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 PtaSULTR1;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 PtaSULTR4;2 expression). During early S deficiency, whole plant distribution of S was impacted, as indicated by increasing expression of the phloem-localized sulphate transporter PtaSULTR1;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 PtaATPS3/4 (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...
and the expression of miR395 was shown to be induced upon post-transcriptionally regulated by microRNA395 (miR395), Nikiforova et al. (APS) reductase (APR) (Bolchi 2011). At SULTR1;2 SULTR4;2 seems to be more susceptible to S depletion than Arabidopsis 2007). Still considerable differences were observed between Different studies reported a cell-specific expression pattern of AtAPS, with enhanced activity of AtAPS (Kawashima et al., 2009, 2011). 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 AtSULTR1;1 and AtSULTR1;2. Expression of both genes was negatively affected by cytokinins in roots of Arabidopsis thaliana (Maruyama-Nakashita et al., 2004b; Werner et al., 2010). In contrast, Ohkama et al. (2002) showed a positive transcriptional regulation of AtAPRI and AtSULTR2;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 nitrate 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 KNO₃, 1.3 mM Ca(NO₃)₂·4H₂O, 0.3 mM MgSO₄·7H₂O, 1.5 mM KCl, 0.25 mM KH₂PO₄, 2.3 mM MnCl₂·4H₂O, 10 μM H₃BO₃, 0.08 μM CuCl₂·2H₂O, 0.2 μM ZnCl₂, 0.2 μM Na₂MoO₄·4H₂O, 0.04 μM CoCl₂·6H₂O, 22.5 μM Na₂-EDTA, 22.5 μM FeCl₃, pH 5.5] including 0.15% fungicide solution (Proplant, Dr. Stählern GmbH, Stade, Germany). Plants were cultivated in a greenhouse (26±2 °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 MgSO₄, but with 1.8 mM Ca(NO₃)₂·4H₂O, 22.5 mM MnCl₂·4H₂O, 0.22 mM H₃BO₃, 0.04 mM CuCl₂·2H₂O, 0.22 mM ZnCl₂, 0.22 mM Na₂MoO₄·4H₂O, 0.04 mM CoCl₂·6H₂O, 22.5 mM Na₂-EDTA, 22.5 mM FeCl₃, pH 5.5) including 0.15% fungicide solution (Proplant, Dr. Stählern GmbH, Stade, Germany). 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 tenth 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.

![Graph showing sulphate content in the flow-through solution during sulphur depletion treatment.](https://academic.oup.com/jxb/article-abstract/63/5/1873/526316)

**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 (NanoDrop® 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 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 PtaATPS1/2, PtaATPS3/4, PtaANT (Antegumenta),
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 penalty of 11 and a gap extension of 1.

|        | AtAPS1 (At1g22890) | 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 Ptβtub (β-tubulin) were prepared according to sequence information in the Populus trichocarpa genome database (http://genome.igi-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 and a gap extension of 1. Because each 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 (INV’α, Invitrogen) and the cloned gene segment was sequenced (Eurofins). The sequence for elongation factor 1α (EF1α) from Arabidopsis thaliana was used as a reference for the alignment of the corresponding gene sequences of P. tremula × P. alba (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 Kopriwa et al. (2004), and the sequence for elongation factor 1β (EF1β) 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 μg of ground plant tissue were mixed with 1 ml of extraction buffer [2% (w/v) PVP K40, 100 mM TRIS-HCl, pH 8.0, 25 mM Na2EDTA, 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/isoamylic alcohol (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 complemented to miRNA sequences were radio-labelled with [γ-32P]ATP using T4 polynucleotide kinase (Fermentas). The oligonucleotide probes used were 5′-GAGTCTTCCACTCCCTTG-3′ (miR395) and 5′-ACGGGATTCTGCAATTCACAC-3′ (miR395). The oligonucleotide sequence for detecting miR395 corresponds to the mature sequence of miR395a of P. trichocarpa (accession no. MIMAT0002031; taken from the microRNAs database ‘miBase’ (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 Tm of the oligonucleotide.

Detection of RNA cleavage products by RNA ligase-mediated 5′–3′ random amplification of cDNA ends (RLM 5′–3′ RACE)

RLM 5′–3′ RACE-PCR was performed with a modified GeneRacer Kit (Invitrogen) protocol (Llave et al., 2002). PCRs were performed using the GeneRacer forward primer 5′-CGACTGAGACGAGGAGCAGACTGA-3′ and the gene-specific reverse primer 5′-TCTTATCACCTCCTGAAGCTGCTGCAATC-3′ followed by a PCR with the nested GeneRacer forward primer 5′-GGACACTGACATGGACTGAGAGTA-3′ and the nested gene-specific reverse primer 5′-CTACATGGAAGTAAACCCAGGGAGCT-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

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of deionized H₂O 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 H₂O and analysed directly. In each case, 50 μl were injected into the ion chromatography system.

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 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 rp (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, 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’).

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.
Gene expression of sulphur-metabolism related genes

Expression of five SULTR genes, i.e. the root-localized PtaSULTR1;2, the phloem-specific PtaSULTR1;1, the two vacuolar sulphate transporter genes PtaSULTR4;1 and PtaSULTR4;2, and the leaf-localized sulphate transporter PtaSULTR2;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 PtaATPS1/2 and PtaATPS3/4 and the APS reductase gene PtaAPR, was examined.

In all tissues, mRNA levels of the group 1 sulphate transporter gene, PtaSULTR1;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 PtaSULTR1;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. 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).
Transcripts of the leaf-localized PtaSULTR2;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 PtaSULTR2;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 PtaSULTR4;1 reacted differently to S depletion in fine roots, and mature and young leaves. In fine roots and young leaves, transcript levels of PtaSULTR4;1 remained unaffected during the whole period of S depletion (Fig. 3A4, B4). In mature leaves, transcript levels of PtaSULTR4;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 PtaSULTR4;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, PtaSULTR4;2 transcripts were further enhanced after 21 d of S depletion.

As found for the expression of the putative vacular SULTR, transcript levels of PtaATPS1/2 and PtaATPS3/4 also reacted differently to S depletion in fine roots, and young and mature leaves. Expression of PtaATPS1/2 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 PtaATPS3/4 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 PtaATPS1/2 (see Fig. 4A1), while PtaATPS3/4 expression did not change significantly in young leaves (Fig. 4B2).
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\(\text{APR}\) 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\(\text{APR}\) 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\(\text{EF1b}\), Pta\(\text{bTub}\), and Pta\(\text{ANT}\), 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\(\text{APS1}\) and At\(\text{APS4}\) (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\(\text{ATPS3}\) and Pta\(\text{ATPS4}\) were predicted to be targets of miR395 (data not shown), and miR395-dependent cleavage products of Pta\(\text{ATPS3/4}\) 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 \(\text{trans}\)-zeatins (tZs), \(\text{cis}\)-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 were increased in young leaves after 21 d of S depletion (Fig. 7B1) while contents of cZ riboside 0-\(\beta\)-glucoside (cZROG) were increased after 5 d (Fig. 7B3). In mature leaves, contents of cZ 0-\(\beta\)-glucoside (cZOG) and cZROG decreased after 21 d of...
S sulphur 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 leaves.

**Fig. 5.** (A) Validation of PtaATPS3/4 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 PtaATPS3/4 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.
leaves where contents of DZ9G were elevated after 5 d and after 21 d of S depletion (Fig. 8C1). 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
performed. In fine roots, young leaves, and mature leaves, transcript levels of \textit{PtaSULTR1;1}, \textit{PtaSULTR4;2}, and \textit{PtaAPR} were strongly negatively correlated with sulphate contents (Table 2). Further strong negative correlations with sulphate contents were found for transcript levels of \textit{PtaSULTR1;2} in fine roots and for \textit{PtaSULTR4;1}, \textit{PtaATPS1/2} and \textit{PtaATPS3/4} in mature leaves. Transcript levels of \textit{PtaSULTR2;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 ($p=0.63$, $P=0.0007$), and sucrose contents correlated negatively with sulphate contents ($p=–0.53$, $P=0.0061$).

**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

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**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.
PtaSULTR1;2 in fine roots and of PtaSULTR4;2 and PtaAPR in fine roots, mature leaves, and young leaves (Fig. 11A). In addition, increasing transcript levels of PtaSULTR2;2, PtaSULTR4;1, PtaATPS3;1/2, and PtaATPS3;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 PtaATPS3;1/2 and PtaATPS3;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 PtaATPS genes are of importance for the regulation of S metabolism during S depletion. In young leaves, transcript levels of PtaSULTR2;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 shown from several other studies (Smith et al., 1997; Takahashi et al., 1997; Vidmar et al., 2000; Buchner et al., 2004a; 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, PtaSULTR1;2 (Supplementary fig. 1 of Dür et al., 2010), and of PtaAPR 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 PtaSULTR4;2 is likely to be an indication of increased sulphate re-mobilization from the vacuolar sulphate pool (Kataoka et al., 2004; Zuber et al., 2004) as observed in Brassica and Arabidopsis (Buchner et al., 2004a; Kataoka et al., 2004; Parmar et al., 2007; Abdallah et al., 2010). This assumption is supported by the finding that high transcript abundance of PtaSULTR4;2 correlated with low sulphate contents in leaves during seasonal growth (Dür et al., 2010). Up-regulation of SULTR4;2 induced by S depletion occurred earlier and was stronger than that of SULTR4;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). BnSULTR4;1 expression was induced only in mature leaves but not in young leaves (Dubousset et al., 2009). In addition, PtaSULTR4;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 (Dür 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 PtaSULTR1;2, PtaSULTR4;2, and PtaAPR, were further enhanced in all tissues upon early S deficiency and this was accompanied by increasing transcript levels of the phloem-specific PtaSULTR1;1 (Fig. 11B). In addition, enhanced expression of PtaATPS3;1/2 in fine roots and decreased expression of the leaf-localized PtaSULTR2;2 (Supplementary fig. 1 of Dür et al., 2010) in young leaves were detected as tissue-specific responses. At the metabolite level, OSH contents decreased
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 PtaSULTR2;2 in leaves seem controversial, but might be under developmental control. This is indicated by up-regulation of PtaSULTR2;2 transcripts in mature leaves and down-regulation in young leaves upon S depletion. Controversial reactions of SULTR2;2 expression upon

**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.
miR395a was very low. PtaATPS1/2 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 PtaATPS1/2 differs from that of PtaATPS3/4. The predicted amino acid sequences of the two ATPS isoforms of *P. trichocarpa*, PtaATPS1 and PtaATPS2, were most similar to the protein sequence of AtAPS2 that is proposed to be the only cytosolic ATPS in *A. thaliana* (Hatzefeld 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 thios and proteins were reduced in aps1 mutants and APS4-RNAi *Arabidopsis* plants under S depletion (Kawashima et al., 2011). Furthermore, AtAPS3 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).

Sensitivity might depend on different local expression patterns and can explain the higher levels of PtaATPS3/4 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 PtaATPS1/2 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 PtaATPS1/2 differs from that of PtaATPS3/4. The predicted amino acid sequences of the two ATPS isoforms of *P. trichocarpa*, PtaATPS1 and PtaATPS2, were most similar to the protein sequence of AtAPS2 that is proposed to be the only cytosolic ATPS in *A. thaliana* (Hatzefeld 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 thios and proteins were reduced in aps1 mutants and APS4-RNAi *Arabidopsis* plants under S depletion (Kawashima et al., 2011). Furthermore, AtAPS3 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).

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

|                      | Fine roots | Young leaves | Mature leaves |
|----------------------|------------|--------------|---------------|
| **PtaSULTR1;1**      | -0.81      | -0.85        | -0.91         |
| **PtaSULTR1;2**      | -0.77      | -0.66        | -0.91         |
| **PtaSULTR2;2**      | 0.68       | -0.85        | -0.91         |
| **PtaSULTR4;1**      | -0.75      | -0.85        | -0.91         |
| **PtaSULTR4;2**      | -0.69      | -0.85        | -0.91         |
| **PtaATPS1;2**       | -0.70      | -0.85        | -0.91         |
| **PtaATPS2;4**       | -0.52      | -0.85        | -0.91         |
| PtaAPR               | -0.79      | -0.78        | -0.91         |
| Glutathione          | 0.89       | -0.73        | -0.91         |
| Sucrose              | -0.75      | -0.73        | -0.91         |
| cZROG                | -0.79      | -0.85        | -0.91         |
| DZR                  | 0.75       | -0.73        | -0.91         |
| IAA                  | 0.59       | -0.73        | -0.91         |
| ABA                  | 0.65       | -0.73        | -0.91         |

- **ρ**, Pearson correlation coefficient; *p*, Spearman’s 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 PtaATPS3/4 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. PtaATPS3/4 has been identified as an *in vivo* target of miR395a by 5′ RACE. In mature leaves, transcript levels of PtaATPS3/4 were still higher than in the controls after 21 d of S depletion. In *Arabidopsis*, miR395 targeted AtAPS1, AtAPS3, AtAPS4, and AtSULTR2;1 (Jones-Rhoaides and Bartel, 2004; Jones-Rhoaides et al., 2006; Kawashima et al., 2009), but the cell type-specific expression pattern was different for miR395 and AtSULTR2;1 in roots (Kawashima et al., 2009; Liang et al., 2010). Moreover, post-transcriptional regulation via miR395 seems to affect primarily transcripts of AtAPS4 and only to a lesser extent transcripts of AtAPS1 and AtAPS3 (Liang et al., 2010; Kawashima et al., 2011). Thus, different sensitivities of PtaATPS3 and PtaATPS4 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 PtaATPS3/4 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.

### 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) 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.
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) led to increasing transcript levels of AtSULTR1;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. (2004b). The authors prevented up-regulation of AtSULTR1;1 and AtSULTR1;2 in Arabidopsis roots by application of benzyladenine, an artificial active cytokinin. When tZ or tZR was applied exogenously to Arabidopsis plants, increasing AtAPR and AtSULTR2;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 PtaAPR 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

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**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, dihydrozeatin; GSH, glutathione; IAA, indole-3-acetic-acid; tZ, trans-zeatin; tZR, tZ riboside. For abbreviations of genes (in italics) see text.
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, PtaSULTR4:2, clearly indicates enhanced sulphate remobilization already during S limitation from both leaf and root vacuoles. Comparable results were obtained with vegetative B. napus (Dubouset 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 TaeSULTR4:1 and TaeSULTR1:1 in the wheat shoot (Buchner et al., 2010), and the expression pattern of TaeSULTR1:1 is comparable with the expression pattern of PtaSULTR1:1 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 PtaAPR and PtaATPS1/2 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 (EF1β) (1), β-tubulin (bTub) (2), and Anteportina (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 carotenoids (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 PtaATPS1/1, PtaATPS2/4, PtaANT, and PtaTub.

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.

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