Assimilatory Sulfate Reduction in C_3, C_3-C_4, and C_4 Species of *Flaveria*[1]

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The activity of the enzymes catalyzing the first two steps of sulfate assimilation, ATP sulfurylase and adenosine 5′-phosphosulfate reductase (APR), are confined to bundle sheath cells in several C_4 monocot species. With the aim to analyze the molecular basis of this distribution and to determine whether it was a prerequisite or a consequence of the C_4 photosynthetic mechanism, we compared the intercellular distribution of the activity and the mRNA of APR in C_3, C_3-C_4, C_4-like, and C_4 species of the dicot genus *Flaveria*. Measurements of APR activity, mRNA level, and protein accumulation in six *Flaveria* species revealed that APR activity, cysteine, and glutathione levels were significantly higher in C_4-like and C_4 species than in C_3 and C_3-C_4 species. ATP sulfurylase and APR mRNA were present at comparable levels in both mesophyll and bundle sheath cells of C_4 species *Flaveria trinervia*. Immunogold electron microscopy demonstrated the presence of APR protein in chloroplasts of both cell types. These findings, taken together with results from the literature, show that the localization of assimilatory sulfate reduction in the bundle sheath cells is not ubiquitous among C_4 plants and therefore is neither a prerequisite nor a consequence of C_4 photosynthesis.

Assimilatory sulfate reduction is a pathway used by prokaryotes, fungi, and photosynthetic organisms to convert inorganic sulfate to sulfide, which is further incorporated into carbon skeletons of amino acids to form Cys or homo-Cys (Brunold, 1993). In this pathway, sulfate is first activated by ATP sulfurylase (ATPS) forming adenosine 5′-phosphosulfate (APS). In higher plants, APS is reduced by APS reductase (APR) to sulfite, which is further reduced to the level of sulfide by sulfite reductase (Bick and Leustek, 1998; Suter et al., 2000). APS can also be phosphorylated by APS kinase to phosphoadenosine 5′-phosphosulfate, which is utilized for synthesis of a wide range of sulfated compounds in reactions catalyzed by a variety of sulfotransferases (Varin et al., 1997). Thus, APR is a key step in sulfate assimilation and as such, the enzyme is highly regulated, e.g. by light, sulfur and nitrogen supply, heavy metals, or chilling (Rüegsegger et al., 1990; Neuenschwander et al., 1991; Brunold, 1993; Brunner et al., 1995; Kopriva et al., 1999, 2000).

C_4 plants are characterized by an intercellular compartmentation of CO_2 and nitrate assimilation between mesophyll and bundle sheath cells (Black, 1973; Moore and Black, 1979). Also, sulfate assimilation was proposed to be restricted to the bundle sheath cells of C_4 plants (Gerwick et al., 1980; Schmutz and Brunold, 1984). Several groups reported that 75% to 100% of total leaf ATPS activity in maize (*Zea mays*) is confined to bundle sheath cells (Gerwick et al., 1980; Passera and Ghisi, 1982; Schmutz and Brunold, 1984). These findings were extended to 17 other C_4 species where 95% to 100% of total leaf ATPS activity was found in chloroplasts of bundle sheath cells. Also, the next enzyme in the sulfate reduction pathway, APR, was found exclusively or almost exclusively in bundle sheath cells of maize (Schmutz and Brunold, 1984; Burgener et al., 1998), whereas the activities of the subsequent enzymes of the pathway, sulfite reductase and O-acetyl-Ser-(thiol) lyase, were found in both cell types at comparable levels (Passera and Ghisi, 1982; Burnell, 1984; Schmutz and Brunold, 1985). In accordance, in maize the mRNAs for APR, ATPS, and sulfite reductase accumulated in bundle sheath only, whereas the mRNA for *O*-acetyl-Ser-(thiol) lyase was also detected in mesophyll cells (Kopriva et al., 2001). Cultivation of maize plants at 12°C resulted in a prominent increase of APR mRNA and activity in bundle sheath cells. In addition, after chilling mRNAs for APR and sulfite reductase, as well as low APR activity, were detected in mesophyll cells. Therefore, it seems that chilling stress is able to affect not only the levels but also the intercellular distribution of mRNAs for enzymes of the sulfate assimilation (Kopriva et al., 2001).

The functional significance of the compartmentation of sulfate assimilation in bundle sheath cells of

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maize and other C_4 species is not yet clear. A possible explanation could be the reduced O_2 concentrations in bundle sheath cells compared with mesophyll cells, which might prevent oxidation of the reaction intermediates of sulfate assimilation, SO_3^{2-} and S^{2-}, or, alternatively, higher availability of Ser, resulting from exclusive localization of Gly decarboxylase and Ser hydroxymethyltransferase in bundle sheath cells (Burgener et al., 1998). As a first step to elucidate the importance of this compartmentation, the question arose of whether the exclusive or almost exclusive localization of the first two enzymes of sulfate assimilation was a prerequisite or a consequence of C_4 photosynthesis. To answer this question we turned to the *Flaveria* genus. The genus *Flaveria* (Flaveriinae-Asteraceae) is unique because, beside C_3 and C_4 species, a relatively large number of C_3-C_4 intermediates exist in this genus (Powell, 1978; Bauwe, 1984; Ku et al., 1991). The levels of C_4 enzymes in *Flaveria* spp. are correlated with the degree of C_4 photosynthesis based on the initial products of photosynthesis and CO_2 compensation points (Ku et al., 1991; Rosche et al., 1994; Marshall et al., 1996). This means that a continual gradation exists among *Flaveria* spp. both in the physiology and biochemistry of photosynthesis (Monson and Moore, 1989). Here, we report on activities, mRNA, protein accumulation, and inter- and intracellular localization of ATPS and APR in six species of the genus *Flaveria* with C_3, C_3-C_4 intermediate, and C_4 photosynthesis.

RESULTS

ATPS and APR Activity, Protein Accumulation, and Thiol Levels in Different *Flaveria* Spp.

ATPS and APR activities were measured in young fully developed leaves of *Flaveria cronquistii* (C_3), *Flaveria pringlei* (C_3), *Flaveria anomala* (C_3-C_4), *Flaveria palmeri* (C_4-like), *Flaveria trinervia* (C_4), and *Flaveria australasica* (C_4). As shown in Figure 1A, the APR activity in C_4-like and C_4 species *F. palmeri*, *F. trinervia*, and *F. australasica* was significantly higher than in the C_3 species. ATPS activities showed a similar pattern, with the highest activity in *F. palmeri*, but due to great variations between different experiments the differences were not significant (data not shown). The APR protein accumulation was addressed by western analysis with antisera against APR from Arabidopsis. Similar to APR enzyme activities, the highest APR protein amount was detected in *F. palmeri* (Fig. 1B) and the lowest in the C_3 species. Also, Cys and glutathione (GSH) concentrations in young leaves were lower in C_3 species then in C_4 and C_4-like species, analogous to the distributions of APR activity and protein amount (Fig. 1, C and D). There was a strong correlation between the foliar GSH concentrations and measured APR activities among the six *Flaveria* spp. analyzed ($r = 0.906$).

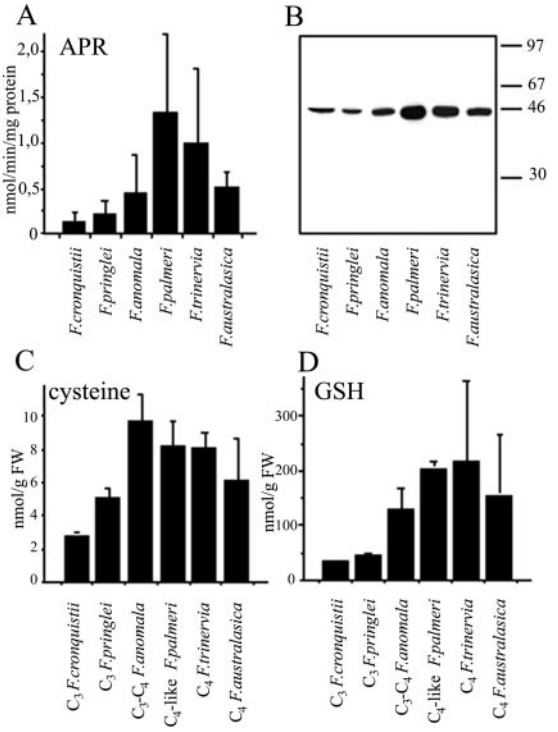

Figure 1. Assimilatory sulfate reduction in young leaves of six *Flaveria* species with different types of photosynthesis. A, APR activity. The results are presented as mean values + SD from six independent measurements. B, Western-blot analysis of APR. Ten micrograms of leaf proteins was resolved on 12% (w/v) SDS-PAGE gel and transferred onto nitrocellulose membrane. APR was immunologically detected using antisera against recombinant APR2 from Arabidopsis. C, Cys content. The results are presented as mean values + SD from three to six independent measurements. D, GSH content. The results are presented as mean values + SD from three to six independent measurements.

Expression Analysis

The levels of ATPS and APR mRNA were determined in young, fully developed leaves of five *Flaveria* spp. by northern blotting. Partial cDNAs for ATPS and APR were cloned from the C_4 dicot *F. trinervia* by reverse transcriptase (RT)-PCR with degenerate primers against conserved domains (Suter et al., 2000). To avoid a bias caused by hybridization with a probe from a C_4 species only, cDNA for APR was isolated also from the C_3 species *F. cronquistii*. Both ATPS and APR mRNA levels, quantified with a densitometer, were very similar in all species; only slightly higher levels were observed in C_4 and C_4-like species than in C_3 and C_3-C_4 species (Fig. 2). The results of hybridizations with an APR probe from C_3 and C_4 species were essentially identical (data not shown). As controls, the filters were hybridized with cDNA probes coding for Rubisco SSU and PEPCase from *F. trinervia*. The differences in expression of these genes among the different species corresponded to the expected pattern, i.e. mRNA levels for Rubisco SSU were highest in C_3 species and de-

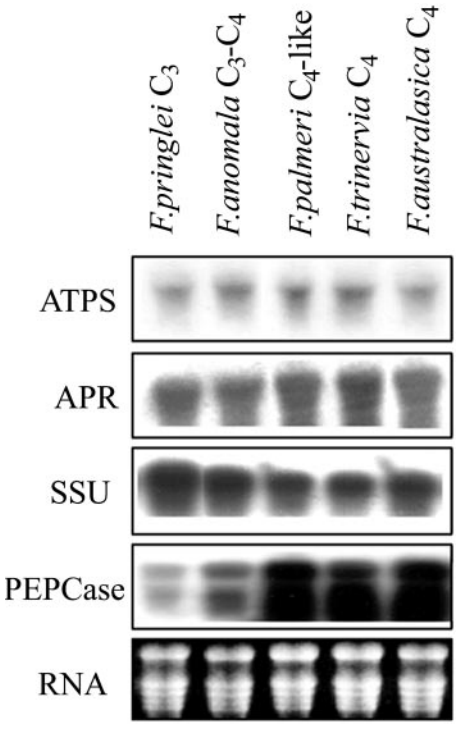

Figure 2. Northern-blot analysis. Total RNA was extracted from young leaves of five *Flaveria* spp., separated on 1% (w/v) agarose in the presence of formaldehyde, blotted onto Hybond-N nylon membrane, and hybridized with ^{32}P-labeled cDNA fragments of ATPS, APR, Rubisco small subunit (SSU), and phosphoenolpyruvate carboxylase (PEPCase) from *F. trinervia*. Ethidium bromide-stained RNA is shown as a control of loading and RNA intactness.

creased toward the C_4 ones, whereas PEPCase mRNA was very abundant in C_4-like and C_4 species but hardly detectable in C_3 species. Therefore, it seems that the variations in ATPS and APR activity (Fig. 1A) in the different species are not regulated solely at the transcriptional level.

To compare the intercellular distribution of ATPS and APR mRNA in maize and *Flaveria* spp., we isolated RNA from mesophyll and bundle sheath cells from two C_4 species, *F. trinervia* and *F. australasica*, and subjected it to northern analysis. Although the RNA preparations from the two cell types were cross contaminated, the quantification of the northern data surprisingly revealed that the mRNAs for ATPS and APR were present in both cell types at about the same levels (Fig. 3A). For both enzymes the relative transcript levels in mesophyll and bundle sheath cells (M/BS) were 1.1 to 1.2, as expected for mRNAs present in both cell types. In contrast, the mRNA of the marker enzyme for bundle sheath cells, Rubisco SSU, was detected predominantly in the RNA prepared from this cell type (M/BS = 0.5). PEPCase mRNA correspondingly was detected at a higher level in the RNA from mesophyll cells (M/BS = 2.1).

In Situ RNA Hybridization

Because the results of northern analysis with bundle sheath and mesophyll RNA in C_4 *Flaveria* differed significantly from maize (Kopriva et al., 2001) we wanted to confirm them using in situ RNA hybridizations with cDNA for APR from *F. trinervia*. As controls, in situ hybridizations were performed with cDNAs for Rubisco SSU and PEPCase, from *F. trinervia*. These hybridizations resulted in expected patterns of expression, i.e. bundle sheath-specific localization of Rubisco mRNA and mesophyll-specific expression of PEPCase (Fig. 4) in the C_4 and C_4-like species. In contrast, in all species analyzed, including C_4 and C_4-like *F. trinervia* and *F. palmeri*, no cell-specific expression pattern was observed and the APR mRNA was localized in both bundle sheath and mesophyll cells at comparable levels (Fig. 4), thus confirming the results of northern analysis.

Immunolocalization of APR

The presence of APR mRNA in the leaf mesophyll cells of *F. trinervia* does not necessarily mean that the mRNA is translated and the protein is active there.

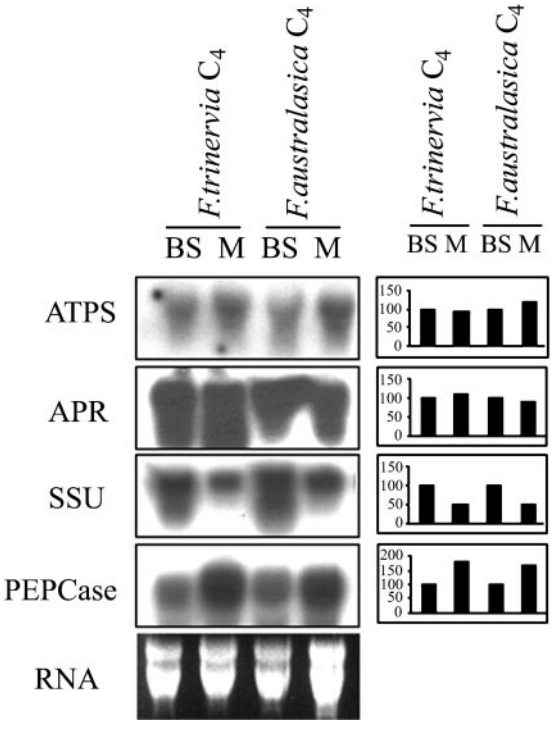

Figure 3. Northern-blot analysis of mesophyll- and bundle sheath-specific RNA. Total RNA was extracted from mesophyll and bundle sheath cells of young leaves of the C_4 species *F. trinervia* and *F. australasica*, separated on 1% (w/v) agarose in the presence of formaldehyde, blotted onto Hybond-N nylon membrane, and hybridized with ^{32}P-labeled cDNA fragments of ATPS, APR, Rubisco SSU, and PEPCase from *F. trinervia*. Ethidium bromide-stained RNA is shown as a control of loading and RNA intactness. Right, Results of densitometric quantification of the northern blots. 100%, Represents mRNA level in bundle sheath cells of the particular species.

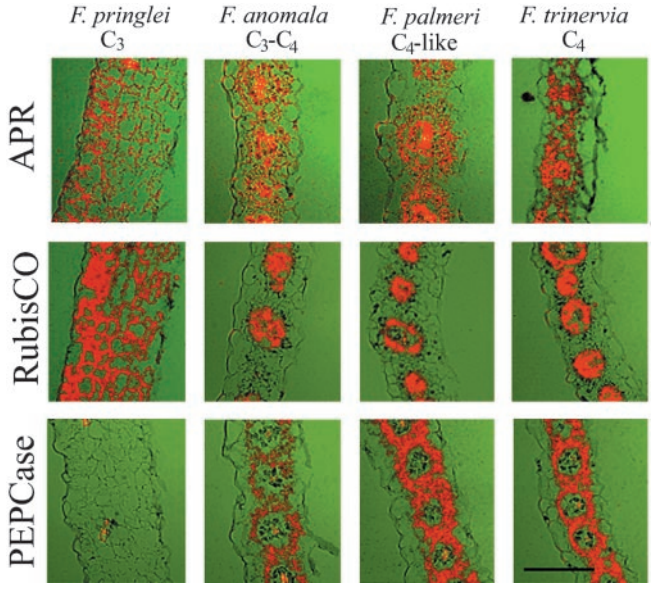

Figure 4. Expression of APR, Rubisco SSU, and PEPCase in leaves of four *Flaveria* spp. Transverse sections (7 μm) of young leaves from *F. pringlei* (C_3), *F. anomala* (C_3-C_4), *F. palmeri* (C_4-like), and *F. trinervia* (C_4) were analyzed by in situ hybridization. The sections were hybridized with probes specific for the indicated genes. Bar = 100 μm.

Therefore, antisera against APR2 from Arabidopsis (Kopriva et al., 1999) were used for immunogold electron microscopy on ultrathin leaf sections from three species: *F. pringlei*, *F. anomala*, and *F. trinervia*, to investigate the cellular distribution and spatial localization of this enzyme. APR protein was detected in chloroplasts of *F. pringlei* and *F. anomala* and in chloroplasts of both mesophyll and bundle sheath cells of *F. trinervia* (Fig. 5). In plastids of all three species, APR was localized approximately 30% to stroma and 70% to thylakoid membranes (data not shown). Treatment of the leaf sections with rabbit pre-immune sera did not result in any significant labeling as well as treatment with sera immunoprecipitated with purified recombinant APR. Because in all previous experiments we observed a strong correlation between APR protein accumulation and activity (Kopriva et al., 1999; Koprivova et al., 2000), we conclude that sulfate assimilation takes place in both mesophyll and bundle sheath cells in the C_4 dicot *F. trinervia*.

DISCUSSION

The aim of this study was to determine whether the well-documented bundle sheath-specific localization of sulfate assimilation in C_4 plants (Gerwick et al., 1980; Schmutz and Brunold, 1984; Burgener et al., 1998) was a prerequisite or a consequence of C_4 photosynthesis. Analogously to the results achieved with maize (Kopriva et al., 2001), we expected that the APR and ATPS mRNA and protein would be localized exclusively in bundle sheath cells in the C_4 *Flaveria* spp. and in all photosynthetic cells in C_3 species. From the distribution of ATPS and APR mRNA in C_3-C_4 intermediate and C_4-like species, we thus would obtain information on the significance of the cell-specific localization of sulfate assimilation for C_4 photosynthesis. However, both APR mRNA and protein were clearly detected in both types of cells in the C_4 species *F. trinervia*. These results are surprising because in several reports the activity of ATPS and APR were localized exclusively or almost exclusively to bundle sheath cells of various C_4 species (Gerwick et al., 1980; Schmutz and Brunold, 1984; Burgener et al., 1998). However, *F. trinervia* is a dicot and in all previous reports only monocot species were analyzed. We conclude, therefore, that the previously observed bundle sheath-specific localization of sulfate assimilation is not a general feature of C_4 photosynthesis. On the other hand, the bundle sheath localization of APR is not a feature of all monocots because in the C_3 monocot species *Triticum aestivum*, APR and ATPS were localized in both mesophyll and bundle sheath cells (Schmutz and Brunold, 1984). In

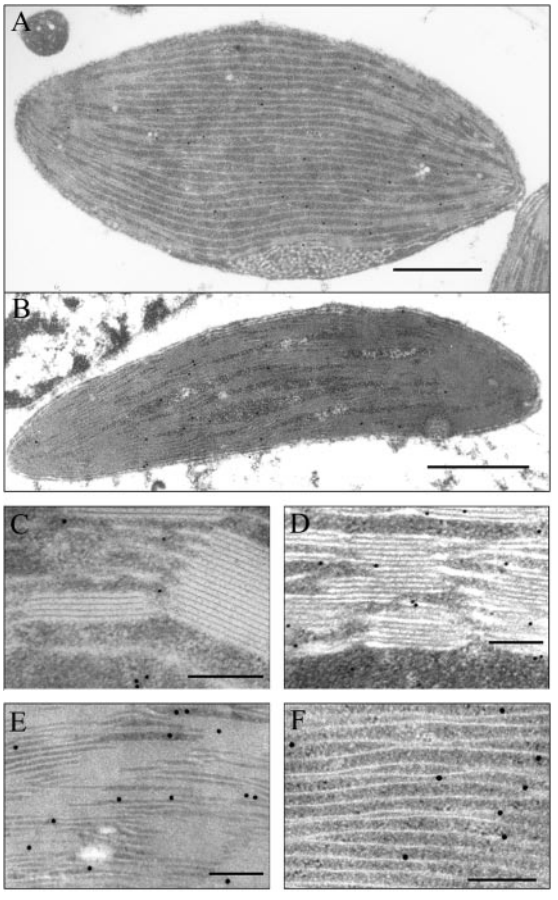

Figure 5. Immunolocalization of APR in different *Flaveria* spp. A, *F. trinervia* (C_4) bundle sheath chloroplast; B, *F. trinervia* (C_4) mesophyll chloroplast; C, *F. pringlei* (C_3) part of chloroplast; D, *F. anomala* part of mesophyll chloroplast; E, *F. trinervia* (C_4) part of mesophyll chloroplast; F, *F. trinervia* (C_4) part of bundle sheath chloroplast. Bar = 1 μm in A and B; bar = 0.2 μm in C through F.

fact, APR and ATPS are not the only enzymes in which cell-type specific expression in C_4 dicots differs from C_4 monocots. Bundle sheath cells of monocot NADP-malic enzyme C_4 species lack photosystem II (Sheen and Bogorad, 1986); however, photosystem II polypeptides were found in both cell types at approximately the same levels in *F. trinervia* (Ketchner and Sayre, 1992).

The APR activities measured at normal conditions in C_3 species vary substantially from 0.3 to 0.8 nmol min^{-1} mg^{-1} protein in hybrid poplar (*Populus tremula* × *P. alba*; Hartmann et al., 2000) and 5 to 10 nmol min^{-1} mg^{-1} protein in Arabidopsis (Kopriva et al., 1999; Koprivova et al., 2000) to 15 to 25 nmol min^{-1} mg^{-1} protein in *Lemna minor* (Neuenschwander et al., 1991). In maize, a C_4 species, APR activities of 0.6 to 1.5 nmol min^{-1} mg^{-1} protein were determined (Brunner et al., 1995). The APR activities measured in the different *Flaveria* spp. thus correspond well to those determined in other plant species. The APR activity in C_4-like and C_4 *Flaveria* spp. was significantly higher than in C_3 and C_3-C_4 species. On the other hand, ATPS activities in C_4 species were not significantly different from C_3 ones (Gerwick et al., 1980). Also, the GSH concentrations in *Flaveria* spp. are in the range measured in other plant species. APR is highly regulated by sulfur demand (Brunold, 1993); therefore, it is not surprising that there was a strong correlation between the foliar GSH concentrations and measured APR activities among the six *Flaveria* species analyzed. The reason for higher GSH concentrations in C_4 *Flaveria* species than in C_3 ones is not clear yet because the plants were grown under identical conditions. It could be speculated, however, that this difference is due to genetic adaptation to conditions of temperature and light stress to which C_4 species are more exposed in their natural habitats than the C_3 ones.

Isolated chloroplasts are capable of reducing sulfate (Trebst and Schmidt, 1969) and, correspondingly, the ATPS and APR activities were localized in spinach (*Spinacia oleracea*) chloroplasts (Schmidt, 1976; Fankhauser and Brunold, 1978; Lunn et al., 1990). No APR activity was measured in peroxisomes, mitochondria, or cytosol (Fankhauser and Brunold, 1978), whereas a cytosolic ATPS isoform could be identified in leaves of spinach and Arabidopsis (Lunn et al., 1990; Rotte and Leustek, 2000). Both APR and ATPS also appear to be exclusively localized to proplastids of root cells (Brunold and Suter, 1989). Recombinant APR from *Catharanthus roseus* was imported into intact pea (*Pisum sativum*) chloroplasts and correctly processed there (Prior et al., 1999), indicating again plastid localization for APR. In addition, in western analysis of pea chloroplast fractions APR protein was detected in stroma but not in any of the membrane fractions (Prior et al., 1999). In accordance with the former reports, immunogold labeling revealed the APR protein in chloroplasts of all three *Flaveria* spp. analyzed.

It is surprising that the APR signal was detected prevalently associated with the chloroplast thylakoid membranes. The reduction of APS does not directly rely on photosynthetic products, the electrons are supplied by glutathione (Bick et al., 1998), so that APR itself would not get any advantage by association with the thylakoids. However, the preceding and subsequent enzymes in the pathway, ATPS and sulfite reductase, directly utilize photosynthetic products formed at the thylakoids, i.e. ATP and reduced ferredoxin, respectively. Thus, it is plausible to hypothesize that the enzymes of assimilatory sulfate reduction form a multi-enzyme complex associated with the thylakoid membranes similarly as was proposed for Calvin cycle enzymes (Süss et al., 1993). In addition, the association of the enzymes of sulfate assimilation in a complex would lead to channeling of the reaction intermediates and thus prevent release of highly reactive and cytotoxic sulfite. It was already shown that two last enzymes of this pathway, O-acetyl-Ser(thiol) lyase and Ser acetyltransferase associate in a multienzyme complex (Bogdanova and Hell, 1997; Droux et al., 1998). The second enzymatic function of APR, namely the GSH-dependent reduction of dehydroascorbate (Bick et al., 1998), alternatively might be responsible for the association of APR with thylakoids because vast amounts of dehydroascorbate are produced at the thylakoid membranes during the photoprotective xanthophyll cycle (Demmig-Adams and Adams, 1996). The existence of a specific dehydroascorbate reductase is a matter of controversy (Foyer and Mullineaux, 1997; Morell et al., 1997). The results presented here indicate that APR might be a good candidate.

In conclusion, our findings taken together with results from the literature show that the localization of assimilatory sulfate reduction in the bundle sheath cells is not ubiquitous among C_4 plants and is therefore neither a prerequisite nor a consequence of C_4 photosynthesis.

MATERIALS AND METHODS

Plant Material

The seeds and/or plants of *Flaveria cronquistii*, *Flaveria pringlei*, *Flaveria anomala*, *Flaveria palmeri*, *Flaveria trinervia*, and *Flaveria australasica* were provided by Prof. P. Westhoff (University of Düsseldorf, Germany). Seeds were sown in soil and plants were grown in a greenhouse at 16-h-light/8-h-dark cycle and a temperature of 25°C ± 3°C.

Enzyme Assays

For extractions, young fully developed leaves of 5- to 6-week-old *Flaveria* spp. plants were used. Two-hundred milligrams of leaf material was homogenized in 2 mL of 50

mM $NaKPO_4$ buffer (pH 8) supplemented with 30 mM Na_2SO_3, 0.5 mM 5′-AMP, and 10 mM dithioerythritol (Imhof, 1994), using a glass homogenizator. APR activity was measured in extracts as the production of [^{35}S]sulfite, assayed as acid volatile radioactivity formed in the presence of [^{35}S]APS and dithioerythritol (Brunold and Suter, 1990). The protein concentrations in the extracts were determined according to Bradford (Bradford, 1976) with bovine serum albumin as a standard. The ATPS activity was determined in the same extracts, diluted one-fifth with extraction buffer, by measurement of ATP production from APS and inorganic pyrophosphate using an ATP meter (Schmutz and Brunold, 1982).

Western-Blot Analysis

Aliquots of 10 μg protein from the extracts for APR measurements were subjected to SDS-PAGE and electrotransferred to nitrocellulose filter (Schleicher and Schuell, Dassel, Germany). The blots were analyzed with antisera against recombinant APR2 from Arabidopsis (Kopriva et al., 1999) and developed with the SuperSignal Western Blotting System (Pierce, Lausanne, Switzerland). The western analysis was performed on two independent protein preparations with the same results.

Thiol Measurements

Young leaves were extracted with 0.1 M HCl and the extracts were centrifuged for 30 min at 4°C. The thiols in the supernatant were reduced by bis-(2-mercaptoethylsulfone) (Bernhard et al., 1998) and labeled by monobromobimane (Kranner and Grill, 1996). Total Cys and glutathione were analyzed by reversed-phase HPLC as described by Schupp and Rennenberg (1988) and modified by Rüegsegger and Brunold (1992).

Isolation of RNA and Northern Blotting

Young leaves were pulverized with mortar and pestle in liquid nitrogen and total RNA was isolated by phenol extraction and selective precipitation with LiCl. Mesophyll and bundle sheath specific RNA was isolated from C_4 species *F. trinervia* and *F. australasica* by a procedure described by Westhoff et al. (1991). Electrophoresis of RNA was performed on formaldehyde-agarose gels at 120 V. RNA was transferred onto Hybond-N nylon membranes (Amersham Pharmacia Biotech, Freiburg, Germany) and hybridized with ^{32}P-labeled cDNA probes for ATPS and APR from *F. trinervia*. The membranes were washed four times at different concentrations of SSC in 0.1% (w/v) SDS for 20 min, the final washing step being 0.5× SSC, 0.1% (w/v) SDS at 65°C, and exposed to an x-ray film (medical RX, Fuji, Dielsdorf, Switzerland) at −80°C for 2 to 3 d. The autoradiograms were quantified with a densitometer GS-670 (Bio-Rad, Glattbrugg, Switzerland) using the software Molecular Analyst.

Cloning of cDNA for APR and ATPS from *F. trinervia*

The cDNAs for APR were cloned from *F. trinervia* and *F. cronquistii* RNA by RT-PCR with degenerate oligonucleotide primers derived from domains conserved among plant APRs and bacterial phosphoadenosine 5′-phosphosulfate reductases (Suter et al., 2000). The ATPS cDNA fragment was amplified from *F. trinervia* total RNA by RT-PCR with degenerate primers against conserved domains. The PCR products were cloned into pCR plasmids by the TA cloning kit (Invitrogen, Groningen, The Netherlands) and sequenced on both strands (Microsynth, Balgach, Switzerland).

In Situ RNA Hybridization

For the generation of probes, the cDNA fragments of Rubisco SSU and PEPCase were amplified by RT-PCR from *F. trinervia* total RNA, cloned into pCR plasmid, and their identity was controlled by sequencing. In situ hybridization experiments were performed on young fully developed leaves of several *Flaveria* spp. according to the protocol described by Fleming et al. (1993), with modifications described by Reinhardt et al. (1998). After development, the slides were stained in toluidine blue and viewed on an LSM 310 microscope (Carl Zeiss AG, Oberkochen, Germany). Images were taken under bright-field light (shown in false green color) and overlaid with epifluorescence images taken under polarized light exhibiting the silver grain signal (shown in false red color). For each probe, control hybridizations were performed with the corresponding sense probes, with the signals obtained negligible compared to the antisense probes.

Immunogold Localization

One-millimeter^2 leaf sections of *F. pringlei*, *F. anomala*, and *F. trinervia* were vacuum infiltrated for a short time with 2% (v/v) formaldehyde and 0.5% (v/v) glutaraldehyde in 50 mM cacodylate buffer (pH 7.2), and kept 2.5 h at room temperature in the same medium. Samples were washed with buffer for 15 min followed by three washes for 15 min with distilled water. Dehydration of samples was done stepwise by increasing the concentration of ethanol and concomitantly lowering the temperature (progressive lowering of temperature) using an automated freeze substitution unit (Leica, Benzheim, Germany). The steps of progressive lowering of temperature substitution were performed as follows: 30% (v/v), 40% (v/v), and 50% (v/v) ethanol for 30 min at 4°C; 60% (v/v) and 75% (v/v) ethanol for 1h at −15°C; and 90% (v/v) ethanol and two times 100% (v/v) ethanol for 1 h at −35°C. The samples were subsequently infiltrated with Lowycryl HM20 resin (Plano GmbH, Marburg, Germany) as follows: 33% (v/v), 50% (v/v), and 66% (v/v) resin in ethanol for 5 h each and then 100% (v/v) resin overnight. Samples were transferred into gelatin capsules, kept there for 3 h in fresh resin, and polymerized at −35°C for 3 d under indirect UV light. The embedded samples were cut into ultrathin sections with a thickness of 70 to 90 nm on an ultramicrotome (Ultra cut F;

Reichert) and mounted on copper grids, followed by immunogold labeling with 15 or 10 nm (*F. pringlei*) gold protein-A as described (Süss et al., 1993), except that the thin sections were slowly agitated during incubation to improve antibody labeling. For controls, APR antiserum was replaced by pre-immunoserum or serum that was incubated with purified recombinant APR. The sections were stained with uranyl acetate and lead citrate prior to examination in a Zeiss CEM 920A transmission electron microscope at 80 kV.

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