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Interaction of Sulfate Assimilation with Nitrate Assimilation as a Function of Nutrient Status and Enzymatic Co-Regulation in *Brassica juncea* Roots

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The interaction of sulfate assimilation with nitrate assimilation in *Brassica juncea* roots was analyzed by monitoring the regulation of ATP sulfurylase (AS), adenosine-5′-phosphosulfate reductase (AR), sulfite reductase (SiR), and nitrite reductase (NiR). Depending on the status of sulfur and nitrogen nutrition, AS and AR activities and mRNA levels were increased by sulfate starvation but decreased by nitrate starvation. The activation of AS and AR by sulfate starvation was inhibited by sulfate/nitrate starvation. However, the rise in steady-state mRNA levels for AS and AR by sulfate starvation was not affected by sulfate/nitrate starvation. SiR gene expression was slightly activated by both sulfate starvation and sulfate/nitrate starvation, but was decreased by nitrate starvation. Although NiR gene expression was little affected by sulfate starvation, it was diminished significantly by either nitrate or nitrate/sulfate starvation. Cysteine (Cys) also decreased AS and AR activities and mRNA levels even when plants were simultaneously starved for sulfate; in contrast, both SiR and NiR gene expressions were only slightly, if at all, affected under the same conditions. This supports our conclusion that Cys, the end-product of sulfate assimilation, is the key regulatory signal. Moreover, SiR and NiR apparently are not the linking step in the co-regulation of sulfate and nitrate assimilation in plants.

Keywords: APS reductase, ATP sulfurylase, cysteine, nitrite reductase, sulfite reductase

Plants utilize inorganic sulfate by reducing it to Cys through the sulfate assimilatory pathway (Fig. 1). First, extracellular inorganic SO_4 is taken up into cells via several transporters (Hawkesford and Belcher, 1991). Then, this intracellular sulfate is activated by AS through the formation of adenosine-5′-phosphosulfate (APS) (Lappartient and Touraine, 1996; Logan et al., 1996). APS is reduced via the formation of an organic thiosulfate with reduced glutathione by AR (Setya et al., 1996). Further reduction to sulfide is catalyzed by a ferroxin-dependent SiR (Bruhl et al., 1996). The incorporation of sulfide into Cys is the final step in the pathway, and is catalyzed by Cys synthetase (O-acetylserine thiol lyase; OAS-TL) from O-acetylserine (OAS) and sulfide. OAS is produced by serine acetyltransferase (SAT) from acetyl-CoA and serine (Hesse et al., 2004).

Numerous studies have established that a regulatory interaction exists between sulfate and nitrate assimilations (Friedrich and Schrader, 1978; Brunold and Suter, 1984; Zenk, 1984; Barney and Bush, 1985a, b; Haller et al., 1986; Suter et al., 1986; Brunold et al., 1987; Takahashi and Saito, 1996; Hesse et al., 2004). Because plants use most assimilated sulfate and nitrate for protein synthesis at a 25:1 N to S molar ratio, that interaction has been proposed as the mechanism that coordinates the flow of these two essential elements (Reuveny and Filner, 1977; Reuveny et al., 1980). This has been especially well demonstrated for nitrate reductase (NR) and AS from cultured tobacco cells (Reuveny et al., 1980; Prosser et al., 2001). When these cells are provided with growth-limiting concentrations of sulfate, the rate of development for NR is proportional to the sulfate concentration in the medium. Likewise, cells cultivated with growth-limiting concentrations of nitrogen as nitrate have a rate of de-repression for AS proportional to the media nitrate concentration (Reuveny et al., 1980). In contrast, NR activity is decreased in maize plants cultivated without a sulfur source (Reuveny et al., 1980). Therefore, based on their results with cultured tobacco cells, Reuveny and Filner (1977) have proposed a scheme that integrates the regulation of assimilatory sulfate and nitrate reduction (Fig. 1), in which each pathway is down-regulated by its own

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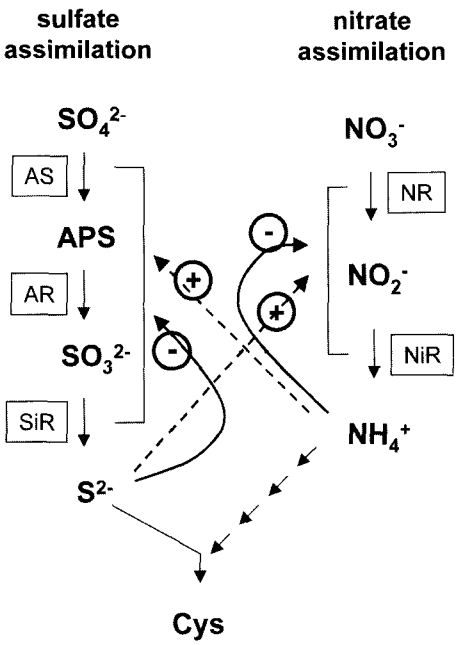

Figure 1. Schematic diagram of regulatory coupling between sulfate and nitrate assimilatory pathways. (+) and (–) represent positive and negative regulatory mechanisms, respectively.

end products and up-regulated by the products of the other pathway. Many subsequent experiments have produced results that support this hypothesis. For example, in *Lemna minor* L., glutamine, the end product of nitrate assimilation, activates AR while AS is unaffected (Suter et al., 1986), but nitrate starvation represses NR, AR, and AS activities (Brunold and Suter, 1984; Koprivia et al., 2000). Furthermore, in Paul's Scarlet Rose cell cultures, AR activity is enhanced by NH_4^+ and diminished by Cys-feeding, although that cysteine-induced decrease is less pronounced in the presence of NH_4^+ (Haller et al., 1986).

Even though all of the above results support this regulatory hypothesis, it has not been well studied how assimilatory sulfate and nitrate reduction are coordinated at the molecular level (Brunold and Rennenberg, 1997; Koprivia et al., 2002). In addition, it has been suggested that SiR and NiR may be the coordinating enzymes between sulfate and nitrate assimilation because each contains siroheme as a cofactor (Sakakibara et al., 1996; Hell, 1997). Therefore, the objective of our study was to analyze the possible interactions and regulation by various enzymes in the assimilation of sulfate and nitrate in the roots of *Brassica juncea*.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Plants of *B. juncea* (cv. 426308) were grown for 20 d in a hydroponic system that comprised a polyethylene pot with four plants and 500 mL of a nutrient medium. This medium (adjusted to pH 6.7 with KOH) contained 1.6 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1 mM KNO_3, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM KH_2PO_4, 50 μM $FeCl_2 \cdot 4H_2O$, 50 μM Na_2EDTA, 11 μM H_3BO_4, 2.5 μM $MnCl_2 \cdot 4H_2O$, 0.15 μM $H_2MoO_4 \cdot 2H_2O$, 0.1 μM $CuCl_2$, and 0.2 μM $ZnCl_2$. Plants were reared in a growth chamber at 23°C, and with a 12-h photoperiod under a light intensity of 100 μE $m^{-2}s^{-1}$. The nutrient solution was exchanged every 3 d. These plants were used for Cys treatment, sulfate starvation, nitrate starvation, and sulfate/nitrate starvation experiments.

Northern Blot Analysis

Total RNA was isolated from plants using a buffer containing guanidinium thiocyanate (Chomsczynsky and Sacchi, 1987). Twenty μg of RNA was electrophoresed on a 1.0% (w/v) agarose gel with formaldehyde (Lehrach et al., 1977). The gel was rinsed several times with water and then treated for 15 min with a solution containing 0.05 N NaOH and 10 mM NaCl. The RNA was transferred to a nylon membrane that had been pre-treated with 50 mM Tris-HCl (pH 7.2) for 10 min, and was pre-hybridized at 65°C for 8 h in a solution of 1.0 M Na^+ (supplied as Na_2HPO_4; pH 7.2), 1 mM EDTA, and 7% (w/v) SDS. Hybridization was carried out in a prehybridization buffer at 65°C for 16 h. The membrane was then washed at maximum stringency in 100 mM Na^+, 1 mM EDTA, and 1% (w/v) SDS at 65°C. Probes were labeled by the random primer method using [α-^{32}P]dCTP (3000 Ci $mmol^{-1}$). The following cDNAs served as probes: APS1 (Leustek et al., 1994), encoding ATP sulfurylase; APR1 (Setya et al., 1996), for APS reductase; SiR (EST Z37681; Bruhl et al., 1996), for sulfite reductase; EST B64XP (T04000), for beta-tubulin; and NiR (D13812), encoding nitrite reductase. The EST clones were provided by the Arabidopsis Biological Resource Center (Ohio State University).

AR Assay

Extracts were prepared by grinding root tissues (frozen in liquid nitrogen) with 50 mM Tris-HCl (pH 8.5).

After centrifugation for 20 min at 15800g, the supernant was used for protein measurements and enzyme assays. The protein concentration in each extract was measured by the assay of Bradford (1976), using BSA (bovine serum albumin) as the standard. AR was measured at 30°C. The reaction was contained in a 100 μL volume of 100 mM Tris-HCl (pH 8.5), 500 mM Na_2SO_4, 1 mM EDTA, 5 mM DTT, the plant tissue extract, and 25 μM [^{35}S]APS (~500 Bq · nmol^{-1}). This assay measured the formation of [^{35}S]SO^{2-}_3 from APS by its conversion to volatile SO_2 after the addition of acid. At the end of the incubation, Na_2SO_3 was added to 40 mM, then H_2SO_4 was added to 3 M. The uncapped reaction tubes were placed in scintillation vials containing 1 mL Tri-n-octylamine. All vials were then capped tightly and incubated overnight at room temperature (RT) to allow the volatilized SO_2 to be absorbed by the Tri-n-octylamine. The reaction tubes were then removed from the vials and 3 mL of scintillation fluid was added (Ready-Safe; Beckman, USA).

AS Assay

Root tissue was extracted by grinding in 50 mM Tri-HCl (pH 8.0). Following centrifugation for 20 min at 15800g, the supernatant was used for the enzyme assay. This assay measured APS-dependent ATP synthesis (reverse reaction), as described by Renosto et al. (1993). The reaction mixture contained 200 mL of extract, 5 mM $MgCl_2$, 1 mM glucose, 0.3 mM NADP, 2 units hexokinase, 1 unit glucose-6-phosphate dehydrogenase, 1 mM NaPPi, 55 mM APS, and 50 mM Tris-HCl (pH 8.0). Production of NADPH was measured for 2 min at RT with a Beckman DU 640 spectrophotometer, monitoring the increase in absorbance at 349 nm. The background rate was measured in a reaction lacking APS and Ppi; this value was subtracted from the rate measured with the substrate.

RESULTS

In 20-d-old *B. juncea* roots, both AS and AR activities rose in response to sulfate starvation (Fig. 2). AR activity increased progressively up to 48 h, while AS activity increased up to 24 h, but then declined after 48 h to the control level. When plants were starved of both nitrate and sulfate, however, enzymatic activity was diminished. We also measured the steady-state level of mRNA for AR and AS in sulfate- and sulfate/nitrate-starved plants, and compared that with the

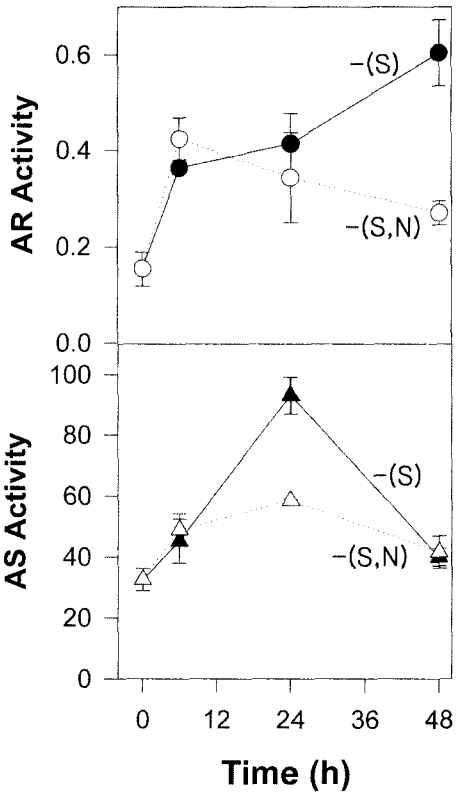

Figure 2. AR and AS activities were measured in roots of 20-d-old *B. juncea* plants at various times after transfer either to sulfate-free media (closed circles and triangles) or to media lacking sulfate and nitrate (open circles and triangles). Activity values are expressed in nmol · min^{-1} · mg^{-1} protein as mean ± standard error of three replicates.

steady-state level of mRNA for SiR and NiR (Fig. 3). AR gene expression was equally induced by sulfate and sulfate/nitrate starvations. The level of increase for sulfate starvation was closely correlated with the rise in enzyme activity (Fig. 2, 3), as was the decline in activity and mRNA level due to nitrate starvation (Fig. 4). However, while nitrate starvation inhibited the sulfate starvation-induced increase in AR activity (Fig. 2), it did not lessen the accumulation of mRNA (Fig. 3). A similar pattern was apparent for AS. In comparison, the SiR mRNA level was slightly increased by both sulfate starvation and sulfate/nitrate starvation (Fig. 3). Although the NiR mRNA level was not significantly changed by sulfate starvation, it did show a significant drop in response to sulfate/nitrate starvation (Fig. 3). Both NiR and SiR gene expressions were significantly decreased by nitrate starvation, as had been observed with AS and AR (Fig. 4)

The influence of nitrate starvation on the repression of sulfate assimilation enzymes was determined at the level of gene expression and enzyme activity

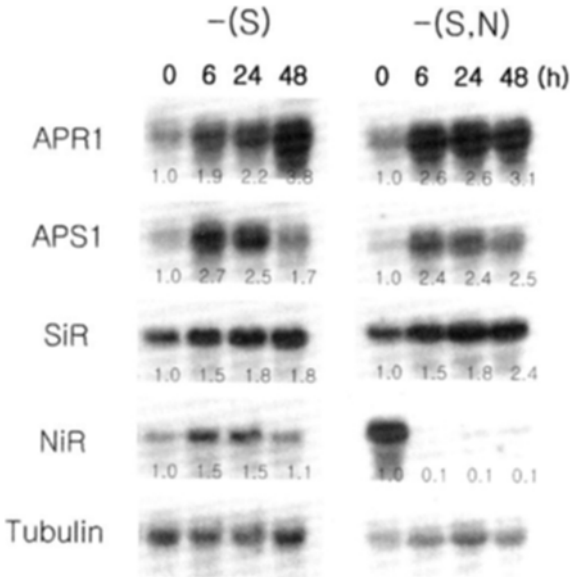

Figure 3. Effect of sulfate starvation or sulfate and nitrate starvation on mRNA levels for sulfate and nitrate assimilation genes. AR (APR1), AS (APS1), SiR, and NiR mRNA levels were measured in roots of 20-d-old *B. juncea* plants at various times after transfer to media for sulfate starvation (left blots) or sulfate plus nitrate starvation (right blots). Eight plants were used for RNA isolation, and 20 μg total RNA was electrophoresed, blotted, and sequentially analyzed with probes for ARP1, APS1, SiR, NiR, and then tubulin. The tubulin probe was used as control for RNA loading. Number below each lane indicates relative intensity of signal, corrected for RNA loading. Control is assigned a value of 1.0.

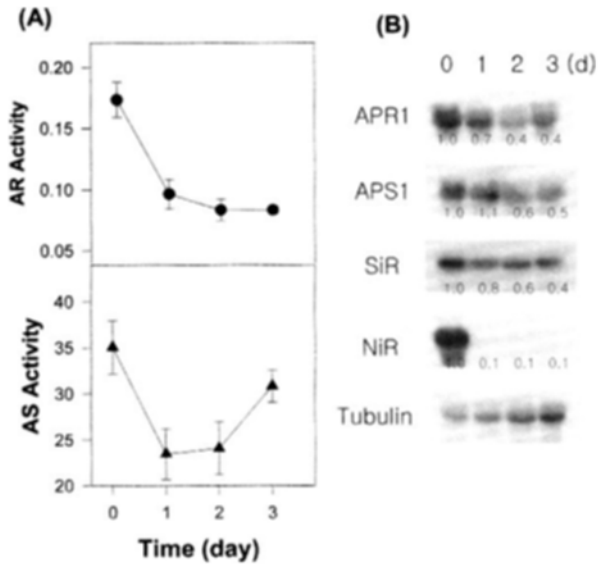

Figure 4. Effect of nitrate starvation on sulfate assimilatory enzymes and genes. **(A)** AR and AS activity were measured in roots of 20-d-old *B. juncea* plants at various times after transfer to nitrogen-free media. Activity values are expressed in nmol · min^{-1} · mg^{-1} protein as mean ± standard error of three replicates. **(B)** Effect of nitrate starvation on steady-state level of mRNA for sulfate assimilation genes and NiR. Roots samples from 20-d-old plants were analyzed at various times after removal of nitrogen from growth media. Eight plants were used for RNA isolation, and 20 μg total RNA was electrophoresed, blotted, and sequentially analyzed with probes for AR (APR1), AS (APS1), SiR, NiR, and then tubulin. The tubulin probe was used as control for RNA loading. Number below each lane indicates relative intensity, corrected for RNA loading. Control is assigned a value of 1.0.

(Fig. 4); this effect was deterred significantly by sulfate/nitrate starvation (Fig. 2, 3). We hypothesized that this nitrate-starvation effect was due to the inhibition of protein synthesis by the depletion of amino acids, finally resulting in the accumulation of Cys, a sulfur-containing amino acid. Because Cys is known to repress sulfate assimilation enzymes, we explored whether it is a more important signal than sulfate in regulating sulfate assimilation by placing 1 mM Cys in a sulfate-free medium. There, AR and AS declined significantly in their activity and mRNA level, while SiR and NiR showed no significant changes in mRNA levels (Fig. 5). The response by the sulfate-assimilating genes and the NiR gene was similar under these Cys treatments, with or without the addition of sulfate in the media (Fig. 6).

DISCUSSION

Although AR and AS enzyme activities and gene expression were activated by sulfate starvation (Fig. 2, 3), their activation was inhibited by nitrate/sulfate starvation (Fig. 2). Nevertheless, the induction of gene expression by sulfate starvation was not inhibited by nitrate/sulfate starvation (Fig. 4). SiR gene expression was slightly activated by sulfate starvation, but this activation was not affected by nitrate/sulfate starvation. NiR gene expression was little affected by sulfate starvation, but was significantly decreased by nitrate/sulfate starvation (Fig. 3). Finally, nitrate starvation repressed the activities of AR, AS, SiR, and NiR (Fig. 4). All these observations suggest that the effect of nitrate starvation on the repression of sulfate assimilation enzymes is due to the inhibition of protein synthesis by the depletion of amino acids. This then results in an increased Cys pool, which further represses the sulfate assimilation enzymes. It is well known that nitrogen starvation inhibits protein synthesis, such that the pools of sulfate, Cys, and methionine are generally high (Smith, 1980). Addi-

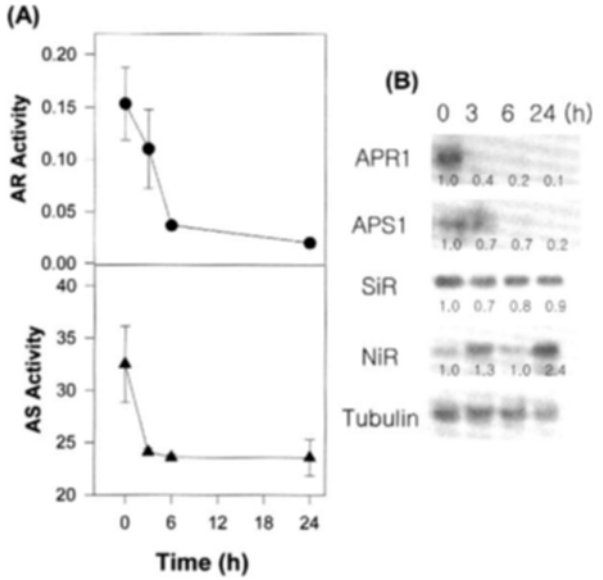

Figure 5. Effect of Cys feeding on AS and AR activity, and on expression of sulfate assimilatory genes and NiR gene after removing sulfate from media containing *B. juncea* roots. Twenty-day-old plants were sampled at various times after removing sulfate and then treating with 1 mM Cys in media. **(A)** Activity values are expressed in nmol · min^{-1} · mg $protein^{-1}$ as mean ± standard error of three replicates. **(B)** Eight plants were used for RNA isolation, and 20 μg total RNA was electrophoresed, blotted, and sequentially analyzed with probes for AR (APR1), AS (APS1), SiR, NiR, and then tubulin. The tubulin probe was used as control for RNA loading. Number below each lane indicates relative intensity of signal that was corrected with tubulin. Control is assigned a value of 1.0.

Figure 6. Effect of Cys feeding on steady-state levels of mRNA for AR (APR1), AS (APS1), SiR, and NiR in roots of *B. juncea*. Twenty-day-old plants were sampled at various times after treatment with 1 mM Cys. Eight plants were used for RNA isolation, and 20 μg total RNA was electrophoresed, blotted, and sequentially analyzed with probes. The tubulin probe was used as control for RNA loading. Number below each lane indicates relative intensity of signal that was corrected with tubulin. Control is assigned a value of 1.0.

tionally, treatment with cycloheximide, an inhibitor of protein synthesis, causes the accumulation of Cys and the repression of AR activity (Brunold, 1978). Our nitrate/sulfate starvation results suggest that the expression of the AR and AS genes increases in response to sulfate starvation, which then decreases the Cys pool. However, the inhibition of enzyme activation is due to the inhibition of protein synthesis, again a result of the depletion of amino acids. Thus, nitrate assimilation seems to affect sulfate assimilation via protein synthesis, which in turn affects the Cys pool. Here, we confirmed the importance of Cys in regulating sulfate assimilation (Fig. 5, 6). Previously, we reported that Cys also represses the sulfate assimilation enzymes in the absence of sulfate (Lee, 2005).

Based on these results, we propose that the role of SiR and NiR as coordinating enzymes between sulfate and nitrate assimilations is not plausible. Therefore, because SiR is not a key regulatory enzyme in sulfate assimilation, even though NiR is, there is no evidence of significant regulation by sulfur nutrients.

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