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Leaf age-dependent differences in sulphur assimilation and allocation in poplar (*Populus tremula* × *P. alba*) leaves

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Abstract

^{35}S-sulphate was flap-fed to poplar leaves of different leaf development stages – young developing, expanding, mature, and old mature poplar leaves. ^{35}S-sulphate was taken up independent of the leaf development stage. Whereas young development leaves did not export the ^{35}S taken up, export increased with increasing leaf development stage. Expanding leaves allocated the exported ^{35}S mainly into apical tree parts (73–87%) and only to a minor extent (13–27%) in basipetal direction. Neither lower trunk sections nor the roots were sinks for the exported ^{35}S. Expanding and developing leaves, but not the shoot apex, were the main sinks for the ^{35}S allocated in apical direction. In contrast, mature and old mature leaves exported the ^{35}S taken up mainly in basipetal direction (65–82%) with the roots constituting the main sinks. The ^{35}S allocated into apical tree parts was found in expanding and developing leaves, but only to a minor extent in the shoot apex. Apical allocated ^{35}S was identified as sulphate. Apparently the demand of young developing leaves for reduced sulphur was not fulfilled by mature leaves. Therefore, reduced sulphur for growth and development of young developing leaves must be supplied from other sources.

***In vitro* activity of enzymes involved in assimilatory sulphate reduction was measured to investigate whether demand for reduced sulphur by young leaves is met by their own sulphate reduction. ATP sulphurylase and APS reductase activities were not significantly lower in developing than in mature leaves. Sulphite reductase and serine acetyltransferase activities were highest in developing leaves; *O*-acetylserine (thiol) lyase activity was similar in all leaf developing stages. Apparently, young developing poplar leaves are able to produce their own reduced sulphur for growth and development. Whether other sources such as storage tissues and/or roots are involved in reduced sulphur supply to developing leaves remains to be elucidated.**

Key words: Poplar trees, glutathione, sulphate, sulphur nutrition, APS reductase, ATP sulphurylase, serine acetyltransferase, *O*-acety-L-serine (thiol) lyase, sulphite reductase, long-distance transport, leaf development.

Introduction

Perennial plants exhibit specific adaptations as a result of seasonal growth dynamics that include long-distance transport between source and sink tissues. In deciduous trees from the temperate zone, starch and storage proteins are mobilized during spring to supply developing leaves with reduced nitrogen, carbohydrate and other sources of energy (Sauter and van Cleve, 1994; Stepien *et al.*, 1994; Schneider *et al.*, 1994). Additionally, reduced sulphur is mobilized from storage tissues to fulfil the developing leaves' demand for reduced sulphur. The latter is indicated by increasing levels of cysteine (Rennenberg *et al.*, 1994) and glutathione (Schneider *et al.*, 1994) in xylem saps before and during bud break. Experiments involving feeding ^{35}S-sulphate to mature beech leaves revealed that sulphate, thiols and insoluble reduced sulphur, possibly incorporated into proteins, are stored

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Abbreviations: CHES, 2-(cyclohexylamino)-ethansulphonacid; DTNB, 5,5′-dithiobis(2-nitrobenzolacid); γ-EC, γ-glutamylcysteine; mBBr, monobromobimane; OAS, *O*-acetylserine; PVPP, polyvinylpolypyrrolidone; PVP, polyvinylpyrrolidone.

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during winter in wood and bark ray parenchyma cells (Herschbach and Rennenberg, 1996). During spring the stored sulphur is mobilized and transported to the swelling buds and expanding leaves. In contrast, the development of the current year's spruce sprouts is dependent on reduced sulphur supplied from last year's needles (Schupp and Rennenberg, 1992). Spruce trees with 4–12 needle generations develop one new flush, usually from April to June. Beech trees develop a single flush during spring within a relatively short period of time with the consequence that all leaves are in the same development stage. Therefore, the observed differences between beech and spruce could either reflect (1) a general difference between deciduous and evergreen trees or (2) different growth patterns.

In cottonwood (Dickson, 1989) and oak (Alaoui-Sossé *et al.*, 1994, 1996; Dickson and Tomlinson, 1996) which, respectively, continuously develop new leaves or two flushes of leaves during spring and summer, current photosynthate is allocated from mature to developing leaves as is also the case for coniferous trees (Ericsson, 1978; Hanson and Beck, 1994). In cottonwood, carbohydrate allocation strictly depends on the development stage of the leaf (Dickson, 1989). Expanding leaves are sink organs for current photosynthate from expanding leaves. Old mature leaves are source organs for the carbohydrate supply to stem and root tissues (Dickson, 1989). A similar relationship was observed in oak during development of the second flush (Dickson and Tomlinson, 1996). Leaves from the first flush allocated current photosynthate into the developing second flush. After full expansion of the second flush, the preference of carbohydrate allocation out of the first flush changed from apical to basipetal transport. This transition was accompanied by changes in sugar concentrations and activity of enzymes with regulatory functions in sucrose metabolism (Alaoui-Sossé *et al.*, 1994, 1996). These results demonstrate the dependence of developing leaves on carbohydrates synthesised in mature leaves and show changes in the source–sink relationship during leaf maturity.

The present study was performed with poplar that shows indeterminant growth in order to elucidate whether sulphur assimilation and allocation are dependent on leaf age in deciduous trees. Such a dependency is to be expected since sulphate reduction is thought to occur in the chloroplasts of leaves and — like the assimilation of carbohydrate — is dependent on photosynthetic energy supply. In contrast to their role of non-participation in carbohydrate assimilation, it appears that the roots can contribute to sulphur reduction in trees (Herschbach and Rennenberg, 1997). Thus, both the leaves and the roots may contribute to the demand for reduced sulphur exhibited by developing leaves, which are generally considered unable to fulfil their own needs for reduced sulphur in growth and development.

Materials and methods

Plant material

Non-mycorrhizal poplar trees (*Populus tremula* × *P. alba*; INRA clone 717 1B4) were micropropagated as described previously (Strohm *et al.*, 1995; Noctor *et al.*, 1996), transferred into a soil mixture, and grown in a greenhouse under long day conditions (light period: 16 h) in pots of 10 cm each in height, length and width. The soil mixture consisted of 1 part silica sand, particle size 0.06–0.2 mm, 1 part sterilized commercial soil and 2 parts Perlite (Agriperl, Perlite-Dämmstoff-GmbH, Germany). Trees were fertilized every 2 weeks with 200 ml commercial fertilizer (3 g l^{-1}, Hakaphos blau, COMPO GmbH, Germany; 15% N; 10% P_2O_5; 15% K_2O; 2% MgO; 0.01% B; 0.02% Cu; 0.05% Fe; 0.05% Mn; 0.001% Mo; and 0.015% Zn). After approximately 8 weeks on soil poplar plants were used for the experiments. At this time, the fresh weight of the roots and the shoot amounted to 16.5 ± 6.2 g and 37.2 ± 6.5 g, respectively. The number of leaves ranged from 25–29 and shoot length from 59–66 cm.

Feeding of ^{35}S-sulphate to the leaves

[^{35}S]-Sulphate was fed to leaves using the flap-feeding technique of Biddulph (Biddulph, 1956). Four different stages of leaf maturity, i.e. young developing leaves (stage 1), expanding leaves approximately 70% expanded (stage 2), mature (stage 3), and old mature leaves (stage 4) were used for the feeding experiments (Fig. 1). For feeding [^{35}S]-sulphate, a flap was cut into a leaf submerged in phosphate buffer (50 μM K_2HPO_4/KH_2PO_4 buffer, pH 6.2). The flap was dipped immediately into a tube containing 20 μl 50 μM phosphate buffer with carrier-free [^{35}S]-sulphate ($0.7–1.4 \times 10^6$ Bq or $3.7–5.1 \times 10^6$ Bq; NEN, Dreieich, Germany). The feeding solution was taken up completely within 10–40 min. After a total incubation time of 6 h at room temperature (28 ± 3 °C) and 150 ± 30 μE m^{-2} s^{-1} PAR (Osram, HPS L 65W/150 ultra white and Osram, L Fluora 35W/77R, Osram, Munich, Germany) incubation was terminated by cutting off the fed leaf and removing the flap from the remaining leaf. Subsequently, poplar trees were divided into leaves, trunk and roots. The latter were further dissected into bark and wood and immediately frozen in liquid nitrogen. Each sample was stored at −24 °C until analysis.

^{35}S-sulphur analysis

Analysis of ^{35}S-sulphur was performed as described previously (Herschbach and Rennenberg, 1996). Each frozen sample was ground into powder in a mortar under liquid nitrogen. For extraction of acid-soluble ^{35}S compounds, aliquots of approximately 100 mg were exposed to 1 ml 0.1 N HCl and 50 mg insoluble polyvinylpolypyrrolidone (PVPP) on a rotary shaker for 30 min at 8 °C. After centrifugation for 15 min at 23 000 *g* at 4 °C (Hettich Universal 30 RF, Hettich, Tuttlingen, Germany) 400 μl supernatant was transferred into scintillation vials (Mini Poly Q-Vials, Beckman Instruments, Munich, Germany). The remaining pellets were extracted twice with 1 ml 0.1 N HCl and each supernatant was transferred separately into a scintillation vial. For liquid scintillation counting (Wallac System 1409, Wallac Oy, Turku, Finland) 4 ml scintillation fluid (OptiPhase HiSafe 2, Canberra Packard, Frankfurt, Germany) was added to each sample. Samples were counted at 73–91% efficiency and were corrected for quenching.

The extracted pellets were used to determine the amount of ^{35}S incorporated into acid-insoluble material such as proteins and other high molecular weight cellular compounds. For discoloration of leaf and bark tissues 200 μl H_2O_2 (35% Merck,

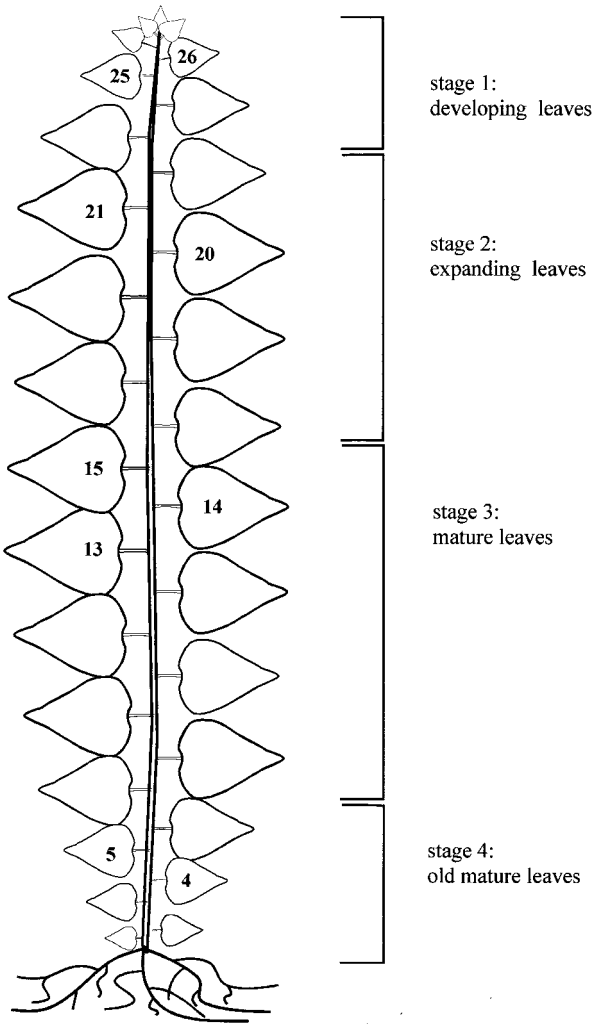

Fig. 1. Schematic view of an 8-week-old poplar with 29 leaves.

Darmstadt, Germany) were added. The mixture was dried 5–7 d at 50 °C and the resulting pellet was dissolved in 500 µl H_2O. Bleached leaf and bark samples were solubilized with a tissue solubilizer (Soluene 350, Canberra Packard, Frankfurt, Germany) for 24 h at 50 °C. 200 µl isopropanol was added and samples were transferred into 20 ml scintillation vials (Zinsser Analytik, Frankfurt, Germany). Wood pellets were dissolved with a tissue solubilizer overnight at 50 °C and subsequently 200 µl isopropanol was added before the transfer of suspensions into 20 ml scintillation vials. After addition of 300 µl H_2O_2 wood samples were bleached overnight at room temperature. For scintillation counting 15 ml liquid scintillation fluid (OptiPhase HiSave 2, Canberra Packard, Frankfurt, Germany) was added to each sample. Samples were counted at 60–90% efficiency and were corrected for quenching.

Collection of phloem exudates

Phloem exudates were sampled from poplar trees to which mature leaves (stage 3; 5.1×10^6 Bq ^{35}S-sulphate) were fed with ^{35}S-sulphate as described previously (Herschbach *et al.*, 1998). For this purpose bark slices of approximately 150 mg bark fresh weight were separated from the wood, washed in 2 mM EDTA and exuded in 2 mM EDTA, 1 mM cyanide at pH 5.8. To prevent destruction of thiols by phenols, polyvinylpolypyrrolidone (PVPP) was added at a PVPP : bark fresh weight ratio of 1 : 1. After 5 h at 4 °C exudation was complete (Herschbach *et al.*, 1998). Phloem exudates were centrifuged for 15 min at 23 000 *g* at 4 °C. The supernatant was frozen in liquid nitrogen and stored at −24 °C until analysis.

Identification of ^{35}S thiols in phloem exudates

Thiols in phloem exudates were analysed by the procedure reported previously (Herschbach *et al.*, 1998). Phloem exudates were centrifuged for 10 min at 23 000 *g* at 4 °C. 350 µl aliquots of the supernatant were adjusted to pH 8.3 ± 0.2 by the addition of 120 µl 0.5 M CHES (2-(*N*-cyclohexylamino)-ethane-2-sulphonic acid), pH 8.4. Reduction of thiols was initiated by addition of 30 µl 15 mM dithiothreitol (DTT) and terminated after 60 min by addition of 45 µl 30 mM monobromobimane (mBBr). After 15 min derivation was stopped by acidification with 120 µl 15% (v/v) acetic acid to stabilize mBBr thiol derivatives. Aliquots of 300 and 50 µl, respectively, were subjected to HPLC analysis as described previously (Schupp and Rennenberg, 1988).

Identification of ^{35}S-thiols in bark, wood and root tissues

Thiols in bark, wood and roots were extracted as described previously (Strohm *et al.*, 1995). 100 mg frozen powder was transferred into 1.2 ml 0.1 N HCl containing 100 mg insoluble PVPP (Sigma, Deisenhoven, Germany) pre-cooled at 4 °C. Samples were centrifuged for 20 min at 23 000 *g* at 4 °C. Supernatants from the first HCl extraction were used to identify low molecular weight thiols (Schupp *et al.*, 1992). For this purpose 350 µl supernatant was added to 250 µl 500 mM CHES (pH 9.2). For reduction of thiols 20 µl 15 mM DTT was added and the mixture incubated for 60 min at room temperature. Subsequently, mBBr (30 µl, 30 mM) was added. Derivation was terminated after 15 min by addition of 100 µl acetic acid (30%). Aliquots of 300 µl and 50 µl, respectively, were subjected to HPLC analysis for separating thiols as described previously (Schupp and Rennenberg, 1988). After HPLC analysis fractions of the eluate were collected at 1 min intervals and ^{35}S-radioactivity was determined by liquid scintillation counting after the addition of 4 ml scintillation fluid (OptiPhase HiSave 3, Canberra Packard, Frankfurt, Germany). Radioactive thiols in the eluate were identified by comparison with the fluorescence peaks of mBBr derivatives and by ^{35}S-cysteine, ^{35}S-glutathione and ^{35}S-sulphate standards.

Extraction of enzymes

For extraction of ATP sulphurylase, *O*-acetylserine (thiol) lyase, serine acetyltransferase, sulphite reductase, and acid invertase 500 mg leaf material was cut into small pieces and transferred into 5 ml ice-cooled extraction buffer A. Buffer A contained 0.1 M TRIS/HCl pH 8.0, 0.1 M KCl, 20 mM $MgCl_2$, 1% (w/v) Tween 80, 2% (w/v) polyvinylpyrrolidone (PVP K30), and 10 mM dithioerythritol (DTE). For the determination of APS reductase activity 100 mg leaf material was extracted in 5 ml ice-cooled extraction buffer B. Buffer B contained 0.1 M KH_2PO_4/K_2HPO_4 pH 7.7, 5 mM EDTA, 0.5 mM AMP, 1% (w/v) Tween 80, 5% (w/v) polyvinylpyrrolidone (PVP K30), 10 mM DTE, and 10 mM L-cysteine. Homogeneity was achieved by use of dispersing tools (Ultraturrax T25, IKA Labortechnik, Germany; and Polytron, Kinematic, Littau, Switzerland) twice for 5 s and once for 10 s at 15 000 rpm. All enzymes were measured in crude extracts after filtration.

Table 1. *Uptake, recovery and export of the ^{35}S fed to leaves of approximately 8-week-old poplar at different developing stages via flap feeding*

Data given are from single experiments.

Developing stage of the fed leaf	^{35}S-Sulphate taken up by the fed leaf (% of the ^{35}S fed)	Recovery of ^{35}S supplied (% of the ^{35}S fed)	^{35}S exported out of the fed leaf (% of the ^{35}S taken up)	^{35}S allocated apical (% of the ^{35}S exported)	^{35}S allocated basipetal (% of the ^{35}S exported)
Stage 2	92	100	0.1	74	27
ca. 70%	76	91	1.4	87	13
expanded	56	59	0.1	77	23
Stage 3	92	93	0.8	31	69
Mature	104	116	0.6	36	65
	80	86	0.9	27	73
	82	84	4.1	19	82
Stage 4	96	104	1.7	41	59
Old mature	65	80	2.8	34	66
	67	81	1.0	49	51

In vitro *ATP sulphurylase (ATPSase; EC 2.7.7.4) activity*

ATP sulphurylase activity was determined luminometrically by measuring the ATP production from APS and inorganic PP_i (reverse reaction) according to Schmutz and Brunold (Schmutz and Brunold, 1982). A 20 µl aliquot of a 1 : 5 diluted leaf extract was added to 100 µl 165 µM $Na_4P_2O_7$ and 20 µl 0.1 mM APS. The reaction was initiated by the addition of 100 µl luciferine/luciferase reagent (ATP Bioluminescence Assay Kit CLS II, Boehringer Mannheim, Mannheim, Germany). Initial ATP production was measured luminometrically (Biolumat LB 9500C, Berthold, Wildbad, Germany) over a 30 s interval. ATP production was linear until 120 s (data not shown). For quantification, ATP standards of 0.008–0.17 nmol ATP were measured.

In vitro *APS reductase (APSRase; EC 1.8.99.-) activity*

APS reductase activity was determined through the formation of ^{35}S-sulphite from AP^{35}S in the presence of the artificial carrier DTE according to Brunold and Suter (Brunold and Suter, 1983). The enzyme assay contained 50 µl 1 M TRIS/HCl pH 9, 200 µl 2 M $MgSO_4$, 80 µl extraction buffer B, 10 ml 0.2 M DTE, and 50 µl 1 : 2 diluted leaf extract. The reaction was initiated by the addition of 10 µl 3.75 mM AP^{35}S (46–75 kBq µmol^{-1}). After 29 min incubation at 37 °C the reaction tube was transferred into a 20 ml scintillation vial containing 1 ml 1 M triethanolamine pH 9–11. After 30 min 200 µl 1 M H_2SO_4 was added liberating ^{35}S-sulphite as $^{35}SO_2$. After 16 h at room temperature the reaction tube was removed from the scintillation vial. The trapping solution was mixed with 2 ml scintillation fluid (Quickszint Flow 306, Zinsser, Frankfurt, Germany) and ^{35}S radioactivity was detected by liquid scintillation counting (Betamatic, Kontron Instruments).

In vitro *sulphite reductase (SiRase; EC 1.8.7.1) activity*

In vitro activity of sulphite reductase was assayed with the artificial electron donor system dithionite/methylviologen for reduction of sulphite to sulphide. Because of the high endogenous activity of OASase in the extract the sulphide formed was coupled to cysteine formation as described previously (Schupp and Rennenberg, 1992). The enzyme assay contained 200 µl leaf extract, 450 µl 400 mM Tricine/NaOH pH 7.4, 150 µl 4.6 mM methylviologen, and 50 µl 120 mM OAS pH 6.0. The reaction was initiated by the addition of 150 µl 40.2 mM $Na_2S_2O_4$ 148.8 mM $NaHCO_3$ solution. After 15 min at 37 °C the reaction was terminated by the addition of 200 µl 50 mM CHES pH 8.4 and 30 µl 30 mM mBBr to 60 µl of the enzyme assay. After 15 min at room temperature derivation of cysteine was stopped by addition of 250 µl 10% (v/v) acetic acid. The mBBr cysteine derivative was quantified with a fluorescence detector after separation by reversed phase HPLC as described previously (Bosma *et al.*, 1991).

In vitro *serine acetyltransferase (SATase, EC 2.3.1.30) activity*

Measurement of serine acetyltransferase was based on coupling OAS formation from L-serine and acetyl-CoA by serine acetyltransferase with cysteine formation by *O*-acetylserine (thiol) lyase as described previously (Nakamura *et al.*, 1987). The enzyme assay contained 120 µl leaf extract, 20 µl TRIS/HCl pH 8.0, 20 µl 40.5 mM Na_2S, and 20 µl 300 mM serine. The reaction was initiated by the addition of 20 µl 30 mM acetyl-CoA. After 5 min incubation at 30 °C the reaction was terminated by the addition of 200 µl 50 mM CHES pH 8.0 and 40 µl 30 mM mBBr. Derivation was terminated after 15 min with 40 µl 15% (v/v) acetic acid. The mBBr cysteine derivative was quantified with a fluorescence detector after separation by reversed phase HPLC as described previously (Schupp and Rennenberg, 1992).

In vitro O-*acetylserine (thiol) lyase (OASase; EC 4.2.99.8) activity*

OAS lyase activity was determined through cysteine formation from OAS and sulphide (Pieniazek *et al.*, 1973), and measured photometrically through the formation of a ninhydrin derivative (Gaitonde, 1967). The enzyme assay contained 20 µl leaf extract, 200 µl 1 M TRIS/HCl pH 7.5, 50 µl 0.2 M DTE and 160 µl 50 mM OAS. The reaction was initiated with addition of 50 µl 162 mM Na_2S. After 5 min at 30 °C 200 µl enzyme assay was transferred into a tube in an ice-water bath and mixed with 200 µl ninhydrin reagent (250 mg ninhydrin, 4 ml HCl_{conz}, 16 ml 98% (v/v) acetic acid). After boiling 5 min of 400 µl ethanol was added and tubes were cooled to 4 °C in an ice-water bath. The absorption of the ninhydrin derivative was measured at 546 nm. For quantification, 10–660 nmol cysteine standards were subjected to the same procedure.

In vitro *acid invertase (EC 3.2.1.26) activity*

Activity of acid invertase was determined by glucose formation according to Schneider *et al.* (Schneider *et al.*, 1996). The enzyme assay contained 100 µl leaf extract and 900 µl H_2O; pH was adjusted to 4.5 with 100 µl 400 mM citric buffer pH 4.5. After 5 min at 37 °C the reaction was initiated by the addition of 100 µl 2 M sucrose. Glucose and fructose formation were stopped after 30 min by neutralization with 0.5 M NaOH within

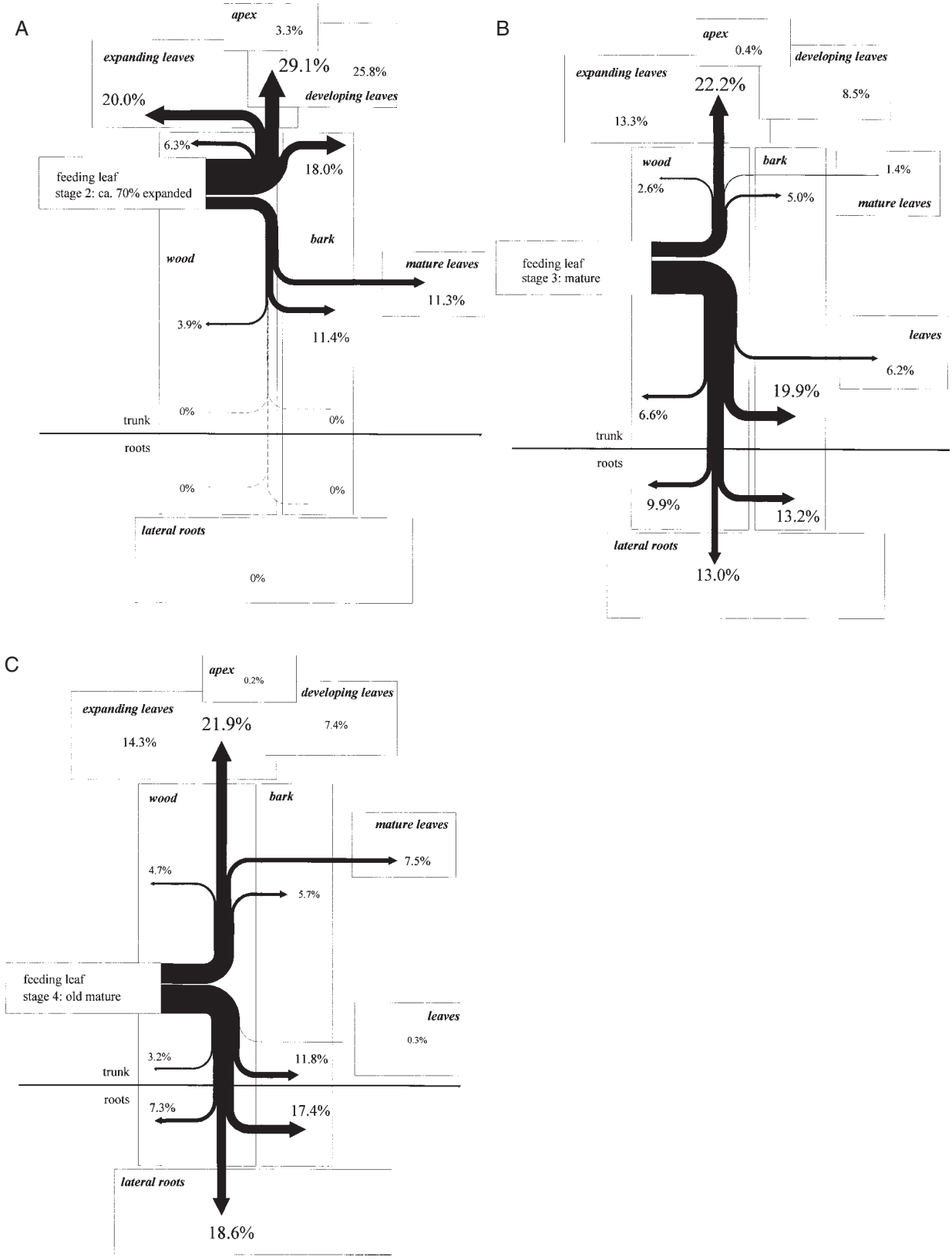

Fig. 2. Influence of leaf maturity on ^{35}S allocation. ^{35}S-sulphate was fed via flap feeding (Biddulph, 1956) to leaves of 8-week-old poplar trees at three different stages of maturity (expanding $n=3$ (A); mature $n=4$ (B); old mature $n=3$ (C)). Trees were dissected into leaves, bark and wood of various trunk sections; lateral roots; and bark and wood of main root sections. The absolute distribution pattern of the ^{35}S-radioactivity exported out of the fed leaf is given. The data shown represent a typical experiment having similar results to those of the independent experiments indicated.

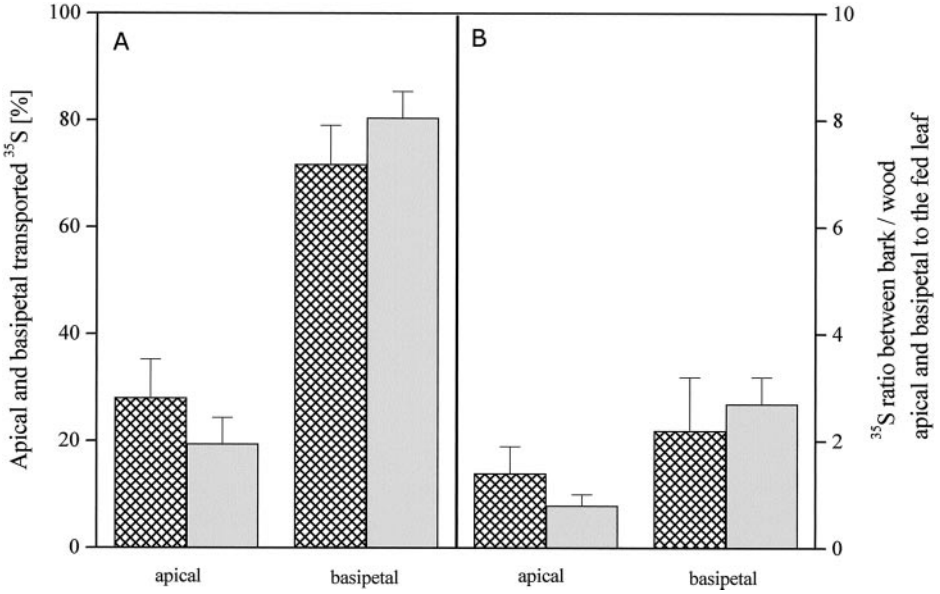

Fig. 3. Influence of stem girdling on ^{35}S-sulphur allocation. Feeding of ^{35}S-sulphate to a mature leaves (stage 3) of approximately 8-week old poplar trees was performed as described in Fig. 2. Apical to the fed mature leaf approximately 1 cm of the bark was peeled off to interrupt phloem transport. The absolute distribution of the ^{35}S exported out of the fed mature leaf into apical and basipetal tree parts as well as into bark and wood is given for three girdled (▨) and 4 control (▦) poplar trees.

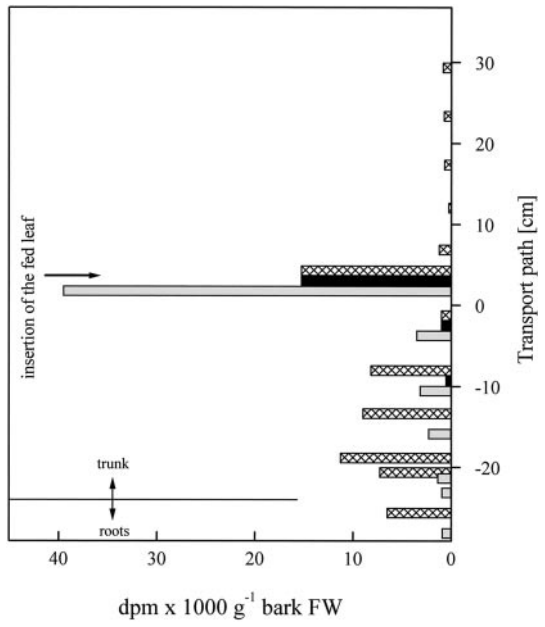

Fig. 4. Identification of the ^{35}S allocated in the phloem. ^{35}S-sulphate was fed to a mature leaf (stage 3) of an approximately 8-week-old poplar tree via flap feeding as described in Fig. 2. Bark pieces were selected along the tree axis and phloem exudates were sampled by incubation in an EDTA solution. Thiols were reduced with DTT, derivatized with mBBr and separated by reversed phase HPLC. ^{35}S-sulphate (▨), ^{35}S-cysteine (■) and ^{35}S-glutathione (▦) were detected in HPLC eluates. Data given represent one experiment.

the range of pH 6.5–9 and 2 min boiling at 95 °C. Glucose and fructose content were analysed by use of a D-glucose/D-fructose test kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Protein determination

Soluble protein in 100 µl aliquots of leaf extract was precipitated with 100 µl trichloroacetic acid (10%, w/v) for 10 min at room temperature. After centrifugation for 5 min at 4883 *g* (Rotina 48R, Hettich, Tuttlingen, Germany) the remaining pellets were dissolved in 1 ml 1 M KOH. The protein content of this solution was determined as described previously (Bradford, 1976). For this purpose aliquots of 50 µl were diluted with 750 µl H_2O and subsequently 200 µl Bradford reagent was added. Solutions containing 2–8 µg bovine serum albumin served as protein standards.

Data analysis

Statistical analysis was performed using Dunkan's multifactorial analysis with the statistic program SPSS (SPSS for Windows).

Results

Influence of leaf maturity on sulphur allocation

^{35}S-sulphate was fed to the leaves of approximately 8-week old poplar plants at four different leaf development stages for 6 h (Fig. 1, Table 1). Young developing leaves took up only small amounts of the feeding solution (data not shown). ^{35}S detected in soluble and insoluble compounds apical and basipetal to the fed leaf was close to the detection limit. Apparently, the ^{35}S taken up was not transported out of young developing leaves. Independent of the developing stage, expanding, mature

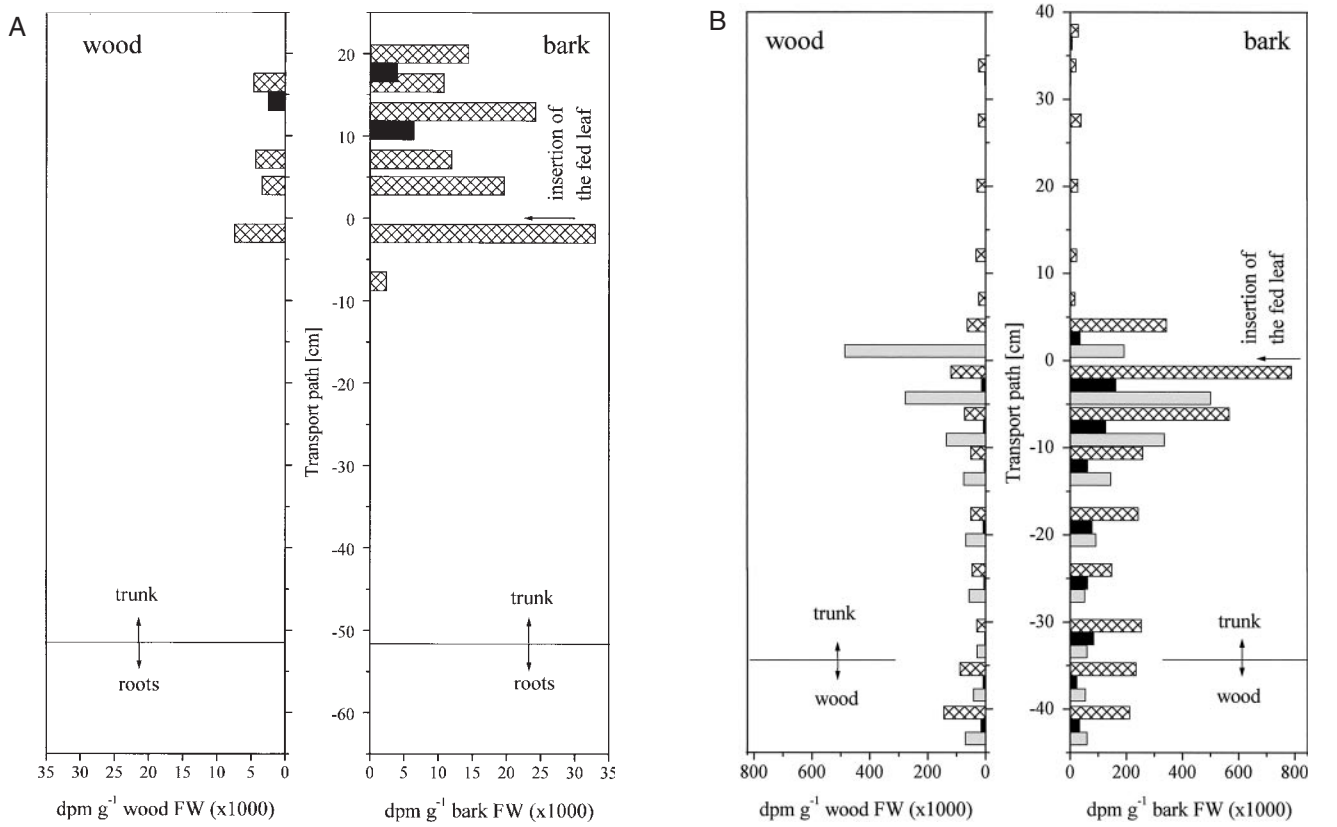

Fig. 5. Identification of the ^{35}S found in bark and wood tissues. ^{35}S-sulphate was fed to expanding (A, stage 2) and mature leaves (B, stage 3) of approximately 8-week old poplar trees via flap feeding as described in Fig. 2. The tree axis was dissected into approximately 15 sections. Each section was separated into bark and wood. Acid-soluble thiols were extracted, reduced with DTT, derivatized with mBBr and separated by reversed phase HPLC. ^{35}S-sulphate (▨), ^{35}S-cysteine (■) and ^{35}S-glutathione (▤) were detected in HPLC eluates. Data given show one experiment at each of development stages 2 and 3.

or old mature leaves took up $81 \pm 15\%$ of the supplied ^{35}S-sulphate (Table 1). Export ranged between 0.1% and 4.1% and was not significantly different among expanding, mature and old mature leaves. The low ^{35}S export rates out of the fed leaf were comparable with the export in beech (Herschbach and Rennenberg, 1995) and oak (Schulte *et al.*, 1998).

Expanding leaves (stage 2; Table 1) allocated 73–87% from the ^{35}S exported into apical parts and 13–27% into basipetal parts of the tree (Table 1). Developing and expanding leaves rather than the shoot apex were the predominant sinks (Fig. 2A). In the example shown in Fig. 2A, 25.8% of the ^{35}S exported was translocated into developing and 20.0% into expanding leaves. Comparable amounts were detected along the apical transport path, i.e. 18.0% in bark and 6.3% in wood tissues. ^{35}S transported in basipetal direction was found in mature leaves (11.3%) and in the corresponding bark tissues (11.4%). Old mature leaves and the corresponding stem tissues as well as roots did not serve as sinks for the ^{35}S exported out of expanding leaves (Fig. 2A). Similar results were observed with 14-week-old poplar trees having 44–48 leaves (data not shown).

The distribution of the exported ^{35}S changed dramatically when mature leaves, rather than expanding leaves, were fed. Only 19–36% of the exported ^{35}S was allocated into apical parts of the tree; the highest proportion (65–82%) of the exported ^{35}S was found basipetal to the fed leaf (Table 1). Similar results were observed with 14-week-old poplar trees (data not shown). Main sinks for the apical transported ^{35}S were expanding and developing leaves (Fig. 2B) rather than the shoot apex. The main sinks of the basipetal allocated ^{35}S were the stem bark (19.9%), root bark (13.2%) and fine roots (13%). The ^{35}S found in the stem wood and in the wood of main roots was comparable with that determined in old mature leaves and amounted to 6.2–9.9% of the exported ^{35}S (Fig. 2B). The distribution pattern of the ^{35}S exported out of old mature leaves was similar to that of mature leaves (Fig. 2C). The main proportion of the ^{35}S transported in an apical direction was found in expanding (14.3%) and developing leaves (7.4%). 7.5% of the exported ^{35}S was measured in mature leaves apical to the fed leaf. Root bark tissues (17.4%) as well as lateral roots (18.6%) were the main sinks for the ^{35}S transported in basipetal direction (Fig. 2C). In stem as well as in root

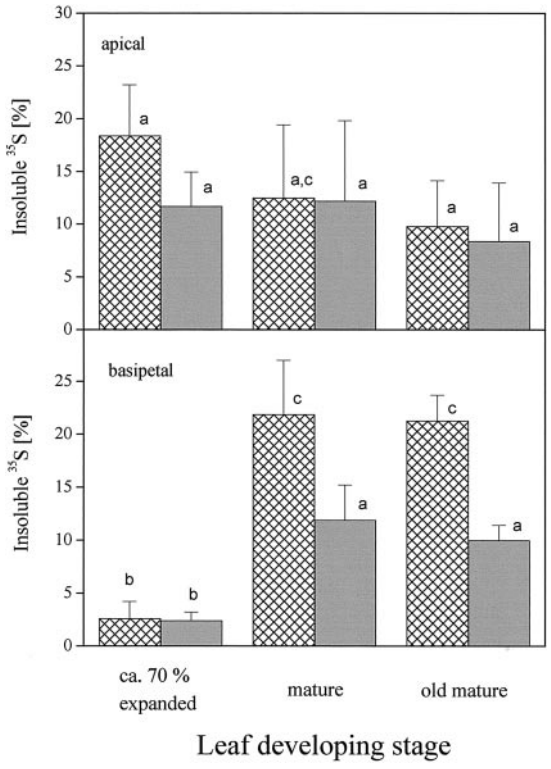

Fig. 6. Metabolization of the ^{35}S exported out of the fed leaf. ^{35}S-sulphate was fed to expanding (stage 2, $n=3$), mature (stage 3, $n=4$) and old mature leaves (stage 4, $n=3$) of approximately 8-week-old wild-type poplar trees via flap feeding as described in Fig. 2. The tree axis was dissected into leaves, bark and wood sections, and roots. Acid-soluble ^{35}S was extracted from powdered samples and insoluble ^{35}S was extracted from the remaining pellets with a tissue solubilizer. The percentage of insoluble ^{35}S from total ^{35}S found in each sample is given. Indices indicated significant differences at $P<0.05$ between bark (▦) and wood (▩) as well as between apical and basipetal sections. Data shown are the mean values calculated from the independent experiments indicated.

sections, bark tissues were stronger sinks than wood tissues independent of the developmental stage of the fed leaf.

Sulphur exchange between phloem and xylem

To investigate the contribution of phloem to xylem exchange to apical allocation of ^{35}S, the stem was girdled apical to the fed leaf. 1 cm bark was removed apical to mature leaves (stage 3). Export of ^{35}S out of mature leaves was $2.2\pm1.1\%$ in girdled trees and not significantly different from ungirdled controls ($1.6\pm1.7\%$). The girdle did not influence the distribution pattern of the exported ^{35}S (Fig. 3). The percentage of ^{35}S allocated in apical direction ranged from 17–36% independent of girdling. Basipetal allocation of ^{35}S was not enhanced as a consequence of interrupted phloem transport apical to the fed leaf and ranged from 65–83%. Apparently, ^{35}S transported into apical parts of the tree was rapidly exchanged between phloem and xylem. The distribution of ^{35}S between bark and wood was not influenced by stem girdling (Fig. 3), neither in apical (0.7–1.9) nor in basipetal (1.6–3.7) parts of the stem. These results demonstrate that ^{35}S can be transported from the xylem back into bark tissues and thus is subject to bi-directional exchange between bark and wood.

Identification of translocated sulphur compounds

Of the ^{35}S-sulphate taken up by mature leaves $1.7\pm0.9\%$ was found in cysteine, $3.6\pm2.3\%$ in glutathione, 13.9% in acid-insoluble sulphur compounds such as protein, and 23.2–39.1% could not be identified. Analysis of phloem exudates showed that sulphate was exclusively transported in apical direction (Fig. 4) whereas reduced sulphur, mainly as glutathione, as well as sulphate were transported in basipetal direction. The ^{35}S glutathione content in phloem exudates per gram fresh bark decreased with increasing distance from the fed leaf, indicating increasing specific labelling of the transport pool of glutathione with continuing incubation. The ^{35}S-sulphate/^{35}S-glutathione ratio was 5.3 ± 2.0 and did not vary along the transport path (data not shown). Analysis of bark and wood tissues showed similar results (Fig. 5). Only ^{35}S-sulphate was identified in apical tree sections independent of the maturity of the leaf fed (Fig. 5A, B). Because ^{35}S-cysteine was not found along the apical transport path, ^{35}S-cysteine identified in the youngest bark and wood tissues must be attributed to sulphate reduction in developing leaves (Fig. 5A). Upon feeding mature leaves (stage 3), ^{35}S was transported to the roots (Figs 2B; 5B). The ^{35}S allocated in basipetal direction was identified as sulphate, cysteine and glutathione independent of the tissue analysed (Fig. 5B). In bark and wood tissues ^{35}S in glutathione decreased with increasing distance from the fed leaf, but accumulated slightly in wood tissues. ^{35}S-sulphate in wood tissues decreased along the trunk, but accumulated in the root wood. In the bark ^{35}S-sulphate decreased along the trunk and remained unchanged in root bark tissues. In both bark and wood tissues the ^{35}S-sulphate/^{35}S-glutathione ratio increased along the trunk from apical to basipetal direction (data not shown). In wood tissues the ^{35}S-sulphate/^{35}S-glutathione ratio increased from 0.4 in apical to 2.1 in basal trunk sections, whereas in bark tissues this ratio was generally higher and increased from 1.6 in apical to 4.2 in basal trunk sections (data not shown).

Metabolization of the ^{35}S exported out of the fed leaves varied depending on the leaf developmental stage of the fed leaf (Fig. 6). When an expanding leaf was fed, of the exported ^{35}S $11.7\pm3.2\%$ and $18.0\pm4.8\%$ was metabolized into insoluble ^{35}S in bark and in wood tissues respectively. Significantly lower percentages were metabolized in bark ($2.4\pm0.8\%$) and wood ($2.6\pm1.6\%$) tissues basipetal to the fed leaf. When mature and old mature leaves were

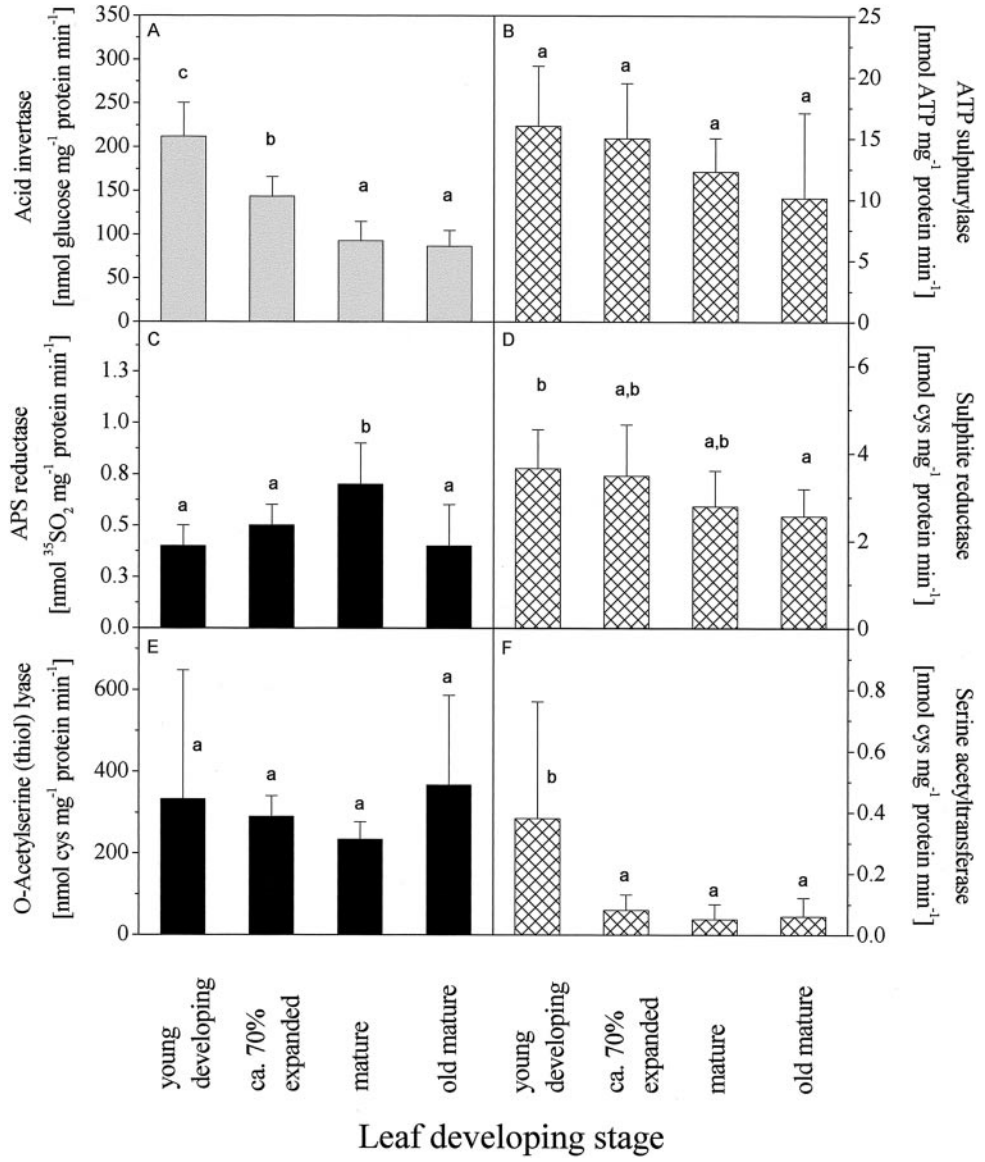

Fig. 7. *In vitro* activity of the enzymes involved in the assimilatory sulphate reduction pathway in dependence of leaf maturity. Acid invertase (A) ATP sulphurylase (B), APS reductase (C), sulphite reductase (D) *O*-acetylserine (thiol) lyase (E), and serine acetyltransferase (F) were extracted and their activities determined in young developing (stage 1), expanding (stage 2), mature (stage 3) and old mature poplar leaves (stage 4) as described in the Materials and methods section. Data given are means ±SD of 4–6 independent experiments with one poplar tree each.

fed, wood tissues basipetal to the fed leaf incorporated significantly more ^{35}S into insoluble compounds compared to basipetal bark tissues (Fig. 6).

In vitro *activities of enzymes involved in the assimilatory sulphate reduction*

The *in vitro* activities of enzymes involved in assimilatory sulphate reduction were determined in poplar leaves at different stages of leaf maturity. ATP sulphurylase activity declined slightly, but not significantly, from 16 ± 5 nmol ATP mg^{-1} protein min^{-1} in developing leaves to 10 ± 7 nmol ATP mg^{-1} protein min^{-1} in old mature leaves (Fig. 7B). APS reductase activity showed a significant maximum in mature leaves (stage 3, Fig. 7C); however, the activities in developing and old mature leaves were similar (0.4 ± 0.1 and 0.4 ± 0.2 nmol $^{35}SO_2$ mg^{-1} protein min^{-1}, respectively). Sulphite reductase activity declined significantly from 3.7 ± 0.9 in young developing leaves to 2.6 ± 0.6 nmol cys mg^{-1} protein min^{-1} in old mature leaves (Fig. 7D). *O*-acetylserine (thiol) lyase activity was similar in all leaf developmental stages investigated and ranged from 200 to 700 nmol cys mg^{-1} protein min^{-1} (Fig. 7E). Serine acetyltransferase activity was generally close to the detection limit of the applied method (30–50 pmol cys mg^{-1} protein min^{-1}). Highest activity was determined in developing poplar leaves ($n = 12$,

Fig. 7F). The protein content per gram fresh leaf weight was similar in all leaf developmental stages studied and ranged from 10–47 mg protein g^{-1} fresh leaf weight (data not shown).

Acid invertase activity was measured as an indication of leaf maturity, since the activity of this enzyme declines with increasing leaf age in oak (Alaoui-Sossé *et al.*, 1996) and citrus leaves (Schaffer *et al.*, 1987). In poplar, the highest acid invertase activity was found in young developing (212 ± 39 nmol glucose mg^{-1} protein min^{-1}) and expanding leaves (143 ± 22 nmol glucose mg^{-1} protein min^{-1}; Fig. 7A). In mature and old mature leaves, lowest *in vitro* acid invertase activity was determined and amounted to 92 ± 22 and 86 ± 18 nmol glucose mg^{-1} protein min^{-1}, respectively. Similar results were obtained for alkaline invertase but absolute values were 10 times lower than those determined for acid invertase (data not shown).

Discussion

The dependency of the allocation pattern of ^{35}S on leaf maturity in poplars determined in the present study is similar to the ^{14}C photosynthate distribution pattern observed in cottonwood after $^{14}CO_2$ labelling (Dickson, 1989). ^{35}S in expanding leaves was mainly transported into developing tissues and ^{35}S in mature and old mature leaves was allocated both to developing leaves and to storage tissues. This result demonstrates that the source–sink relationship of ^{35}S depends on leaf maturity and leaf position as previously found for ^{14}C photosynthate (Dickson, 1989). Mature cottonwood leaves showed transport of an equal amount of ^{14}C photosynthate into apical as well as into basipetal tree parts and old mature leaves exported the main proportion into basal tree parts. The ^{14}C photosynthate allocated in apical direction could supply energy for growth and development of developing leaves, whereas the basipetal translocated ^{14}C is used for storage as well as root growth and development. Correspondingly, reduced sulphur transported from mature to developing leaves may fulfil the demand for reduced sulphur for protein synthesis necessary in growth and development. The ^{35}S transported in an apical direction was found to be sulphate rather than reduced sulphur. Apparently, young developing poplar leaves are not supplied with reduced sulphur from mature leaves. This conclusion is supported by previous investigations with beech (Herschbach and Rennenberg, 1995, 1996) and oak (Schulte *et al.*, 1998). ^{35}S-sulphur fed to mature beech and oak leaves was not allocated into apical tree parts in beech and oak, irrespective of whether or not a new sprout developed; ^{35}S-sulphate, ^{35}S-glutathione and ^{35}S-cysteine were only transported into basipetal storage tissues of the trunk.

A cycling pool of reduced sulphur was not observed in beech and oak (Herschbach and Rennenberg, 1995; Schulte *et al.*, 1998) even after prolonged chase periods (Herschbach and Rennenberg, 1996). Consistent with these observations reduced sulphur is not exchanged between phloem and xylem in poplar (Figs 4, 5); however, ^{35}S-sulphate is transported into apical parts of poplar trees (Figs 4, 5) and exchanged between phloem and xylem (Fig. 3). Apparently a cycling pool of sulphate exists in poplar trees. Sulphur redistribution experiments exhibiting sulphur deficiency in herbaceous plants showed similar results (Smith and Lang, 1988; Sunarpi and Anderson, 1996, 1997; Blake-Kalff *et al.*, 1998). In oilseed rape under sulphur deficiency the sulphate pool was diminished in older leaves to supply young developing leaves with sulphur (Blake-Kalff *et al.*, 1998). Neither glucosinolates nor glutathione constituted a major source of sulphur during sulphur deficiency in oilseed rape, and remobilization from insoluble sulphur was not observed. In combination with nitrogen deficiency, sulphur starvation in soybean stimulated hydrolysis of proteins and the subsequent export of hGSH synthesized from insoluble sulphur (Sunarpi and Anderson, 1997). Smith and Lang (Smith and Lang, 1988) found that 90% of suphur transported out of mature soybean leaves could be attributed to sulphate, and export of glutathione to developing leaves appears negligible. The authors concluded that most of the sulphate entering mature leaves was directly reloaded into the phloem. These findings support the general assumption that reduced sulphur for growth and development of developing leaves is not supplied by older mature leaves.

The demand for reduced sulphur in developing poplar leaves must therefore be fulfilled by other sources. It may be supplied (1) by the young leaves' own assimilatory sulphate reduction, (2) by mobilization of reduced sulphur in storage tissues of the stem as observed in beech during spring (Herschbach and Rennenberg, 1996) and/or (3) by assimilatory sulphate reduction in the roots. Investigation of the *in vitro* activity of the enzymes involved in assimilatory sulphate reduction showed that all enzymes were active in developing poplar leaves (Fig. 7). In developing poplar leaves ATP sulphurylase activity declined slightly with increasing leaf maturity, APS reductase showed a maximum activity in mature leaves and sulphite reductase activity decreased significantly with increasing leaf age (Fig. 7). In *Pisum sativum* ATP sulphurylase, APS reductase and sulphite reductase were present at high specific activities in young leaves and declined with increasing leaf age (von Arb and Brunold, 1986). In contrast, in developing spruce needles, ATP sulphurylase activity increased during bud swelling but declined rapidly after bud break (Schupp and Rennenberg, 1992). APS reductase activity exhibited peak

values before bud break, but was low during later needle development for the whole growing season and sulphite reductase activity increased with increasing needle age (Schupp and Rennenberg, 1992). From these findings it was concluded that young developing spruce needles are not able to reduce sulphur for protein synthesis in sufficient amounts. Since ATP sulphurylase and APS reductase activities were not significantly lower in developing than in old mature poplar leaves and sulphite reductase activity was higher in young than in mature leaves, it has been concluded that these enzymes may not limit sulphate reduction (Brunold, 1993) in young developing poplar leaves. This assumption is supported by the finding that sulphate was transported to developing poplar leaves and that cysteine was labelled in bark and wood tissues of the shoot apex (Fig. 5).

To synthesize cysteine, sulphide is exchanged by the *O*-acetyl-group of *O*-acetylserine in the reaction catalysed by *O*-acetylserine (thiol) lyase. In spruce needles *O*-acetylserine (thiol) lyase activity increased continuously with increasing needle maturity (Schupp and Rennenberg, 1992); in poplar leaves *O*-acetylserine (thiol) lyase activity remained constant during different developmental stages as was previously found in *Pisum sativum* (von Arb and Brunold, 1986). Therefore, this enzyme is unlikely to limit cysteine formation. *O*-Acetylserine is produced by serine acetyltransferase activity which couples sulphur and nitrogen metabolism. Serine acetyltransferase is associated with *O*-acetylserine (thiol) lyase in a multienzyme complex to enable increased cysteine building velocity (Ruffet *et al*., 1994). The activity of serine acetyltransferase is generally low. This enzyme is thought to play a key role in the regulation of cysteine synthesis (1) by supplying OAS for cysteine synthesis which can, in turn, stimulate ATP sulphurylase and APS reductase activity (Neuschwander *et al*., 1991) and (2) by cysteine-mediated feedback inhibition of the enzyme dependent on its subcellular localization (Noji *et al*., 1998). As in spruce, serine acetyltransferase activity was 1000 times lower than *O*-acetylserine (thiol) lyase activity in the present investigation of poplar; in other studies 300 times lower activity was observed (Schupp and Rennenberg, 1992; Ruffet *et al*., 1994). Serine actetyltransferase activity in developing poplar leaves was significantly higher than that observed in mature leaves. It can therefore be concluded that young developing poplar leaves are able to produce cysteine for protein synthesis in appreciable amounts. Nevertheless, it remains unclear as to whether the cysteine demand of developing poplar leaves is completely fulfilled by their own cysteine synthesis, or supplemented by other sources, such as the mobilization of cysteine from storage tissues of the trunk and cysteine synthesis in the roots. Further studies are required to provide more detailed information on the supply of reduced sulphur to developing poplar leaves.

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