. alba*: DQ906929 (Pta*SULTR1;1*), DQ174472 (Pta*SULTR1;2*), DQ174474 (Pta*SULTR2;2*), DQ906930 (Pta*SULTR4;1*), DQ906935 (Pta*SULTR4;2*), HM853668 (Pta*ATPS1/2*), HM853669 (Pta*ATPS3/4*), AY353089 (Pta*APR*), FJ372570 (Pta*Ef1b*), HM853671 (Pta*bTub*), and HM853670 (Pta*ANT*).

## Results

### Changes in anion, thiols, and carbohydrate contents

Sulphate contents used as an indicator of the S status in the plants decreased successively in fine roots, young leaves, and mature leaves in response to S depletion (Fig. 2A1, B1, C1). The strongest decrease was found in young leaves, where sulphate contents declined significantly already after 2 d of S depletion. After 9 d of S depletion, the sulphate content in fine roots, and young and mature leaves was almost halved compared with the control (0 d of S depletion). After 21 d of S depletion, sulphate contents in young and mature leaves were close to zero, whereas considerable amounts of sulphate were still present in fine roots.

Phosphate contents were determined to obtain an indication of the general nutrient status of the plant. Phosphate contents varied in response to S depletion in fine roots and young leaves, but not in mature leaves (Fig. 2A2, B2, C2). After 2 d and 5 d of S depletion, phosphate contents decreased in fine roots, and after 2, 5, and 14 d in young leaves. None of these changes coincided with the successive decline of the sulphate contents.

Contents of GSH declined in fine roots, and in young and mature leaves with decreasing sulphate contents (Fig. 2A3, B3, C3). The strongest decline in GSH content was observed in young leaves where a first significant reduction was already found after 5 d of S depletion and a second after 21 d. In fine roots and mature leaves, a significant decrease of the GSH content was first observed after 21 d of S depletion. Comparable changes were not observed for cysteine or γ-EC contents (Supplementary Fig. S1A1-2, B1-2, C1-2 at *JXB* online).

The sugars glucose, fructose, and sucrose were determined because of their regulatory influence on sulphate metabolism (Kopriva *et al.*, 1999; Hesse *et al.*, 2003; Kopriva and Rennenberg, 2004). Sucrose contents in fine roots did not change in response to S depletion except for a transient decline after 2 d of S depletion (Fig. 2A4) that coincided with the decrease in the phosphate, but not the sulphate content. In contrast, sucrose contents in young leaves increased with decreasing sulphate contents (Fig. 2B4). A first significant increase was found after 9 d, and a second after 14 d and 21 d of S depletion. In mature leaves, a clear tendency of changes in sucrose contents was not observed during S depletion (Fig. 2C4). Glucose and fructose contents remained unaffected in roots, except for a peak value after 5 d of S depletion
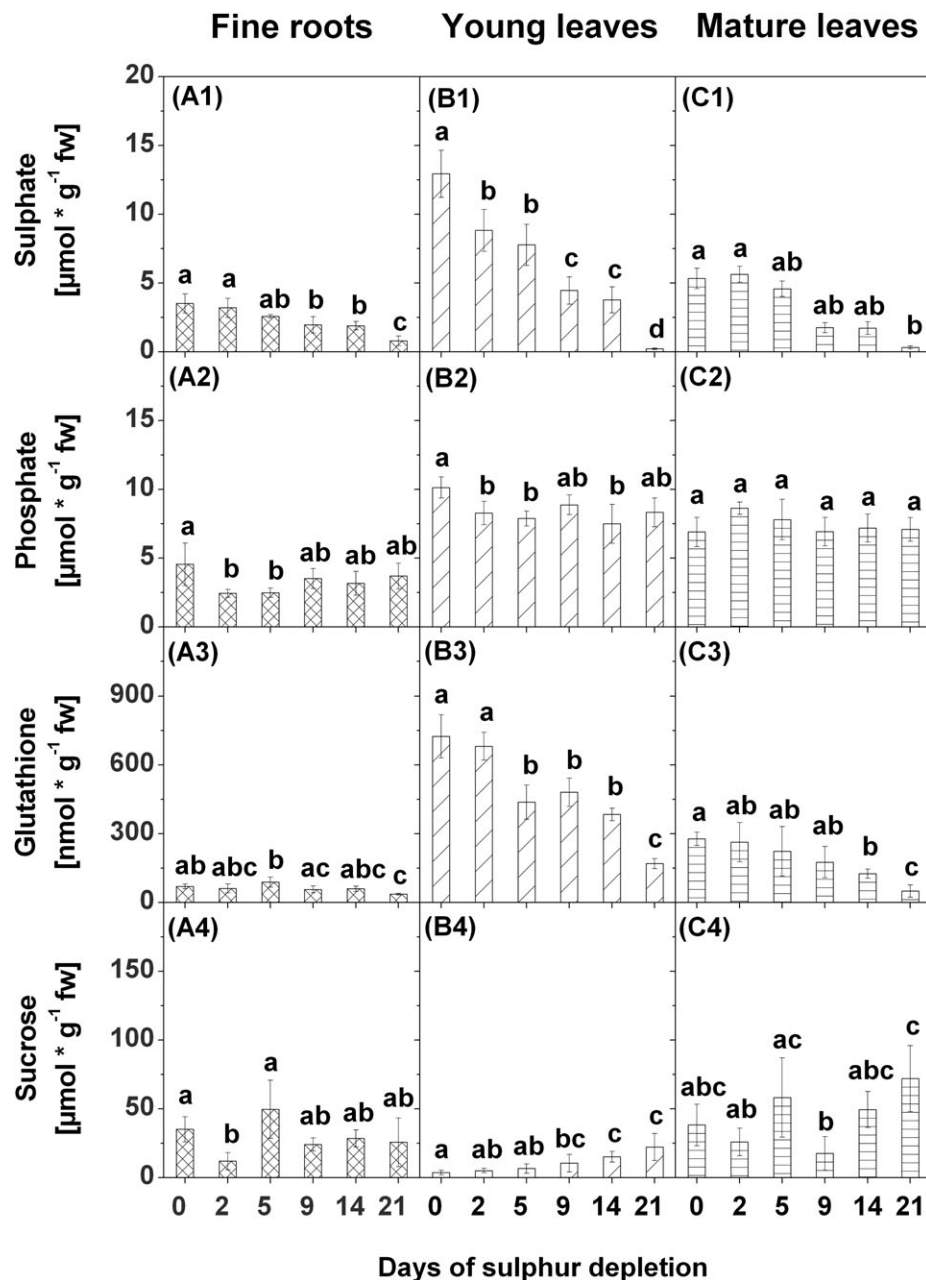
**Fine roots     Young leaves     Mature leaves**



**Fig. 2.** Sulphate (1), phosphate (2), glutathione (3), and sucrose (4) contents in fine roots (A), young leaves (B), and mature leaves (C) of poplars treated with sulphur depletion and of control plants (day 0). Data presented are the means ±SD of five replicates where each replicate consisted of two pooled plants. Lower case letters indicate significant differences at *P* < 0.05 between different time points within one tissue. fw, fresh weight

(Supplementary Fig. S1A3–4 at *JXB* online). In young and mature leaves, the contents of these sugars fluctuated strongly during the treatment but declined after 21 d of S depletion (Supplementary Fig. S1B3–4, C3–4).

### Gene expression of sulphur-metabolism related genes

Expression of five SULTR genes, i.e. the root-localized Pta*SULTR1;2*, the phloem-specific Pta*SULTR1;1*, the two vacuolar sulphate transporter genes Pta*SULTR4;1* and Pta*SUTR4;2*, and the leaf-localized sulphate transporter Pta*SULTR2;2*, was analysed (see Supplementary fig. 1 of Dürr *et al.*, 2010). Furthermore, expression of genes

encoding enzymes involved in sulphate reduction, i.e. the ATP sulphurylase genes Pta*ATPS1/2* and Pta*ATPS3/4* and the APS reductase gene Pta*APR*, was examined.

In all tissues, mRNA levels of the group 1 sulphate transporter gene, Pta*SULTR1;1*, increased first after 21 d of S depletion (Fig. 3A1, B1, C1) when almost no sulphate was left (see Fig. 2A1, B1, C1). In contrast, expression of the root-specific Pta*SULTR1;2* (see Supplementary fig. 1 of Dürr *et al.*, 2010) increased in fine roots already after 9 d and further after 21 d of S depletion (Fig. 3A2). This pattern coincides with the first and the second decrease of the root sulphate content.
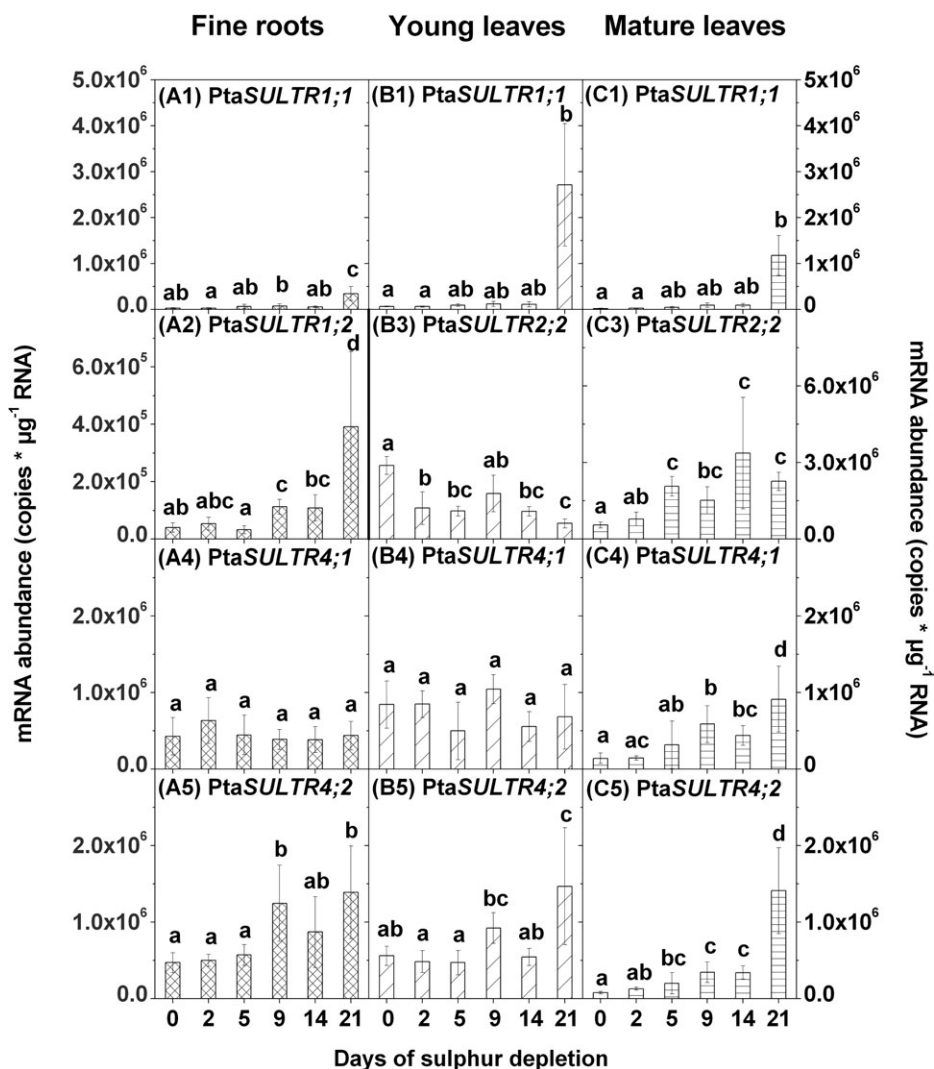
**Fig. 3.** Changes in gene expression of sulphate transporter genes in fine roots (A), young leaves (B), and mature leaves (C) due to sulphur depletion treatment. Controls are represented by day 0. Expression of Pta*SULTR1;2* (2) was analysed in fine roots only (left *y*-axis), expression of Pta*SULTR2;2* (3) solely in leaf tissues (right *y*-axis), and expression of Pta*SULTR1;1* (1), Pta*SULTR4;1* (4), and Pta*SULTR4;2* (5) was measured in fine roots, young leaves, and mature leaves. mRNA abundance was related to total RNA. Data presented are the means ±SD of five replicates where each replicate consisted of two pooled plants. Lower case letters indicate significant differences at *P* < 0.05 between different time points within one tissue.

Transcripts of the leaf-localized Pta*SULTR2;2* (Supplementary fig. 1 of Dürr *et al.*, 2010) reacted in the opposite way in young and mature leaves (Fig. 3B3, C3). While transcript levels of Pta*SULTR2;2* were reduced after 2, 5, 14, and 21 d of S depletion in young leaves, levels in mature leaves increased with decreasing sulphate contents, with the first significant increase after 5 d of S depletion.

Expression of the putatively vacuolar sulphate transporter Pta*SULTR4;1* reacted differently to S depletion in fine roots, and mature and young leaves. In fine roots and young leaves, transcript levels of Pta*SULTR4;1* remained unaffected during the whole period of S depletion (Fig. 3A4, B4). In mature leaves, transcript levels of Pta*SULTR4;1* continuously increased, with a first significant increase after 5 d and a second increase after 21 d of S depletion (Fig. 3C4). Expression of Pta*SULTR4;2* was significantly enhanced in

all tissues after 9 d of S depletion when the sulphate content was almost halved (Fig. 3A5, B5, C5). In both leaf types, but not in the roots, Pta*SULTR4;2* transcripts were further enhanced after 21 d of S depletion.

As found for the expression of the putative vacuolar SULTR, transcript levels of Pta*ATPS1/2* and Pta*ATPS3/4* also reacted differently to S depletion in fine roots, and young and mature leaves. Expression of Pta*ATPS1/2* increased in fine roots after 5 d and 21 d, and in mature leaves already after 9 d of S depletion (Fig. 4A1, C1). No changes were observed in young leaves (Fig. 4B1). In contrast, transcript levels of Pta*ATPS3/4* increased continuously in mature leaves for up to 14 d of S depletion (Fig. 4C2). In fine roots, a peak was detected after 5 d of S depletion (Fig. 4A2) similar to expression of Pta*ATPS1/2* (see Fig. 4A1), while Pta*ATPS3/4* expression did not change significantly in young leaves (Fig. 4B2).
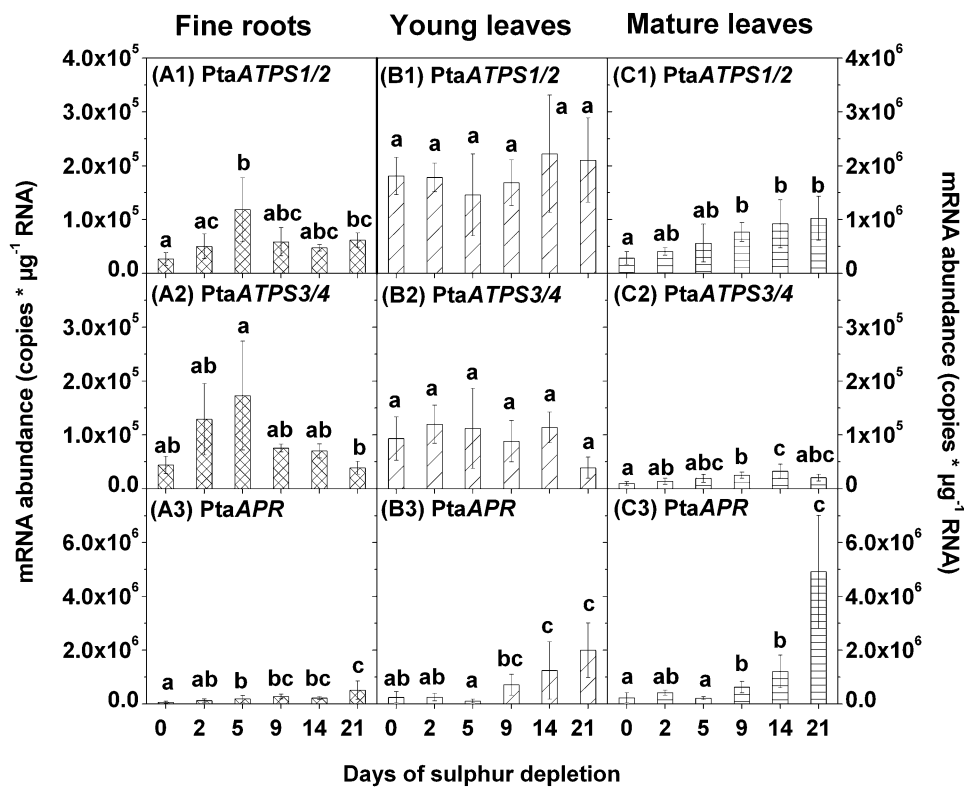
**Fig. 4.** Changes in gene expression of ATP sulphurylase 1/2 (Pta*ATPS1/2*) (1), 3/4 (Pta*ATPS3/4*) (2), and APS reductase (Pta*APR*) (3) in fine roots (A), young leaves (B), and mature leaves (C) due to sulphur depletion treatment. Controls are represented by day 0. Expression levels of Pta*ATPS1/2* are related to different y-axes (A to the left y-axis B and C to the right y-axis). mRNA abundance was related to total RNA. Data presented are the means ±SD of five replicates where each replicate consisted of two pooled plants. Lower case letters indicate significant differences at $P < 0.05$ between different time points within one tissue.

Pta*APR* transcript levels increased in fine roots, and young and mature leaves continuously with decreasing sulphate contents (Fig. 4A3, B3, C3). The first increase of Pta*APR* transcripts appeared in fine roots after 5 d, and in mature and young leaves after 9 d of S depletion. After 21 d, transcript levels of Pta*APR* increased further in all three tissues.

Gene expression data were not normalized by using expression of reference genes, but transcripts of three reference genes, namely Pta*EF1b*, Pta*bTub*, and Pta*ANT*, were analysed (Supplementary Fig. S2 at *JXB* online). No changes in gene expression of these genes were observed in fine roots and young leaves. In mature leaves, the expression patterns of all three reference genes were different from each other.

### The role of miR395 during S depletion

In previous studies, miR395 was shown to regulate gene expression of At*APS1* and At*APS4* (Jones-Rhoades and Bartel, 2004; Maruyama-Nakashita *et al.*, 2006; Kawashima *et al.*, 2009, 2011), and its expression was demonstrated to be induced under S depletion (Jones-Rhoades and Bartel, 2004; Kawashima *et al.*, 2009, 2011). In poplar, Pta*ATPS3* and Pta*ATPS4* were predicted to be targets of miR395 (data not shown), and miR395-dependent cleavage products of Pta*ATPS3/4* were obtained by 5′ RACE-PCR (Fig. 5A). Therefore, expression of miR395a was examined in fine roots, young leaves, and mature leaves upon S depletion. In

all tissues, expression of miR395a was detected first after 21 d of S depletion (Fig. 5B, C). The strongest expression of miR395a was found in mature leaves followed by young leaves (Fig. 5C). In fine roots, expression of miR395a could only be demonstrated with a highly sensitive method, but even then the expression of miR395a was very weak compared with the expression in young leaves (Fig. 5B).

### Responses of plant hormones to S depletion

To examine the importance of plant hormonal regulation during S depletion in poplar, internal cytokinin, auxin, gibberellic acid, and ABA contents were measured in fine roots, young leaves, and mature leaves (Figs 6–9, for details see Supplementary Tables S3, S4, and S5 at *JXB* online). Differences in response to S depletion were found in contents of *trans*-zeatins (tZs), *cis*-zeatins (cZs), and dihydrozeatins (Figs 6–8). Two peak values were detected for contents of tZ and of tZ riboside (tZR) in young leaves after 5 d and 21 d of S depletion (Fig. 6B1–2). In fine roots, contents of tZR were increased after 5 d of S depletion only, while they were elevated after 2 d in mature leaves (Fig. 6A2). Among the cZs, contents of cZ increased in young leaves after 21 d of S depletion (Fig. 7B1) while contents of cZ riboside *O*-β-glucoside (cZROG) were increased after 5 d (Fig. 7B3). In mature leaves, contents of cZ *O*-β-glucoside (cZOG) and cZROG decreased after 21 d of
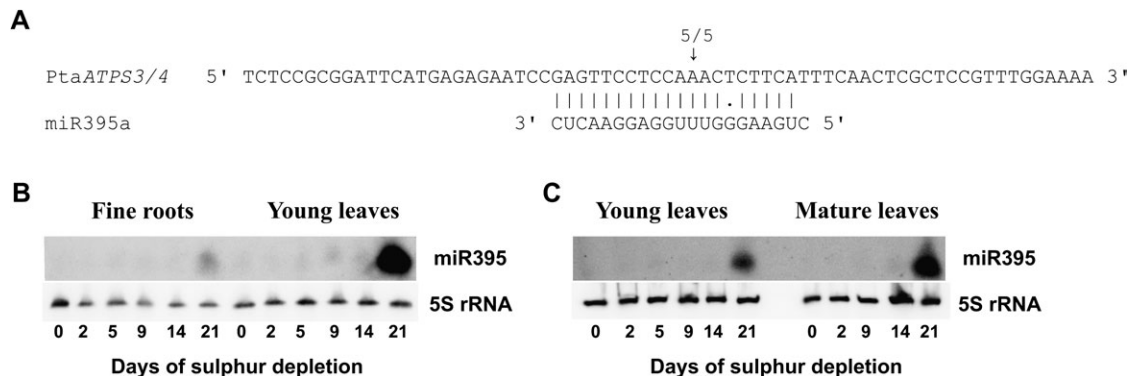
**A**

```
                                          5/5
                                           ↓
PtaATPS3/4   5' TCTCCGCGGATTCATGAGAGAATCCGAGTTCCTCCAAACTCTTCATTTCAACTCGCTCCGTTTGGAAAA 3'
                                      ||||||||||||||.|||||
miR395a                          3' CUCAAGGAGGUUUGGGAAGUC 5'
```



**Fig. 5.** (A) Validation of Pta*ATPS3/4* as the target of miR395. Validation was carried out by rapid amplification of 5′ cDNA ends (5′ RACE). RNA from mature leaves after 0 d or 21 d of sulphur depletion treatment and a primer pair for Pta*ATPS3/4* products were used (see materials and methods). Each sample derived from two plants pooled together. Arrows indicate the 5′ ends of cleavage products mapped inside the displayed sequence including the number of cloned and sequenced RACE products with the respective 5′ end. Target sequences are shown on top of the miR395a sequence. (B, C) Expression of miR395a in fine roots, young leaves, and mature leaves of poplar due to sulphur depletion treatment. Northern blot analysis was carried out with 15 µg of total RNA from fine roots and young leaves using a high-sensitive membrane (Amersham-Hybond-NX) (B) or with 70 µg of total RNA from young leaves and mature leaves using a less sensitive membrane (Amersham-Hybond-N+) (C). All tissues derived from pooled samples of two poplar plants. 5S rRNA was used as loading control.
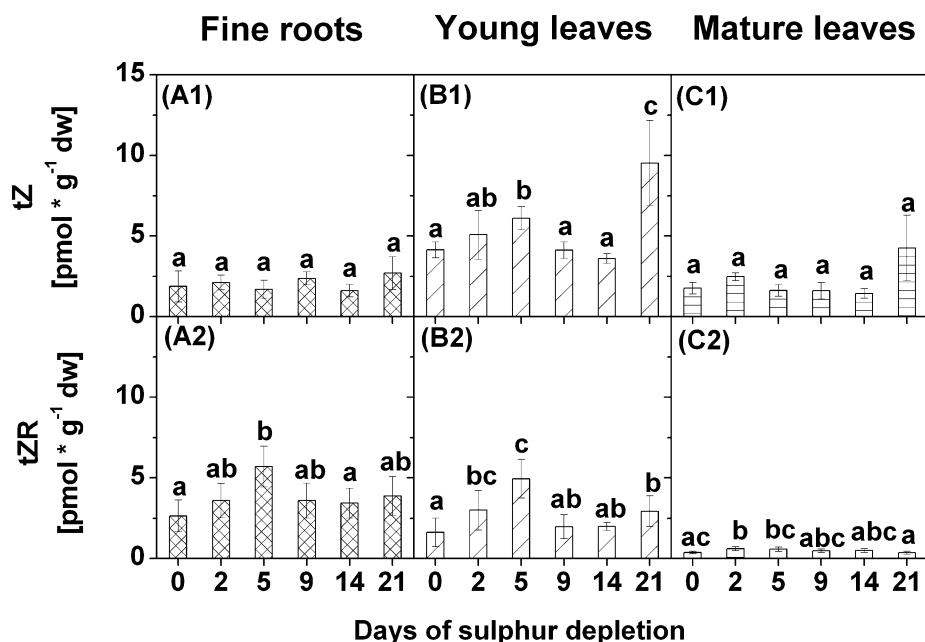


**Fig. 6.** Contents of *trans*-zeatin (tZ) (1) and of tZ riboside (tZR) (2) in fine roots (A), young leaves (B), and mature leaves (C) of poplars treated with sulphur depletion and of control plants (day 0). Data presented are the means ±SD of five replicates where each replicate consisted of two pooled plants. Lower case letters indicate significant differences at $P < 0.05$ between different time points within one tissue. dw, dry weight

S depletion (Fig. 7C2–3). Differences in contents of cZ, cZOG, and cZROG were also found in fine roots (Fig. 7A1–3) but not in relation to the control values. Significant differences exist for cZ between 2 d and 5 d of S depletion and for cZOG and cZROG between 2 d and 21 d of S depletion.

Several dihydrozeatins were increased in fine roots, young leaves, and mature leaves after 21 d of S depletion. However, the composition of individual dihydrozeatins differed greatly between tissues. In fine roots, dihydrozeatin riboside (DZR) and DZR phosphates (DZRPs) were

detected, but DZR was found only at the end of the S depletion treatment (Fig. 8A1–2). Contents of both dihydrozeatins increased with decreasing sulphate contents, although this increase was not statistically significant. Both dihydrozeatins were also increased in young leaves, as was dihydrozeatin 9-*N*-glucoside (DZ9G) (Fig. 8B1–3). However, contents of all three dihydrozeatins fluctuated strongly during S depletion treatment in young leaves, with peak values after 5 d (DZR and DZRPs) or after 2 d (DZ9G). A comparable pattern was observed for DZ9G in mature
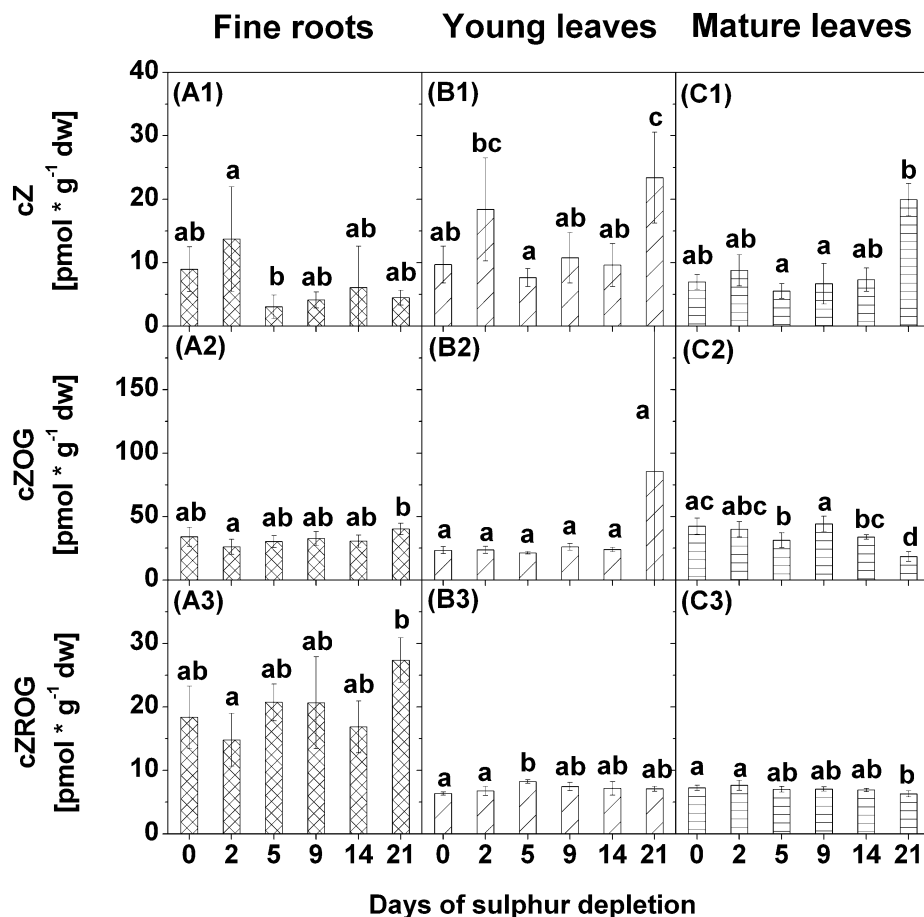
## Fine roots      Young leaves    Mature leaves



**Fig. 7.** Contents of *cis*-zeatin (cZ) (1), of cZ *O*-β-glucoside (cZOG) (2), and of cZ riboside *O*-β-glucoside (cZROG) (3) in fine roots (A), young leaves (B), and mature leaves (C) of poplars treated with sulphur depletion and of control plants (day 0). Data presented are the means ±SD of five replicates where each replicate consisted of two pooled plants. Lower case letters indicate significant differences at *P* < 0.05 between different time points within one tissue. dw, dry weight

leaves where contents of DZ9G were elevated after 5 d and after 21 d of S depletion (Fig. 8C3). Contents of DZR did not change in mature leaves during S depletion (Fig. 8C1), and DZRPs were not detected at all (Fig. 8C2).

Contents of the auxin indole acetic acid (IAA) did not change in fine roots in response to S depletion (Fig. 9A1), but tended to decrease with decreasing sulphate contents in mature leaves (Fig. 9C1). In young leaves, IAA contents first increased until 5 d of S depletion and then decreased to values lower than found in the controls after 14 d and 21 d of S depletion (Fig. 9B1). Contents of ABA decreased in young and mature leaves after 14 d or 21 d of S depletion, respectively (Fig. 9B2, C2). In fine roots, ABA contents were reduced after 2 d of S depletion, but then returned to the level of the controls (Fig. 9A2).

*Analyses of xylem sap and phloem exudates — indications of a whole plant regulation*

Sulphate, phosphate, and metabolite contents as well as plant hormone contents were also analysed in xylem sap and phloem exudates. Sulphate contents in xylem sap and in phloem exudates decreased continuously upon S depletion. A first significant decrease was observed after 14 d of

S depletion (Fig. 10A1, B1). Phosphate contents in xylem sap were enhanced after 5 d and 9 d of S depletion, but subsequently decreased again to the level of the controls (Fig. 10A2). In phloem exudates, no differences in phosphate contents were found in response to S depletion (Fig. 10B2).

Contents of GSH did not change in xylem sap due to S depletion (Fig. 10A3), whereas GSH contents in phloem exudates were diminished with decreasing sulphate contents (Fig. 10B3). This reduction became significant after 14 d of S depletion.

In xylem sap, sucrose contents were more or less unaffected, except for a slight increase after 5 d of S depletion (Fig. 10A4). In contrast, sucrose contents in phloem exudates increased after 21 d of S depletion (Fig. 10B4).

For contents of cytokinins, auxins, gibberellic acids, and ABA, no significant differences were found either in xylem sap or in phloem exudates in response to S depletion (see Supplementary Table S6 and S7 at *JXB* online).

*Correlation analyses*

To underline the connection between decreasing sulphate contents and changes in the transcript levels, metabolite and plant hormone contents, correlation analyses were
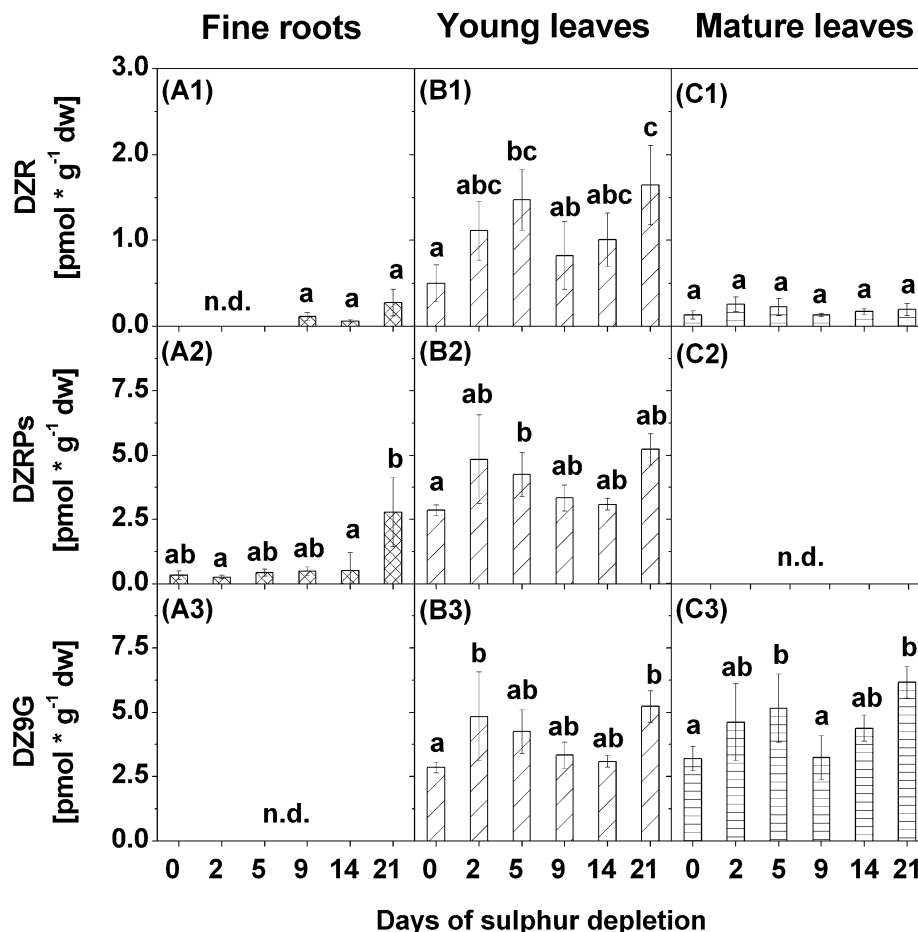
**Fig. 8.** Contents of dihydrozeatin riboside (DZR) (1), dihydrozeatin riboside phosphates (DZRPs) (2), and dihydrozeatin-9-*N*-glucoside (DZ9G) (3) in fine roots (A), young leaves (B), and mature leaves (C) of poplars treated with sulphur depletion and of control plants (day 0). Data presented are the means ±SD of five replicates where each replicate consisted of two pooled plants. Lower case letters indicate significant differences at *P* < 0.05 between different time points within one tissue. dw, dry weight; n.d., not detected

performed. In fine roots, young leaves, and mature leaves, transcript levels of Pta*SULTR1;1*, Pta*SULTR4;2*, and Pta*APR* were strongly negatively correlated with sulphate contents (Table 2). Further strong negative correlations with sulphate contents were found for transcript levels of Pta*SULTR1;2* in fine roots and for Pta*SULTR4;1*, Pta*ATPS1/2* and Pta*ATPS3/4* in mature leaves. Transcript levels of Pta*SULTR2;2* were positively correlated with sulphate contents in young leaves and negatively in mature leaves. Although GSH contents decreased in all tissues with decreasing sulphate contents, a positive correlation with sulphate contents was found only in young and mature leaves, but not in fine roots. Moreover, sucrose contents in young leaves correlated negatively with sulphate contents. Among the different plant hormones, negative correlations with sulphate contents were found for DZR contents in fine roots and in young leaves. A positive correlation with sulphate contents was detected for contents of cZROG in mature leaves, for IAA in young leaves, and for ABA in both leaf types.

No significant correlations with sulphate contents were found in xylem sap. In phloem exudates, a positive correlation with sulphate contents was found for GSH contents

($\rho$=0.63, *P*=0.0007), and sucrose contents correlated negatively with sulphate contents ($\rho$= –0.53, *P7*=0.0061).

## Discussion

*Different responses upon 'S limitation' and 'early S deficiency'*

The present experiment investigated early events of S depletion at a high temporal resolution in different plant tissues and the transport tissues xylem and phloem. A broad spectrum of genes of S metabolism (sulphate transporters and genes of sulphate assimilation), of metabolites, and of plant hormone contents was analysed and correlated with the sulphate content. The observed changes in response to S depletion can be separated into two phases, denominated as 'S limitation' and 'early S deficiency'.

S limitation was defined by halved sulphate contents compared with the controls (0 d of S depletion). This corresponds in fine roots, mature leaves, and young leaves to day 9 of S depletion (although sulphate contents in young leaves were significantly reduced already before). S limitation was accompanied by increasing transcript levels of
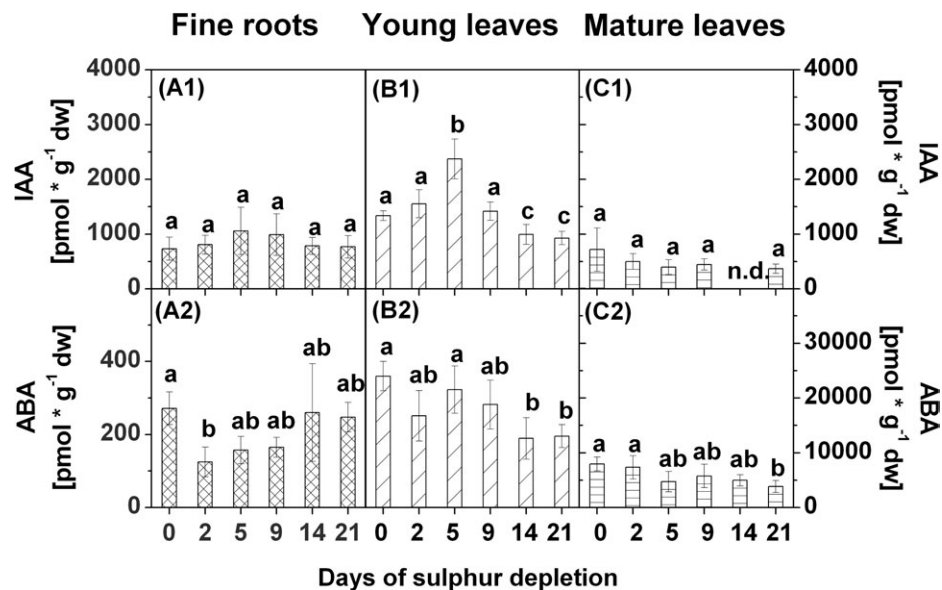
**Fig. 9.** Contents of indole-3-acetic acid (IAA) (1) and of abscisic acid (ABA) (2) in fine roots (A), young leaves (B), and mature leaves (C) of poplars treated with sulphur depletion and of control plants (day 0). Data presented are the means ±SD of five replicates where each replicate consisted of two pooled plants. Lower case letters indicate significant differences at $P < 0.05$ between different time points within one tissue. dw, dry weight; n.d., not detected

Pta*SULTR1;2* in fine roots and of Pta*SULTR4;2* and Pta*APR* in fine roots, mature leaves, and young leaves (Fig. 11A). In addition, increasing transcript levels of Pta-*SULTR2;2*, Pta*SULTR4;1*, Pta*ATPS1/2*, and Pta*ATPS3/4* in mature leaves as well as a decline in GSH and an increase in sucrose contents in young leaves were identified as tissue-specific responses to S limitation (Fig. 11A). The peak expression of Pta*ATPS1/2* and Pta*ATPS3/4* in fine roots after 5 d of S depletion could not be related to the decreasing sulphate contents and was therefore not regarded as an S limitation response. Further experiments have to show if those peaks in expression of the Pta*ATPS* genes are of importance for the regulation of S metabolism during S depletion. In young leaves, transcript levels of Pta*SULTR2;2* were also reduced before 9 d of S depletion but increased again at day 9. Therefore, this reaction in young leaves could not clearly be defined as a response to S limitation.

As known from several other studies (Smith *et al.*, 1997; Takahashi *et al.*, 1997; Vidmar *et al.*, 2000; Buchner *et al.*, 2004*a*; Koralewska *et al.*, 2007, 2009; Barberon *et al.*, 2008), transcripts of group 1 *SULTR* genes responsible for sulphate uptake and of *APR* strongly react to S depletion. This is consistent with the findings in poplar. The up-regulation of the corresponding SULTR from poplar, Pta*SULTR1;2* (Supplementary fig. 1 of Dürr *et al.*, 2010), and of Pta*APR* indicates an improvement in the sulphate uptake capacity in fine roots and in the sulphate reduction capacity in all tissues already during S limitation. The elevated expression of Pta*SULTR4;2* is likely to be an indication of increased sulphate re-mobilization from the vacuolar sulphate pool (Kataoka *et al.*, 2004; Zuber *et al.*, 2010) as observed in *Brassica* and *Arabidopsis* (Buchner *et al.*, 2004*a*; Kataoka *et al.*, 2004; Parmar *et al.*, 2007;

Abdallah *et al.*, 2010). This assumption is supported by the finding that high transcript abundance of Pta*SULTR4;2* correlated with low sulphate contents in leaves during seasonal growth (Dürr *et al.*, 2010). Up-regulation of *SULTR4;2* induced by S depletion occurred earlier and was stronger than that of *SUTLR4;1* in mature leaves. It can be assigned to tissue-specific differences in the responsiveness of vacuolar *SULTR* genes as observed in *Arabidopsis* and *Brassica* (Kataoka *et al.*, 2004; Dubousset *et al.*, 2009). Bn*SULTR4;1* expression was induced only in mature leaves but not in young leaves (Dubousset *et al.*, 2009). In addition, Pta*SULTR4;1* expression in poplar is more likely to be controlled by leaf development than by the sulphate content itself as no correlation with the sulphate content was observed during seasonal growth (Dürr *et al.*, 2010).

The second denominated phase was characterized by an almost depleted soluble sulphate pool. This phase is reached in all tissues after 21 d of S depletion. As basic plant characteristics such as tree height, growth increment, and chlorophyll contents (Supplementary Fig. S3, S4 at *JXB* online) were not affected by 21 d of S depletion, the observed changes were denoted as early S deficiency responses. The effect of prolonged S depletion was much stronger in young and mature leaves than in fine roots. Nevertheless, the most dominant responses to S limitation, the increasing transcript levels of Pta*SULTR1;2*, Pta-*SULTR4;2*, and Pta*APR*, were further enhanced in all tissues upon early S deficiency and this was accompanied by increasing transcript levels of the phloem-specific Pta-*SULTR1;1* (Fig. 11B). In addition, enhanced expression of Pta*ATPS1/2* in fine roots and decreased expression of the leaf-localized Pta*SULTR2;2* (Supplementary fig. 1 of Dürr *et al.*, 2010) in young leaves were detected as tissue-specific responses. At the metabolite level, GSH contents decreased
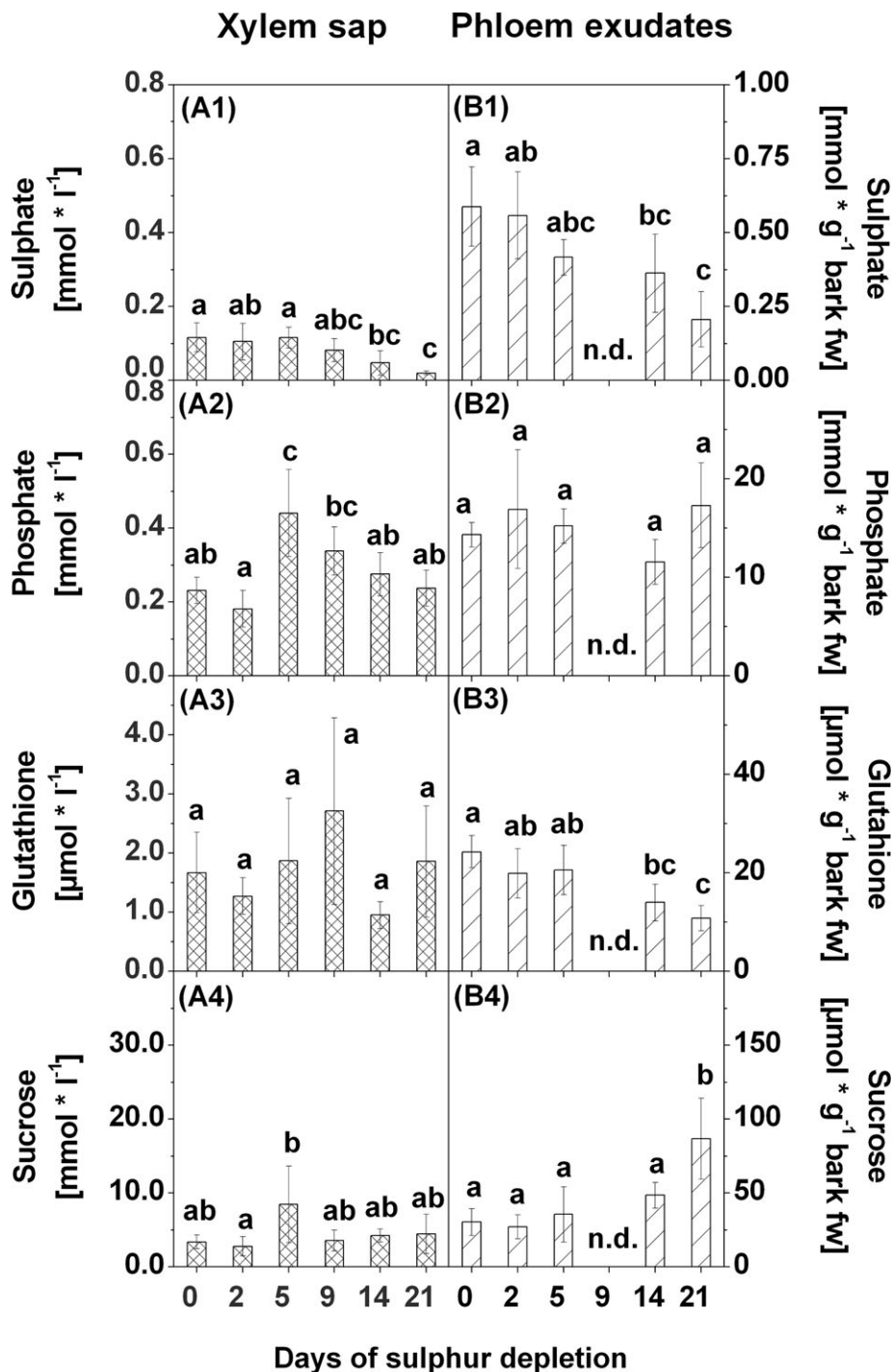
**Fig. 10.** Sulphate (1), phosphate (2), glutathione (3), and sucrose (4) contents in xylem sap (A) and phloem exudates (B) of poplars treated with sulphur depletion and of control plants (day 0). Data presented are the means ±SD of five replicates where each replicate consisted of two pooled plants. Lower case letters indicate significant differences at $P < 0.05$ between different time points within one tissue. Contents were related to litres of xylem sap or bark fresh weight (fw) of the bark piece used for phloem exudation, respectively. n.d., not detected

further in young leaves and were also reduced in fine roots and mature leaves (Fig. 11B). In both leaf types, a strong correlation was found between sulphate and GSH contents. All these reactions are generally described as specific reactions to S depletion (Smith *et al.*, 1997; Lappartient and Touraine 1997; Vidmar *et al.*, 1999; Hirai *et al.*, 2003; Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003;

Hopkins *et al.*, 2005; Koralewska *et al.*, 2007, 2009; Parmar *et al.*, 2007; Barberon *et al.*, 2008). Only responses of the Pta*SULTR2;2* in leaves seem controversial, but might be under developmental control. This is indicated by up-regulation of Pta*SULTR2;2* transcripts in mature leaves and down-regulation in young leaves upon S depletion. Controversial reactions of *SULTR2;2* expression upon

**Table 2.** Correlation analysis of glutathione contents, sucrose contents, mRNA abundance, and of plant hormone contents with the sulphate content in fine roots, young leaves, and mature leaves

Only correlation coefficients with ρ or $ρ_s$ >0.5 or ρ or $ρ_s$ less than –0.5 and corresponding *P*-values <0.01 are presented.

| | Fine roots | | | Young leaves | | | Mature leaves | | |
|---|---|---|---|---|---|---|---|---|---|
| | ρ | *P*-value | n | ρ or $ρ_s$ | *P*-value | n | $ρ_s$ | *P*-value | n |
| Pta*SULTR1;1* | –0.81 | <0.0001 | 30 | –0.85* | <0.0001 | 30 | –0.91* | <0.0001 | 30 |
| Pta*SULTR1;2* | –0.77 | <0.0001 | 30 | | | | | | |
| Pta*SULTR2;2* | | | | 0.68 | <0.0001 | 30 | –0.66* | 0.0001 | 30 |
| Pta*SULTR4;1* | | | | | | | –0.75* | <0.0001 | 30 |
| Pta*SULTR4;2* | –0.69 | <0.0001 | 30 | –0.68 | <0.0001 | 30 | –0.85* | <0.0001 | 30 |
| Pta*ATPS1/2* | | | | | | | –0.70* | <0.0001 | 30 |
| Pta*ATPS3/4* | | | | | | | –0.52* | 0.0035 | 30 |
| Pta*APR* | –0.79 | <0.0001 | 30 | –0.78 | <0.0001 | 30 | –0.73* | <0.0001 | 30 |
| Glutathione | | | | 0.89 | <0.0001 | 30 | 0.74* | <0.0001 | 30 |
| Sucrose | | | | –0.75 | <0.0001 | 30 | | | |
| cZROG | | | | | | | 0.57* | 0.0011 | 30 |
| DZR | –0.75 | 0.0047 | 12 | –0.54 | 0.0019 | 30 | | | |
| IAA | | | | 0.59 | 0.0006 | 30 | | | |
| ABA | | | | 0.65 | 0.0001 | 30 | 0.52* | 0.0033 | 30 |

ρ, Pearson correlation coefficient, $ρ_s$. Spearmans rank correlation coefficient (indicated by *), *n*, number of replicates.

S depletion were also observed with herbaceous plants. *SULTR2;2* expression is induced in leaves of *A. thaliana* and *B. napus* (Takahashi *et al.*, 2000; Parmar *et al.*, 2007) but not in *Brassica oleracea* (Buchner *et al.*, 2004a).

## Increasing miR395 expression is related to early S deficiency

During early S deficiency, post-transcriptional regulation of Pta*ATPS3/4* via miR395a might be relevant in all analysed plant tissues. When sulphate nearly disappeared in leaves, miR395a expression increased. In fine roots, where the decrease in sulphate was not as strong, induction of miR395a was very low. Pta*ATPS3/4* has been identified as an *in vivo* target of miR395a by 5′ RACE. In mature leaves, transcript levels of Pta*ATPS3/4* were still higher than in the controls after 21 d of S depletion. In *Arabidopsis*, miR395 targeted At*APS1*, At*APS3*, At*APS4*, and At*SULTR2;1* (Jones-Rhoades and Bartel, 2004; Jones-Rhoades *et al.*, 2006; Kawashima *et al.*, 2009), but the cell type-specific expression pattern was different for miR395 and At*SULTR2;1* in roots (Kawashima *et al.*, 2009; Liang *et al.*, 2010). Moreover, post-transcriptional regulation via miR395 seems to affect primarily transcripts of At*APS4* and only to a lesser extent transcripts of At*APS1* and At*APS3* (Liang *et al.*, 2010; Kawashima *et al.*, 2011). Thus, different sensitivities of Pta*ATPS3* and Pta*ATPS4* transcripts for post-transcriptional regulation via miR395a cannot be excluded in the present study with poplar. Sensitivity might depend on different local expression patterns and can explain the higher levels of Pta*ATPS3/4* in mature leaves after 21 d of S depletion compared with the control levels. Cell-specific localization via either *in situ* hybridization or the use of reporter gene constructs will help to address this question in future studies.

In fine roots and mature leaves, the increase in Pta*ATPS1/2* transcript levels could support sulphate activation for further use in the sulphate reduction pathway during S deficiency. This could be of particular importance if the subcellular localization of Pta*ATPS1/2* differs from that of Pta*ATPS3/4*. The predicted amino acid sequences of the two ATPS isoforms of *P. trichocarpa*, PtcATPS1 and PtcATPS2, were most similar to the protein sequence of AtAPS2 that is proposed to be the only cytosolic ATPS in *A. thaliana* (Hatzfeld *et al.*, 2000). Bioinformatic prediction of the subcellular localization of PtcATPS1 and PtcATPS2 from *P. trichocarpa* using the program TargetP (www.cbs. dtu.dk/services/TargetP/; Emanuelsson *et al.*, 2007) proposed a cytosolic localization for these PtcATPS sequences (data not shown). The predicted amino acid sequences of PtcATPS3 and PtcATPS4 were more closely related to the chloroplastic AtAPS1, AtAPS3, and AtAPS4 and were predicted to contain a putative chloroplastic transit peptide in the N-terminus of their amino acid sequences (data not shown). Thus, it appears likely that sulphate activation in the cytosol by PtaATPS1/2 may become more important when sulphate supply is reduced due to decreasing influx but counteracted by a higher sulphate efflux from the vacuole. This view is consistent with the observation that sulphate fluxes into thiols and proteins were reduced in *aps1* mutants and APS4-RNAi *Arabidopsis* plants under S depletion (Kawashima *et al.*, 2011). Furthermore, At*APS3* transcripts increased upon S depletion in roots and shoots of *Arabidopsis* so that sulphate activation can be maintained at S deficiency by AtAPS3 (Liang *et al.*, 2010).

## Changes in plant hormone levels are related to early S deficiency

In the present experiment, early responses to S deficiency are not only connected to almost extinguished sulphate contents, decreasing GSH contents, and increasing transcript levels of genes related to S metabolism. In leaves, early S deficiency is also accompanied by changes in cytokinin contents, whereas none of the changes in fine roots could be related to one of the two phases of S depletion. The active cytokinin forms tZ, tZR and cZ (Sakakibara, 2006) increased in young leaves during early S deficiency (Fig. 11B). The peaks of tZ and tZR contents after 5 d of S depletion were not regarded as S limitation responses because of the subsequent decrease in the contents of these cytokinins (Fig. 11A). In mature leaves, the contents of the storage forms cZOG and cZROG which can be activated by conversion into cZ (Sakakibara, 2006) decreased (Fig. 11B). This may indicate cytokinin activation of cZs in mature leaves, although the increase of cZ contents itself was not significant. In contrast, the increase of cZ in young leaves cannot be attributed to cytokinin activation because cZOG and cZROG contents did not change in the phase of early S deficiency.
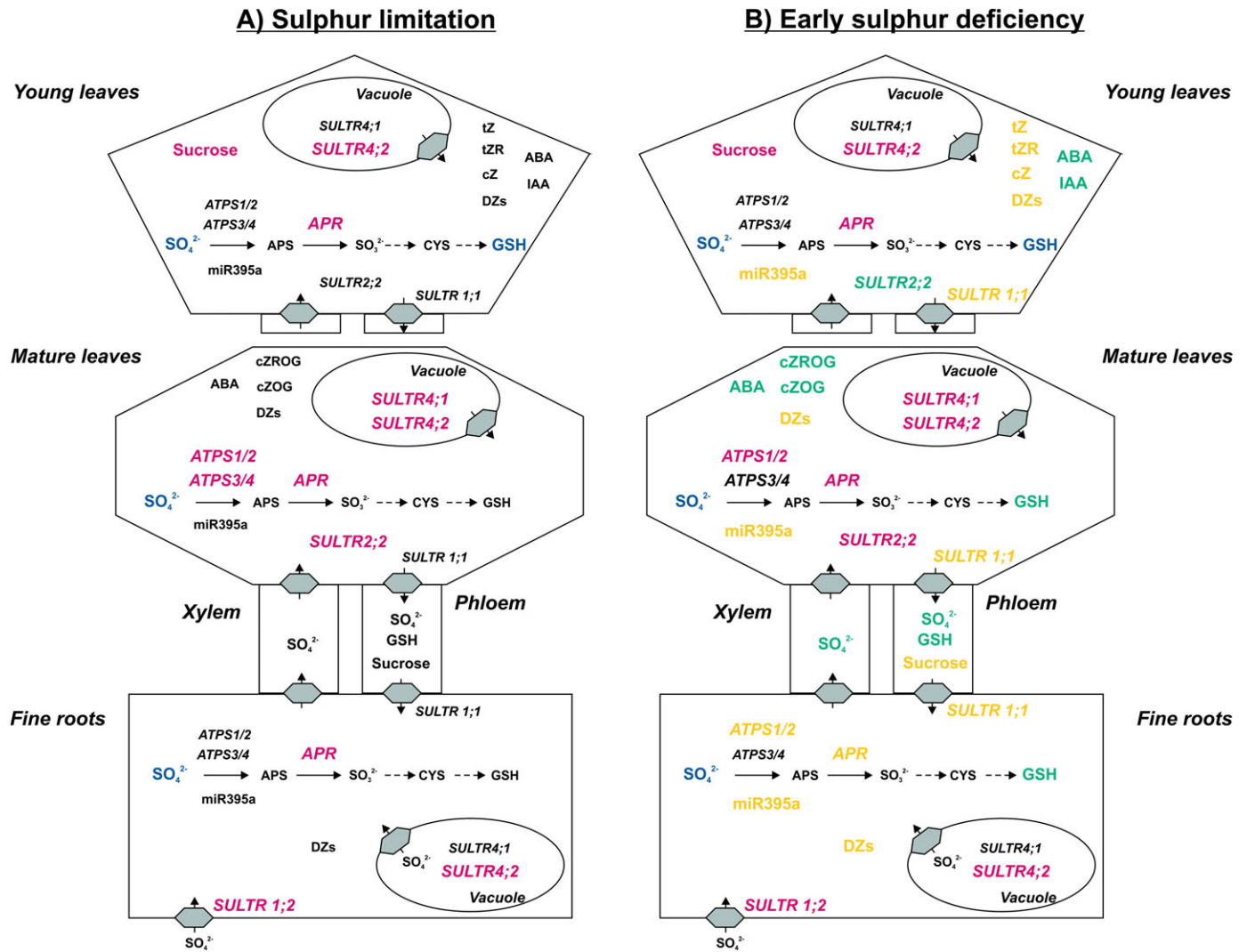
**Fig. 11.** Schematic overview of the changes in sulphur metabolism of poplar during sulphur limitation (A) and during early sulphur deficiency (B). Changes in expression of sulphur-metabolism related genes, metabolite or plant hormone contents during sulphur limitation are labelled in red (increase) or blue (decrease). Additional changes appearing first during early sulphur deficiency are marked in yellow (increase) or blue green (decrease). ABA, abscisic acid; APS, adenosine 5′-phosphosulphate; CYS, cysteine; cZ, *cis*-zeatin; cZOG, cZ O-β-glucoside; cZROG, cZ riboside O-β-glucoside; DZs, dihydrozeatins; GSH, glutathione; IAA, indole-3-acetic-acid; tZ, *trans*-zeatin; tZR, tZ riboside. For abbreviations of genes (in italics) see text.

cZs are assumed to possess less physiological activity than tZs (Schmitz *et al.*, 1972; Kamínek *et al.*, 1987). However, Gajdošová *et al.* (2011) hypothesized that cZs are of special importance under growth-limiting conditions for sustaining basic cytokinin signalling. Increasing active cZ during early S deficiency may support this hypothesis. Overexpression of a cytokinin oxidases (CKX) let to increasing transcript levels of At*SULTR1;2* in *Arabidopsis* roots (Werner *et al.*, 2010). As active cytokinin contents were drastically diminished in these *Arabidopsis* plants, it was hypothesized that active cytokinins block the up-regulation of *SULTR* expression in roots under normal S conditions. Only if active cytokinins decline due to inactivation/degradation repression of *SULTR* transcription upon S depletion thought to be abolished. This is consistent with findings from Maruyama-Nakashita *et al.* (2004*b*). The authors prevented up-regulation of At*SULTR1;1* and At*SULTR1;2* in *Arabidopsis* roots by

application of benzyladenine, an artificial active cytokinin. When tZ or tZR was applied exogenously to *Arabidopsis* plants, increasing At*APR* and At*SULTR2;2* transcript levels were observed in rosette leaves independent of the S supply (Ohkama *et al.*, 2002). This positive regulation of S-responsive genes by cytokinins is consistent with the increasing tZ and tZR contents in young leaves of poplar during early S deficiency. However, Ohkama *et al.* (2002) hypothesized that exogenous cytokinin operates indirectly via sucrose in rosette leaves because they found no increasing cytokinin contents during S depletion. Such a positive effect of sucrose has already been described for APR (Kopriva *et al.*, 1999, 2002) and, thus, is consistent with increasing Pta*APR* transcript levels and sucrose contents in young poplar leaves in the present study.

Furthermore, an increase of dihydrozeatins was found in all tissues studied, namely fine roots, mature leaves, and

young leaves, upon early S deficiency. Dihydrozeatins are biologically very stable cytokinins because of their insensitivity to CKX and because their contents in plant tissues are relatively low compared with tZs and isopentenyladenines (Sakakibara, 2006; Hirose *et al.*, 2008; see also Supplementary Tables S3, S4, and S5 at *JXB* online). Therefore, they are thought to be rather inactive (Armstrong, 1994; Sakakibara, 2006). Nevertheless, increasing DZR contents in fine roots and young leaves strongly correlate with the decline in sulphate contents. Cytokinin ribosides such as DZR are more active than *O*- and *N*-glucosides (Sakakibara, 2006). As recently reported for cZs (Gajdošová *et al.*, 2011), Mok and Mok (2001) proposed that the conversion into dihydrozeatins might help to stabilize the physiological activity of cytokinins. The maintenance of basic cytokinin functions during adaptation of plant metabolism to decreasing S availability could be an explanation for the increasing dihydrozeatin contents observed in all tissues during early S deficiency.

Considering other plant hormones, almost consistent reactions were observed in mature and young poplar leaves upon early S deficiency. In both tissues, ABA and IAA contents declined in parallel with decreasing sulphate contents. Increasing amounts of ABA are often related to environmental stress, for example to drought or salt stress (Raghavendra *et al.*, 2010). In the present experiment, the observed decline in ABA during early S deficiency is assumed to be part of adaptive processes rather than stress reactions. Therefore, a stress-related role for ABA in this phase might not be relevant. As ABA is believed to have a positive effect on leaf growth of poplar (Arend *et al.*, 2009), decreasing ABA contents might accommodate adaptation of growth processes to early S deficiency.

Auxin assists in tissue development and growth (Vieten *et al.*, 2007), and its decline in young leaves may indicate growth retardation. However, an effect of S depletion on the growth of poplar was not observed (Supplementary Fig. S3 at *JXB* online). In *Arabidopsis* up-regulation of auxin correlates with the *nitrilase3* (*NIT3*) gene that is part of the auxin synthesis pathway from glucosinolate degradation and is increased during S depletion (Grsic *et al.*, 1999; Bak *et al.*, 2001; Kutz *et al.*, 2002; Nikiforova *et al.*, 2003). As poplar does not contain glucosinolates, this pathway does not exist, and changes in auxin contents in young poplar leaves at early S deficiency must account for other, so far unidentified processes.

### Responses to early S deficiency at the whole plant level

It has been reported that S remobilization from old and mature leaves during S depletion is negligible (Adiputra and Anderson, 1995). Already Bell *et al.* (1994, 1995) demonstrated that sulphate efflux from the vacuole during S stress is scarce, but possible during vegetative growth. In the present experiment with poplar, the increasing expression of the putative tonoplast-localized SULTR, Pta*SULTR4;2*, clearly indicates enhanced sulphate remobilization already during S limitation from both leaf and root vacuoles. Comparable results were obtained with vegetative *B. napus* (Dubousset *et al.*, 2009;

Abdallah *et al.*, 2010) and wheat (Shinmachi *et al.*, 2010). Increasing sulphate remobilization from the vacuole in the roots under S limitation counteracts sulphate storage within the vacuole and can maintain sulphate availability for loading into the xylem (Kataoka *et al.*, 2004). This might be the reason for the almost unaffected sulphate contents in xylem sap for up to 9 d of S depletion in poplar.

Another route for the distribution of sulphate remobilized from the vacuole in the plant can be sulphate loading into the phloem. This process requires expression of the SULTR involved in phloem loading, especially in mature leaves (Dürr *et al.*, 2010) that are thought to be sources for the sulphate transported into developing leaves of the apex and into roots in poplar (Hartmann *et al.*, 2000). In the present study, expression of the phloem-localized Pta-*SULTR1;1* increased in leaves during early S deficiency and, thus, could suggest an improved capacity for phloem loading of sulphate for its transport to developing leaves and roots. Also in wheat, S depletion is accompanied by increased expression of Tae*SULTR4;1* and Tae*SULTR1;1* in the wheat shoot (Buchner *et al.*, 2010), and the expression pattern of Tae*SULTR1;1* is comparable with the expression pattern of Pta*SULTR1;1* in poplar (Dürr *et al.*, 2010). These results again indicate an enhanced phloem loading capacity of sulphate in combination with increased sulphate efflux from the vacuole (Buchner *et al.*, 2010). Together these processes are expected to be responsible for a steady sulphate supply of sink tissues such as developing grains in wheat as total S in grains did not change significantly upon S depletion (Shinmachi *et al.*, 2010). Still S remobilization from roots and leaves in poplar is not sufficient to maintain the sulphate content in young, developing leaves upon S depletion. The reduced sulphate and GSH contents in phloem exudates during early S deficiency indicate insufficient re-mobilization of S. Young poplar plants show continuous growth and, thus, developed new leaves throughout the S depletion treatment of 21 d. Since developing leaves are the main sinks for sulphate from mature leaves (Anderson and Anderson, 1996a, b, 1997), it can be assumed that the remobilized sulphate from vacuoles is completely transported to the developing leaves around the apex to maintain shoot growth. Consequently, vacuolar storage of sulphate in young leaves cannot be established.

Moreover, nutrient deficiency generally induces enhanced root growth (López-Bucio *et al.*, 2003). Consequently, root sink strength for nutrients and carbon which are delivered from the shoot via phloem transport increases. In *Brassica*, sulphate remobilized from the vacuole of mature leaves was mainly found in roots and supported root growth upon S depletion (Abdallah *et al.*, 2010). As the sulphate pool of fine roots of poplar was considerably less affected than the sulphate pool in young and mature leaves at early S deficiency, these results are consistent. Therefore, it can also be assumed that in poplar remobilization of sulphate in mature leaves predominantly promoted sulphate contents of the roots. This assumption is supported by increased sucrose contents in phloem exudates indicating higher carbon flow to the roots for growth.

The reduced sulphate and GSH pools in fine roots, leaves, and phloem exudates, however, clearly indicate reduced S availability and S cycling within the poplar plants at early S deficiency (Hartmann *et al.*, 2000; Herschbach *et al.*, 2011). Still GSH remained unaffected in the xylem sap. Whether increased sulphate reduction in the roots, indicated by the increased Pta*APR* and Pta*ATPS1/2* transcript levels, supports the supply of reduced S to the shoot under S depletion remains to be investigated in future studies. In conclusion, S depletion clearly affects S metabolism of poplar on the transcriptional and metabolite level, including changes of endogenous plant hormones in a time series of events, but its impact on plant growth seems to be less severe than in herbaceous plants such as *Arabidopsis* or *Brassica*.

## Supplementary data

Supplementary data are available at *JXB* online.

**Figure S1.** Cysteine (1), γ-glutamyl-cysteine (γ-EC) (2), glucose (3), and fructose (4) contents in fine roots (A), young leaves (B), and mature leaves (C) of poplar treated with sulphur depletion and of control plants (day 0).

**Figure S2.** Changes in gene expression of three reference genes, i.e. *elongation factor 1β* (*Ef1b*) (1), *β-tubulin (bTub)* (2), and *Aintegumenta* (*ANT*) (3), in fine roots (A), young leaves (B), and mature leaves (C) due to sulphur depletion treatment.

**Figure S3.** Leaf number, plant height, and plant growth of poplar treated with sulphur depletion and of control plants (day 0).

**Figure S4.** Contents of chlorophyll *a* (1), chlorophyll *b* (2), and carotinoids (3) in young (A) and mature leaves (B) of poplar treated with sulphur depletion and of control plants (day 0).

**Table S1.** Sequences of oligonucleotide primer pairs used for cloning the gene sequences of Pta*ATPS1/2*, Pta*ATPS3/4*, Pta*bTub*, and Pta*ANT*.

**Table S2.** Oligonucleotide primer sequences used for qRT-PCR analysis and resulting PCR fragment lengths.

**Table S3.** Contents of all analysed plant hormones in fine roots of poplar treated with sulphur depletion.

**Table S4.** Contents of all analysed plant hormones in young leaves of poplar treated with sulphur depletion.

**Table S5.** Contents of all analysed plant hormones in mature leaves of poplar treated with sulphur depletion.

**Table S6.** Contents of all analysed plant hormones in xylem sap of poplars treated with sulphur depletion.

**Table S7.** Contents of all analysed plant hormones in phloem exudates of poplars treated with sulphur depletion.

## Acknowledgements

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