

Cyst(e)ine Is the Transport Metabolite of Assimilated Sulfur from Bundle-Sheath to Mesophyll Cells in Maize Leaves¹

Marta Burgener, Marianne Suter, Stephanie Jones, and Christian Brunold*

Institute of Plant Physiology, Altenbergrain 21, CH-3013 Berne, Switzerland

The intercellular distribution of the enzymes and metabolites of assimilatory sulfate reduction and glutathione synthesis was analyzed in maize (*Zea mays* L. cv LG 9) leaves. Mesophyll cells and strands of bundle-sheath cells from second leaves of 11-d-old maize seedlings were obtained by two different mechanical-isolation methods. Cross-contamination of cell preparations was determined using ribulose biphosphate carboxylase (EC 4.1.1.39) and nitrate reductase (EC 1.6.6.1) as marker enzymes for bundle-sheath and mesophyll cells, respectively. ATP sulfurylase (EC 2.7.7.4) and adenosine 5'-phosphosulfate sulfotransferase activities were detected almost exclusively in the bundle-sheath cells, whereas GSH synthetase (EC 6.3.2.3) and cyst(e)ine, γ -glutamylcysteine, and glutathione were located predominantly in the mesophyll cells. Feeding experiments using [³⁵S]sulfate with intact leaves indicated that cyst(e)ine was the transport metabolite of reduced sulfur from bundle-sheath to mesophyll cells. This result was corroborated by tracer experiments, which showed that isolated bundle-sheath strands fed with [³⁵S]sulfate secreted radioactive cyst(e)ine as the sole thiol into the resuspending medium. The results presented in this paper show that assimilatory sulfate reduction is restricted to the bundle-sheath cells, whereas the formation of glutathione takes place predominantly in the mesophyll cells, with cyst(e)ine functioning as a transport metabolite between the two cell types.

Maize (*Zea mays* L.), as a typical C₄ plant, is characterized by a compartmentation of carbon assimilation into specific cell types, with CO₂ initially being fixed into malate in the MC and then transported into the BSC, where the formation of glycerate 3-phosphate is localized (Black, 1973). Conversely, the reduction of nitrate occurs exclusively in the MC (Moore and Black, 1979). This division of labor is a primary factor contributing to high rates of carbon assimilation (Black, 1973) and nitrogen use efficiency (Brown, 1978) in C₄ plants.

The intercellular compartmentation of sulfate assimilation is less well documented. Sulfite reductase (EC 1.8.7.1) and *O*-acetyl-L-Ser sulfhydrylase (EC 4.2.99.8) activities were found in both cell types at comparable levels (Passera and Ghisi, 1982; Burnell, 1984; Schmutz and Brunold, 1984, 1985). ATPase (EC 2.7.7.4), the first enzyme in the path-

way of assimilatory sulfate reduction, is, however, reported to be predominantly or even exclusively located in the BSC (Gerwick and Black, 1979; Gerwick et al., 1980; Passera and Ghisi, 1982; Burnell, 1984; Schmutz and Brunold, 1984; Ghisi et al., 1986). In addition, the second step in sulfate reduction, catalyzed by APSSTase (Brunold and Rennenberg, 1997), an enzyme that may be identical to 5'-adenylsulfate reductase recently described by Setya et al. (1996), is essentially restricted to the BSC (Schmutz and Brunold, 1984).

There are no published results concerning the intercellular compartmentation of GSH synthesis. This tripeptide plays an important role in the plant's defense system (Rennenberg and Brunold, 1994). It is involved in various stress situations, such as heavy metal stress (Nussbaum et al., 1988; Rügsegger and Brunold, 1992; Galli et al., 1996), xenobiotic stress (Farago et al., 1994), and chilling responses (Kocsy et al., 1996). GSH is also an essential factor in the regulation of sulfur nutrition in plants (Brunold and Rennenberg, 1997). Its long-distance transport mediates distribution of reduced sulfur according to the requirements of individual plant organs, and controls sulfate influx into the plant (Rennenberg and Lamoureux, 1990; Herschbach and Rennenberg, 1994; Lappartient and Touraine, 1996). There is also evidence for membrane transport of GSH in an active, carrier-mediated process (Rennenberg and Lamoureux, 1990). These facts, together with the localization of enzymes involved in assimilatory sulfate reduction, raise the question of the nature of thiol compounds transported from BSC to MC in C₄ plants.

In this paper we present evidence that cyst(e)ine is the transport metabolite and that GSH synthesis takes place predominantly in MC.

MATERIALS AND METHODS

Fifty maize (*Zea mays* L. cv LG 9, Limagrain, Ennezat, France) kernels were soaked for 24 h in aerated tap water at room temperature and then transferred to a pot containing 5000 cm³ of moist Perlite (Samen Mauser, Berne, Switzerland). After 3 d in the dark, the seedlings were cultivated in a 16-/8-h photoperiod at 25/20°C with a PPFD of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 70% RH. One liter of nutrient solution (Hen-

¹ This work was supported by the Swiss National Science Foundation. The term "Cys" is used when it is clear that cystine is not involved; "cyst(e)ine" is used for an undefined mixture of Cys and cystine. The concentrations are expressed in all cases relative to Cys.

* Corresponding author; e-mail chbrunold@pfp.unibe.ch; fax 41-31-332-20-59.

Abbreviations: APS, adenosine 5'-phosphosulfate; APSSTase, adenosine 5'-phosphosulfate sulfotransferase; ATPase, adenosine triphosphate sulfurylase; BSC, bundle-sheath cell(s); γ EC, γ -glutamylcysteine; MC, mesophyll cell(s); NR, nitrate reductase; RuBP, ribulose-1,5-bisphosphate.

schel, 1970), modified according to the method of Nussbaum et al. (1988), was added every 3rd d. Second leaves were harvested 10 d after imbibition in the middle of the light period.

Cell Isolation

MC and BSC extracts were obtained by two different mechanical-isolation methods.

Method A Modified after Schmutz and Brunold (1984)

One gram of leaves was cut vertically across the veins in approximately 1-mm segments with a razor blade and transferred to 10 mL of medium containing 2% cellulase and 2% pectinase for protoplast isolation, according to the method of Mills and Joy (1980). The plant material was infiltrated by applying a vacuum and then incubated for 20 min at 30°C. The predigested leaf strips were thoroughly rinsed and then homogenized in 8 mL of 0.1 M Tris-HCl (pH 8.0) containing 20 mM MgCl₂, 100 mM KCl, and 10 mM dithioerythritol. The extraction was carried out using a Sorvall Omni Mixer for 10 s at 100 V (6,700 rpm) and twice for 15 s at 170 V (11,500 rpm). The homogenate was filtered through a 60- μ m nylon net. The filtrate contained the broken MC. The plant material on the nylon net was resuspended in 8 mL of extraction buffer and homogenized again twice for 20 s at 240 V (16,250 rpm), and was then filtered and rinsed. The bundle-sheath strands on the nylon net were collected and broken in 4 mL of extraction buffer using a cooled glass homogenizer.

Method B Using a Roller Device according to the Method of Leegood (1985)

The harvested leaves were cut into 3- to 4-cm sections. Leaf segments, placed on 100 μ L of extraction buffer, were rolled once, thus squeezing out MC. The sap from two leaves was collected with a pipette and resuspended in 300 μ L of extraction buffer. The rolled leaf laminas were extracted with a cooled glass homogenizer in 4 mL of extraction buffer. The activities of these extracts as well as their thiol content were corrected for pure BSC given that NR is restricted to MC (Moore and Black, 1979; Vaughn and Campbell, 1988).

All extracts were filtered through two layers of 100% viscose fleece (Milette, Migros, Switzerland). Cross-contamination of the cell preparations was determined using Rubisco and NR as marker enzymes for BSC and MC, respectively.

Enzyme Assays

Rubisco activity was determined according to the method of Buchanan and Schürmann (1973) by measuring the nonvolatile radioactivity produced from RuBP and H¹⁴CO₃⁻ and by following the modifications given by Wyss and Brunold (1979). Incubation was for 10 min at 30°C. NR measurement was carried out according to the method of Neyra and Hagemann (1975) with modifications

described by Kast et al. (1995). ATPase activity was determined by measuring production of ATP from APS and PPi with a luciferin-luciferase system (Schmutz and Brunold, 1982) using a Lumac/3M Biocounter (model M 2010, Lumac, Basel, Switzerland). APSTase activity was measured as the production of [³⁵S]sulfite, assayed as acid volatile radioactivity formed from [³⁵S]APS (Brunold and Suter, 1990) in the presence of dithioerythritol. GSH synthetase activity was measured as previously reported (Klapheck et al., 1987; Rügsegger and Brunold, 1992) by the quantification of the reaction product after reverse-phase HPLC separation of its monobromobimane derivative.

The activities of Rubisco, NR, APSTase, and GSH synthetase were determined immediately after extraction. ATPase activity, which was stable in the crude extract up to at least 6 h (data not shown), was routinely measured 1 h after the homogenization of the plant material.

Determination of RuBP

RuBP was determined by the incorporation of ¹⁴CO₂ into an acid-stable product as described by Doulis et al. (1997).

Determination of Cyst(e)ine, γ EC, and GSH

Thiols were separated and quantified by reverse-phase HPLC after reduction with NaBH₄ and fluorescent labeling with monobromobimane (Newton et al., 1981; Schupp and Rennenberg, 1988) as previously described (Rügsegger and Brunold, 1992). Recoveries of 88, 90, and 98% were determined for cyst(e)ine, γ EC, and GSH, respectively, and were used for calculating the actual amounts of the thiols in the plant material. The measured values were related to average protein content of extracts.

Tracer Experiment

For [³⁵S]sulfate labeling, second leaves were excised and placed into 0.5 mL of nutrient solution containing 75 instead of 750 μ M sulfate and 5.55 \times 10⁶ Bq of [³⁵S]sulfate. The incubation took place under cultivation conditions. At different times, leaves were immediately extracted with isolation method B. The sections that had been in contact with the nutrient solution were excised and discarded. The experiments were repeated with the method described for determination of thiols, including the acidic extraction.

Measurement of ³⁵S Label in Thiols and Sulfide

The determination of the radioactive sulfide content required an alkaline-extraction method. The extraction buffer consisted of 200 mM 2-(cyclohexyl-amino)-ethanesulfonic acid-NaOH (pH 8.4) containing 1 mM Na₂EDTA. Three hundred microliters of extract was reduced on ice with 30 μ L of freshly prepared NaBH₄ for 30 min. For derivatization, 220 μ L of this mixture was added to 20 μ L of 15 mM monobromobimane and kept in the dark at room temperature for 15 min. The reaction was stopped with 165 μ L of 5% (v/v) acetic acid.

A 50- μ L aliquot of each sample was separated by HPLC, as described for the inactive thiols, using a modified gradient of methanol (0–14% methanol in 15 min and then 14–51% methanol in 30 min). Fractions of 0.75 mL were collected in scintillation vials. Two milliliters of Ultima Gold XR scintillation cocktail (Packard, Zürich, Switzerland) was added per fraction, and the radioactivity was counted in a Betamatic V liquid-scintillation counter (Kontron, Zürich, Switzerland).

³⁵S Labeling of Isolated Bundle-Sheath Strands

Two grams of 1-mm leaf segments was blended in 16 mL of medium according to the method of Valle and Heldt (1991) in a polytron with 1-s (20,000 rpm) and 20-s (15,000 rpm) bursts. The bundle-sheath strands were collected on a 280- μ m nylon net, washed thoroughly, and resuspended at a level of approximately 50 μ g chlorophyll mL⁻¹ resuspending medium (0.3 M sorbitol, 20 mM Tricine-KOH (pH 7.5), 4 mM MgCl₂, 10 mM KCl, 2 mM KH₂PO₄, 1 mM ADP, 10 mM glycerate 3-phosphate, 5 mM glutamate, 5 mM malate, 2 mM *O*-acetyl-L-Ser, and 0.2 mM K₂SO₄). The bundle-sheath strands were incubated after the addition of 1.85×10^7 Bq [³⁵S]sulfate mL⁻¹ at 30°C with an irradiance of approximately 1500 μ mol m⁻² s⁻¹. At different times, supernatants of the suspended bundle-sheath strands were obtained by a 5-min centrifugation at 10,000 rpm at 4°C. The supernatant was treated with 0.3 mM DTT for reducing possibly existing thiols, derivatized, and separated by HPLC as described for cyst(e)ine, γ EC, and GSH. The injection volume was 100 μ L. Fractions of 0.375 mL were collected and determined as in the tracer experiment with second leaves.

The incubated BSC were washed extensively and extracted in 0.1 N HCl. The determination of ³⁵S-labeled substances was performed as described for the supernatant.

Protein Determination

The protein content of the extracts was measured according to the method of Bradford (1976) with BSA as the standard.

Chlorophyll Determination

Acetone was added to the bundle-sheath strands and the mixture was shaken several times for 30 min in the dark. Chlorophyll was measured in the clear supernatant after centrifugation using the procedure of MacKinney (1941).

Statistical Analysis

The Mann-Whitney rank sum test (SigmaStat for Windows, version 1.0, 1992–1994, Jandel, San Rafael, CA) was used to determine significant differences in the enzyme activities and concentrations of metabolites between extracts of BSC and MC.

Chemicals

Monobromobimane was obtained from Calbiochem and γ EC was obtained from Nacalai Tesque (Kyoto, Japan). [³⁵S]APS was prepared according to the method of Li and Schiff (1991), using ATPase from Sigma and [³⁵S]sulfate from the Radiochemical Centre (Amersham). All other chemicals were purchased from Fluka.

RESULTS

Rubisco was assayed as a marker for BSC and NR was assayed as a marker for MC to determine the level of cross-contamination in the cell preparations. The activity of Rubisco indicated that there was less than 10% of BSC in the mesophyll extracts obtained by method A (Fig. 1A) and less than 5% in those obtained with the roller device (Fig. 1B). Based on the distribution of NR activity, procedure A yielded very pure BSC extracts with a contamination of about 5% (Fig. 1A), whereas the rolled leaf laminas in method B still contained an appreciable amount of MC (Fig. 1B).

The distribution of ATPase and APSase (the two first enzymes of assimilatory sulfate reduction) between BSC and MC is presented in Figure 1A. Both are predominantly located in BSC. The APSase activity in the MC extracts was at the same relative level as Rubisco activity, indicating that this enzyme is exclusively active in the BSC. The relative level of ATPase activity detected in the MC extract is higher than expected from contaminating BSC, indicating that this enzyme of sulfate assimilation is active in both cell types, with more than 90% of total activity in BSC.

Figures 1B and 2 show the compartmentation of GSH synthetase and thiols. After correction for contamination, only 22% of the total GSH synthetase activity was located in BSC (Fig. 1B). Also, the amounts of the thiols cyst(e)ine, γ EC, and GSH in the BSC extracts were significantly lower compared with those in the MC preparations (Fig. 2). The content of RuBP in the MC extract was used to calculate the degree of contamination by low-*M_r* compounds originating in the BSC (Doullis et al., 1997). The RuBP distribution between extracts of BSC and MC was similar to that of Rubisco (data not shown), indicating that the concentrations of the thiols were not a consequence of leaky plasma membranes.

Mixing of extracts from BSC and MC gave additive activities of all measured enzymes, indicating that no inactivator was present in either cell type and that the activities were comparable to those of the whole leaves, showing that no activity was lost during cell separation (data not shown).

The distribution of the activities of the two first enzymes of assimilatory sulfate reduction indicated an almost exclusive localization of this pathway in BSC, whereas GSH synthesis predominantly took place in MC. This opened the question of intermediates being synthesized in BSC and transported into MC. We addressed this question by feeding excised leaves with [³⁵S]sulfate and analyzing the radioactivity of the thiols in the MC in a time-course exper-

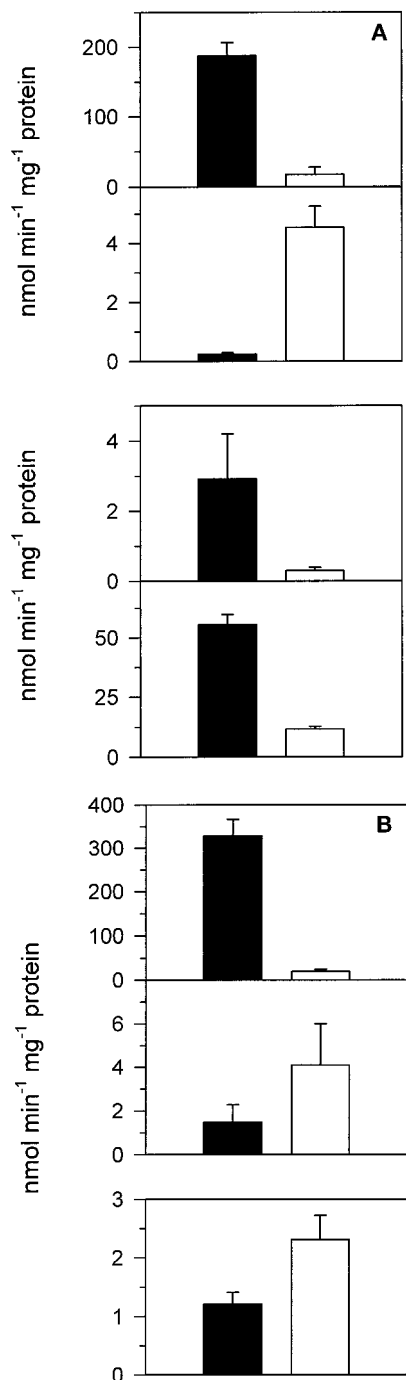


Figure 1. A, Intercellular localization of activities of Rubisco (first panel), NR (second panel), APSSase (third panel), and ATPase (fourth panel) in second leaves of 11-d-old maize plants. MC (open bars) and BSC (black bars) extracts were obtained by isolation procedure A. Mean values \pm SD of four different experiments are presented. The values of the extracts of the two cell types differ significantly from each other ($P \leq 0.05$). B, Intercellular localization of activities of Rubisco (top), NR (middle), and GSH synthetase (bottom) in MC (open bars) and BSC (black bars) extracts obtained by isolation method B. Means \pm SD of five independent experiments are presented. The values of the extracts of the two cell types differ significantly from each other ($P \leq 0.05$).

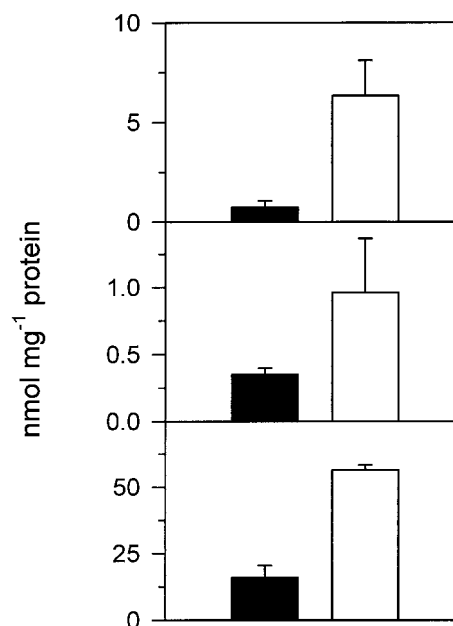


Figure 2. Contents of cyst(e)ine (top), γ EC (middle), and GSH (bottom) in BSC (black bars) and MC (open bars). Cell types were obtained by isolation procedure B. The values of BSC were corrected for contamination by MC. Means \pm SD of four independent experiments are presented. The values of the two cell types differ significantly from each other ($P \leq 0.05$).

iment. The percentage of ³⁵S-radiolabeled thiols in MC extracts from such an experiment is presented in Figure 3A. Seventy-five percent of the radioactivity of the thiols in MC was detected in cyst(e)ine after a 5-min incubation, whereas after 90 min the label was found at a comparable percentage in GSH. The recovery of cyst(e)ine, γ EC, and GSH was 36 to 37% in comparison with the acidic procedure as a standard method for thiol extraction (data not shown). Only negligible amounts of [³⁵S]sulfide were detected at all times, although the recovery rate of sulfide with the alkaline-extraction method was as high as those of the thiols at any time. The repetition of the experiment, including the acidic extraction, resulted in the same time course of radioactivity in the thiols (Fig. 3B). The amount of ³⁵S-radiolabeled thiols in the BSC during the incubation time was very low compared with those in MC (Fig. 3C). After a 90-min incubation, 75% of the total radioactivity in the thiols was measured in GSH of MC. Calculated on the basis of the specific activity of [³⁵S]sulfate fed to the leaves, this percentage corresponded to 0.13 nmol GSH mg⁻¹ protein.

Taken together with the distribution of the enzyme activities involved, these results indicate that cyst(e)ine is the thiol transported from BSC to MC, where it is used for GSH synthesis. To corroborate this result, we incubated isolated bundle-sheath strands in a medium containing [³⁵S]sulfate. These experiments resulted in only one radioactive product detectable in the resuspending medium. Figure 4 shows the HPLC chromatographs of ³⁵S-labeled substances in the resuspending medium for different incubation times. The

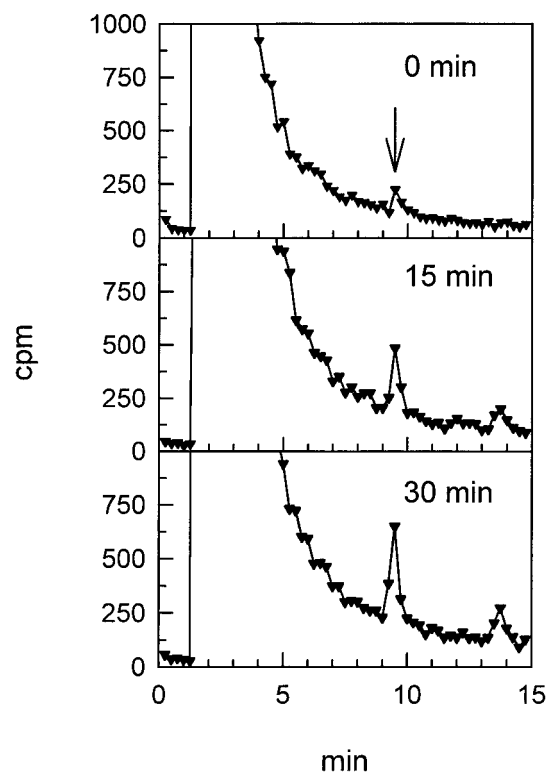
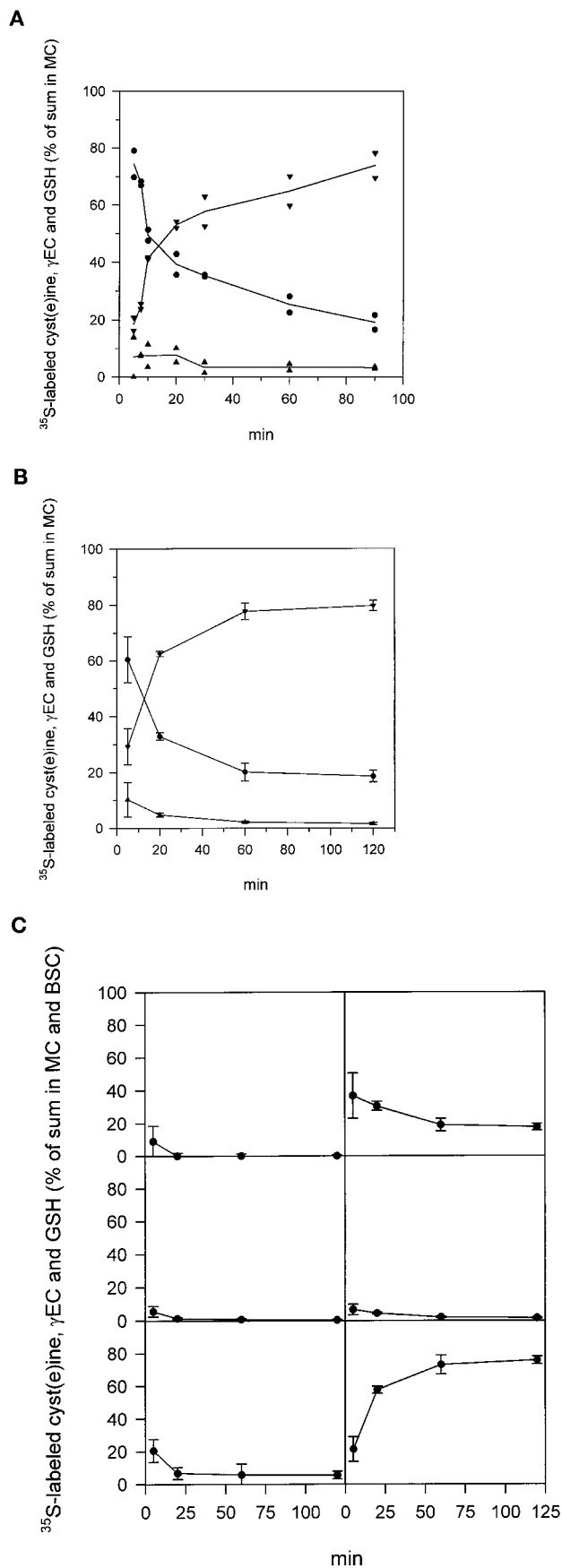


Figure 4. Separation of ^{35}S compounds in the incubation medium of isolated bundle-sheath strands by HPLC. Incubation with ^{35}S sulfate was for 0, 15, and 30 min. The arrow indicates the retention time of the monobromobimane derivative of cyst(e)ine.

peak with a retention time of 2.5 min represents the supplied ^{35}S sulfate, and the signal at 13.5 min resulted from the washing of the column. The only other substance detected with appreciable radioactivity was identified as cyst(e)ine by injecting monobromobimane derivatives of Cys, γEC , and GSH and measuring them with a fluorescence detector. Calculated on the basis of the specific activity of ^{35}S sulfate fed to the isolated BSC, the amounts of synthesized cyst(e)ine in the 15- and 30-min incubations were 1.2 and 2.4 nmol mg^{-1} chlorophyll, respectively. The small peak at the beginning of incubation corresponding to 0.5 nmol cyst(e)ine mg^{-1} chlorophyll can be most likely ex-

Figure 3. A, Radioactivity in cyst(e)ine (●), γEC (▲), and GSH (▼) in MC as a percentage of total radioactivity in the three compounds after an alkaline-extraction method. Radioactivity was supplied as ^{35}S sulfate by incubating 11-d-old second leaves for 5 to 90 min. Values of two independent experiments at each time are presented. B, Radioactivity in cyst(e)ine (●), γEC (▲), and GSH (▼) in MC as a percentage of total radioactivity in the three compounds after an acid-extraction method. Means \pm SD of four independent experiments at each time are presented. C, Radioactivity in cyst(e)ine (top), γEC (middle), and GSH (bottom) in BSC (left) and MC (right) as a percentage of the sum of the three compounds in both cell types after acidic extraction. The values of BSC were corrected for contamination by MC. Mean values \pm SD of four independent experiments at each time are presented.

plained by synthesis of the product during separation of BSC from resuspending medium. In a parallel experiment, in which the BSC were centrifuged first and the resulting supernatant was incubated with [^{35}S]sulfate, no cyst(e)ine was detectable (data not shown).

To exclude the possibility that the above-mentioned results were due to leaky plasma membranes we measured RuBP as a marker (Doulis et al., 1997). The similar amounts of RuBP that were detected in BSC before and after the incubation indicated that the chloroplasts and plasma membranes of these cells were not leaky for all low- M_r molecules caused by the exposure (data not shown).

DISCUSSION

Because of their lower stability, MC disrupt preferentially in a mechanical isolation procedure. This characteristic makes it possible to obtain very pure MC extracts within seconds using a roller device (Leegood, 1985). The rapid extraction method is, therefore, excellent for short-time tracer experiments and measurements of enzyme activities in MC. If pure extracts of both MC and BSC are of importance, then the leaves should be predigested in a medium for protoplast isolation (Mills and Joy, 1980) before the cell types are separated mechanically (Schmutz and Brunold, 1984).

Bundle-sheath strands consisting of a segment of vascular bundle surrounded by BSC of high functional integrity and metabolic competence can be obtained easily by blending leaf segments in an appropriate medium with a polytron (Valle and Heldt, 1991). The intact plasmodesmata that originally connected the bundle-sheath cytosol with MC are permeable to molecules up to a molecular mass of about 900 D (Valle et al., 1989). This means that the highly intact BSC are accessible to substrates added to the resuspending medium, thus enabling metabolic studies.

The findings presented in this paper suggest a cooperation between BSC and MC in sulfate reduction and GSH synthesis, as shown in Figure 5. The scheme compares the intercellular localization of sulfur assimilation with the compartmentation of reactions involved in carbon and nitrogen assimilation. The distribution of ATPase and APSSase activities in maize was consistent with previous results, which indicated a preponderant or even exclusive localization of assimilatory sulfate reduction to BSC (Burnell, 1984; Schmutz and Brunold, 1984, 1985; Ghisi et al., 1986). The high percentage of GSH synthetase activity in MC indicates that this cell type is the main site for synthesis of the tripeptide in maize leaves and correlates with the high levels of GSH determined in MC, as compared with BSC.

Information about intermediates of sulfate assimilation transported from BSC to MC was provided by feeding intact leaves with [^{35}S]sulfate and then rapidly extracting the MC. The immediate appearance of ^{35}S -labeled cyst(e)ine in the MC indicated that this amino acid is the molecule transporting reduced sulfur. This hypothesis was corroborated by the finding that isolated bundle-sheath strands released cyst(e)ine into the resuspending medium.

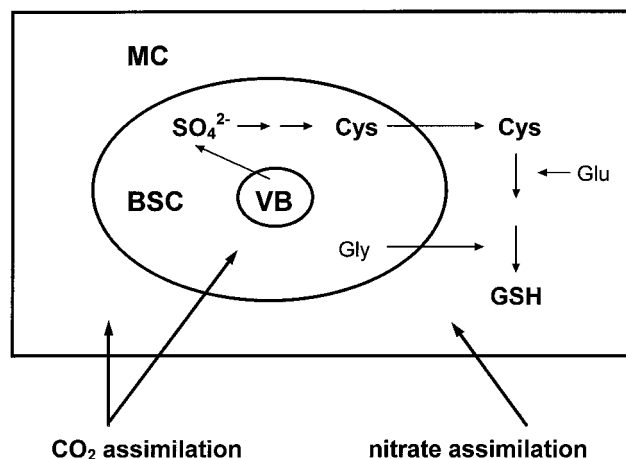


Figure 5. Diagram of the hypothetical intercellular distribution of CO_2 , nitrate, and sulfate assimilation and GSH synthesis in maize leaves. Cys and Gly exported from BSC and Glu formed in MC are used for GSH synthesis. VB, Vascular bundle.

Since no cyst(e)ine was detectable in a parallel experiment in which the BSC were centrifuged first, and only the resulting supernatant of the suspension was incubated with [^{35}S]sulfate, we can be sure that the [^{35}S]cyst(e)ine was synthesized in the intact BSC and then transported into the resuspension medium. In a physiological context this cyst(e)ine would be exported into MC.

The compartmentation of CO_2 and nitrate assimilation in C_4 plants leads to important advantages with regard to carbon (Black, 1973) and nitrogen use efficiency (Brown, 1978). The physiological reason for the cell-type-specific localization of sulfate assimilation and GSH synthesis between BSC and MC is not clear. Sulfate assimilation is located in BSC. These enclose the vascular bundles, which are the source of sulfate originating from the soil. It is plausible that reduced levels of PSII (Sheen and Bogorad, 1988; Pfündel et al., 1996) and correspondingly lower O_2 concentrations in BSC compared with MC could prevent autooxidation of the reaction intermediates of assimilatory sulfate reduction, i.e. sulfite and/or sulfide. A second reason might be that Gly decarboxylase and Ser hydroxymethyl transferase (enzymes synthesizing Ser, the precursor of Cys) are localized exclusively in BSC of C_4 plants (Gardeström et al., 1978; Ohnishi and Kanai, 1983; Becker et al., 1993). Since Gly is also produced in this cell type (Martin et al., 1983; Farineau et al., 1984; Yamaya and Oaks, 1988), GSH synthesis in the MC could proceed using Cys and Gly formed in BSC and Glu from MC (Fig. 5). A predominant localization of GSH synthesis and correspondingly high levels of GSH in MC would be advantageous for the plant to react against reactive oxygen species (Foyer et al., 1994; Doulis et al., 1997), which are probably produced preferentially in this cell type in various stress situations (Rennenberg and Brunold, 1994) and during pathogen attack (Low and Merida, 1996).

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