

RESEARCH PAPER

# Redox states of glutathione and ascorbate in root tips of poplar (*Populus tremula* × *P. alba*) depend on phloem transport from the shoot to the roots

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Received 8 October 2009; Revised 24 November 2009; Accepted 27 November 2009

## Abstract

Glutathione (GSH) and ascorbate (ASC) are important antioxidants that are involved in stress defence and cell proliferation of meristematic root cells. In principle, synthesis of ASC and GSH in the roots as well as ASC and GSH transport from the shoot to the roots by phloem mass flow is possible. However, it is not yet known whether the ASC and/or the GSH level in roots depends on the supply from the shoot. This was analysed by feeding mature leaves with [<sup>14</sup>C]ASC or [<sup>35</sup>S]GSH and subsequent detection of the radiolabel in different root fractions. Quantitative dependency of root ASC and GSH on shoot-derived ASC and GSH was investigated with poplar (*Populus tremula* × *P. alba*) trees interrupted in phloem transport. [<sup>35</sup>S]GSH is transported from mature leaves to the root tips, but is withdrawn from the phloem along the entire transport path. When phloem transport was interrupted, the GSH content in root tips halved within 3 d. [<sup>14</sup>C]ASC is also transported from mature leaves to the root tips but, in contrast to GSH, ASC is not removed from the phloem along the transport path. Accordingly, ASC accumulates in root tips. Interruption of phloem transport disturbed the level and the ASC redox state within the entire root system. Diminished total ASC levels were attributed mainly to a decline of dehydroascorbate (DHA). As the redox state of ASC is of particular significance for root growth and development, it is concluded that phloem transport of ASC may constitute a shoot to root signal to coordinate growth and development at the whole plant level.

**Key words:** Ascorbate, glutathione, phloem transport, poplar, redox state, root growth.

## Introduction

Ascorbate (ASC) and glutathione (GSH) are important antioxidants that exhibit numerous functions in stress defence, regulation of plant metabolism, as well as growth and development (May *et al.*, 1998; Meyer and Hell, 2005; Mullineaux and Rausch, 2005; Halliwell, 2006; Noctor, 2006; Meyer, 2008; Foyer and Noctor, 2009). Both ASC and GSH are involved in root development due to their function in redox regulation. However, this function is executed in an independent way because one cannot compensate for the absence of the other (Sánchez-Fernández *et al.*, 1997; Potters *et al.*, 2002, 2004; Jiang and Feldman, 2005). Maintenance of the root quiescent centre (QC) is accompanied by low total ASC content and high ascorbate oxidase activity (Kerk and Feldman, 1995; Liso

*et al.*, 2004); as a result the total ASC pool is dominated by dehydroascorbate (DHA) (Jiang *et al.*, 2003). ASC is necessary for the transition from G<sub>1</sub> to S in the cell cycle (Liso *et al.*, 1988).

As a consequence, any changes in ASC content affect cell cycle activity. Hence, the G<sub>1</sub> state is extended when the ASC content of the cells in the QC is low, as reviewed in Potters *et al.* (2002). Application of ASC induced cell division in *Allium cepa* roots (Liso *et al.*, 1988). In the tobacco cultivar Bright Yellow 2 (BY-2), ASC stimulated cell division while DHA decreased the mitotic index (de Pinto *et al.*, 1999) and slowed down cell cycle progression (Potters *et al.*, 2004). The latter, however, was only observed when DHA was added in the G<sub>1</sub> phase (Potters *et al.*, 2004). ASC treatment

of *Arabidopsis* roots resulted in a complete loss of a QC marker. Lee *et al.* (2007) concluded that ASC treatment might change the maintenance of cell type identities in roots, and affected cell type-specific gene expression. When auxin transport was inhibited by a specific inhibitor, DHA in the QC declined to approximately one-third compared to the controls, whereas ASC increased; these reactions were accompanied by an activation of the distal region in the QC (Jiang *et al.*, 2003). These examples clearly demonstrate the importance of the ASC to DHA ratio and its adjustment for root growth and development.

GSH, though less intensively studied, is also important for root growth and development (Potters *et al.*, 2002). GSH enhanced the number of meristematic cells undergoing mitosis, while depletion of GSH had the opposite effect (Sánchez-Fernández *et al.*, 1997). Inhibition of GSH synthesis by the specific inhibitor buthionine sulphoximine (BSO) resulted in reduced root formation (Cobbett *et al.*, 1998). In accordance with this, a mutant from *Arabidopsis* (*rml1*) deficient in  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -ECS) is unable to establish roots (Vernoux *et al.*, 2000).  $\gamma$ -ECS catalyses the first step of glutathione synthesis. Thus this mutant revealed a markedly diminished GSH content.

Root hair development depends on formation of reactive oxygen species (ROS) by NADPH oxidase (Foreman *et al.*, 2003). As ASC and GSH are involved in ROS detoxification (Noctor and Foyer 1998), the amounts of ASC and/or GSH as well as the maintenance of its redox state seem to be important for root hair growth. Indeed, in *Arabidopsis* roots, the GSH level is linked to root hair tip growth, and redox-dependent modulation is thought to be a crucial element in adjusting growth and development to the environment conditions (Sánchez-Fernández *et al.*, 1997). In addition to the influences of ASC on cell division within the QC, it may also be involved in lateral root development, since a very low ASC content *Arabidopsis* mutant (*vtc2*) exhibited altered root growth with the number and length of lateral roots being increased (Olmos *et al.*, 2006). As ASC removes ROS (Foyer and Halliwell, 1976; Noctor and Foyer, 1998), the low ASC in roots of the *vtc2* mutant can improve lateral root development (Foreman *et al.*, 2003; Olmos *et al.*, 2006) probably by retaining ROS. Therefore, low ASC contents mediated by ROS scavenging under stress conditions may improve growth, as discussed by Olmos *et al.* (2006). From these observations it can be hypothesized that ASC and/or GSH transported from the shoot to the roots may affect the levels of these antioxidants in the roots and thereby function as a shoot to root signal for growth and development. A prerequisite for such a function is that the ASC and GSH level in the root tip depends on its long-distance transport from the shoot.

The highest level of ASC synthesis takes place in the leaves, but ASC synthesis seems to be apparent in all plant cells (see Hancock *et al.*, 2003). Feeding of L-galactono-1,4-lactone, the precursor of ascorbate (Smirnoff *et al.*, 2001), results in increased ASC contents mainly in mature leaves of *Medicago sativa* (Franceschi and Tarlyn, 2002) but also in *Cucurbita maxima* roots (Liso *et al.*, 2004). ASC is

a widespread constituent of phloem sap, and isolated phloem strands are competent for ASC biosynthesis (Hancock *et al.*, 2003). Transport of ASC from leaves to sink tissues such as root tips and floral tissues has been demonstrated for three herbaceous plant species (Franceschi and Tarlyn, 2002). Since sulphate assimilation is a light-dependent process (Brunold, 1990) and because cysteine formation limits GSH synthesis (Strohm *et al.*, 1995) it is assumed that GSH is mainly synthesized in the leaves. This is supported by the finding that GSH synthesis can be stimulated with increasing light intensity (Ogawa *et al.*, 2004). However, glutathione production has also been found in other plant organs including the roots (Vauclare *et al.*, 2002). Like ASC, GSH is a regular constituent of phloem sap (Rennenberg *et al.*, 1979; Bonas *et al.*, 1982; Lappartient and Touraine, 1996; Bourgis *et al.*, 1999; Hartmann *et al.*, 2000; Kuzuhara *et al.*, 2000; Schulte *et al.*, 2002) and is transported from mature leaves to the roots (Rennenberg *et al.*, 1979; Bonas *et al.*, 1982; Hartmann *et al.*, 2000). However, it has not been established whether ASC and/or GSH levels in the roots and, hence, root growth and development depend on *in situ* synthesis of these antioxidants or its long-distance transport from the shoot in the phloem. The aim of the present study was to address these questions by two different approaches. Radio-labelled ASC or GSH was fed to a mature poplar leaf and the distribution of radioactivity in different root fractions was determined. In girdling experiments, where phloem transport to the root was interrupted at the transition between stem and root, the dependency of the ASC and GSH levels in different root fractions on shoot-derived ASC and GSH was determined.

## Materials and methods

### *Plant material and growth conditions*

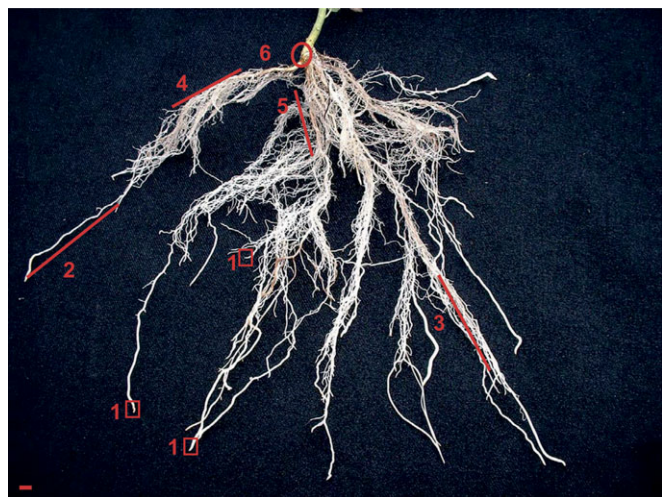
Seedlings of the poplar hybrid *Populus tremula* × *P. alba* clone 717 1B4 (Institute National de la Recherche Agronomique, INRA) were micropropagated as described by Strohm *et al.* (1995) and Noctor *et al.* (1996). After 4 weeks, cuttings were transferred onto quartz sand (0.7–2 mm, Götz and Moritz, Freiburg, Germany) and were grown in a greenhouse (26 ± 5 °C) under long day (16 h light) conditions and a light intensity that varied from 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  to 600  $\mu\text{mol m}^{-2} \text{s}^{-1}$  depending on the weather conditions. At full sunlight the greenhouse was shaded automatically. Seedlings were watered with 1/4 modified Hoagland solution combined with Long-Ashton medium (Strohm *et al.*, 1995) consisting of 1.25 mM KNO<sub>3</sub>, 2.5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.5 mM MgSO<sub>4</sub>, 4.5 mM MgCl<sub>2</sub>, 0.25 mM KH<sub>2</sub>PO<sub>4</sub>, 2.3  $\mu\text{M}$  MnCl<sub>2</sub>, 10  $\mu\text{M}$  H<sub>3</sub>BO<sub>3</sub>, 0.08  $\mu\text{M}$  CuCl<sub>2</sub>, 0.2  $\mu\text{M}$  ZnCl<sub>2</sub>, 0.2  $\mu\text{M}$  Na<sub>2</sub>MoO<sub>4</sub>, 0.04  $\mu\text{M}$  CoCl<sub>2</sub>, 22.5  $\mu\text{M}$  FeCl<sub>2</sub>, and 22.5  $\mu\text{M}$  Na<sub>2</sub>EDTA. Plants were harvested after 8 weeks of growth. Since the bark of deciduous trees includes the phloem, interruption of phloem transport can be achieved by peeling off the bark (Mason and Maskell, 1928). In girdling experiments, 2 cm of the bark was peeled off at the stem–root transition around the entire plant.

### *Feeding of [<sup>35</sup>S]GSH and L-[<sup>14</sup>C]ASC to the leaves*

[<sup>35</sup>S]GSH was fed to leaves using the flap feeding technique of Biddulph (1956). A flap was cut into a mature leaf so that the

connection to the main vein was maintained in the direction of the petiole. For this purpose, the first two cuts of  $\sim 1$  mm were made lateral to the main leaf vein. The third cut was made to release the leaf vein from the leaf lamina. The flap that contained part of the main vein remained connected in the direction of the petiole. In this way GSH and ASC were fed directly into the phloem, and phloem transport out off the fed leaf was facilitated. During cutting of the flap, the leaf was submerged in potassium phosphate buffer ( $50 \mu\text{M K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  buffer, pH 6.2). After cutting, the flap was dipped immediately into a test tube containing the feeding solution, i.e.  $15.6 \mu\text{l}$  of  $[^{35}\text{S}]\text{GSH}$   $\{30 \mu\text{Ci } [^{35}\text{S}]\text{GSH}$  (Hartmann Analytic GmbH, Braunschweig, Germany) prepared from an aqueous solution containing  $1075 \text{ Ci mmol}^{-1}$  GSH and  $10 \text{ mM}$  dithiothreitol (DTT) or  $15 \mu\text{l}$  of  $[^{14}\text{C}]\text{ascorbic acid}$   $\{30 \mu\text{Ci}$  of  $\text{L-}[^{14}\text{C}]\text{ASC}$  (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) prepared from solid ASC with  $8.5 \text{ mCi mmol}^{-1}$  in  $50 \mu\text{M K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  buffer pH 6.2}. The feeding solutions were taken up completely within  $30 \pm 5$  min.

After a total incubation time of 2, 3, or 5 h in the case of  $[^{35}\text{S}]\text{GSH}$  feeding, or after 5 h in the case of  $[^{14}\text{C}]\text{ASC}$  feeding at room temperature ( $25 \pm 3 \text{ }^\circ\text{C}$ ) and  $600 \pm 30 \mu\text{E m}^{-2} \text{ s}^{-1}$  PAR (Osram, HPS L 65W/150 ultra white and Osram, L Fluora 35W/77R, Osram, Munich, Germany) at plant height, incubation was terminated by cutting off the fed leaf. Subsequently, poplar trees were dissected into the apex, and the first, second, third, seventh, and 11th leaves counted from the apex. The trunk section basal to the fed leaf was divided into sections of 2 cm in length that were separated into bark and wood. The root system was separated into six root fractions of different developmental stages (Fig. 1). Fraction 6 (R6) was the main root that appears green. Smaller roots that showed secondary growth and appeared red were combined in fraction 5 (R5). Roots with secondary growth that appeared white constituted fraction 4 (R4). Fraction 3 (R3) contained roots with a diameter of  $\sim 0.5$ – $1$  mm and small side roots. Long white roots without side roots that were  $\sim 1$  mm in



**Fig. 1.** The root system of an 8-week-old poplar plant grown in sand culture. The root system was dissected into six fractions of different developmental stages. Fraction 6 (R6) was the main root that appears green. Smaller roots that revealed secondary growth and appeared red were indicated as fraction 5 (R5). Roots with secondary growth which appeared white constituted fraction 4 (R4). Fraction 3 (R3) contained the roots with a diameter of  $\sim 0.5$ – $1$  mm and developed small side roots. The long white roots without side roots that were  $\sim 1$  mm in diameter comprised fraction 2 (R2). The root tips were sampled whenever possible and were combined in fraction 1 (R1). The bar indicates 5 mm.

diameter were combined in fraction 2 (R2). Root tips were sampled whenever possible and were combined in fraction 1 (R1). All samples were immediately frozen in liquid nitrogen and stored at  $-24 \text{ }^\circ\text{C}$  until analysis.

#### $^{35}\text{S}$ and $^{14}\text{C}$ analyses

$^{35}\text{S}$  and  $^{14}\text{C}$  radioactivity was determined in 20–100 mg of powdered (under liquid nitrogen) plant tissue as described by Herschbach and Rennenberg (1996). After solubilization with a tissue solubilizer (1 ml of Soluene 350, Packard Instruments, Frankfurt, Germany), samples were bleached with  $200 \mu\text{l}$  of  $\text{H}_2\text{O}_2$  (30%) overnight. After adding 5 ml of scintillation fluid (HiSafe 3, Packard Instruments, Frankfurt, Germany), radioactivity was determined using a liquid scintillation counter (Wallac System 1409, Wallac, Turku, Finland). Data were corrected for quenching.

#### Analyses of thiols and $^{35}\text{S}$ -labelled metabolites

Thiols, i.e. cysteine,  $\gamma$ -EC, and GSH, were extracted, derivatized, and quantified as described by Strohm *et al.* (1995) and Herschbach *et al.* (2000). A 30 mg aliquot of leaf material powdered under liquid nitrogen or 100 mg of root and bark powder was homogenized in  $750 \mu\text{l}$  of  $0.1 \text{ M HCl}$  containing 50 mg of insoluble polyvinylpyrrolidone (PVPP). Samples were centrifuged ( $14\,000 \text{ g}$ , 15 min) and  $120 \mu\text{l}$  of the clear supernatant was added to  $180 \mu\text{l}$  of  $200 \text{ mM CHES}$  buffer (pH 9.3). Reduction of reduced disulphides was performed with  $30 \mu\text{l}$  of  $15 \text{ mM DTT}$  for 1 h at room temperature. Thiols were derivatized with  $20 \mu\text{l}$  of  $30 \text{ mM}$  monobromobimane and stabilized by adding  $240 \mu\text{l}$  of 10% (v/v) acetic acid after 15 min of derivatization. Aliquots of  $150 \mu\text{l}$  were taken to separate bimane conjugates by HPLC analysis (SUPERCOSILTM LC-18,  $25 \text{ cm} \times 4.6 \text{ mm}$ ,  $5 \mu\text{m}$ , Sigma-Aldrich) as described by Schupp and Rennenberg (1988) using 10% (v/v) methanol, 0.25% (v/v) acetic acid (pH 3.9) as solvent A and 90% (v/v) methanol, 0.25% (v/v) acetic acid (pH 3.9) as solvent B. Bimane derivatives were detected by fluorescence detection at 480 nm after excitation at 380 nm (Schupp and Rennenberg, 1988) and quantified by the use of external standards. During this analysis  $[^{35}\text{S}]\text{sulphate}$  eluted prior to cysteine. To determine the amount of  $^{35}\text{S}$  in thiols, 1 ml fractions of the HPLC eluate were collected. After addition of 4 ml of scintillation fluid (HiSafe 3, Packard Instruments, Frankfurt, Germany), radioactivity was determined by liquid scintillation counting and classified by comparison with the fluorescent detector output.

#### Analyses of $^{14}\text{C}$ -labelled metabolites

For  $^{14}\text{C}$ -labelled metabolite analysis in plants fed  $[^{14}\text{C}]\text{ASC}$  a combination of HPLC analysis and liquid scintillation counting was applied. For this purpose, ASC was determined as described by Polle *et al.* (1990). ASC and DHA were extracted from 100 mg of root or wood tissue or from 50 mg of leaf tissue in  $500 \mu\text{l}$  of meta-phosphoric acid (5%, v/v) plus 50 mg of PVPP at  $4 \text{ }^\circ\text{C}$ . Total ASC was determined after enzymatic oxidation of ASC to DHA by ascorbate oxidase. Aliquots of  $50 \mu\text{l}$  of tissue extracts were diluted with  $100 \mu\text{l}$  of sodium acetate ( $200 \text{ mM}$ , pH 6.2). After addition of  $15 \mu\text{l}$  of ascorbate oxidase ( $1 \text{ mg ml}^{-1}$  in  $200 \text{ mM}$  sodium acetate pH 6.2) the mixture was incubated for 15 min at  $37 \text{ }^\circ\text{C}$ . Thereafter,  $100 \mu\text{l}$  of  $3.7 \text{ mM}$  sodium acetate were added and the mixture was kept further for 30 min at room temperature. Thereafter, DHA was derivatized after addition of  $50 \mu\text{l}$  of *o*-PDA (*o*-phenyldiamine,  $1 \text{ mg ml}^{-1}$  ethanol) during 30 min at room temperature in the dark. The final volume was adjusted to  $655 \mu\text{l}$ . A  $150 \mu\text{l}$  aliquot was taken to separate the DHA derivative by isocratic HPLC analysis with a solvent consisting of  $80 \text{ mM K}_2\text{HPO}_4$  and 20% (v/v) methanol (pH 7.8 adjusted with orthophosphoric acid) on a reversed phase column (ODS  $15 \times 4.6 \text{ mm}$ ,  $5 \mu\text{m}$  Ultrasphere<sup>TM</sup>, Beckman Lincoln, Krefeld, Germany). Fluorescence of the DHA derivative was measured at 450 nm after

excitation at 350 nm. DHA was quantified using external standards. Radioactivity within the eluate was determined with a liquid scintillation counter in 1 min fractions after adding 4 ml of scintillation fluid (HiSafe 3, Packard Instruments, Frankfurt, Germany).

#### ASC assay

ASC in non-radioactive samples was determined photometrically using the method of Okamura (1980) as described by Haberer *et al.* (2007). ASC and DHA were extracted from 20–25 mg of plant tissue, which was frozen and powdered under liquid nitrogen, with 500  $\mu$ l of meta-phosphoric acid (5%; w/v) at 4 °C on ice. The mixture was stirred and centrifuged (14 000 g, 30 min, 4 °C). The supernatant (100  $\mu$ l) was neutralized with triethanolamine (20  $\mu$ l, 1.5 mM) and mixed with sodium phosphate buffer (100  $\mu$ l, 150 mM, pH 7.4). The ASC in the assay was measured directly, while total ASC was measured after complete reduction by DTT (50  $\mu$ l, 10 mM, 30 min). Excess DTT was removed with *N*-ethylmaleimide (50  $\mu$ l of NEM, 0.5%). Samples for ASC analysis were treated in the same way as described by Haberer *et al.* (2007). ASC reduces ferric ions to ferrous ions which coupled with 2,2'-dipyridyl to form a complex with a characteristic absorption at 525 nm, allowing quantification (Okamura, 1980). DHA was then calculated by subtraction of readings for ASC from readings for total ASC.

#### Data analysis

Significant differences in ASC, DHA, and GSH contents between treatments ( $n=3$ ) and between root sections ( $n=3$ ) of girdled trees were analysed with the statistics program SPSS 16.0 for windows (Chicago, IL, USA). Prior to the test of significance with the Turkey test, the normality and homogeneity of the data were tested. Normality of the data was tested with the Kolmogorov–Smirnov test that includes correction of significance after Lilliefors and Shapiro–Wilk. Homogeneity of variance was tested with the Levene test. If homogeneity was not given, values were transferred using the natural logarithm. If homogeneity was still not given, the Games–Howell test was applied. Significant differences at  $P < 0.05$  are indicated.

## Results

### Translocation of $^{35}\text{S}$ after [ $^{35}\text{S}$ ]GSH feeding to a leaf

One leaf of poplar plants (one of the ninth to 14th leaf counted from the apex) was fed with [ $^{35}\text{S}$ ]GSH by flap feeding (Biddulph, 1956) and the subsequent  $^{35}\text{S}$  transport out of the fed leaf was terminated after 2, 3, and 5 h. Along the transport path, the highest labelling in bark and wood was detected just below the fed leaf, and labelling decreased with increasing distance from the fed leaf (data not shown). After 2 h of incubation, only low levels of  $^{35}\text{S}$  were detected in the root tips or any other root fraction (Fig. 2A).  $^{35}\text{S}$  reached the root tips 3 h after starting [ $^{35}\text{S}$ ]GSH feeding. A slight enrichment of  $^{35}\text{S}$  in the root tips compared to the roots of fraction 2 was observed after 5 h of incubation. As the root tips only amounted to 4.2–8.7% of total root mass (Fig. 2B), the distribution of  $^{35}\text{S}$  within the root system was calculated (Fig. 2C). From this calculation, it appears that within the root system,  $^{35}\text{S}$  is preferentially found in the roots of fraction R3 and in the main root (fraction R6). This distribution correlates well with the biomass of

different root fractions that was highest in roots of fraction R3 followed by the main root (R6) (Fig. 2B).

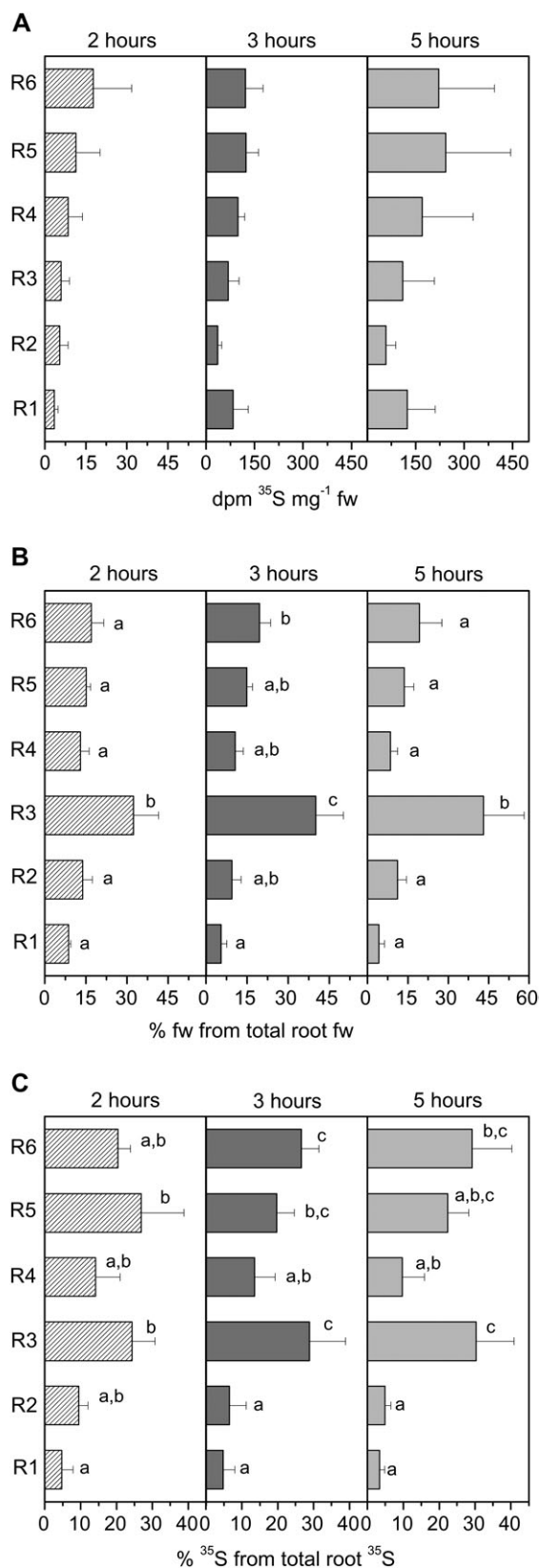
Along the transport path from the fed leaf to the roots, the [ $^{35}\text{S}$ ]GSH to [ $^{35}\text{S}$ ]sulphate ratio decreased. This indicates that with increasing distance from the fed leaf, the amount of labelled GSH declined in comparison with the amount of labelled sulphate. Whereas the [ $^{35}\text{S}$ ]GSH to [ $^{35}\text{S}$ ]sulphate ratio in the fed leaf ranged from 1.0 to 1.5, it amounted to 0.2 in the bark, 0.4 in the wood, and ranged from 0.17 to 0.33 in the roots.

### GSH contents in roots after stem girdling

Poplar plants were girdled at the transition of the stem to the roots to disrupt glutathione transport in the phloem. Glutathione contents in roots of different fractions were analysed 6 h, 24 h, 3 d, and 5 d after girdling (Fig. 3). Roots from control poplar plants without a bark girdle were harvested twice; first at time zero and secondly after 120 h, i.e. 5 d after starting the experiment. The GSH content without girdling was similar in roots of fraction R4, R5, and R6, but only half as high in roots of fraction R3 and R2 (Fig. 3). GSH contents in the root tip (R1) were  $\sim 30\%$  higher compared to the main root (R6). Disruption of phloem transport did not affect the glutathione content of the roots of fraction R2, R3, R4, R5, or R6, but GSH in the root tips (fraction R1) declined with increasing time of disrupted phloem transport. After 3 d of disruption of phloem transport, the GSH content of the root tips was diminished to about half the level determined prior to girdling. Compared with control roots which were harvested on day 0 and day 5, the GSH content in root tips after 5 d of phloem transport interruption was diminished to 40% and 60%, respectively. GSSG contents in roots were not affected by girdling in any root fraction either 6 h or 5 d of girdling (data not shown).

### Translocation of $^{14}\text{C}$ after [ $^{14}\text{C}$ ]ASC feeding to a leaf

After 5 h incubation, the  $^{14}\text{C}$  from [ $^{14}\text{C}$ ]ASC fed to a mature leaf (16th or 18th counted from the apex) was detected mainly in the root tips (Fig. 4A). Preferential translocation of  $^{14}\text{C}$  label to the root tip was also obvious when the absolute distribution of  $^{14}\text{C}$  within the root system was calculated. Although the contribution of root tips to total root fresh weight was only  $2.5 \pm 0.5\%$  (Fig. 4B), the proportion of  $^{14}\text{C}$  within the root tip fraction amounted to  $56.5 \pm 25.0\%$  (Fig. 4C). This means that root tips are the preferential sink for the  $^{14}\text{C}$  transported from the fed leaf to the root system.  $^{14}\text{C}$  was also detected in the apex ( $46 \pm 48$  dpm  $\text{mg}^{-1}$  fw) and in young developing leaves ( $76 \pm 81$  dpm  $\text{mg}^{-1}$  fw); however, at amounts comparable with those found in the main root fraction (R6) ( $53 \pm 39$  dpm  $\text{mg}^{-1}$  fw). In both the apex and the main root, the ASC determined by HPLC analysis correlated with the  $^{14}\text{C}$  in the corresponding fraction of the HPLC eluate (data not shown). Apparently, [ $^{14}\text{C}$ ]ASC is transported in both the acropetal and basipetal direction. These results indicate that [ $^{14}\text{C}$ ]ASC is



**Fig. 2.** <sup>35</sup>S in root fractions (A), relative root fresh weight of each fraction (B), and relative <sup>35</sup>S distribution between root fractions (C). (A) <sup>35</sup>S radioactivity in the six root fractions (see Fig. 1) 2, 3, and 5 h after feeding [<sup>35</sup>S]GSH to a mature leaf. (B) Contribution of the six different root fractions to total root fresh weight. Total root fresh weight of the individual poplar plants was set to 100% and

transported from the fed leaf to the roots by phloem transport, but, in contrast to [<sup>35</sup>S]GSH, ASC was not taken up from the phloem along the transport path and was preferentially transported to the root tips.

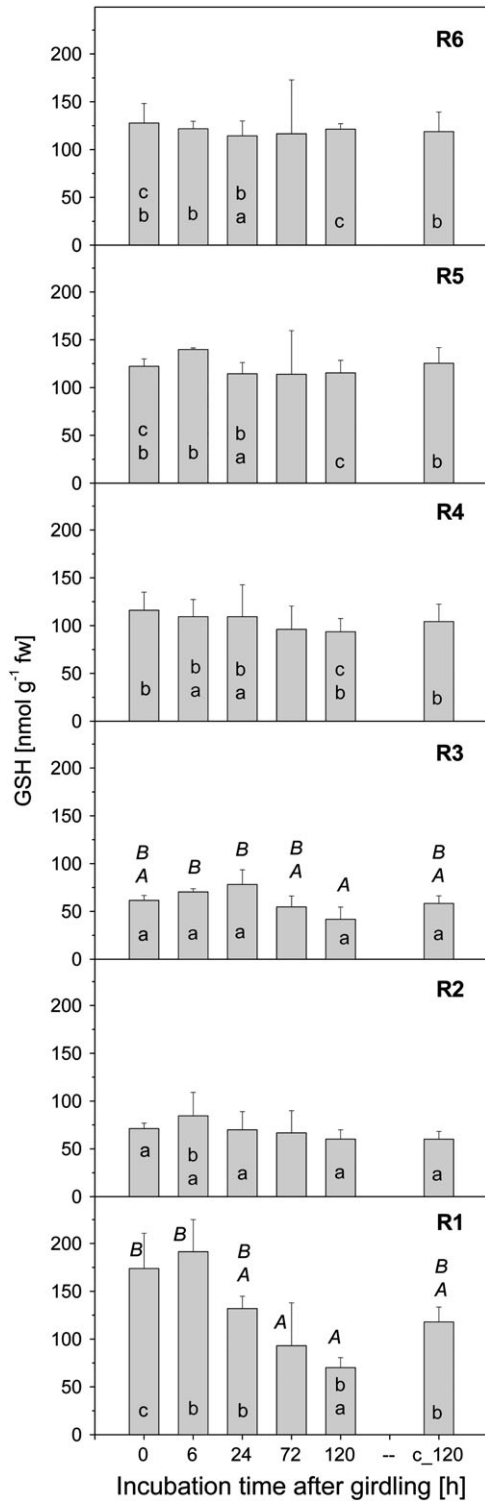
#### ASC contents in roots after stem girdling

ASC and DHA were analysed in the root system after disruption of phloem transport by girdling (Fig. 5). Total ASC levels in roots reached only 10–40% of the levels in leaves (~ 6–8 μmol ASC g<sup>-1</sup> leaf fw). The lowest amounts of total ASC were found in roots of fractions R2 and R3, while total ASC levels were higher in roots of fractions R6, R5, R4, and R1 (Fig. 5). Girdling resulted in a significant decline in total ASC in root tips (R1) and in roots from fraction R3 and R6. In these fractions, total ASC was diminished to ~ 40% of that in controls after 5 d of girdling. The contribution of oxidized to total ASC was 30–60% prior to girdling and 6 h after phloem disruption by girdling. This was also true for the root tips, where 40% and 50% of the ASC was found to be oxidized, respectively (Fig. 5). After 24 h of girdling the contribution of DHA to total ASC declined significantly. After 120 h of phloem disruption by girdling, the pool of DHA was significantly diminished in all root fractions. This was clearly an effect of girdling, as non-girdled control poplar plants did not show such an effect.

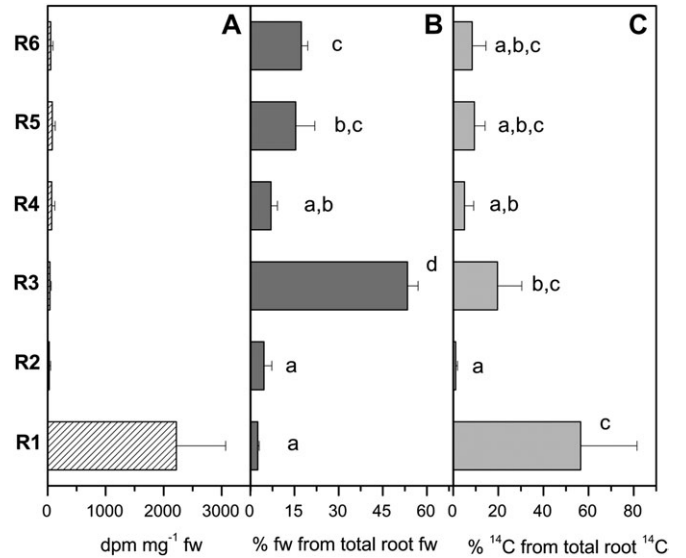
## Discussion

In poplar plants ASC is transported from mature leaves to the roots. As previously observed for herbaceous plants (Franceschi and Tarlyn, 2002) leaf fed [<sup>14</sup>C]ASC accumulated in poplar root tips. In contrast to the two herbaceous species *Medicago sativa* and *Arabidopsis* where young leaves, flower buds, and siliques are strong sinks for the applied [<sup>14</sup>C]ASC, this was not observed with poplar. Although [<sup>14</sup>C]ASC labelling was found in the poplar apex (46±48 dpm mg<sup>-1</sup> fw) and in young developing leaves (76±81 dpm mg<sup>-1</sup> fw) 5 h after feeding, this labelling was negligible compared to the labelling of root tips (R1) (2219±849 dpm mg<sup>-1</sup> fw). Whether flowers or seeds of poplar are sinks for ASC from mature leaves could not be answered in the present study, because poplar trees switch from juvenile growth to reproductive growth after only 7–10 years (Hsu *et al.*, 2006). Thus, it cannot be excluded that during flowering and seed development phloem

the percentage of each fraction was calculated. (C) The contribution of <sup>35</sup>S detected in each of the six root fractions to total root <sup>35</sup>S radioactivity per root system of poplar plants fed with [<sup>35</sup>S]GSH to a mature leaf. Total <sup>35</sup>S determined in the whole root system was set to 100%. The contribution of each root fraction was calculated. Data given are mean values from three poplar plants investigated per time point. Different indices indicate significant differences between root fractions at *P* < 0.05.



**Fig. 3.** GSH contents in the six different root fractions of 8-week-old poplar plants girdled for different periods of time at the transition between stem and root. c\_120 indicates an additional control harvested at day 5 after starting the experiment. Data given are mean values  $\pm$ SD from three plants at the time indicated. (a) Indices indicate significant differences between the six root fractions during one harvest time at  $P < 0.05$ ; (A) indicates significant differences between sampling dates along one root fraction at  $P < 0.05$ . The absence of indices indicated that significant differences were not found.



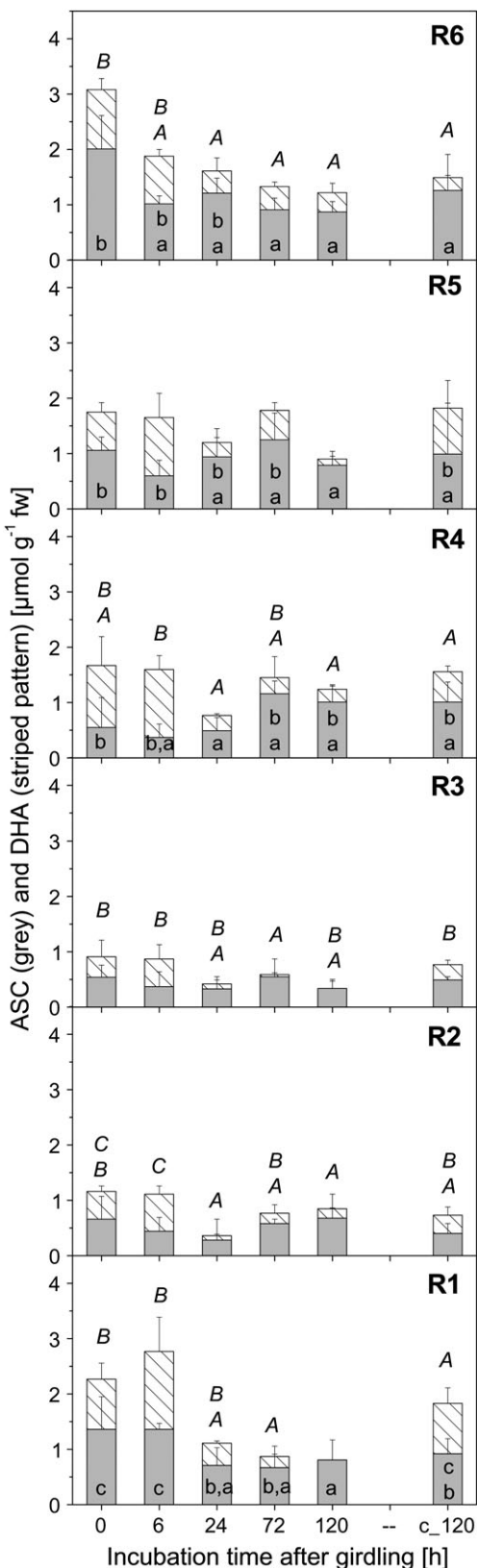
**Fig. 4.** Distribution of <sup>14</sup>C within the root system 5 h after [<sup>14</sup>C]ASC was fed to a mature leaf. Data given in A are mean values of dpm mg<sup>-1</sup> fresh weight from the three trees analysed. Data given in B are mean values  $\pm$ SD of the percentage of each root fraction from total root fresh weight. Data given in C represent the amount of <sup>14</sup>C from each root fraction relative to total <sup>14</sup>C determined in the whole root system that was set to 100%. Different indices indicate significant differences between root fractions at  $P < 0.05$ .

transport into apical sink tissues also takes place in poplar, as found for herbaceous plants.

During ASC transport to the roots ASC was not withdrawn from the phloem and was thus not stored in bark or wood tissues, or in any other root fraction. This was indicated by a lack of [<sup>14</sup>C]ASC accumulation in these tissues (data not shown). Thus it can be concluded that root tips of poplar trees are a preferential sink of shoot-derived ASC. Contrasting results were found for GSH. Along the transport path, <sup>35</sup>S from the <sup>35</sup>S fed as GSH to a mature leaf decreased continuously up to the main root and accumulated in the bark and wood of the trunk (data not shown). Simultaneously, the proportion of radiolabelled sulphate compared to GSH increased. Previous experiments also showed that radiolabelled GSH in the phloem declined during its transport from source to sink, whereas sulphate remained more or less constant (Hartmann *et al.*, 2000). Nevertheless, GSH is a regular sulphur constituent of phloem exudates from poplar (Herschbach *et al.*, 1998) and thus it is transported from the shoot to the roots (present study). Thus, although GSH was transported up to fine roots, specific accumulation of [<sup>35</sup>S]GSH in root tips was not found 5 h after [<sup>35</sup>S]GSH was fed to a mature leaf. In conclusion, root tips of poplar trees are a preferential sink of shoot-derived ASC, but not for shoot-derived GSH or sulphate.

Beside transport to the roots it must be considered that ASC could be synthesized in roots. When *Cucurbita maxima* roots were fed with the ASC precursor L-galactono-1,





**Fig. 5.** Total ascorbate (complete bar), ASC (grey parts), and DHA (striped pattern) in different root fractions of 8-week-old poplar plants girdled for different periods of time at the transition between stem and root. Total ascorbate and ASC were determined photometrically as described by Haberer *et al.* (2007). DHA contents were calculated as the difference between these two

4-lactone, the ASC level increased (Liso *et al.*, 2004); and the enzyme catalysing the final step of ascorbate synthesis (L-galactono-1,4-lactone dehydrogenase) was detected both in leaves and in roots (Groten *et al.*, 2005; Matamoros *et al.*, 2006). ASC levels in roots are usually low compared to leaves (Franceschi and Tarlyn, 2002; Matamoros *et al.*, 2006) as also found in the present study ( $7.0 \pm 1.1 \mu\text{mol ASC g}^{-1}$  fw in leaves versus  $2.3 \pm 0.3 \mu\text{mol ASC g}^{-1}$  fw in fine roots). The girdling experiments with poplar indicate that — despite ASC synthesis in roots (Liso *et al.*, 2004; Groten *et al.*, 2005; Matamoros *et al.*, 2006) — even these low root ASC levels are largely maintained by phloem transport of ASC. Approximately half of the ASC was lost in root fractions R1, R3, R5, and R6 24 h after disruption of phloem transport. This decline is consistent with the ASC turnover rate of  $2.5\% \text{ h}^{-1}$  previously reported in *Arabidopsis* leaves (Conklin *et al.*, 1997). In germinating pea seedlings an even faster turnover of ASC of  $13\% \text{ h}^{-1}$  has been reported (Pallanca and Smirnoff, 2000). Phloem transport interruption simultaneously induced a decline in DHA content of poplar roots. Apparently, phloem transport of ASC plays a decisive role in ASC homeostasis in poplar roots.

Kerk and Feldmann (1995) established a direct correlation between the ASC redox state and cell proliferation rates. High DHA contents blocked the transition from  $G_1$  to S in the QC of *Zea mays* roots that correlated with low ASC contents in the QC and prevented cell proliferation, while higher ASC levels induced cell division in surrounding meristematic initials. In tobacco cell cultures (BY-2), peak values of ascorbate, but not GSH, coincided with a peak in the mitotic index (de Pinto *et al.*, 1999). Similar to decreasing DHA during the  $G_1$  phase that may shorten the cell cycle in tobacco cell cultures (Kato and Esada, 1999), the reduction of DHA in roots as found in the present study after girdling could trigger cell division and thus may change root growth and development. This was supported by an ASC-deficient mutant (*vtc2*) that revealed changes in the root system (Olmos *et al.*, 2006). The most noticeable root phenotype is the aberrant gravitropic response of the primary root and lateral roots of the *vtc2* mutant. Thus, phloem-transported ASC may be considered as a signal controlling root growth and development. Still, a phloem-transported signal that controls root ASC synthesis cannot be excluded from the present experiments. However, ASC re-synthesis in the roots from ASC degradation products synthesized in the fed leaf is unlikely because specific labelling of  $^{14}\text{C}$  coincides with the HPLC ASC peak (data not shown).

parameters. c\_120 indicates an additional control harvested at day 5 after starting the experiment. Data given are means  $\pm$  SD from three individual plants. (a) Indices indicate significant differences of total ASC within one root fraction between treatments at  $P < 0.05$  and (A) indicates significant differences of DHA of a given root fraction between treatments at  $P < 0.05$ . Absence of indices indicates that significant differences were not found.

The low shoot-derived GSH supply to root tips may be the result of significant sulphate assimilation so that the roots are largely independent of reduced sulphur from the shoot. Tips of maize roots showed the highest levels of adenosine 5'-phosphosulphate reductase activity (Kopriva *et al.*, 2001), the enzyme that catalyses the regulatory step for sulphate reduction (Kopriva and Koprivova, 2004; Martin *et al.*, 2005). GSH synthesis from sulphate has been demonstrated in excised root of *Arabidopsis* (Vauclare *et al.*, 2002) and poplar roots (Scheerer *et al.*, 2009). Therefore, it seems possible that roots are self-sufficient in sulphate reduction and thus in GSH synthesis. Another explanation for the lack of preferential translocation of shoot-derived GSH to the root tip may be GSH degradation in the fed leaf and GSH re-synthesis in the roots. As cysteine is not the main transport form of reduced sulphur in the phloem of poplar (Herschbach *et al.*, 1998, 2000) and radiolabelling of the cysteine pool was not observed in phloem exudates from poplar (Hartmann *et al.*, 2000), it seems improbable that cysteine is the transport form of reduced sulphur from mature leaves to roots. Hence GSH re-synthesis from shoot-derived cysteine within the roots seems unlikely, but GSH synthesis from shoot-derived glutamate and/or glycine cannot be excluded. Nevertheless the GSH content in root tips declined after 3 d of phloem transport interruption, indicating that part of the GSH in root tips originates from the shoot. The observed decline suggests a contribution of long-distance transport to the compensation of GSH turnover in the root tips of  $\sim 0.5\% \text{ h}^{-1}$ . Apparently, this low rate of delivery by phloem transport is required to maintain the GSH level in the root tips that do not seem from these experiments to be completely self-sufficient in GSH synthesis. As an alternative explanation, a shoot signal delivered by phloem transport may be necessary for full capacity of GSH biosynthesis in root tips.

It seems from the present experiments that poplar root tips are dependent on GSH and ascorbate from the shoot. However, the rates are different (around 10% losses of GSH, but even 50% loss of total ASC after 24 h phloem interruption). As the ASC content in roots was one order of magnitude higher than that of GSH it may be speculated that the effect of ASC on redox-dependent root growth is more relevant. Maintenance of the cellular redox state does not only depend on the concentration of an antioxidant, but — among other factors — also on the rate constant of its conversion in disturbing reactions. As the rate constant for superoxide radical scavenging is higher for GSH than for ASC (Rennenberg and Polle, 1994), ASC may be a less potent chemical antioxidant than GSH at similar concentrations. This may be overcome by higher ASC concentrations, as frequently observed in plant tissues including roots (Rennenberg and Polle, 1994; Noctor, 2006; Rennenberg *et al.*, 2007), thereby supporting the relevance of ASC for redox-dependent root growth under these conditions. This is supported by an *Arabidopsis* mutant (*vtc*) low in ASC. This mutant showed changes in hormone levels and primary root development, and also changed stress sensitivity. It can therefore be assumed that the ASC level is one

possible internal signal allowing plants to respond to environmental stimuli by adjusting growth and development (Kotchoni *et al.*, 2009).

Since both GSH and ASC are subject to compartmentation, the subcellular distribution of GSH and ASC seems to be essential for its functions in plant growth and development. This point is even more important when it is considered that both antioxidants affect root growth and development in different ways. Whereas ASC/DHA seems more effective in cell proliferation, GSH affects cell size determination in tobacco cell cultures (de Pinto *et al.*, 1999) and root hair formation that could not be mimicked by ASC in *Arabidopsis* roots (Sánchez-Fernández *et al.*, 1997). The latter corresponds to high GSH contents in the root epidermis and in rapidly dividing cells (Fricker *et al.*, 2000). Nevertheless, GSH depletion blocks cell proliferation in BY-2 tobacco cell cultures (Vernoux *et al.*, 2000). Potters *et al.* (2002) concluded that a sufficient GSH concentration is necessary for regular cell division. This view is supported by results from studies with *Arabidopsis* mutants. Whereas root development was not found in the *rml1* mutant that exhibited only 3% of the GSH detected in control roots (Vernoux *et al.*, 2000), cell division in roots, i.e. normal root development, was observed in the *cad2-1* mutant which exhibits 30% of the GSH compared to the control (Howden *et al.*, 1995).

From these studies it can be concluded that not only the redox state but also the absolute amounts of GSH and ASC are important for the development of plants in a changing environment. The present study shows that shoot to root transport in the phloem is required to maintain the GSH and ASC levels in the roots. Thus, phloem transport of these antioxidants may constitute an important signal for the adjustment of root growth and development to changing environmental conditions. Further experiments under distinct environmental growth conditions that include transgenic plants modified in phloem transport of ASC or GSH and in root ASC or GSH biosynthesis are required to test these assumptions.

## Acknowledgements

Financial support by the University of Freiburg within the programme 'Support of evaluated research projects' is gratefully acknowledged.

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