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Translocation of Sulfate in Soybean (*Glycine max* L. Merr)[1]

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ABSTRACT

Sulfate translocation in soybean (*Glycine max* L. Merr) was investigated. More than 90% of the sulfate entering the shoot system was recoverable in one or two developing trifoliate leaves. In young plants, the first trifoliate leaf contained between 10 to 20 times as much sulfate as the primary leaves, even though both types of leaf had similar rates of transpiration and photosynthesis. We conclude that most of the sulfate entering mature leaves is rapidly loaded into the phloem and translocated to sinks elsewhere in the plant. This loading was inhibited by carbonylcyanide *m*-chlorophenylhydrazone and selenate. At sulfate concentrations below 0.1 millimolar, more than 95% of the sulfate entering primary leaves was exported. At higher concentrations the rate of export increased but so did the amount of sulfate remaining in the leaves. Removal of the first trifoliate leaf increased two-fold the transport of sulfate to the apex, indicating that these are competing sinks for sulfate translocated from the primary leaves. The small amount of sulfate transported into the mesophyll cells of primary leaves is a result of feedback regulation by the intracellular sulfate pool, not a consequence of their metabolic inactivity. For example, treatment of plants with 2 millimolar aminotriazole caused a 700 nanomoles per gram fresh weight increase in the glutathione content of primary leaves, but had no effect on sulfate aquisition.

In a previous study of sulfate transport into soybean plants, we showed that more than 90% of the newly tansported sulfate in the shoot system was localized in a single developing leaf (16). Since the rate of transpiration of mature and developing leaves is not markedly different, similar amounts of sulfate must initially be drawn into both kinds of leaf. The ultimate accumulation of most of the sulfur in the developing leaf must therefore require translocation of sulfur. Several studies, with a variety of plants, show that sulfur is translocated in phloem as inorganic sulfate or sulfite (after exposure of plants to SO_2 [7]) or as organic reduced sulfur, principally glutathione (3, 12). Biddulph *et al.* (2) suggested that the differential accumulation of sulfur in developing leaves was due to sulfate metabolism and incorporation of sulfur into amino acids and protein. However, the idea that immobilization of sulfur in a particular plant organ requires metabolic conversion to immobile molecules, such as proteins and sulfolipids, is not valid. For instance, when wheat plants are transferred from [^{35}S]sulfate to unlabeled sulfate, the transport of label to the shoot stops immediately, even though 75% of the label in the roots is still present as sulfate (9).

Our objective was to characterize sulfur translocation in soybean, with particular emphasis on the form in which sulfur is translocated and the explanation of its final distribution.

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MATERIALS AND METHODS

Plants and Experimental Protocol. Soybean (*Glycine max* [L.] Merr) plants, raised from seed were grown in potting soil in a growth chamber, on a regime of 12 h light, intensity 400 μmol $m^{-2}s^{-1}$ at 27°C and 12 h dark at 21°C. Plants were harvested, the soil was washed from the roots, and the plants were placed overnight in an aerated solution composed of 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 1 mM $MgCl_2$, and 2 mM $(NH_4)_2HPO_4$, adjusted to pH 6.0 (25 plants/500 ml). Four intact plants were placed in 200 ml of the above medium suplemented with $Na_2[^{35}S]O_4$ and exposed to light (400 μmol m^{-2} s^{-1}) at 27°C. Because sodium selenate and CCCP[2] inhibit sulfate transport into roots, experiments with these compounds were done using shoot systems from which the roots were excised just prior to the experiment. CCCP was dissolved in 95% (v/v) ethyl alcohol and 0.2 ml added to 200 ml of sulfate-containing medium. When aminotriazole was used to stimulte glutathione accumulation, the light intensity was increased to 700 μmol m^{-2} s^{-1}.

Determination of Radioactivity. Discs (1.5 cm diameter) were cut from the lamina of the leaf with a cork borer, placed in scintillation vials with 1 ml 2% NaOCl (40% v/v commercial bleach), and the cuticle broken by tapping gently with a metal rod (18). Tissue was completely bleached and partially digested by incubating at 60°C for 1 to 4 h. Ammonium hydroxide (0.2 ml 4 M) was added to neutralize the sodium hypochlorite, and after 30 min at room temperature 10 ml ScintiVerse E (Fisher Scientific Co.) liquid scintillation cocktail was added. Samples were kept in the dark for 2 h before counting in a Beckman LS 3801 liquid scintillation counter.

Transpiration. Rates of transpiration and CO_2 fixation were measured using an ADC model LCA2 IR gas analyzer and a PLC-N Parkinson Leaf Chamber (P. K. Morgan Instruments, Dallas, TX). The air flow was adjusted to 250 ml min^{-1}, light intensity was 300 μmol m^{-2} s^{-1}, ambient CO_2 ranged from 345 to 370 ppm, and the temperature was 25°C. Duplicate measurements were taken from 10 plants at 11 AM, 1 PM, and 3 PM.

Other Assays. Leaf tissue was homogenized in 5 volumes 5% (w/v) sulfosalicylic acid with a small amount of sand, a further 5 volumes of sulfosalicylic acid were added, and the brei centrifuged at 1000*g* for 10 min to sediment insoluble material. GSH in the supernatant was measured by the coupled GSH reductase assay (15).

Sulfate and sulfur amino acids present in the supernatant were separated by cation exchange chromatography. A 5 ml aliquot of the supernatant was passed through a Dowex 50 H^+-form column (3.5 × 0.8 cm), and the column washed twice with 5 ml distilled water. Amino acids were eluted with 2 × 5 ml 3 M NH_4OH. Radioactivity present in these samples was determined following the addition of 10 ml ScintiVerse E to 5 ml of the effluent or eluate.

[2] Abbreviation: CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

Table I. *Distribution of Newly Transported Sulfur in Soybean Plants of Different Ages*

Plants were placed in a medium containing 0.1 mM $Na_2[^{35}S]O_4$ (3.7 × 10^4 Bq/μmol) for 24 h. Values represent the means of duplicate samples from 4 plants ± SD.

Leaf Sampled	wt	[^{35}S]	wt	[^{35}S]	wt	[^{35}S]	wt	[^{35}S]
	mg	*nmol/g fresh wt*	*mg*	*nmol/g fresh wt*	*mg*	*nmol/g fresh wt*	*mg*	*nmol/g fresh wt*
Primary	230 ± 5	170 ± 150	230 ± 50	20 ± 15	260 ± 30	16 ± 6	Not measured	
Trifoliate								
First	Not developed		430 ± 50	250 ± 160	660 ± 90	26 ± 15	Not measured	
Second	Not developed		Not developed		520 ± 100	430 ± 260	940 ± 90	6 ± 3
Third	Not developed		Not developed		80 ± 20	1600 ± 700	1200 ± 160	60 ± 40
Fourth	Not developed		Not developed		Not developed		630 ± 210	800 ± 360
Fifth	Not developed		Not developed		Not developed		76 ± 15	1800 ± 1100

RESULTS AND DISCUSSION

Effect of Plant Age on Sulfur Distribution. Most of the newly transported sulfate entering the shoot system of a soybean plant was localized in the developing leaves (Table I). Ion exchange chromatography showed that, when the transport period was less than 6 h, more than 90% of the label was present as sulfate. The fact that relatively small amounts of label were present in fully expanded leaves indicates that very little of the sulfate entering leaves is transported into mesophyll cells. If newly transported [^{35}S]sulfate entered the mesophyll cells and mixed with the existing pool of sulfate, label would be incorporated into S-containing metabolites which as will be discussed later, was not the case.

The initial acquisition of sulfate by plant shoots is dependent upon the rate of transpiration (9, 10); however, the final distribution in the shoot system is not. In a plant with a single well developed trifoliate leaf, sulfate was primarily localized in the trifoliate leaf, even though this leaf was of similar size to the primary leaves and had slightly lower rates of transpiration and photosynthesis than the primary leaves (Table II). We conclude that sulfate entering mature leaves via the transpiration stream is retrieved from the apoplasm by the phloem tissue and subsequently transported to sink tissues. These sinks may either be leaves whose volume is increasing, thus requiring additional sulfate to maintain a steady state pool size, almost totally expanded leaves which require sulfur for the synthesis of proteins and sulfolipids, or roots. The translocation of sulfate in phloem has been demonstrated in several plants, using both physiological (11) and anatomical methods (1). For intact soybeans placed in 0.1 mM sulfate, sulfate was translocated out of the primary leaves as rapidly as it entered (Table III). Specifically, sulfate present in the primary leaves, after exposure to radioactive sulfate for 2 h, was not chased out of the leaves by unlabeled sulfate. The amount of radioactive sulfate in the trifolite leaves increased during the 8 h chase; however, this sulfate did not come from the primary leaves, but must represent the accumulation of sulfate which came from the roots or was in transit at the initiation of the chase. We think that radioactive sulfate in the primary leaves was not chased out because it represents sulfate transported into mesophyll cells. The immobility of intracellular sulfate reported here supports earlier work with roots (9) and leaves (4).

Table II. *Rates of Transpiration, Photosynthesis, and Sulfur Acquisition by Primary and Trifoliate Leaves of Soybean*

Plants with a single well developed trifoliate leaf were placed in a medium containing $Na_2[^{35}S]O_4$ for 6 h (9 AM–3 PM). Reported values are the means of 20 measurements (duplicate samples from 10 plants). Rates of transpiration and photosynthesis were measured at 11 AM, 1 PM, and 3 PM; reported values are the mean of 60 measurements (duplicate values from 10 plants at 3 times) ± SD.

	Primary Leaf	Trifoliate Leaf
Weight (mg)	220 ± 20	230 ± 30
[^{35}S] (nmol/g fresh wt)	14 ± 12	200 ± 59
Transpiration (mmol/m^2 · s)	2.1 ± 0.5	1.8 ± 0.5
Photosynthesis (μmol/m^2 · s)	4.2 ± 1.0	3.5 ± 1.1

Inhibition of Sulfate Redistribution. Phloem loading of sucrose has been extensively investigated, and is characterized by being energy dependent, saturable, and specific (8). In principle, sulfate loading may share some or all of these characteristics. CCCP was chosen as an inhibitor of proton gradient establishment, because of its ability to inhibit sulfate influx into plant cells at concentrations which do not stimulate efflux (13). Our aim was to inhibit movement of sulfate into phloem without stimulating efflux of sulfate from mesophyll cells. At high concentrations of CCCP, there was a small inhibition of water entry as evidenced by the decline in weight of primary leaves (Table IV). CCCP inhibited the translocation of sulfate by the phloem, as evidenced

Table III. *Time Course of Sulfur Distribution in Primary and Trifoliate Soybean Leaves following Short-Term Exposure to* $Na_2[^{35}S]O_4$

Plants placed in a medium containing 0.1 mM $Na_2[^{35}S]O_4$ for 2 h were transferred to the same medium with unlabeled sulfate, and the distribution of label measured at 2 h intervals for 8 h. Individual primary leaves weighed 390 ± 20 mg and trifoliate leaves 90 ± 20 mg. Values are the means of 20 samples ± SD.

Time	Primary Leaves		Trifoliate Leaf	
h	*nmol/g fresh wt*	*total, nmol*	*nmol/g fresh wt*	*total, nmol*
0	12 ± 5	9 ± 4	150 ± 40	14 ± 3
2	12 ± 4	9 ± 4	170 ± 50	15 ± 5
4	13 ± 8	10 ± 7	240 ± 70	22 ± 8
6	16 ± 8	13 ± 6	200 ± 40	18 ± 6
8	16 ± 8	13 ± 7	320 ± 90	29 ± 10

Table IV. *Inhibition of Sulfate Redistribution by CCCP*

Excised shoots of soybean were placed in medium containing 10 μM $Na_2[^{35}S]O_4$ supplemented with the indicated concentrations of CCCP for 6 h. Values are the means of duplicate samples from 10 plants ± SD.

CCCP	Primary Leaves			Trifoliate Leaf		
	wt	[^{35}S]	Total [^{35}S]	wt	[^{35}S]	Total [^{35}S]
μM	*mg*	*nmol/g fresh wt*	*nmol*	*mg*	*nmol/g fresh wt*	*nmol*
0	320 ± 40	1 ± 0	1 ± 0	200 ± 40	42 ± 3	8 ± 2
10	300 ± 60	9 ± 4	5 ± 1	220 ± 50	32 ± 3	7 ± 2
50	290 ± 30	26 ± 5	15 ± 4	220 ± 60	31 ± 4	7 ± 2
250	260 ± 60	27 ± 6	14 ± 4	170 ± 50	22 ± 2	4 ± 1

Table V. *Effect of Sulfate Concentration on Sulfate Redistribution*

Excised shoots were placed in medium containing the indicated amounts of $Na_2[^{35}S]O_4$, in the presence or absence of 50 μm CCCP, for 4 h. Values are the mean ± SD [^{35}S] contents in the primary leaves of duplicate samples from 8 plants.

Sulfate Concentration	Control	50 μM CCCP	Minimum Exported
μM	*nmol/g fresh wt · h*		
25	0.2 ± 0.1	8 ± 2	8
	ND[a]	ND	ND
50	0.8 ± 0.7	23 ± 4	22
	0.5 ± 0.3	14 ± 6	13
100	ND	ND	
	0.8 ± 0.6	38 ± 5	37
250	64 ± 24	130 ± 20	66
	13 ± 13	ND	
500	137 ± 33	248 ± 26	111
	97 ± 26	250 ± 19	153
1000	455 ± 70	460 ± 59	5
	410 ± 81	ND	
2000	1100 ± 170	1100 ± 118	0
	930 ± 90	1200 ± 68	270

[a] Not determined.

by the accumulation of sulfate in primary leaves (Table IV). Although the concentration (50 μM) required to effectively inhibit transport was much higher than that (1 μM) which totally inhibits sulfate transport into cultured tobacco cells (13). Inhibition of sulfate translocation by CCCP does not necessarily mean that sulfate loading *per se* requires the establishment of a proton gradient. Bulk flow of materials in the phloem is primarily due to the loading of sucrose and the subsequent entry of water in response to a water potential gradient. Dissipation of proton gradients in the sieve cells would inhibit sucrose loading, and thus reduce the rate of bulk flow of all mobile molecules.

Because CCCP inhibits redistribution of sulfate, the difference between the amount of sulfate present in the primary leaves in the presence and absence of this proton ionophore is a minimum measure of the amount of sulfate being translocated. At sulfate concentrations below 0.1 mM, more than 90% of the sulfate entering primary leaves was translocated out of the leaf to the developing trifoliate leaves (Table V). The efficiency of this process *versus* transport into mesophyll cells is a combination of several factors, among which are the following; first, transport of sulfate into mesophyll cells will be inhibited by the existing sulfate pool. This feedback regulation of sulfate transport is well established in cultured cells (14), carrot storage roots (5, 6) and intact plants (4). Although cellular sulfate exchanges with medium sulfate in carrot (5, 6), usually symplastic sulfate in leaves and roots is relatively immobile and cannot be chased out (4, 9); second, bulk flow in phloem will maintain a sulfate gradient across the plasma membrane of the sieve cells tending to facilitate additional sulfate entry. In the concentration range, 0.1 to 0.5 mM, the rate of export increased, but so did the amount of sulfate remaining in the primary leaves. The export capacity of the phloem was saturated, in the sense that more sulfate entered the leaf in the xylem than was exported from the leaf in the phloem; this is not synonymous with saturation of a transporter system in the kinetic sense. In the millimolar range, accumulation of large amounts of sulfate in the primary leaves obscured differences between CCCP-treated and untreated plants.

Selenate, which is a competitive inhibitor of sulfate transport into plant cells (13), inhibited the redistribution of sulfate (Table VI), resulting in increased amount of sulfate in the primary leaves and a decreased amount in the trifoliate leaves.

Modification of Sulfate Redistribution. The previous experiments establish that trifoliate leaves serve as sinks for sulfate transported out of the primary leaves. The effect that removal of these sinks has on sulfate distribution is shown in Table VII. Independent of the distribution in the entire plant, removal of the first trifoliate leaf always resulted in a marked increase in the sulfate transported to the developing second trifoliate leaf. In experiment 2, removal of the first trifoliate leaf inhibited transport of sulfate out of the primary leaves, but this result is atypical. Usually, removal of this leaf either has little effect upon the sulfate in the primary leaves (experiment 1) or causes a two-fold increase (not shown). These results indicate that trifoliate leaves compete as sinks for sulfate transported from the primary leaves, and that removal of one sink results in the acquisition of more sulfate by an alternate sink.

Fully expanded primary leaves accumulate very little sulfate, because the pools of sulfate and sulfur amino acids are not expanding and the net synthesis of sulfolipids and proteins is minimal. Treatment of plants with aminotriazole, however, stimulates glutathione synthesis and therefore requires an increase in the rate of sulfate reduction (15).

We used aminotriazole as a compound which might influence the distribution of sulfate. One effect of aminotriazole was a reduction of transpiration and also a decline in the total amount of sulfate entering the shoot system, which agrees with previous work using barley (17). Aminotriazole increased the fraction of the total [^{35}S] which was recoverable as amino acids (Table VIII). However, it is clear that the pool of sulfate in the leaves at the start of the experiment is the primary source of sulfur for glutathione synthesis. Specifically, for primary leaves treated with aminotriazole for 4 h there was an increase in glutathione of 740 nmol/g fresh weight, but less than 1.5 nmol/g fresh weight was labeled. Even in trifoliate leaves, where more label entered the amino acid pool, the glutathione was primarily synthesized from unlabeled sulfate.

CONCLUSION

High rates of sulfate acquisition by primary leaves was only observed in plants lacking a trifoliate leaf, indicating that the former acquire their sulfate early, when the leaves are expanding and net synthesis of protein and sulfolipid is occurring. Subsequently, sulfate drawn into the primary leaves by transpiration

Table VI. *Inhibition of Sulfate Redistribution by Selenate*

Excised shoots were placed in a medium containing 10 μM $Na_2[^{35}S]O_4$, in the presence or absence of Na_2SeO_4, for 6 h. Values are the mean [35S] contents in nmol/g fresh wt of duplicate samples from 10 plants ± SD. Mean weights of leaves; primary, 150 mg; first trifoliate, 180 mg; developing trifoliate, 40 mg.

	0 Na_2SeO_4		0.1 mM Na_2SeO_4		
	[35S]	Total [35S]	[35S]	Total [35S]	Difference[a]
	nmol/g fresh wt	*nmol*	*nmol/g fresh wt*	*nmol*	*%*
Primary leaves	11 ± 2	3.3 ± 0.6	6 ± 3	4.8 ± 1.0	+ 45
First trifoliate	25 ± 6	4.5 ± 1.1	30 ± 5	5.4 ± 1.2	+20
Developing trifoliate	16 ± 10	0.6 ± 0.3	13 ± 4	0.5 ± 0.2	−19
	0 Na_2SeO_4		1.0 mM Na_2SeO_4		
Primary leaves	5 ± 5	1.5 ± 1.4	23 ± 5	6.9 ± 2.0	+360
First trifoliate	34 ± 7	6.1 ± 1.6	28 ± 11	5.0 ± 2.4	−18
Developing trifoliate	24 ± 9	1.0 ± 0.4	10 ± 3	0.4 ± 0.2	−58

[a] Difference between selenate treated and control plants.

Table VII. *Effect of Removing the First Trifoliate Leaf on the Distribution of Sulfate in the Shoot.*

Plants were placed in a medium containing 0.1 mM $Na_2[^{35}S]O_4$ for 6 h. The first trifoliate leaf was excised from one set of plants. Values are the mean [35S] contents, in nmol/g fresh wt, of samples from 10 plants ± SD. Mean weights of leaves in the first experiment were primary leaf (291 mg), first trifoliate leaf (199 mg), second trifoliate leaf (91 mg) and in the second experiment, 301 mg, 266 mg, and 39 mg, respectively.

	Entire Plant		Minus First Trifoliate	
	[35S]	Total [35S]	[35S]	Total [35S]
Experiment 1	*nmol/g fresh wt*	*nmol*	*nmol/g fresh wt*	*nmol*
Primary leaves	9 ± 4	5 ± 3	8.0 ± 3	5 ± 2
First trifoliate	32 ± 13	6 ± 3		
Second trifoliate	103 ± 26	9 ± 2	200 ± 90	18 ± 9
Experiment 2				
Primary leaves	14 ± 4	8 ± 4	106 ± 98	65 ± 57
First trifoliate	300 ± 110	80 ± 27		
Second trifoliate	160 ± 67	6 ± 3	350 ± 150	14 ± 9

Table VIII. *Effect of Aminotriazole on the Distribution of Newly Transported Sulfur*

Plants were placed in a medium containing 0.1 mM $Na_2[^{35}S]O_4$ in the presence or absence of 2 mM aminotriazole, for 4 or 8 h. Homogenates of the leaves were subjected to ion-exchange chromatography to yield an anionic sulfate fraction and a cationic amino acid fraction. The total glutathione present in the leaves was also measured.

Time	Leaf	Aminotriazole	Sulfate	S-Amino Acid	GSH
h		*2 mM*	*[35S] nmol/g fresh wt*		*nmol/g fresh wt*
4	Primary	−	10 ± 1	1.5 ± 0.4	160 ± 50
		+	6 ± 2	1.2 ± 0.3	900 ± 300
4	First trifoliate	−	200 ± 95	12.4 ± 0.7	300 ± 70
		+	160 ± 20	21.3 ± 0.9	1000 ± 200
8	Primary	−	15 ± 1	1.7 ± 0.6	450 ± 150
		+	6 ± 0	1.7 ± 0.6	900 ± 30
8	First trifoliate	−	230 ± 40	13.6 ± 3.0	540 ± 90
		+	120 ± 40	33.3 ± 8.2	1400 ± 300

was exported via the phloem to sinks elsewhere in the plant. Export was inhibited by CCCP and selenate, suggesting that it is mediated by a specific carrier and is directly or indirectly dependent upon the establishment of a proton gradient.

One outstanding question is the importance of reduced organic sulfur-containing molecules as mobile forms of sulfur. In previous studies, where $Na_2[^{35}S]O_3$ was applied to leaflets of *Vicia* (7) or $Na_2[^{35}S]O_4$ was flap-fed to tobacco (12) and castor bean plants (3), more than 90% of the sulfur transported out of the leaf was recoverable in the stem as sulfate. Fractionation of the 35S-organic compounds in the stem indicated that glutathione was the major component (67–70% of the total), with the remainder being cysteine (2–8%) and methionine (27–30%) in tobacco and cysteine (3–22%), methionine (3–17%), and unidentified compounds (10–25%) in castor bean. The results presented here indicate that, when mature leaves were exposed to sulfate (<0.1 mM) for short periods (4 h), most of the sulfate was exported rather than transported into mesophyll cells where it could be used to synthesize amino acids (Tables V and VIII). Consequently, even if glutathione is an important mobile form of sulfur, it could not be demonstrated directly in a short-term labeling experiment. Despite this observation, we conclude, on the basis

of indirect evidence, that glutathione export from mature leaves is not quantitatively important. For instance, we calculated that, at a sulfate concentration of 0.1 mM, 37 nmol of labeled sulfate was transported from 1 g of primary leaf each hour (Table V). Because primary leaves can be considered to be steady state systems, export of S-metabolites would require import of an equivalent amount of sulfate, which would be indicated in one of two ways. If the [^{35}S]sulfate mixed with the total sulfate pool in the leaf, dilution of label would occur and the sulfate pool would acquire [^{35}S] at approximately the same rate as the loss of [^{32}S] for metabolite synthesis. Only 10 nmol of [^{35}S]sulfate was accumulated per g fresh weight in a 4 h period (Table VIII). Alternatively, if the [^{35}]sulfate primarily mixed with a small cytoplasmic or chloroplastic pool of sulfate, which is used for the synthesis of amino acids, less dilution of label would occur, and therefore more label would appear in terminal metabolites such as glutathione. This is the more likely scenario, based on previous work (5, 6), but less than 2 nmol of [^{35}S] was recoverable in 4 h in a glutathione pool which varied from 155 to 900 nmol/g fresh weight (Table VIII). These results are consistent with the conclusions of other workers that more than 90% of the sulfur transported in phloem is present as sulfate (11).

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