Short Communication

Overexpression of γ-Glutamylcysteine Synthetase, but not of Glutathione Synthetase, Elevates Glutathione Allocation in the Phloem of Transgenic Poplar Trees

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Phloem exudates were collected along the tree axis at different heights of wild type and transgenic poplar. Overexpression of bacterial γ-glutamylcysteine synthetase (γ-ECS), but not of glutathione synthetase (GSS), significantly enhanced Cys, γ-EC and GSH in phloem exudates. Since growth of poplar is not affected by either γ-ECS or GSS overexpression, phloem transport of thiols seems to be controlled by the source rather than the sink strength.

Key words: Cysteine — γ-Glutamylcysteine — Glutathione — Methionine — Phloem exudate — Transgenic Poplar (Populus tremula × P. alba).

Glutathione is a product of plants’ primary metabolism synthesized by two ATP dependent reactions similar to bacteria, fungi and animals (Bergmann and Rennenberg 1993, Rennenberg 1995). In the first reaction a γ-peptide bond is ligated by γ-glutamylcysteine synthetase (γ-ECS, EC 6.3.2.2) between L-glutamate and L-cysteine. Subsequently, γ-glutamylcysteine (γ-EC) is coupled to glycine in a regular α-peptide bond by glutathione synthetase (GSS, EC 6.3.2.3). None of these enzymes has been purified to homogeneity from plants, but both enzymes were found to be localized in the cytoplasm and in plastids as well (Bergmann and Rennenberg 1993).

Since glutathione participates in a wide range of cellular processes, including various stress responses and detoxification reactions (Rennenberg and Brunold 1994), as well as storage and transport of reduced sulfur (Rennenberg 1995, Herschbach and Rennenberg 1997), glutathione biosynthesis seems to be strictly regulated. From physiological and biochemical studies it appears that the availability of cysteine limits glutathione synthesis (Rüegsegger and Brunold 1992, Strohm et al. 1995). When this limitation is overcome, e.g. by H2S fumigation, glycine supply may also become limiting for glutathione synthesis, especially in the absence of photosynthetic glycine production during darkness (Buwalda et al. 1990). Although not demonstrated to operate in vivo, feedback inhibition of γ-ECS by the endproduct GSH seems to control cellular glutathione levels (Hell and Bergmann 1990, Schneider and Bergmann 1995).

Recent studies with transgenic poplar (Populus tremula × P. alba) trees overexpressing the bacterial gene for γ-ECS (gshI) or GSS (gshII) in the cytosol provided a more detailed view of the regulation of glutathione biosynthesis (Rennenberg and Polle 1994, Foyer et al. 1995, Strohm et al. 1995, Noctor et al. 1996, Brunold and Rennenberg 1998, Rennenberg 1997). Feeding cysteine to leaves of wild type and transgenic poplar overexpressing GSS enhanced glutathione contents; but its cellular level of ca. 1 mM was not exceeded, even when sufficient cysteine was available (Strohm et al. 1995). Since this limitation was overcome by feeding γ-EC, the reaction catalyzed by γ-ECS seems to be rate limiting under these conditions (Strohm et al. 1995). Overexpression of γ-ECS, but not of GSS enhanced the glutathione level of the leaves without affecting the redox state of glutathione (Noctor et al. 1996). Apparently, overexpression of γ-ECS partially can overcome cysteine limitation (Strohm et al. 1995, Noctor et al. 1996). Since feeding of cysteine further enhanced glutathione accumulation in the leaves, cysteine also seems to limit glutathione synthesis to some extent in poplar plants overexpressing γ-ECS. Feedback inhibition of γ-ECS by glutathione did not prevent glutathione accumulation in the leaves and, therefore, cannot be considered a regulatory mechanism in poplar leaves in vivo.

Although the γ-ECS activities in leaves of transgenic poplar plants overexpressing γ-ECS were enhanced by a factor of 24 to 80, glutathione accumulated only three-fold in the presence of sufficient amounts of substrates (Noctor et al. 1996). It may therefore be assumed that a major part of the γ-ECS in the transformants is down-regulated in vivo.
by mechanisms so far unknown, or most of the surplus glutathione produced is rapidly degraded. Alternatively, part of the surplus glutathione produced in the leaves may be exported in the phloem. To test the latter hypothesis, phloem exudates were collected from the bark of wild type and transgenic poplar plants overexpressing γ-ECS or GSS. The composition and contents of thiols in phloem exudates were analyzed and compared with soluble sugars.

**Plant material**—Poplar trees (*Populus tremula x P. alba*) which expressed either bacterial glutathione synthetase or γ-EC-synthetase in the cytosol were produced as described by Strohm et al. (1995) and Noctor et al. (1996), respectively. Transformed and wild type poplar trees were micropropagated (Strohm et al. 1995), transferred into a soil mixture and grown in a greenhouse under long day conditions in pots with 16 cm in height and 19 cm in diameter. The soil mixture consisted of 1 part silica sand, particle size 0.06 to 0.2 mm, 1 part sterilized commercial soil and 2 parts perlite (Agriperl, Perlite-Dammstoff-GmbH, Germany). Trees were fertilized every 2 weeks with 200 ml of a 3 g liter\(^{-1}\) solution of a commercial fertilizer (Hakaphos blau, COMPO GmbH, Germany; 15% N; 10% P\(_2\)O\(_5\); 15% K\(_2\)O; 2% MgO; 0.01% B; 0.02% Cu; 0.05% Fe; 0.05% Mn; 0.001% Mo; 0.015% Zn). After 4–5 months the stem of the trees was 165±10 cm in height and branches were not developed. Because of the continuous growth pattern of poplar trees, leaves were inserted along the entire stem. The lowest stem section (0–35 cm) contained the oldest leaves with severe symptoms of senescence. The following two sections, 30–35 cm each, contained mature leaves showing slight symptoms of senescence and mature leaves without symptoms of senescence were inserted at the stem section from 100–135 cm height. The shoot apex (135–165 cm) contained developing young leaves.

**Collection of phloem exudate**—Phloem exudates were collected from bark slices of the stems of five 30–35 cm sections and the main root. Bark slices of ca. 150 mg fresh weight bark (1–2 cm\(^2\), 0.5–1.5 mm in thickness) were separated from the wood, washed in 2 mM EDTA and exuded in different incubation solutions. The incubation solution for the thiols Cys, γ-EC and GSH contained 2 mM EDTA and 1 mM cyanide at pH 5.8. To prevent destruction of thiols from phenols, polyvinylpyrrolidone (PVPP) was added at a PVPP/bark fresh weight ratio of 2. Cyanide was omitted from the incubation solutions for exportation of soluble sugars and methionine. Incubation was carried out at 4°C for 5 h. After 5 h incubation was nearly completed as indicated by the release of sucrose from bark slices (data not shown). Cyanide in the incubation solution did not affect exudation of soluble sugars (data not shown). Acid invertase was measured as contamination marker of the apoplastic space and the cytosol as described by Schneider et al. (1996). Invertase activity in phloem exudates amounted to 4.3±1.2% of that of untreated bark slices of poplar trees (100±16%, 1.27±0.37 μmol g FW\(^{-1}\) min\(^{-1}\)). This contamination is similar to previous results with bark slices of beech and spruce (Schneider et al. 1996).

**Analysis of sulfur compounds**—Phloem exudates were centrifuged at 16,000 \(\times\) g and 4°C for 10 min. Aliquots of 300 μl of the supernatant were adjusted to pH 8.3±0.2 by adding 100 μl 1 M CHES (2-cyclohexylenaminioethansulfonacid), pH 8.4. Reduction of thiols was initiated by addition of 20 μl 15 mM DTT and terminated after 60 min by addition of 30 μl 30 mM monobromobimane (mBBr) for derivatization. After 15 min derivatization was stopped by acidification with 50 μl acetic acid that also stabilizes the mBBr thiol derivatives. Aliquots of 100 μl were subjected to HPLC analysis and thiols were separated and quantified by fluorescence detection as described by Schupp and Rennenberg (1998). Peaks were identified and quantified using a standard solution containing 0.2 mM Cys, 0.1 mM γ-EC and 1 mM GSH in 0.01 M HCl. As control selected samples were exposed to 30 μl 15 mM DTNB (5,5'-dithiobis(2-nitrobenzoic acid) in 100 mM Tris-Cl, pH 7, for 10 min at room temperature prior to mBBr derivatization. DTNB blocked all thiol groups and made them no longer available for mBBr derivatization. Analysis of the control allowed the identification of fluorescent peaks not owing to thiols.

For methionine analysis aliquots of 1.3 ml phloem exudates were freeze-dried. The dried material was resuspended with 100 μl 0.2 M potassium citrate buffer, pH 3.20, and 70 μl were injected into an amino acid analyzer (Biochrom, Pharmacia LKB, Freiburg, Germany). Methionine, δ,δ-cystathionine and homocysteine were separated on a cation exchange column (PEEK column, Potassium, 100×4.6 mm, Laborbedarf und Analysetechnik Karin Grüning, Olching, Germany) with a system consisting of two potassium citrate buffers. Separation was carried out at a flow rate of 16.1 ml h\(^{-1}\) using 0.2 M potassium citrate and a gradient of increasing pH from 3.35 to 4.25 within 24 min. Thereafter, the column was regenerated within 8 min using 0.4 M NaOH, 2,686 mM EDTA and was equilibrated within 19 min with 0.2 M potassium citrate buffer pH 3.35. The methionine, δ,δ-cystathionine and homocysteine separated were subjected to postcolumn derivatization with ninhydrin. The absorption of ninhydrin derivates was recorded at 570 nm. Peaks were identified and quantified using a standard solution containing 500 μM methionine, δ,δ-cystathionine and homocysteine each.

**Sugar analysis**—Aliquots of 50 μl phloem exudates were diluted 1 : 10 with distilled water and 10 μl were injected into an automated sugar analyzer (DX 500, Dionex, Idstein, Germany). Sucrose, glucose and fructose were separated on an anion exchange column (CarboPac PA 1, 4×250 mm, Dionex, Idstein, Germany) with an isocratic NaOH system. Separation was carried out with 36 mM NaOH free of carbohydrate within 37 min at a flow rate of...
1 ml min⁻¹. Sugars were detected by pulsed amperometry. Peaks were identified and quantified using a standard solution containing 10 mM sucrose, glucose and fructose. Subsequent to each separation the column was regenerated with 200 mM NaOH free of carbohydrate and equilibrated for 24 min with 36 mM NaOH at a flow rate of 1 ml min⁻¹. Under the experimental conditions used for phloem exudation, acid hydrolysis of sucrose could not be excluded. Therefore, the amounts of glucose, fructose and sucrose measured were combined as soluble sugars. Along the stem the sucrose to glucose plus fructose ratio increased apical to basipetal from 1 to 2.8.

**Data analysis**—Statistical analysis was performed using Dunkans multifactorial test with the statistic program SPSS (SPSS for Windows, Release 7.0). If data from poplar lines were not significantly different, the significance of differences along the tree axis was determined by pooling the corresponding data from the different poplar lines.

**Sugar concentration in phloem exudates**—The concentrations of sugars and its gradient along the tree axis were not significantly different between the three poplar lines analyzed (Fig. 1). Apparently, overexpression of GSS or γ-ECS did not affect phloem transport of soluble sugars. This finding is consistent with the observation that growth of the poplar lines was not different between the lines in the present (data not shown) and previous studies (Foyer et al. 1995, Strohm et al. 1995, Noctor et al. 1996). Highest amounts of sugars were found in stem sections with mature leaves at 35–135 cm height, and significant lower amounts in those from the shoot apex and main roots (Fig. 1). Similar results were previously reported with castor bean (Vreugdenhil and Koot-Gronsveld 1989) and several other woody plant species (Dickson 1989, 1991, Minchin and Troughton 1980, van Bel 1993). Therefore, the present data confirm previous reports indicating that phloem loading of sucrose is predominantly mediated by mature leaves, in which sucrose synthesis exceeds their own need in growth and development. The concentration of soluble sugars found in the present study ranged between 18 ± 6 to 33 ± 6 μmol (g bark fresh weight)⁻¹. Provided the functional phloem represents ca. 30% of the bark fresh weight, the concentration of sugars in the phloem sap is 60 to 110 mM. In poplar neither the incision (Vreugdenhil and Koot-Gronsveld 1989) nor the aphid technique (Zimmermann 1974) could be used, since phloem sap is not released upon incision and the aphid technique would require sap collection without protectants at room temperature for several hours. Under these conditions rapid degradation would not allow proper thiol analysis.

**Thiols in phloem exudate**—In phloem exudates of wild type and GSS transgenic poplar trees cysteine and glutathione were found to be present in similar amounts. Methionine was found in both poplar lines in lower amounts.
amounts; d,l-cystathionine, homocysteine and γ-glutamylcysteine could not be detected (Fig. 2, 3). The thiol contents (ca. 50-90 nmol soluble S(-II) (g bark fresh weight)^−1) of wild type and GSS overexpressing poplar trees were similar to those of beech phloem exudates collected under similar conditions (Herschbach, unpublished results).

In γ-ECS overexpressing transgenic poplar 2 to 3.5 times higher amounts of cysteine and 4 to 7 times higher amounts of GSH were found in the phloem exudates as compared to wild type plants. In addition γ-EC, the precursor of GSH, was present in considerable amounts (Fig. 3). Methionine was also found in the phloem exudate of poplar overexpressing γ-ECS (Fig. 2). As a consequence of γ-ECS overexpression glutathione became the main long distance transport form of thiols as previously found for herbaceous plants (Rennenberg 1995) and in tracer experiments with other woody species (Herschbach and Rennenberg 1997). Since wild type and transgenic poplar lines did not exhibit differences in growth and enhanced thiol contents in the phloem were found in plants containing enhanced thiol concentrations in the leaves (Noctor et al. 1996), the present results indicate that phloem loading of thiols seemed to be controlled by ‘source’ rather than by ‘sink strength’. From the glutathione accumulation in the leaves (ca. 3-fold) and the much higher increase of the in vitro γ-ECS activity in the leaves of transgenic poplar overexpressing γ-ECS, considerable down-regulation of the enzyme activity in vivo or, alternatively, enhanced GSH turnover, has been proposed (Noctor et al. 1996). The present experiments, however, show that GSH accumulation in the leaves (Noctor et al. 1996) does not reflect glutathione synthesis because of appreciable GSH export.

Despite the absolute amounts of thiols in phloem exudates differed between the poplar lines studied, the distribution pattern of cysteine, methionine and glutathione along the tree axis were similar (Fig. 2, 3). Highest concentrations of these compounds were determined in shoot sections containing the youngest leaves; below these shoot sections cysteine, methionine and glutathione contents decreased continuously (Fig. 2, 3). This distribution pattern is entirely different from that of soluble sugars and of γ-EC (Fig. 1, 3). Thus, source to sink relations for cysteine, methionine and glutathione are different from γ-EC and soluble sugars in poplar trees. Further experiments are required to elucidate as to whether these differences can be attributed to developmentally determined differences in phloem loading and/or unloading.

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References
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Sulfur allocation in the phloem of transgenic poplar 451


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