Plant Physiol. (1996) 111: 147–157

Demand-Driven Control of Root ATP Sulfurylase Activity and SO_4^{2-} Uptake in Intact Canola[1]

The Role of Phloem-Translocated Glutathione

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The activity of ATP sulfurylase extracted from roots of intact canola (*Brassica napus* L. cv Drakkar) increased after withdrawal of the S source from the nutrient solution and declined after refeeding SO_4^{2-} to S-starved plants. The rate of SO_4^{2-} uptake by the roots was similarly influenced. Identical responses were obtained in SO_4^{2-}-fed roots when one-half of the root system was starved for S. The internal levels of SO_4^{2-} and glutathione (GSH) declined after S starvation of the whole root system, but only GSH concentration declined in +S roots of plants from split root experiments. The concentration of GSH in phloem exudates decreased upon transfer of plants to S-free solution. Supplying GSH or cysteine to roots, either exogenously or internally via phloem sap, inhibited both ATP sulfurylase activity and SO_4^{2-} uptake. Buthionine sulfoximine, an inhibitor of GSH synthesis, reversed the inhibitory effect of cysteine on ATP sulfurylase. It is hypothesized that GSH is responsible for mediating the responses to S availability. ATP sulfurylase activity and the SO_4^{2-} uptake rate are regulated by similar demand-driven processes that involve the translocation of a phloem-transported message (possibly GSH) to the roots that provides information concerning the nutritional status of the leaves.

S is predominantly available to higher plants as SO_4^{2-} taken up from soil by the roots. The rate of S uptake by plant roots, especially those of crop species, which have been bred for their fast growth capacity and high harvest potential, is controlled by regulatory processes that operate in such a way that the needs of the whole organism are matched by rates of S uptake. A good illustration of this behavior is provided by the severalfold enhancement of SO_4^{2-} uptake observed in plants previously deprived of S for periods of a few hours to a few days (Smith, 1975, 1980; Lass and Ullrich-Eberius, 1984; Clarkson and Saker, 1989; Hawkesford and Belcher, 1991; Hawkesford et al., 1993; Lee, 1993). Conversely, SO_4^{2-} uptake decreased steadily upon restoration of SO_4^{2-} availability (Smith, 1980; Clarkson et al., 1983, 1992; Clarkson and Saker, 1989). This control exerted on ion-transport systems by the nutritional status is a general feature that is more extensively documented for K^+ (Glass, 1975, 1976; Petterson and Jensén, 1979; Drew and Saker, 1984; De la Guardia et al., 1985; Siddiqi and Glass, 1986, 1987), $H_2PO_4^-$ (Clarkson and Scattergood, 1982; Drew and Saker, 1984; Lee, 1993), and NO_3^- (Lee and Rudge, 1986; Bowman et al., 1989; Lee, 1993). For instance, it has been shown that decreasing or increasing the internal demand for N led to corresponding changes in the NO_3^- uptake rate (see Touraine et al., 1994).

The stimulation of ion uptake rate by element deficiency is believed to contribute to the ability of plant cells to maintain nutrient homeostasis despite frequent variations that occur in the root environment. Demand-driven regulation of ion uptake (see Imsande and Touraine, 1994) has two important traits: (a) it affects the transport rate of one specific ion in roots (Datko and Mudd, 1984; Lee and Rudge, 1986; Clarkson and Saker, 1989; Hawkesford and Belcher, 1991; Lee, 1993); and (b) the underlying process involves a remote control of root activity by shoots. In the case of SO_4^{2-}, this has been shown by experiments in which enhancement of SO_4^{2-} uptake in a given root had been obtained by depriving other roots of S, and the root under observation was continuously fed with SO_4^{2-} (Clarkson et al., 1983). Signaling between such differently fed roots includes the translocation of xylem and phloem saps and exchanges between these saps and leaf cells. The demand-driven control of SO_4^{2-} uptake therefore is likely to originate from the shoot and involve the translocation of a regulatory message via the phloem. One interpretation is that ion uptake by roots is normally repressed and that it is derepressed when the needs for a given element increases so that the uptake rate fits the requirements of the whole plant (e.g. Clarkson and Lüttge, 1991; Imsande and Touraine, 1994). It has been proposed that the signal responsible for the repression of SO_4^{2-} uptake could be either internal SO_4^{2-} (Smith, 1975, 1980; Jensèn and König, 1982; Datko and Mudd, 1984; Lass and Ullrich-Eberius, 1984) or one of the products of its reduction, such as GSH (Rennenberg et al., 1988, 1989; Herschbach and Rennen-

[1] This work was supported in part by a fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche to A.G.L.

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Abbreviations: APS, adenosine-5'-phosphosulfate; BSO, buthionine sulfoximine; γGluCys, γglutamyl-cysteine.

berg, 1991, 1994). Reduced S molecules are translocated in the phloem (Rennenberg et al., 1979; Bonas et al., 1982; Rennenberg, 1982; Schneider et al., 1994), so this may be the route whereby S demand information is conveyed from shoots to roots.

S assimilation and incorporation into organic compounds require reduction of SO_4^{2-}, which in turn needs to be activated by binding to ATP, forming APS. This reaction is activated by ATP sulfurylase (Osslung et al., 1982; Renosto et al., 1993; Klonus et al., 1994; Leustek et al., 1994). Most SO_4^{2-} reduction, as well as APS formation, occurs in mesophyll cells in leaves, where the major part of activity is associated with chloroplasts (Burnell, 1984; Lunn et al., 1990; Renosto et al., 1993). Cacco et al. (1977) reported that the activity of ATP sulfurylase in roots was subjected to variations, fitting the same pattern as the SO_4^{2-} uptake rate, suggesting that ATP sulfurylase activity and SO_4^{2-} uptake may be regulated in a similar way. Here we report a study that demonstrates that a control of ATP sulfurylase activity in canola (*Brassica napus* L.) roots by the nutritional demand operates at the whole plant level. We then address the role of GSH as a phloem-translocated message involved in this regulation.

MATERIALS AND METHODS

Seed Germination and Plant Growth

Seeds of canola (*Brassica napus* L. cv Drakkar) were soaked in aerated-distilled water for 3 h and sowed on gauze stretched over a 0.2 mM $CaSO_4$ solution. Seed germination was carried out at 25°C in the dark. Three days later, seedlings were transferred to 5-L tanks containing a vigorously aerated, basic nutrient solution. From this stage, plants were grown in a controlled environment room at 25°C and 70% RH during the 14-h light period and at 20°C and 75% RH during the 10-h dark period. Fluorescent lamps provided a PPFD of 400 μmol m^{-2} s^{-1} at shoot level during the daytime. The basic nutrient solution contained 2 mM KNO_3, 1 mM $(CaNO_3)_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 100 μM NaFeEDTA, 50 μM KCl, 30 μM H_3Bo_3, 5 μM $MnCl_2$, 1 μM $CuCl_2$, 1 μM $ZnCl_2$, and 100 nM $(NH_4)_6Mo_7O_{24}$. The pH of fresh nutrient solution was close to 5.5. Five days later, plants were transferred to 10-L tanks (15 plants per tank). Solutions were renewed every 3 or 4 d to minimize pH shifts and nutrient depletion. When solutions lacking SO_4^{2-} were used, $MgCl_2$ was substituted for $MgSO_4$. For Cys or GSH treatments, the basic nutrient solution was supplemented with 1 mM Cys or GSH for 0 to 36 h before harvest, as indicated in the text and figure legends.

Experiments were performed on 21-d-old plants. At this stage they had three to five leaves and their roots weighed 0.5 to 1.5 g fresh weight. Roots and shoots were harvested separately, and their fresh weight were recorded. In addition, dry weight was occasionally determined. Each result presented is the mean of at least five replicates, and every experiment was repeated three times.

ATP Sulfurylase Activity

Fresh tissues from roots or leaves were rapidly ground at 4°C in a buffer consisting of 10 mM Na_2EDTA, 20 mM Tris-HCl (pH 8.0), 2 mM DTT, and approximately 0.01 g/mL insoluble PVP, using a 1:4 (w/v) tissue-to-buffer ratio. The homogenate was strained through gauze and centrifuged at 20,000*g* for 10 min at 4°C. The supernatant (crude extract) was used for in vitro ATP sulfurylase assays.

ATP sulfurylase activity was measured using molybdate-dependent formation of pyrophosphate. The reaction was initiated by adding 0.1 mL of crude extract to 0.5 mL of the reaction mixture, which contained 7 mM $MgCl_2$, 5 mM Na_2MoO_4, 2 mM Na_2ATP, and 0.032 units/mL of sulfate-free inorganic pyrophosphatase (Sigma) in 80 mM Tris-HCl buffer (pH 8.0). Another aliquot from the same extract was added to the same reaction mixture except that Na_2MoO_4 was absent. Incubations were carried out side by side at 37°C for 15 min, after which phosphate was determined colorimetrically. The ATP sulfurylase-dependent formation of pyrophosphate was estimated from the difference between the two figures. To verify that interferences due to other pyrophosphate-generating processes were ruled out, we checked that the measured activity was inhibited by SO_4^{2-} as expected from the known stability of the APS-enzyme complex.

Sulfate Uptake

Sulfate uptake was estimated from the rate of ^{35}S incorporation from a labeled nutrient solution over 5 min. Two days before uptake measurement, unstarved plants were transferred to a solution identical to the basic nutrient solution, except that the $MgSO_4$ concentration was lowered to 0.5 mM. For the plants supplied with a SO_4^{2-}-free solution before SO_4^{2-} uptake measurements, 0.5 mM $MgSO_4$ was added to the solution 15 min before the labeling period. At the onset of the loading period, ^{35}S-labeled Na_2SO_4 (Amersham) was added to the uptake solution, and a rapid mixing was ensured through vigorous aeration. Routinely, the specific radioactivity was approximately 5.2 MBq/mmol. Roots were separated from shoots, rinsed for three consecutive 20-s periods in chilled, nonlabeled 0.2 mM $CaSO_4$ solution, and blotted. Root fresh weights were recorded, and tissues were digested in 0.1 N HCl for 1 h (20 mL/g fresh weight). Radioactivity was determined by liquid-scintillation counting (460-C Tri-Carb, Packard Instruments, Downers Grove, IL) and was corrected for quenching by reference to a curve established for plant extracts. Radioactivity translocated to shoots was always negligible (never exceeding 2% of the total activity recovered in the whole plant). Therefore, the corresponding values have been omitted in the presented data.

The method of measuring SO_4^{2-} uptake used herein is a first approximation of the plasmalemma influx, which probably provides overestimation of the actual SO_4^{2-} influx because it includes a significant amount of extracellular SO_4^{2-}. Assuming that extracellular spaces occupy 10% of the tissue volume (Canny and Huang, 1993) and that the average SO_4^{2-} in these spaces is equal to that in the bulk uptake solution (i.e. 0.5 mM), they would contain 50 nmol/g fresh weight of root. If the root extracellular spaces are likened to a cylinder, 30% of this SO_4^{2-} should be

totally lost during washing, and the remaining 70% should be exponentially exchanged with a 30-s half-time. With these assumptions, 17.5% of the 50-nmol g^{-1} fresh weight-labeled SO_4^{2-} accumulated in the extracellular spaces, i.e. 9 nmol g^{-1} fresh weight would remain in roots after a 1-min washing. This is equivalent to an apparent influx, calculated over 1 h, of approximately 100 nmol h^{-1} g^{-1} fresh weight. This might be subtracted from all calculated SO_4^{2-} uptake rates. However, the actual extracellular SO_4^{2-} fraction is not precisely known, so the values given in the figures are not corrected for this overestimation, but the question will be addressed in "Discussion."

Phloem Sap

Petioles of leaves from 21-d-old plants were excised under 20 mM sodium EDTA, pH 7.0, using a sharp blade and were immersed in 1.5 mL of 5 mM sodium EDTA, pH 7.0, for 4 h in darkness and 95% RH. Sulfate and thiols were determined as described below. The volume of phloem exudates collected were not known, and it may have varied independently of phloem-translocation rate, so results are normalized to Suc (i.e. expressed as mol of SO_4^{2-} or thiols per mol of Suc). Suc was determined using an enzymatic method (Boehringer Mannheim).

Determination of Thiols

Leaves and roots were frozen in liquid N immediately after harvesting. Thiols were extracted by grinding 1 to 2 g of fresh weight in 4 mL of a mixture containing 0.1 N HCl, 1 mM Na_2EDTA, and approximately 0.1 g of insoluble PVP. The suspension was centrifuged at 18,000*g* for 10 min at 4°C, and the supernatant was removed for analysis of thiols.

Thiols were determined in extracts and standards using reverse-phase HPLC after reduction and derivatization with monobromobimane (Calbiochem) according to the method described by Schupp et al. (1992). For reduction, 200-μL aliquots were treated with 300 μL of 0.2 M 2-(*N*-cyclohexylamino)ethanesulfonic acid (pH 9.3) and 50 μL of 3 mM DTT. Reduction was carried out in darkness at room temperature for 1 h. For derivatization, 10 μL of 30 mM monobromobimane was added. After 15 min at room temperature in darkness, the reaction was stopped by adding 440 μL of 5% acetic acid. Aliquots from standard solutions (10 μM Cys, 10 μM γGluCys, and 100 μM GSH) were submitted to the same reduction and derivatization protocol. Derivatized compounds were separated on a Hypersil ODS 5-μm 250 × 4.6-mm column (Sigma) eluted with a gradient of 0.25% (v/v) acetic acid in water (pH 3.9) and methanol. Monobromobimane derivatives were detected fluorometrically (Jasco 821-FP [Japan Spectroscopic, Hachioji City, Japan]) at 480-nm emission, 380-nm excitation. It was checked that there is a linear correlation between peak areas and thiols concentrations in the 0 to 8 mM range, and the higher value obtained from tissue extracts was below 2 mM.

Loss of thiols may occur due to degradation because of their known oxidation at room temperature, especially at pH 7, as for phloem sap exudation, or because of enzymatic reactions developed after tissue disruption or sap exudation. Concerning the oxidation of thiols at room temperature and pH 7, it was expected that the reduction step applied before analysis should lead to recovery of Cys and GSH from cystine and GSSG formed in the course of sap exudation. To check this, a standard solution was added to the exudation medium at room temperature in darkness, and the recovery ratios for Cys, γGluCys, and GSH were measured. There was no significant loss of these thiols (recovery ratios: 86, 85, and 95%, respectively). To determine the loss of thiols in the phloem sap, leaves were dipped in an exudation medium plus or minus standard thiol solution. From the differences between the concentrations measured in these two media after 4 h in the usual exudation conditions, the following recovery ratios were calculated: Cys, 86%; γGluCys, 86%; and GSH, 90%. To check that no thiols were lost during tissue extraction, crude extracts were dispatched in two tubes immediately after grinding, and a standard solution was added to one of them. The concentrations of thiols in these internal standards were measured to be between 92 and 95% of those measured in the standard solution analyzed separately. Raw data were corrected according to these ratios.

Analyses of SO_4^{2-}, NO_3^-, and $H_2PO_4^-$

Roots were separated from shoots, rinsed for three consecutive 20-s periods in distilled H_2O, and blotted. SO_4^{2-} was extracted from fresh roots or shoots using 0.1 N HCl for 30 min.

Sulfate was measured according to the turbidimetric method described by Tabatabai and Bremner (1970). Nitrate in nutrient solution was determined by reduction to nitrite on a Cu-Cd column and subsequent diazotization and colorimetric assay using a hand-made autoanalyzer. Phosphate in nutrient solution was determined using a colorimetric method. The reacting solution contained 5 volumes of 0.42% (w/v) $(NH_4)_2MoO_4$ in 1 N H_2SO_4 and 1 volume of 10% (w/v) ascorbic acid in water. The reaction was carried out at room temperature for 30 min and stopped using one-half volume of 10% (w/v) citrate, and the A_{660} was read.

RESULTS

Changes in SO_4^{2-} Uptake and ATP Sulfurylase Activity in Roots in Response to S Starvation

Canola plants were transferred to a SO_4^{2-}-free solution for various periods, ranging from 0 to 3 d, or were first deprived of SO_4^{2-} for 3 d and then resupplied with a fresh complete nutrient solution for various periods up to 36 h. Figure 1 shows that ATP sulfurylase activity in roots increased with the duration of S deprivation, reaching a level 4 times higher in 3-d-starved plants than in plants continuously fed with SO_4^{2-}. Subsequently, it declined sharply when SO_4^{2-} availability was restored. Similarly, the SO_4^{2-} uptake rate was positively affected by SO_4^{2-} withdrawal from the external solution and negatively affected by its restoration. The patterns of ATP sulfurylase activity and

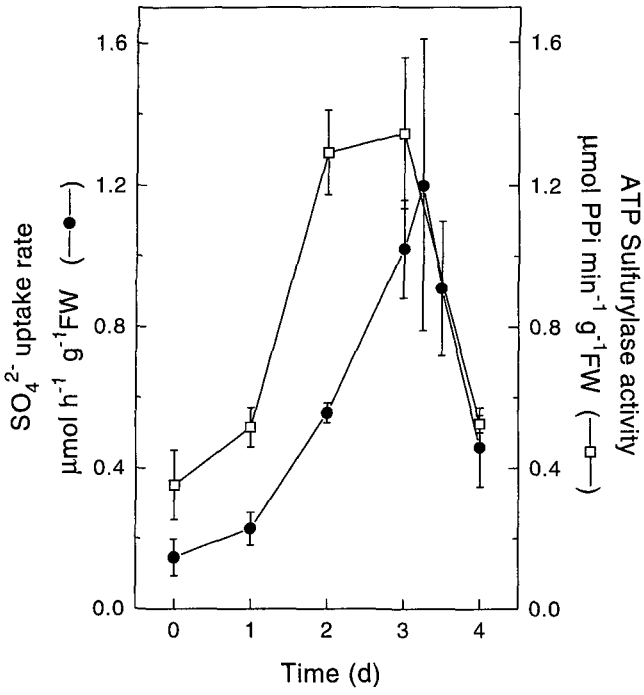

Figure 1. Time courses of ATP sulfurylase activity and SO_4^{2-} uptake with duration of S starvation. Canola plants were hydroponically grown in a complete nutrient solution that contained 1 mM SO_4^{2-}. The plants were transferred from this solution to an S-free, otherwise similar solution for varying periods ranging from 0 to 3 d, and the SO_4^{2-} supply of 1 mM SO_4^{2-} was then restored to some plants. Measurements were on 21-d-old plants. Enzyme activity was assayed in vivo on crude extracts of roots. The rate of SO_4^{2-} uptake was measured as the rate of ^{35}S incorporation over 5 min, and plants were transferred to a one-half strength nutrient solution 15 min before the uptake period. Data are means ± SD of five replicates. FW, Fresh weight.

SO_4^{2-} uptake were similar although the former increased more rapidly than the latter.

S deprivation for up to 3 d had no significant effect on the growth rates of either roots or shoots, as measured by their mean dry weights (Table I) or dry weight/fresh weight ratio (Table I). Roots and shoots grew at relative growth rate of 0.22 d^{-1} and 0.21 d^{-1}, respectively, as estimated from the dry weights of plants harvested every 2 to 3 d between the ages of 16 and 24 d. The duration of culture on an S-free nutrient solution had a considerable impact on the SO_4^{2-} of roots and shoot, although SO_4^{2-} declined more rapidly in roots (Fig. 2). After 3 d on an S-free solution, SO_4^{2-} was hardly detectable in roots, whereas it was 20% of the level measured in shoots of control plants. Internal SO_4^{2-} concentration responded to external SO_4^{2-} restoration with different time courses in roots and shoots (Fig. 2).

The thiols Cys, γGluCys, and GSH are abundant forms of organic S in plant tissues (Rennenberg, 1982; Rennenberg and Lamoureux, 1990), so we investigated whether S starvation and SO_4^{2-} supplied to previously starved plants changed the concentrations of these thiols in roots. Cys, γGluCys, and GSH were predominant in canola (results not shown), and the average concentration of GSH was more than one order of magnitude higher than those of Cys or γGluCys in control plants. In roots, although S starvation caused only slight changes in the levels of Cys and γGluCys, the level of GSH decreased after SO_4^{2-} starvation (Table II). When the SO_4^{2-} supply was restored, the decline in the GSH pool stopped and began to recover. The same pattern was observed in leaf tissues (data not shown).

In phloem exudates, as in root and shoot tissues, the average GSH concentrations were greater than Cys or γGluCys concentrations. Table III shows that, in phloem, GSH and Cys concentrations decreased with S starvation and increased again on SO_4^{2-} restoration. γGluCys concentration in the phloem sap was not affected by S starvation treatments.

To define the degree of specificity of observed effects of S starvation, we investigated the impact of SO_4^{2-} withdrawal on the net uptake rates of two other major anions, NO_3^- and $H_2PO_4^-$. Figure 3 shows that $H_2PO_4^-$ uptake was not significantly affected, whereas NO_3^- uptake declined markedly with S starvation.

Changes in SO_4^{2-} Uptake and ATP Sulfurylase Activity in Split Root Experiments

Sulfate uptake and ATP sulfurylase activity may respond directly to the nutritional status of the tissue, or they may be regulated by a whole plant, demand-driven regulatory mechanism. To discriminate between these possibilities, split root

Table I. *Effects of S starvation on dry weight, fresh weight, and water content of roots and shoots from 21-d-old canola plants*

Canola plants were grown on a complete nutrient solution where S was supplied as 1 mM SO_4^{2-} throughout the culture (Control); or on the same solution for 18 d and then transferred on an S-free solution (3 d −S); or on the S-replete solution for 17 d, transferred on an S-free solution for 3 d, and finally transferred back on the complete nutrient solution for 1 d (3 d −S/1 d +S). Values are means of 10 replicates ± SD.

Treatment	Roots			Shoots		
	Dry wt	Fresh wt	Water content	Dry wt	Fresh wt	Water content
	mg	*g*	*%*	*mg*	*g*	*%*
Control	69 ± 21	1.2 ± 0.4	94 ± 1	359 ± 107	3.2 ± 0.8	89 ± 1
3 d −S	61 ± 24	1.0 ± 0.3	93 ± 2	357 ± 152	2.8 ± 1.1	88 ± 2
3 d −S/1 d +S	60 ± 45	1.0 ± 0.6	94 ± 2	337 ± 195	2.7 ± 1.4	88 ± 3

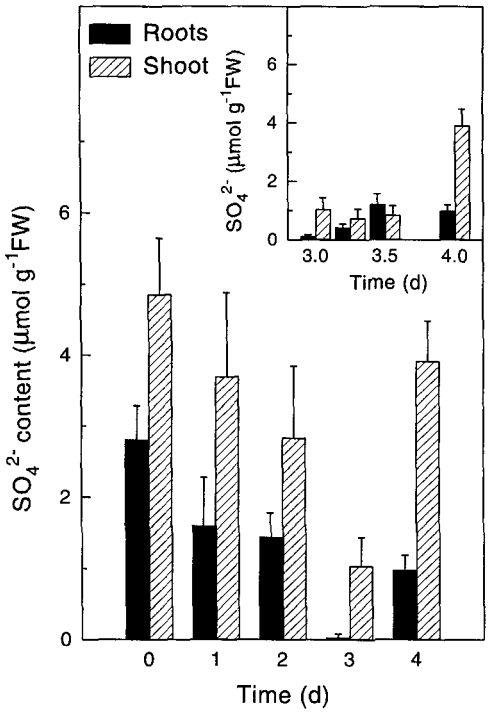

Figure 2. Time courses of the SO_4^{2-} concentration in canola plants with duration of S starvation. Starvation treatments are as described in legend of Figure 1. A detailed time course of SO_4^{2-} concentration variation after the restoration of SO_4^{2-} availability to plants previously deprived of S for 3 d is shown in inset. Data are means ± SD of five replicates. FW, Fresh weight.

experiments were conducted. Root systems were divided into two parts; approximately two-thirds of the whole root system was treated successively with SO_4^{2-}-free and complete nutrient solutions for various periods, and the remainder was continuously supplied with the complete nutrient solution. The roots were harvested from these two parts, referred to as −S and +S roots, respectively; and the shoots were assayed for ATP sulfurylase activity. Additionally, SO_4^{2-} uptake was measured in +S roots.

Starving part of the root system for S enhanced both the ATP sulfurylase activity and SO_4^{2-} uptake rate in the +S portion of the root system (Fig. 4) to nearly the same extent as when the whole root system was deprived of S (compare to Fig. 1). ATP sulfurylase activity and SO_4^{2-} uptake were readily reversible after restoration of SO_4^{2-} to the −S roots. The ATP sulfurylase activity in the shoot was increased by starvation treatments regardless of whether the whole or a part of the root system was fed with S-free solution (results not shown).

Table IV shows that the SO_4^{2-} concentration of −S roots and shoots was decreased by SO_4^{2-} starvation but that there was no change in the SO_4^{2-} concentration of +S roots. In contrast, the level of GSH declined in all tissues (−S, +S roots, and shoots), whereas Cys and γGluCys pools were either slightly affected or not modified at all (Table II). GSH levels recorded in +S roots were close to levels measured in roots of plants in which the whole root system was treated (Table II).

Effect of GSH on ATP Sulfurylase Activity and the SO_4^{2-} Uptake Rate

Both the ATP sulfurylase activity and SO_4^{2-} uptake decreased upon addition of 1 mM GSH to nutrient solutions (Fig. 5). The rate of SO_4^{2-} uptake was rapidly inhibited, thereafter reaching a level that remained somewhat constant. In contrast, ATP sulfurylase activity decreased only after 12 h. As shown in Table V, the presence of 1 mM GSH in nutrient solution resulted in a large and continuous increase in GSH in roots during the entire 36 h tested. The internal concentration of Cys increased during the first 12 h after GSH supply and remained constant thereafter. In contrast, the concentrations of γGluCys and SO_4^{2-} in roots were essentially unchanged within the first 24 h. Internal SO_4^{2-} levels decreased by 30% in the next 12 h, whereas the concentration of γGluCys significantly increased.

To examine the possible role of Cys, 1 mM Cys was supplied to plants, resulting in an increase of internal Cys and GSH levels (Table VI) and a decrease in ATP sulfurylase activity (Fig. 5). To investigate whether Cys or a Cys metabolite was responsible for this regulation, 1 mM BSO, an inhibitor of GSH synthesis, was applied together with Cys. Figure 6 shows that BSO, which had no effect on ATP sulfurylase activity by itself, prevented the Cys-mediated

Table II. *Effects of S starvation on thiol concentrations in roots from 21-d-old canola seedlings*

Whole root system experiments were as follows: canola were grown for 21 d, either on a complete nutrient solution where S was provided as 1 mM SO_4^{2-} throughout the culture (control) or on the same solution for 18 to 20 d and then transferred to an S-free solution (1 d −S, 2 d −S, 3 d −S). Alternatively, plants were grown on the S-replete solution for 17 d, transferred to an S-free solution for 3 d, and finally retransferred to the complete nutrient solution for 1 d (3 d −S/1 d +S). Split root experiments were processed similarly, except that only a part of the root system was subjected to the various treatments, whereas the rest of the roots, which were continuously supplied with the complete nutrient solution, were assayed for their thiol concentrations. Values are means ± SD six replicates.

Treatment	Whole Root System Experiments			Split Root Experiments		
	Cys	γGluCys	GSH	Cys	γGluCys	GSH
	nmol g^{-1} fresh wt					
Control	51 ± 28	24 ± 4	1439 ± 426	50 ± 25	24 ± 3	1436 ± 395
1 d −S	47 ± 22	16 ± 4	952 ± 253	45 ± 11	22 ± 7	1197 ± 296
2 d −S	44 ± 12	20 ± 4	650 ± 44	15 ± 3	8 ± 2	747 ± 274
3 d −S	31 ± 11	12 ± 1	635 ± 198	20 ± 9	20 ± 3	536 ± 68
3 d −S/1 d +S	31 ± 12	17 ± 4	749 ± 98	20 ± 4	18 ± 4	559 ± 95

Table III. *Effect of S starvation on thiol levels in phloem exudates from 21-d-old canola plants*

Canola plants were grown as described in Table I. Phloem exudates were collected in a 5 mM Na-EDTA, pH 7.0, buffer for 4 h in the dark and the leaf lamina at 95% RH. Values are means ± SD of six replicates.

Treatment	SO_4^{2-}	Cys	γGluCys	GSH
		μmol mol⁻¹ Suc		
Control	0.7 ± 0.2	7 ± 2	5 ± 2	195 ± 32
1 d −S	0.8 ± 0.3	6 ± 2	6 ± 3	68 ± 10
2 d −S	1.1 ± 0.2	3 ± 1	4 ± 1	73 ± 18
3 d −S	0.9 ± 0.2	2 ± 1	5 ± 2	66 ± 6
3 d −S/1 d +S	0.8 ± 0.1	4 ± 1	5 ± 1	163 ± 25

inhibition of activity. BSO alone did not inhibit the accumulation of Cys but did inhibit the accumulation of γGluCys and GSH (data not shown).

Providing roots with GSH resulted in a 6-fold increase of GSH concentration in phloem exudates within 12 h (Table VII). Thereafter, the concentration of GSH recovered in phloem exudates remained at this high level. The results presented in Table VII also show that neither Cys nor γGluCys concentrations changed in response to GSH feeding. Split root experiments were designed to examine the possibility that GSH acts as a long-distance message involved in the control of the SO_4^{2-} uptake rate and ATP sulfurylase activity. GSH was supplied at a concentration of 1 mM to two-thirds of a root system. SO_4^{2-} uptake and

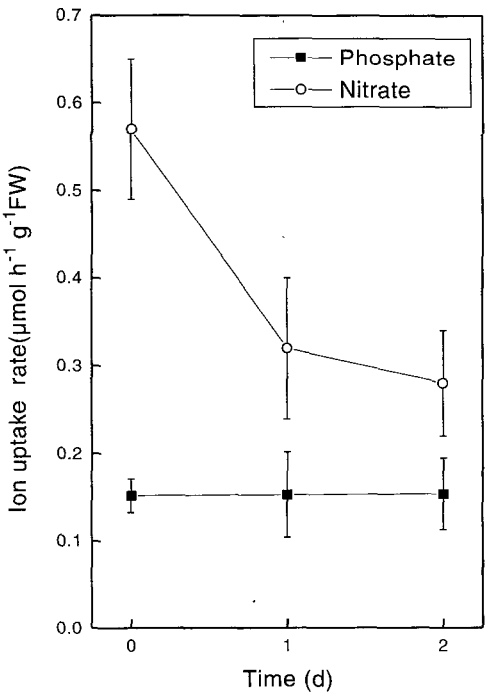

Figure 3. Time courses for NO_3^- and $H_2PO_4^-$ uptake by roots of intact canola plants with duration of S starvation. Plants were grown and treated as described in the legend of Figure 1. Net uptake rates were calculated from the decrease in external concentrations of NO_3^- and $H_2PO_4^-$ for 6-h periods. Data are means ± SD of five replicates. FW, Fresh weight.

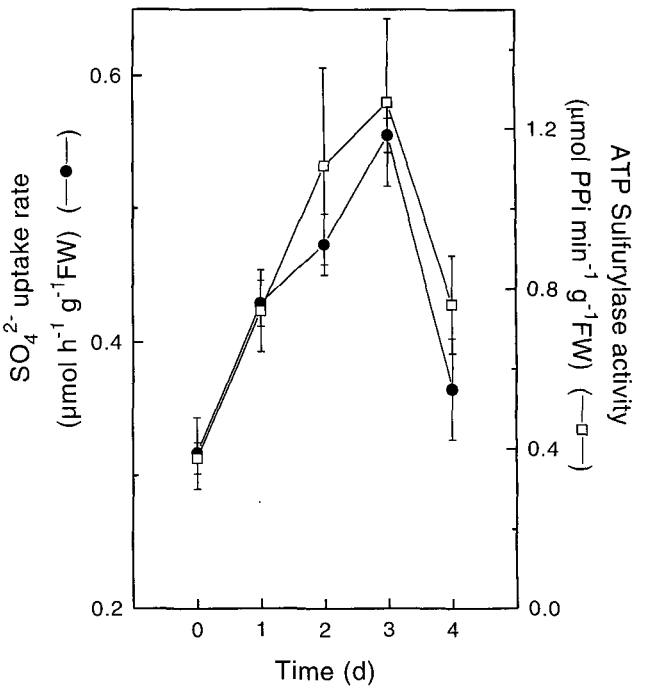

Figure 4. Time courses of ATP sulfurylase activity and SO_4^{2-} uptake in SO_4^{2-}-fed roots of intact canola plants with duration of exposure of the other roots to an S-free solution. Plants divided with root systems in two parts were initially grown on a complete nutrient solution containing 1 mM SO_4^{2-} identically provided to both parts. Then, the solution supplied to one part of the root system (approximately two-thirds of the total root biomass, referred to as "−S roots" in text) was changed to a SO_4^{2-}-free solution for various periods up to 3 d and then changed back to the same SO_4^{2-} replete solution for some plants. The SO_4^{2-} uptake and ATP sulfurylase activity were measured on the other roots (referred as "+S roots" in the text), which were continuously supplied with SO_4^{2-}. Data are means ± SD of five replicates. FW, Fresh weight.

ATP sulfurylase activity were measured in the portion of the root system that was not exposed to GSH. As a result of these treatments, both the SO_4^{2-} uptake rate and ATP sulfurylase activity were inhibited to similar extents to those observed when GSH was supplied to the entire root system (compare Figs. 5 and 7). GSH levels increased throughout the 36-h experimental period, whereas Cys concentrations remained constant from 12 to 36 h, and γGluCys concentrations remained essentially unchanged

Table IV. *Effects of S starvation on the concentrations of SO_4^{2-} in roots and shoots of 21-d-old canola plants*

Canola plants were grown under split root conditions as described in Table II. SO_4^{2-} was determined for +S Roots, −S Roots, and Shoots. Values are means ± SD of five replicates.

Treatment	+S Roots	−S Roots	Shoots
		μmol g⁻¹ fresh wt	
Control	2.1 ± 0.1	2.0 ± 0.3	4.0 ± 1.0
1 d −S	1.8 ± 0.5	1.1 ± 0.2	4.2 ± 0.8
2 d −S	2.2 ± 0.1	0.8 ± 0.1	2.0 ± 0.2
3 d −S	2.3 ± 0.4	Trace	1.8 ± 0.4
3 d −S/1 d +S	2.2 ± 0.2	2.2 ± 0.1	4.4 ± 1.0

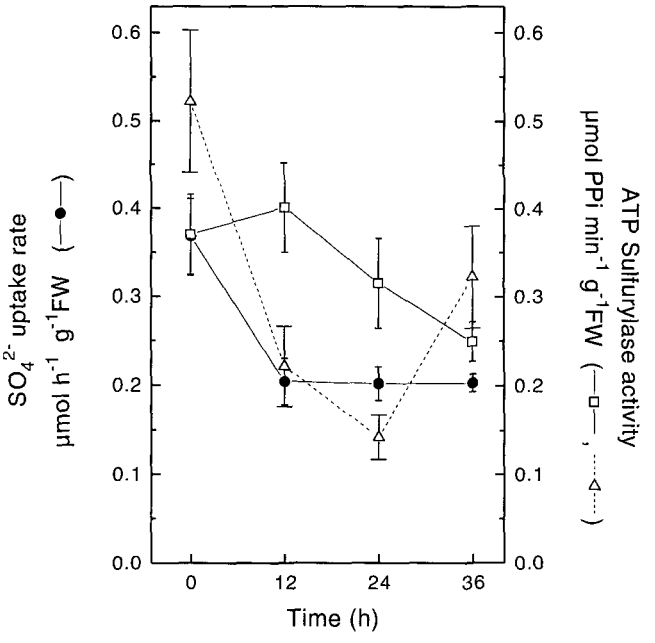

Figure 5. Time course responses of ATP sulfurylase activity and SO_4^{2-} uptake in canola roots in response to GSH or Cys treatment. Plants were grown for 21 d on a complete nutrient solution, into which 1 mM GSH (solid line) or Cys (dashed line) was added 0 to 36 h before harvest. The enzyme activity was determined on crude extracts, measuring the molybdate-dependent formation of pyrophosphate. Sulfate uptake was measured as the rate of ^{35}S incorporation over 5 min. Data are means ± SD of five replicates. FW, Fresh weight.

for the first 24 h (Table V). As observed in experiments in which the entire root system received 1 mM GSH, SO_4^{2-} concentration in roots decreased as a consequence of the depressive effect of this treatment on SO_4^{2-} uptake (Fig. 5).

DISCUSSION

Under adequate S nutrition, canola plants take up SO_4^{2-} at approximately 0.2 µmol h^{-1} g^{-1} fresh weight. Whether this figure is corrected for any assumed remaining extracellular SO_4^{-}, leading to a 0.1 µmol h^{-1} g^{-1} fresh weight influx (see calculations in "Materials and Methods"), it falls into the 0.1 to 0.3 µmol h^{-1} g^{-1} fresh weight range found elsewhere for other species such as *Macroptilium atropurpureum* (Clarkson et al., 1983; Bell et al., 1995), barley and wheat (Clarkson and Saker, 1989; Clarkson et al., 1992), tobacco (Herschbach and Rennenberg, 1994), or mature spinach (Herschbach et al., 1995). Under S deficiency, SO_4^{2-} uptake rates in canola increased considerably (Fig. 1), which is consistent with previous reports (e.g. Lee, 1982, 1993; Hawkesford and Belcher, 1991; Bell et al., 1995). This positive effect is readily reversible upon restoration of SO_4^{2-} (Fig. 1), indicating that SO_4^{2-} uptake is regulated by S demand. Moreover, relative changes in uptake rates over S starvation treatments are likely to be higher than indicated in Figure 1 because all values should be overestimated by 0.1 µmol h^{-1} g^{-1} fresh weight because of the extracellular SO_4^{2-} remaining in roots after washing. The data presented confirm that these changes are not simply due to modified growth rate; no significant variation in biomass production was observed even after a 3-d starvation period (Table I). With regards to ion uptake, this demand-driven regulation specifically concerns SO_4^{2-}; phosphate uptake was unaffected by S starvation (Fig. 3). Incidentally, this also indicates that if a general growth perturbation was occurring because of the withdrawal of SO_4^{2-} from nutrient solution, it would be very unlikely to have a significant impact on ion uptake by roots. On the other hand, the NO_3^- uptake rate declined in response to S depletion (50% decrease within 1–2 d; Fig. 3) rather than increased, as was the case for SO_4^{2-} uptake. Such a behavior of coordinated repression of uptake of other nutrients when the supply of one essential nutrient is lacking was reported earlier using several combinations of major ions, including K^+, NO_3^-, $H_2PO_4^-$, and SO_4^{2-} (e.g. Lee, 1982, 1993; Clarkson and Saker, 1989). In our particular case, the SO_4^{2-} deficiency must have perturbed amino acid synthesis. Considering the trend of maintaining the relative concentration of S-containing amino acids versus total amino acids constant, N demand should consequently decline. The operation of demand-driven control processes on NO_3^- uptake (Imsande and Touraine, 1994; Touraine et al., 1994) could then explain the negative effect of S starvation, involving a direct effect of S starvation on NO_3^- transport systems.

Compared with SO_4^{2-} uptake, the pattern of ATP sulfurylase activity in response to S starvation has received little attention. Studies performed on cell cultures (Reuveny and Filner, 1977; Reuveny et al., 1980; Haller et al., 1986) have

Table V. *Effects of GSH supplied on the concentrations of thiols (Cys, γGluCys, and GSH) and SO_4^{2-} in roots*

Canola plants were grown on a complete nutrient solution where S was supplied as 1 mM SO_4^{2-} throughout the culture (control). The amount of 1 mM GSH was added 0 to 36 h before harvest. Split root experiments were processed similarly, except that only one part of the root system (approximately two-thirds) received GSH, whereas the rest of the root system, in which the concentrations given in this table were determined, was continuously supplied with a GSH-free nutrient solution. Data are means ± SD of six replicates.

Duration of Treatment	Whole Root System Experiments				Split Root Experiments			
	Cys	γGluCys	GSH	SO_4^{2-}	Cys	γGluCys	GSH	SO_4^{2-}
h	*nmol g^{-1} fresh wt*							
0	39 ± 8	17 ± 3	1454 ± 362	2130 ± 650	34 ± 7	16 ± 2	1225 ± 189	2590 ± 1380
12	178 ± 75	26 ± 7	2469 ± 643	2160 ± 280	158 ± 27	15 ± 3	2092 ± 526	1750 ± 440
24	129 ± 23	23 ± 3	4203 ± 1028	2060 ± 450	139 ± 31	22 ± 2	4031 ± 612	1310 ± 330
36	158 ± 40	55 ± 15	6140 ± 687	1430 ± 400	162 ± 45	35 ± 6	6254 ± 702	810 ± 250

Table VI. *Effects of Cys supplied to the whole root system of canola on the concentrations of thiols (Cys, γGluCys, and GSH) in roots*

The culture of canola plants and addition of 1 mM Cys were as described in Table V for GSH treatments. Data are means ± SD of six replicates.

Duration of Treatment	Cys	γGluCys	GSH
h		*nmol g⁻¹ fresh wt*	
0	43 ± 5	28 ± 9	1360 ± 469
12	65 ± 22	30 ± 10	3719 ± 1010
24	89 ± 20	35 ± 5	5951 ± 998
36	94 ± 15	38 ± 6	7945 ± 1864

shown that ATP sulfurylase activity is repressed by readily assimilated S sources, especially SO_4^{2-} ions, and derepressed by S starvation, although to a smaller extent than the activity of APS sulfotransferase, the next enzyme involved in the SO_4^{2-} assimilatory pathway (Haller et al., 1986). By contrast, in *Lemna minor* ATP sulfurylase just was affected significantly by 2 d of S starvation, and this treatment caused a doubling of APS sulfotransferase activity (Brunold et al., 1987). The data reported here clearly demonstrate that the activity of ATP sulfurylase in canola roots is strongly affected by previous S nutrition (Fig.1), demonstrating that regulation of ATP sulfurylase activity does occur in roots and that it is closely similar to the regulation of SO_4^{2-} uptake. This conclusion is consistent with the sole report (Cacco et al., 1977) in which ATP sulfurylase activity and the SO_4^{2-} uptake rate in roots of an intact plant were compared. This study showed that the distribution of ATP sulfurylase activity extracted from a corn root was correlated with the variations in the SO_4^{2-} uptake rate along the same root and that the opaque-2 mutation is accompanied by an identical shift in the locations of both the maximum SO_4^{2-} uptake rate and ATP sulfurylase activity along the root.

Regulation of both SO_4^{2-} uptake and ATP sulfurylase activity appears to be exerted by remote processes that require the transport of information about nutritional status over a long distance. This is suggested by the experiments in which the SO_4^{2-} uptake rate and ATP sulfurylase activity in some roots were affected by modifying the SO_4^{2-} supply to other roots of the same plant (Fig. 4). Similarly, it has been shown that modifying S demand in shoots by H_2S or SO_2 fumigation of spinach leaves inhibited SO_4^{2-} uptake and xylem loading (Herschbach et al., 1995). From the data presented here, we conclude that an overall control of S nutrition operates in the intact plant, acting on both SO_4^{2-} uptake and ATP sulfurylase activity, and involves a phloem-translocated message. Such coordination between the control of ion uptake and assimilatory metabolism by nutritional demand is not a general feature of the demand-driven control of mineral nutrition in higher plants. For instance, whereas the acquisition of NO_3^-, the other major anion that is absorbed by plant roots and whose assimilation requires reduction steps to be performed within plant tissues, is subjected to negative feedback by internal N (Imsande and Touraine, 1994; Touraine et al., 1994), nitrate reductase activity is not enhanced by N starvation. Differences between S and N metabolisms are illustrated by the dramatic decrease observed in nitrate reductase activity from cell cultures of rose when transferred to an N-free medium, whereas these cells exhibited an increase of ATP sulfurylase activity when transferred to an S-free medium (Haller et al., 1986).

Considering that the compound responsible for the regulation of ATP sulfurylase activity and SO_4^{2-} uptake must reflect the S nutritional status, the best candidates are internal SO_4^{2-} itself and the products of its assimilation, as has been proposed in the case of demand-driven control of NO_3^- uptake (Glass, 1988; Imsande and Touraine, 1994; Touraine et al., 1994). Based on the negative correlation between internal SO_4^{2-} and SO_4^{2-} uptake rate (Fig. 2; Table IV), several authors (e.g. Smith, 1975, 1980; Jensen and Konig, 1982; Datko and Mudd, 1984; Lass and Ullrich-Eberius, 1984; Bell et al., 1995) have proposed that internal SO_4^{2-} is the factor responsible for the repression of SO_4^{2-} uptake. Besides internal SO_4^{2-}, a thiol, GSH, also readily responds to S starvation in whole root system experiments (Table II). On the other hand, in split root experiments, S starvation did not significantly affect the concentration of SO_4^{2-} in +S roots, whereas the level of GSH responded to the same extent as in roots from whole root system-treated plants (Table IV). Although this observation favors the GSH hypothesis, the model according to which the internal concentration of SO_4^{2-} controls the SO_4^{2-} uptake rate, and possibly the ATP sulfurylase activity, cannot be dismissed. In this model, the "internal concentration" that would con-

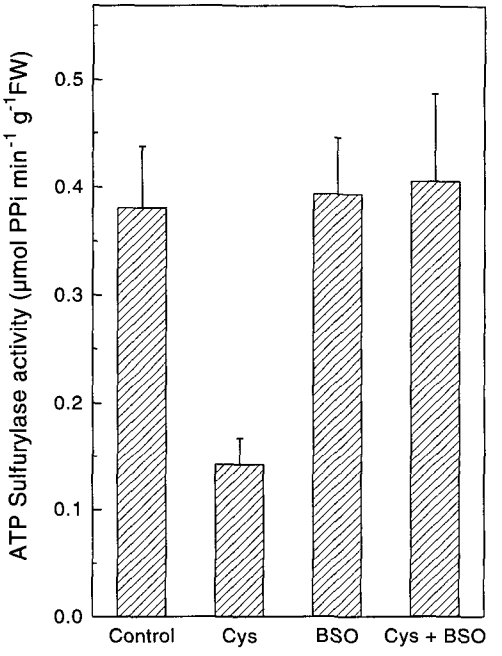

Figure 6. Effect of Cys and BSO on ATP sulfurylase activity in roots of intact canola. The activity of ATP sulfurylase extracted from roots of 21-d-old plants supplied with 1 mM Cys, 1 mM BSO, or both Cys and BSO for 24 h was determined. For comparison, the activity in roots of plants receiving neither Cys nor BSO (Control) is indicated. Data are means ± SD of five replicates. FW, Fresh weight.

Table VII. *Effect of GSH supplied to roots of canola on the concentration of thiols in phloem sap exudates from mature canola plants*

The culture of canola plants and addition of 1 mM GSH were as described in Table V. Phloem exudates have been collected in 5 mM sodium EDTA, pH 7.0, for 4 h in the dark and 95% RH. Data are means ± SD of five replicates.

Duration of Treatment	Cys	γGluCys	GSH
h		*μmol mol⁻¹ Suc*	
0	17 ± 4	50 ± 27	281 ± 72
12	19 ± 5	36 ± 9	1787 ± 450
24	19 ± 11	70 ± 29	1687 ± 582
36	22 ± 9	60 ± 20	2245 ± 342

trol S uptake and assimilation is likely to be the cytosolic concentration. According to Bell et al. (1995), in root cells of *M. atropurpureum* grown with 0.25 mM SO_4^{2-}, the flux of SO_4^{2-} from the vacuole to the cytoplasm is relatively small compared with fluxes exiting the cytoplasm, and hence cytosolic SO_4^{2-} concentration should decline substantially when the roots are deprived of an external SO_4^{2-} source. Following this reasoning and considering the small proportion of root SO_4^{2-} that is located in the cytoplasm (Bell et al., 1995), a large difference in the "active" (cytosolic) concentration of SO_4^{2-} may well occur but may be masked by the larger, average SO_4^{2-} concentration (vacuole plus cytoplasm) in roots, as presented in Table IV. Conversely, other evidence from compartmental analysis in carrot root suggests that SO_4^{2-} content can increase in the vacuole while that in the cytoplasm remains constant in conditions in which the tonoplast influx is reduced but the plasmalemma influx is unchanged (Cram, 1983). Determination of compartment contents would enable establishing the role of SO_4^{2-} and GSH concentrations in specific internal pools on the SO_4^{2-} uptake rate and ATP sulfurylase activity.

Focusing on the interorgan regulation of SO_4^{2-} uptake and ATP sulfurylase activity, the messenger conveying S demand information from shoot to root is, consequently, translocated in the phloem. Both SO_4^{2-} and GSH are translocated within the sieve sap (Rennenberg et al., 1979; Bonas et al., 1982; Rennenberg, 1982; Schneider et al., 1994), so both of them are, a priori, good candidates for this role. However, the analyses of phloem exudates made in canola (Table III) strongly support the hypothesis that GSH is the sought molecule because its concentration in phloem sap dropped by more than 60% within the 1st d after SO_4^{2-} withdrawal from the external solution, whereas the concentration of SO_4^{2-} failed to change. Furthermore, the rapid increase in GSH concentration in phloem sap after SO_4^{2-} restoration (Table III) is again consistent with the hypothesis of the involvement of phloem-translocated GSH in the regulation of S nutrition.

To test the involvement of GSH in controlling ATP sulfurylase activity and SO_4^{2-} uptake, the effect of a 1-mM GSH addition to the nutrient solution was studied. This treatment reduced both the ATP sulfurylase activity and the SO_4^{2-} uptake rate (Fig. 5). Although the impact of GSH on ATP sulfurylase activity is not documented, data on the effect of GSH on SO_4^{2-} uptake are available in the literature, mainly from the extensive studies done on tobacco by Rennenberg's group. According to their data, SO_4^{2-} uptake is repressed by GSH in cell cultures (Rennenberg et al., 1988, 1989), excised roots (Herschbach and Rennenberg, 1991), and intact plants (Herschbach and Rennenberg, 1994). The negative effect of GSH is not recorded in the first hours after the supply of this thiol to cells (Rennenberg et al., 1988), and it appeared to be reversible unless an inhibitor of protein synthesis was present (Rennenberg et al., 1989), so it was concluded that GSH acts by inhibiting de novo synthesis of transport proteins.

A priori, the inhibition of ATP sulfurylase activity by GSH is most probably due to an increase of its internal level rather than simply to its presence in the external solution. The ability of plant cells to take up GSH in the concentration range of 0.01 to 1 mM has been demonstrated unequivocally by Schneider et al. (1992). However, the possibility that the observed effect might be mediated by another compound whose level in root cells would also change subsequently to GSH supply has still to be considered. In this regard, Cys, both a precursor in GSH synthesis and a degradation product of GSH, which is accumulated in GSH-treated plants (Table V), is particularly interesting. Cys is known to inhibit SO_4^{2-} uptake (Herschbach and Rennenberg, 1991, 1994) and ATP sulfurylase activity

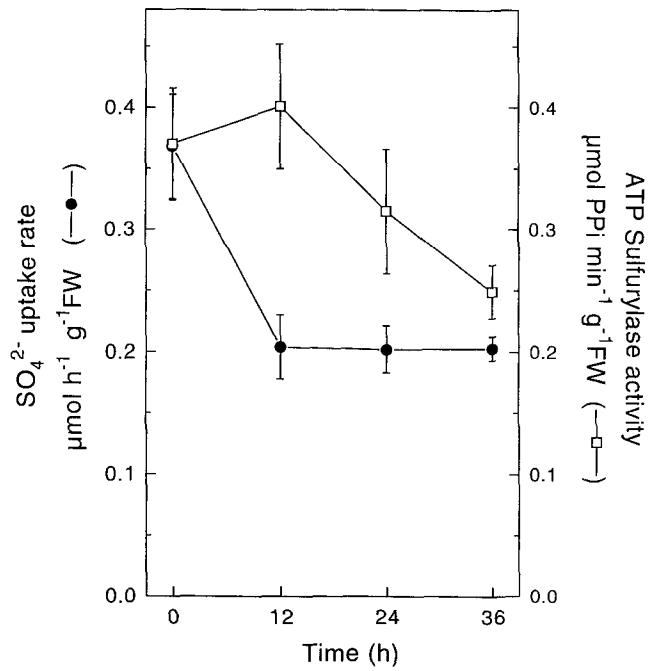

Figure 7. Time course responses of ATP sulfurylase activity and SO_4^{2-} uptake in part of the root systems of intact canola plants to the supply of solution containing 1 mM GSH to the other roots. The experiment was identical to the experiment in Figure 5, except that 1 mM GSH was in the solution supplied to approximately two-thirds of the total root system, and SO_4^{2-} uptake and ATP sulfurylase activity were measured in the other roots, which were continuously fed with the usual, GSH-free nutrient solution. Data are means ± SD of five replicates. FW, Fresh weight.

(Zink, 1984; Haller et al., 1986). On the other hand, BSO counteracted Cys inhibition of ATP sulfurylase activity, so a product of Cys synthesis, possibly GSH, is probably responsible for the inhibition of ATP sulfurylase activity. Consistently, similar conclusions have been drawn for SO_4^{2-} uptake in excised (Herschbach and Rennenberg, 1991) and attached (Herschbach and Rennenberg, 1994) tobacco roots treated with BSO. Taken together, the results from the literature and those presented herein thus demonstrate that GSH has a negative effect on both the SO_4^{2-} uptake rate and ATP sulfurylase activity and that this effect cannot be attributed to the main intermediates of its synthesis, Cys and γGluCys.

Under normal conditions, GSH is the predominant form of S translocation from leaves to roots in canola (Table VII). It has been possible to change its concentration in phloem by modifications of the nutritional status (Table III) and by experimental manipulation (Table VII). Modifying the GSH translocation rate resulted in concomitant decreases of both the ATP sulfurylase activity and SO_4^{2-} uptake rate in roots that received no exogenously supplied GSH (Fig. 7). In conclusion, it may still be a matter of debate whether GSH actually was the primary factor responsible for the control of SO_4^{2-} uptake and ATP sulfurylase activity in roots or whether GSH treatments led to the synthesis of an unknown regulatory factor that would have been the true cause of observed inhibitions. Nevertheless, GSH is clearly involved in the signaling process that operates at the whole plant level.

Received September 25, 1995; accepted January 23, 1996.

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