Plant Physiol. (1984) 74, 866–870
0032-0889/84/74/0866/05/$01.00/0

Intercellular Localization of Assimilatory Sulfate Reduction in Leaves of *Zea mays* and *Triticum aestivum*[1]

Received for publication July 19, 1983 and in revised form December 3, 1983

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ABSTRACT

The intercellular distribution of assimilatory sulfate reduction enzymes between mesophyll and bundle sheath cells was analyzed in maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) leaves. In maize, a C_4 plant, 96 to 100% of adenosine 5′-phosphosulfate sulfotransferase and 92 to 100% of ATP sulfurylase activity (EC 2.7.7.4) was detected in the bundle sheath cells. Sulfite reductase (EC 1.8.7.1) and *O*-acetyl-L-serine sulfhydrylase (EC 4.2.99.8) were found in both bundle sheath and mesophyll cell types. In wheat, a C_3 species, ATP sulfurylase and adenosine 5′-phosphosulfate sulfotransferase were found at equivalent activities in both mesophyll and bundle sheath cells. Leaves of etiolated maize plants contained appreciable ATP sulfurylase activity but only trace adenosine 5′-phosphosulfate sulfotransferase activity. Both enzyme activities increased in the bundle sheath cells during greening but remained at negligible levels in mesophyll cells. In leaves of maize grown without addition of a sulfur source for 12 d, the specific activity of adenosine 5′-phosphosulfate sulfotransferase and ATP sulfurylase in the bundle sheath cells was higher than in the controls. In the mesophyll cells, however, both enzyme activities remained undetectable. The intercellular distribution of enzymes would indicate that the first two steps of sulfur assimilation are restricted to the bundle sheath cells of C_4 plants, and this restriction is independent of ontogeny and the sulfur nutritional status of the plants.

C_4 plants are characterized by an intercellular compartmentation of CO_2 and NO_3^--assimilation between mesophyll and bundle sheath cells (2, 3, 6, 16). Recently, a predominant localization of enzymes of assimilatory sulfate reduction in the bundle sheath cells was proposed (7, 8, 14). This pathway begins with the formation of APS[2] from ATP and SO_4^{2-} via ATP sulfurylase (EC 2.7.7.4) (1, 18). The sulfate of APS is transferred to a carrier (car-SH) to form bound sulfite (car-S-SO_3^-). This reaction is catalyzed by APSSTase. Car-S-SO_3^- is reduced to car-S-S^- by organic thiosulfate reductase (formerly called thiosulfonate reductase [18]). Finally, the thiol is incorporated into OAS by OASSase (EC 4.2.99.8) to form cysteine. Sulfite reductase (EC 1.8.7.1) which catalyzes the reduction of SO_3^{2-} to H_2S does not seem to be involved in the main reduction pathway (18). Its proposed function is to act on SO_3^{2-} liberated from car-S-SO_3^- by thiols or produced from SO_2 taken up from a polluted atmosphere (18).

Gerwick *et al.* (8) reported that 95 to 100% of total leaf ATP sulfurylase is in the bundle sheath strands of several C_4 plants. In variety XL 72 A of *Zea mays* L., about 75% of total ATP sulfurylase was detected in this type of cell (14). Organic thiosulfate reductase/sulfite reductase were 2 to 3 times higher on a protein basis in bundle sheath extracts than in mesophyll extracts of crabgrass leaves (7). In maize (cv XL 72 A), OASSase activity was found predominantly in the bundle sheath cells (14). Collectively these findings indicate that assimilatory sulfate reduction occurs primarily in the bundle sheath cells in C_4 plants and prompted us to determine the intercellular distribution of all enzymes of assimilatory sulfate reduction.

MATERIALS AND METHODS

Plant Material. Seeds of maize (*Zea mays* L. cv Seneca 60) and wheat (*Triticum aestivum* L. cv Kolibri) were imbibed for 24 h in aerated tap water at room temperature and then transferred to moist filter paper. After 3 d in the dark, the seedlings were cultivated on quartz sand watered regularly with nutrient solution (23). Nutrient solution deficient in S was composed by replacing the SO_4^{2-} salts by equimolar amounts of the corresponding Cl^- salts. The plants were grown at 26°C, with a RH of 40% and a light intensity of 46 μE m^{-2} s^{-1} provided by incandescent lamps (Philips TL 40 w).

Cell Isolation. Mesophyll cells and bundle sheath strands were obtained either by a mechanical or by an enzymic isolation procedure. For both procedures, leaves were cut diagonally in approximately 1-mm-wide strips with a razor blade and then transferred to a medium for protoplast isolation according to Mills and Joy (12), containing 2% cellulase and 2% pectinase. Ten ml of medium were used per g leaf strips. The plant material was infiltrated by applying a vacuum. The incubation time was 15 min in the light for the mechanical procedure. The plant material was then homogenized using a Servall Omni Mixer (Ivan Sorvall, Norwalk, CT) for 10 s at 100 v (6,700 rpm) and twice 10 s at 240 v (16,250 rpm) using 8 ml of extraction buffer per g plant material. The extraction buffer was Tris-HCl (pH 8.0) containing 2 mM MgCl and 10 mM DTE. The homogenate was filtered through a 60-μm nylon net. The filtrate contained the broken mesophyll cells. The bundle sheath strands on the nylon net were collected and broken with a cooled glass homogenizer in extraction buffer. Ten ml/1 g plant material was used.

For the enzymic isolation procedure, incubation in the enzyme solution was 2.5 h in the light. Filtration through a 60-μm nylon net resulted in a residue of bundle sheath strands and a filtrate containing the suspended mesophyll protoplasts. This suspension was centrifuged for 5 min at 100*g*, washed once with 2 ml of isolation medium without enzymes, and centrifuged as before. The pellet was resuspended in extraction buffer and homogenized

[1] Supported by grant 3.109.081 from the Swiss National Science Foundation.

[2] Abbreviations: APS, adenosine 5′-phosphosulfate; APSSTase, adenosine 5′-phosphosulfate sulfotransferase; OASSase, *O*-acetyl-L-serine sulfhydrylase; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; PEP, phosphoenolpyruvate; PEPCase, phosphoenolpyruvate carboxylase; OAS, *O*-acetyl-L-serine; RuBP, ribulose bisphosphate; RuBPCase, ribulose bisphosphate carboxylase.

by passing the suspension 10 times through a hypodermic needle (0.9 × 40 mm). The bundle sheath strands on the nylon net were collected and transferred into protoplast isolation medium and shaken for 10 s on a Vortex to remove residual mesophyll cells. The strands were collected on a 60-μm nylon net and broken with a glass homogenizer using 10 ml extraction buffer per g plant material. All extracts were centrifuged for 10 min at 10,000*g* and 0°C. The supernatant fluid was used for the enzyme assays.

Enzyme Assays. ATP sulfurylase activity was estimated according to Schmutz and Brunold (19) by determining the ATP produced from APS and PPi using an ATP meter. APSSTase activity was measured as the production of [^{35}S]sulfite according to Hodson and Schiff (9). [^{35}S]APS instead of [^{35}S]PAPS was used as sulfonyl donor and DTE was included as thiol compound. Preliminary experiments showed that more than 90% of total APSSTase activity was present in the supernatant of the centrifugation at 10,000*g*.

Sulfite reductase was estimated according to von Arb and Brunold (21) with an assay system in which the H_2S formed from SO_3^{2-} and reduced Fd is used for synthesis of cysteine, which serves as a measure of enzyme activity. OASSase was measured according to Pieniazek *et al.* (15) at 30°C. RuBPCase activity was determined according to Buchanan and Schürmann (5) by measuring the nonvolatile radioactivity produced from RuBP and $H^{14}CO_3^-$ and by following the modifications given by Wyss and Brunold (23). Incubation was for 4 min at 30°C. The determination of PEPCase was done according to Lane *et al.* (11). The incubation mixture contained, in a total volume of 125 μl, 80 mM Tris-HCl (pH 8.5), 8 mM reduced GSH, 3 units of malate dehydrogenase, 1.6 mM PEP, 3.2 mM NADH, 32 mM $NaH^{14}CO_3$, and 10 μl extract. Incubation was for 2 min at 30°C. Protein was determined using the protein assay of Bradford with BSA as the standard (4). Chl was determined according to Strain *et al.* (20).

RESULTS

Figure 1 shows the changes in fresh weight, leaf length, protein content, and ATP sulfurylase and APSSTase activity during ontogenesis of the second leaf of *Z. mays*. Maximal leaf length and fresh weight were reached 10 d after imbibition, whereas highest protein content was measured on day 8, followed by a slow decrease. Total ATP sulfurylase activity was essentially constant between 6 and 18 d after imbibition. At the beginning of the experiment, APSSTase activity was very low, reached a maximum on day 10, and started to decrease 3 d later with a half life of about 6 d. These results make it clear that experiments on the intercellular distribution of these two enzymes should be done with plants cultivated for 10 to about 18 d after imbition when both activities are at appreciable levels.

The distribution of ATP sulfurylase and APSSTase and of other enzymes of assimilatory sulfate reduction between bundle sheath strands and mesophyll cells of *Z. mays* is presented in Table I. PEPCase and RuBPCase were taken as marker enzymes for mesophyll cells and bundle sheath strands, respectively. Using the mechanical procedure for isolation of the two cell types, 23% of total RuBPCase activity was found in the mesophyll cell fraction. This percentage is higher than the 8% and 4% of ATP sulfurylase and APSSTase activity, respectively, in this fraction, indicating that the activities of both enzymes resulted from contaminating bundle sheath cells. APSSTase as well as ATP sulfurylase would be restricted to bundle sheath cells as is RuBPCase. This is substantiated by the results from the enzymic isolation procedure, which yielded bundle sheath strands containing 100, 99.6, and 100% of total RuBPCase, ATP sulfurylase, and APSSTase, respectively. Mixing of extracts from bundle sheath cells and mesophyll cells gave additive activities of ATP

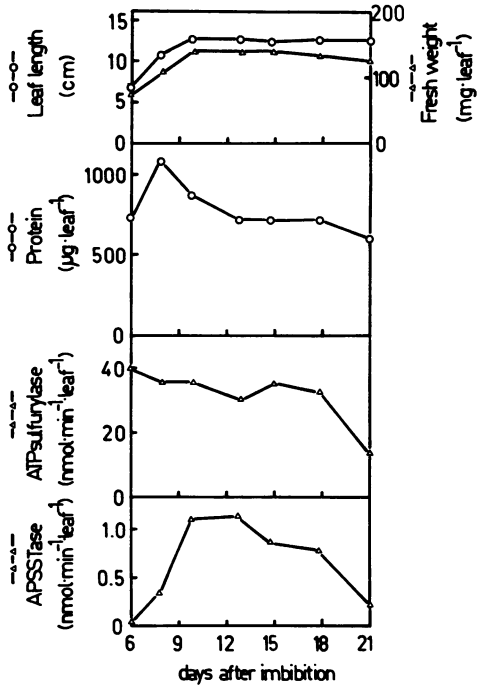

FIG. 1. Changes in length, fresh weight, protein content, and total activity of ATP sulfurylase and APSSTase during development of second leaf blade of maize plants. The parameters were determined from a homogenate of leaves from five plants.

sulfurylase and APSSTase, indicating that no inactivator was present in the mesophyll extracts (data not shown).

Sulfite reductase was found to be enriched in the bundle sheath cells. It was present only at about 30 to 40% of the total leaf activity in the mesophyll cells, whereas OASSase was detected at 60 to 70% in this type of cells. During the enzymic isolation procedure, the specific activity of both ATP sulfurylase and APSSTase decreased enormously. This raised the question whether the deficiency of the mesophyll cells in these enzyme activities was due to inactivation during the enzymic isolation. Therefore, we returned to the mechanical isolation procedure which results in cell fractions with ATP sulfurylase and APSSTase activity which add up to the activity in homogenates of whole leaves.

Figure 2 shows the effect of increasing homogenizing intensity on the distribution of RuBPCase, ATP sulfurylase, and APSSTase between a soluble fraction and a fraction retained by a 60-μm nylon net. With increasing homogenizing intensity, the activity of RuBPCase increased in the soluble fraction from about 10 to 100% of the total activity. This shows that extraction of bundle sheath cells, where RuBPCase is localized, only occurred at appreciable levels when maize leaves were treated with high homogenizing intensities. The activity of the two enzymes of assimilatory sulfate reduction in the two fractions changed in an identical manner as RuBPCase activity, when the homogenizing intensity was increased. This indicates that they are also localized exclusively in the bundle sheath cells.

Figure 3 shows the changes in specific activity of ATP sulfurylase and APSSTase and the Chl content relative to the protein content in leaves of maize plants which had been kept in the dark for 13 d after imbibition and had been transferred to continuous light at the beginning of the experiment. There was a parallel increase in Chl and APSSTase activity in the bundle sheath strands during the first 2 d in the light. Then there was a rapid decrease in APSSTase activity whereas Chl continued to increase. ATP sulfurylase was already present in the dark-grown leaves, increasing by about 100% during greening of the leaves

Table I. *Intercellular Localization of Enzymes in Cell Types Isolated from Maize Leaves*

Plants were examined 15 d after imbibition. After mechanical isolation, bundle sheath strands contained 288 μg proteins/leaf and mesophyll cells contained 272 μg proteins/leaf. For the enzymic isolation procedure, mean values from five experiments ± SE are presented.

Cell Type	Isolation Procedure	PEPCase	RuBPCase	ATP Sulfurylase	APSSTase	Sulfite Reductase	OASSase
		nmol min^{-1} mg^{-1} protein					
Bundle sheath strands	Mechanical	113	179	131	10.8	9.2	3880
Mesophyll cells	Mechanical	1516	52	11	0.5	3.9	9100
Bundle sheath strands	Enzymic	503 ± 131	144 ± 17	32.8 ± 4.4	0.62 ± 0.03	6.3 ± 1.2	454 ± 94
Mesophyll protoplasts	Enzymic	3358 ± 83	0	0.12 ± 0.05	0.000	4.8 ± 1.1	608 ± 243

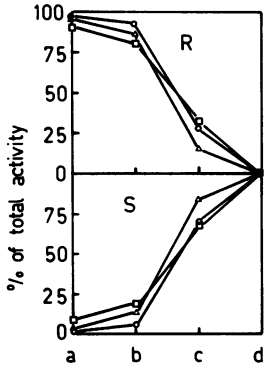

FIG. 2. Distribution of RuBPCase (□——□), APSSTase (○——○), and ATP sulfurylase (△——△) activity between a soluble fraction (S) and a fraction retained by a 60-μm nylon net (R) after homogenizing the second leaf of maize plants 15 d after imbibition. Homogenization was for 10 s at 100 v and twice for 10 s at 150 using a Sorvall Mixer after 10 min incubation in protoplast isolation medium (a) or mixing for 5 s using a Polytron homogenizer on position 2 (b), 4 (c), and at maximal speed (d), respectively. One hundred % activity was 259, 261, 457, and 314 nmol min^{-1} mg^{-1} protein for RuBPCase; 4.4, 1.9, 1.6, and 2.1 nmol min^{-1} mg^{-1} protein for APSSTase; and 176, 57, 45, and 68 nmol min^{-1} protein for ATP sulfurylase with the homogenization procedures a, b, c, and d, respectively.

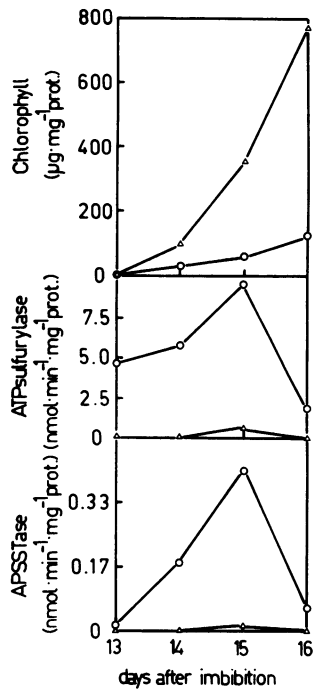

FIG. 3. Changes in Chl and activity of ATP sulfurylase and APSSTase in bundle sheath strands (○——○) and mesophyll cells (△——△) of greening maize leaves. The maize plants were transferred from dark to light 13 d after imbibition. The two types of cells were isolated after enzymic digestion of mesophyll cell walls.

for 2 d and subsequently decreasing parallel to APSSTase activity. At no time could ATP sulfurylase and APSSTase be detected in the mesophyll cells at activities in excess of 7% and 4%, respectively, of total leaf activity.

Table II presents the effect of cultivation on S-deficient nutrient solution on the specific activity of RuBPCase, ATP sulfurylase, and APSSTase in maize leaves. In the bundle sheath strands, ATP sulfurylase and APSSTase were clearly higher than in the controls but could not be detected in the mesophyll protoplasts.

The changes in leaf length, fresh weight, protein content, and activity of ATP sulfurylase and APSSTase of the first leaf of wheat plants from 6 to 17 d after imbibition are presented in Figure 4. Except for APSSTase, there was an increase in all parameters determined during the first 2 d of the experiment. ATP sulfurylase and protein then decreased with half lives of 4 and 10 d, respectively. APSSTase activity was lost from the beginning of the experiment with a half life of about 5 d.

The intercellular distribution of ATP sulfurylase and APSSTase in the first leaf of *T. aestivum* is presented in Table III together with RuBPCase. The three enzyme activities were found to be distributed uniformly between the bundle sheath strands and the mesophyll cells.

DISCUSSION

The results of the present paper show that in *Z. mays* leaves the two first steps of assimilatory sulfate reduction, catalyzed by ATP sulfurylase and APSSTase, are essentially restricted to the bundle sheath cells. This is consistent with the results of Gerwick et al. (8) who detected only 2.4% of ATP sulfurylase in the mesophyll cells of *Z. mays*. In contrast, Passera and Ghisi (14) found 25 to 30% of total ATP sulfurylase activity in the mesophyll cells of *Z. mays* cv XL 72 A. The assay of marker enzymes for each cell type was not reported in the study. Therefore, it is difficult to assess to what extent contaminations may have contributed to this percentage. Nevertheless, it seems possible that

Table II. *Effect of S-Deficient Nutrient Solution on Enzymes from Maize Leaves*

Maize plants were transferred to nutrient solution deficient in S 5 d after imbibition and analyzed 12 d later. Cell types were isolated using the enzymic isolation procedures. The protein content was 2.47 and 2.55 $\mu g\ mg^{-1}$ fresh weight for the S-deficient and the control leaves, respectively. The number in parentheses gives the percentage of the enzyme activities of the treated plants as compared to the controls.

Enzyme	Treatment	Bundle Sheath Cells	Mesophyll Protoplasts
		nmol min^{-1} mg^{-1} protein	
RuBPCase	− S	123 (102)	0
	Control	121	0
ATP sulfurylase	− S	46 (131)	0
	Control	35	2.0
APSSTase	− S	0.40 (308)	0
	Control	0.13	0

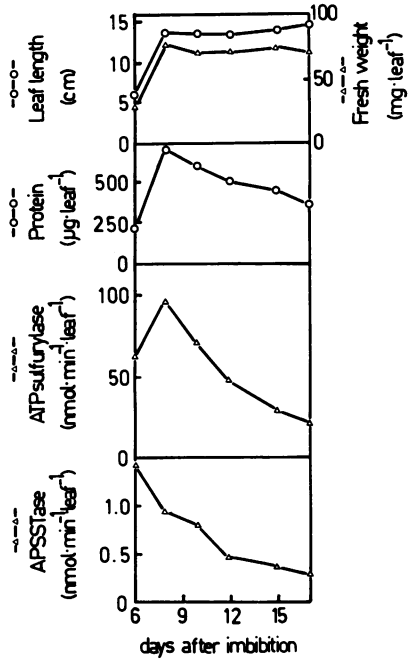

FIG. 4. Changes in length, fresh weight, protein, and activity of ATP sulfurylase and APSSTase from the first leaf of wheat plants. The parameters were determined from a homogenate of leaves from five plants.

in this variety the first step of sulfate reduction can take place at appreciable rates in both cell types.

The intercellular localization of the reduction step is not clear. Using the dithionite assay, Gerwick and Black (7) found the activity of organic thiosulfate reductase/sulfite reductase to be 2 to 3 times higher on a protein basis in bundle sheath extracts than in mesophyll extracts from crabgrass leaves. This is comparable to the intercellular distribution of sulfite reductase in maize leaves. It has been shown, however, that purified sulfite reductase from spinach also reduces NO_2^- (10). It is not clear in our system, and probably also not in the one of Gerwick and Black (7), whether the physiological function of sulfite reductase found in the mesophyll cells is the reduction of sulfite or nitrite. Since nitrate reductase is essentially restricted to mesophyll cells of C_4 plants (13) and since the two first steps of assimilatory sulfate reduction occur exclusively or almost totally in the bundle sheath cells, one can speculate that the physiological function of this enzyme activity is the reduction of nitrite in the mesophyll cells and the reduction of sulfite in the bundle sheath cells.

Table III. *Intercellular Localization of Enzymes in Cell Types Isolated from Wheat Leaves*

Plants were examined 8 d after imbibition. Mechanical isolation resulted in bundle sheath strands containing 310 and mesophyll cells with 290 μg protein/leaf. For the enzymic isolation procedure, mean values from five experiments ± SE are presented.

Cell Type	Isolation Procedure	RuBPCase	ATP Sulfurylase	APSSTase
		nmol min^{-1} mg^{-1} protein		
Bundle sheath strands	Mechanical	431	91.2	0.28
Mesophyll cells		535	84.1	0.20
Bundle sheath strands	Enzymic	330 ± 48	69 ± 9	0.04 ± 0.003
Mesophyll protoplasts		234 ± 31	80 ± 21	0.04 ± 0.007

The last step of assimilatory sulfate reduction can proceed in the bundle sheath and the mesophyll cells in leaves of *Z. mays* since OASSase was detected in both types of cells. This is consistent with the findings of Passera and Ghisi (14). If the other steps of sulfate reduction are restricted to the bundle sheath cells, the function of OASSase in the mesophyll cells could be to trap H_2S escaping from the bundle sheath cells. Light-dependent evolution of H_2S from plants has been reported (22). It is interesting in this connection that detached leaves of maize fed with 100 mM $KHSO_3$ release much less H_2S than leaves of other plants and do not seem to emit H_2S when fed with K_2SO_4 (22).

In previous work with tobacco cells, it has been shown that sulfur starvation derepresses ATP sulfurylase (17). In maize plants cultivated on S-deficient nutrient solution, we could detect a higher specific ATP sulfurylase and APSSTase activity in the bundle sheath strands as compared to controls. However, neither ATP sulfurylase nor APSSTase could be detected in the mesophyll cells of these plants. This indicates that these enzymes are not simply repressed by high sulfate concentration in this type of cells.

In wheat leaves, ATP sulfurylase and APSSTase activity were found at comparable levels in bundle sheath cells and mesophyll cells, indicating that there is no division of function in the C_3 plants with respect to sulfate reduction.

Acknowledgments—We thank C. Fuhrer-Fries for correcting the English of the manuscript; M. Suter for preparing the drawings; and Drs. I. Potrykus, F. Meins, and P. King for helpful discussions and the gift of maize seeds.

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