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ORIGINAL ARTICLE

Sulfur starvation and restoration affect nitrate uptake and assimilation in rapeseed

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Abstract We analyzed the effect of omission of sulfur (S) from the nutrient solution and then restoration of S-source on the uptake and assimilation of nitrate in rapeseed. Incubation in nutrient solution without S for 1–6 days led to decline in uptake of nitrate, activities, and expression levels of nitrate reductase (NR) and glutamine synthetase (GS). The nitrite reductase (NiR) and glutamate synthase (GOGAT) activities were not considerably affected. There was significant enhancement in nitrate content and decline in sulfate content. Evaluation of amino acid profile under S-starvation conditions showed two- to fourfold enhancement in the contents of arginine, asparagine and *O*-acetyl-L-serine (OAS), whereas the contents of cysteine and methionine were reduced heavily. When the S-starved plants were subjected to restoration of S for 1, 3, 5, and 7 days, activities and expression levels of NR and GS recovered within the fifth and seventh days of restoration, respectively. Exogenous supply of metabolites (arginine, asparagine, cysteine, glutamine, OAS, and methionine) also affected the uptake and assimilation of nitrate, with a maximum for OAS. These results corroborate the tight interconnection of S-nutrition with nitrate assimilation and that OAS plays a major role in this regulation. The study must be helpful in developing a nutrient-management technology for optimization of crop productivity.

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Introduction

Food from agriculture supports around six billion people that inhabit this planet today. It is well known that food production and fertilizer application are inextricably linked. To increase the crop productivity per unit area and the total agricultural production so as to meet the demand of ever-increasing population, millions of tons (mt) of fertilizers are applied to the soil. The global demand of N fertilizer has increased substantially since 1940 and is continuing to increase annually by 1% (FAO 2007). Nitrogen (N) is one of the most critical inputs that defines crop productivity and yield under field conditions. The human body contains about 1.5 kg of N atoms. Every human eats nearly three times this quantity of N every year in the form of protein. The current world population consumes some 28 mt of protein-N every year (Fujiwara and Matoh 2009). Nearly 85% of N in food proteins comes from agriculture, either direct through plant-derived foods or via animals fed with plant material. Synthetic fertilizers derived from the Haber–Bosch synthesis of ammonia provide 44–51% of the entire N absorbed by crops. Therefore, roughly 40% of N in foods derives from synthetic ammonia. It has been estimated that there is a sevenfold increase in the use of N fertilizers by doubling of agricultural food production worldwide over the past four decades (Abrol et al. 2007). In view of rising food demands, 30–35 mt of fertilizer N will be required by 2020 in India (NAAS 2005). The global demand of N fertilizer is increasing at an annual rate of 1%, and the input of available N to the Earth's land surface is almost doubled,

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largely through industrial production of nitrogen fertilizer (Gruber and Galloway 2008).

However, the current average nitrogen use efficiency (NUE) in the field is approximately 33%, and a substantial proportion of the remaining 67% is lost into the environment, especially in the intensively cropped areas (Abrol et al. 2007). The un-used fertilizer-N has many environmental effects, including the rising concentrations of nitrous oxide (N_2O) in the Earth's atmosphere (Richardson et al. 2009), its leaching to the ground water causing eutrophication, and human health damage by nitrate in drinking water. N_2O has a 300-fold-greater potential for global warming effect, based on its radioactive capacity compared with that of carbon dioxide (Bates et al. 2008). This concern was reflected in the Nanjing Declaration of the International Nitrogen Initiative (http://www.initrogen.org/nanjing_declaration.0.html), which called for immediate development of a comprehensive approach to optimize N management in every sphere of life. Efficient utilization of fertilizer N is essential to ensure a better return of our investments and minimize the adverse effect of the accumulation of reactive N species in the environment. Efficient plant nutrient use requires a balanced fertilization, optimization of nutrient replenishment, minimum nutrient losses to the environment, and optimization of fertilizer–soil–water interactions.

Sulfur, the least abundant of the six macronutrients required by plants, plays critical roles in the catalytic or electrochemical functions of the biomolecules in cells. Some earlier studies have reported that, under sulfur (S)-deficient conditions, the full potential of nitrogen fertilizer could not be achieved, regardless of top husbandry practices (Ahmad and Abdin 2000). Impairment in the nitrate uptake and assimilation by S-deficiency has been observed in tobacco and spinach in some previous studies (Migge et al. 2000; Prosser et al. 2001). N and S studies have also been done in some grasses like *Brachiaria brizantha* (Batista and Monteiro 2007), ryegrass (Millard et al. 1985), grassland plant mixture (Tallec et al. 2008a), and legumes like *Trifolium repens* L. and *Lolium perenne* L. (Tallec et al. 2008b) and *Vicia faba* (Olivares et al. 1983). However, no report on the effect of restoration of S-supply to the nitrate uptake and assimilation capacity of S-starved rapeseed plant was found. This paper depicts a picture of regulation of nitrate uptake and assimilation by S nutrition in rapeseed at biochemical and molecular levels.

Materials and methods

Plant material and culture treatments

Seeds of rapeseed (*Brassica rapa* L. cv. Pusa Gold), procured from Indian Agricultural Research Institute, New Delhi, India, were surface-sterilized with 0.05% $HgCl_2$, followed by rinsing with double-distilled water. The sterilized seeds were germinated under dark conditions in moist vermiculite for 3 days, and then grown hydroponically in nutrient solution for 12 days under controlled environmental conditions with photosynthetic photon flux density (PPFD) of 450 μmol $m^{-2}s^{-1}$ (16 h day/8 h night), temperature of 28/22°C, and relative humidity of 75%. The composition of nutrient solution was 3.0 mM KNO_3, 2.0 mM $Ca(NO_3)_2$, 1.5 mM $MgSO_4$, 1.0 mM $NH_4H_3PO_4$, 50 μM KCl, 25 μM H_3BO_3, 20 μM NaFeEDTA, 2.0 μM $MnCl_2$, 2.0 μM $ZnCl_2$, 1.0 mM $MgCl_2$, 0.5 μM $CuCl_2$, and 0.5 μM $(NH_4)_6Mo_7O_{24}$ (Ahmad et al. 2005). The pH of the solution was adjusted to 5.5 with 1 M KOH. In S-deficient nutrient solution, $MgSO_4$ was replaced with $MgCl_2$. The levels of S were changed according to the experimental design. Nutrient solution was renewed every 4 days and bubbled with sterile air continuously.

Experimental design

Three experiments were conducted. In experiment I, 15-day-old rapeseed plants grown in complete nutrient solution were transferred to S-deficient nutrient solution for 1 day (1d), 2 days (2d), 3 days (3d), 4 days (4d), 5 days (5d), and 6 days (6d). Rate of nitrate uptake, activities of nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS) and glutamate synthase (GOGAT), amino acid profile, and the contents of nitrate, sulfate, and *O*-acetyl-L-serine (OAS) were analyzed at 1d, 2d, 3d, 4d, 5d, and 6d of S-starvation. The plants were kept on sulfur-deficient nutrient solution for 6d, however, because the first symptoms of senescence were detectable beyond this time period. In experiment II, 1d, 2d, 3d, 4d, 5d, and 6d S-starved rapeseed plants were restored with S supply. Rate of nitrate uptake, activities of NR, GS, NiR and GOGAT, amino acid profile, content of nitrate, sulfate, and OAS were analyzed at 1d, 3d, 5d, and 7d of S-restoration. One set of plants, kept in nutrient solution with S, served as control for both the experiments. In experiment III, plants were grown in the nutrient solution treated with various metabolites, namely, arginine, asparagine, cysteine, glutamine, methionine, and OAS. The uptake and assimilation of nitrate and the activities of the NR and GS were examined by adding individual metabolites (100 μM) in the reaction mixtures of the enzymes.

Nitrate uptake rate

Uptake rate was determined by the disappearance of nitrate from the external solution, which represents net flux into the root. Nitrate was measured by HPLC using a 10× 250-mm analytical anion exchange column (Partisil-10

SAX, Whatman, UK) according to Hunt and Seymour (1985). Nitrate ions were analyzed with UV detector at 210 nm. Sodium phosphate buffer (50 mM/pH3.0) was used as the eluent and the flow rate was kept at 1 mL/min with 25 μL of sample injection volume. Run time was maintained at 15 min. The uptake rate was expressed in micromoles of NO_3^- absorbed per gram of root fresh weight per hour.

In vitro assay of NR activity

Extraction was done by the method of Ahmad and Abdin (1999), whereas Campbell and Samarrelli (1978) were followed for enzyme assay. The reaction mixture contained equal volumes of 25 mM sodium phosphate buffer (pH6.8), 10 mM KNO_3, 100 μM NADH, and the enzyme extract. The reaction was initiated after the enzyme extract was added. After incubation (33°C for 30 min), 0.1 mL aliquot of the reaction mixture was added with 1 mL of sulfanilamide and 0.02% *N*-(1-naphthyl) ethylene-diamine. Absorbance was measured at 540 nm. Enzyme activity was expressed as nanomoles of NO_2^- per milligram of protein per minute.

In vitro assay of NiR activity

Crude homogenates of enzyme were prepared according to Gupta and Beevers (1984). The method of Ida and Morita (1973) was followed for enzyme assay. The reaction mixture contained equal volumes of 100 μmol Tris–HCl buffer (pH7.5), 3 μmol $NaNO_2$, 2 μmol methyl viologen, and the enzyme extract. The reaction was initiated by adding 0.3 mL of freshly prepared 24 μmol sodium dithionite prepared in 0.2 M sodium bicarbonate to the assay mixture. It was incubated at 30°C for 20 min, and the reaction was stopped by shaking the test tube vigorously. One milliliter each of sulfanilamide (1% in 1 N HCl) and 0.02% *N*-(1-naphthyl)-ethylene-diamine was added to 0.1 mL aliquot of the reaction mixture. Absorbance was determined at 540 nm. Enzyme activity was expressed as micromoles of NO_2^- per milligram of protein per minute.

In vitro assay of GS activity

Extraction of the enzyme was done by the method of McNally et al. (1983). For assay, the method of Rhodes et al. (1975) was followed. The reaction mixture contained 100 μmol Tris–HCl buffer (pH8), 10 μmol ATP, 250 μmol sodium glutamate, 100 μmol $MgSO_4$, 10 μmol L-cysteine, and the enzyme extract in a final volume of 3 mL. Reaction was initiated by addition of 10 μmol hydroxylamine. The mixture was incubated at 30°C for 30 min. γ-glutamyl hydroxamate (GH) formed was determined by adding 1.0 mL of ferric chloride reagent. Absorbance was measured at 540 nm. The activity was calculated by using the standard curve of γ-GH. Enzyme activity was expressed in micromoles of γ-GH formed per milligram of protein per minute.

In vitro assay of GOGAT activity

Extraction was done according to the method of Mohanty and Fletcher (1980). The enzyme was assayed following the method of Fowler et al. (1974). Reaction mixture contained 75 μmol Tris–HCl buffer, 10 μmol α-ketoglutarate, 15 μmol L-glutamine, 0.3 μmol NADH, and the enzyme extract. Reaction was initiated by addition of NADH. Enzyme kinetics was studied for 3 min at 340 nm. Enzyme activity was expressed in nanomoles of NADH oxidized per milligram of protein per minute.

Estimation of amino acid profile

Amino acid profiling was done using HPTLC. A Camag TLC system (Switzerland) comprising a Linomat 5 semi-automatic sample applicator, TLC Scanner 3, twin-trough chamber (20×20 cm), winCATS 1.3.2 software, Canon PSG2 digital camera, and Hamilton syringe (100 μL) was used for the study. Standard amino acid kit and pre-coated silica gel 60 F_{254} aluminium sheets (20×20 cm, layer thickness 0.2 mm) were procured from Sigma-Aldrich, Poole, Dorset, UK, and E. Merck, Germany, respectively. Ninhydrin was also procured from Sigma-Aldrich, UK. All solvents and other reagents were of HPTLC grade. Standards were prepared as 0.1 mg mL^{-1} solutions in 60% ethanol. Plant samples were prepared by adding 0.5 g of leaf tissue in 10 mL of 60% alcohol in test tubes and keeping it overnight. The next day, it was filtered through Whatman filter paper (No. 1). The filtrate was completely evaporated by incubating the test tubes in water bath maintained at 40°C. After complete evaporation of alcohol, remaining precipitate was reconstituted in 1 mL 60% alcohol and used further for amino acid analysis. One microliter each of amino acid standards and 8-μL sample per treatment was applied under the flow of nitrogen gas under semiautomatic spotter. TLC plates were developed in solvent system (mobile phase)—*n*-butanol–acetic acid–water (3:1:1 v/v); chamber saturation time, 40 min. Detection of the amino acids was achieved by spraying the developed and air-dried plates with ninhydrin reagent (prepared in butanol). For the quantitative amino acid analysis, the areas of the bands were measured using Camag TLC Scanner III with the tungsten light source set at 615 nm; scanning speed, 10 mm s^{-1}; data resolution, 100 μm $step^{-1}$; loading speed, 80 μL/s. Spotting parameters used were band length, 4.5 mm; space between two bands, 6 mm; distance run, 160–170 mm. The CATS software

automatically produced a calibration curve relating the scan areas of the standard zones to their respective weights (0.1 mg) by polynomial regression and interpolated the weights of sample zones from the curve based on their areas.

Estimation of nitrate and sulfate contents

Extraction of nitrate and sulfate from plants was done by the method of Grover et al. (1978). Nitrate content in the samples was determined by HPLC as described in earlier sections. Sulfate content was estimated by the method of Chesnin and Yien (1950). Results were expressed as micromoles per gram FW.

Estimation of OAS content

Extraction of OAS was done by the method of Buchner et al. (2004). Assay of OAS was carried out by the method of Droux et al. (1998).

RNA extraction and quantitative real-time PCR

For gene expression analysis, total RNA was isolated from 100 mg fresh weight with the RNeasy plant mini kit according to the manufacturer's instructions (Qiagen, Germany). Total RNA was treated with RNase-free DNase I using the DNA free kit (Qiagen, Germany) to remove any contaminating genomic DNA, and then RNA integrity was checked on an agarose gel. Gene expression levels were analyzed by two-step real-time RT-PCR. cDNAs were synthesized from 250 ng total RNA using SuperScript II reverse transcriptase (Invitrogen, USA) and the reaction mixture was diluted 20 times for subsequent PCR. Real-time PCR was performed with a LightCycler (Roche), using SYBR to monitor dsDNA synthesis. As internal standard, the 18 S ribosomal RNA primers/competimers (Ambion, USA) in a ratio of 1:9 were used. Every reaction was set up in five replicates. The program used was as follows: 94°C for 5 min and 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s followed by melting curve analysis. PCR mixtures with final volumes of 20 µL contained 5 µL cDNA, 0.3 mM of each primer, 2.5 mM $MgCl_2$, and 10 µL of QuantiTect SYBR Green Master mix. Data were analyzed using LightCycler 480 basic software (Roche, Germany). A reaction in which reverse transcriptase was omitted in the RT step was used as the control. The relative level of expression was calculated with the following formula: relative expression ratio of the gene of interest is $2^{-\Delta DCT}$ with $\Delta CT = Ct_{gene} - CT_{18S}$. The fragments PCR amplified from total cDNA were gel purified to assure accuracy and specificity.

The primers used were 5' AGTCGCTTGCACG TTACCTG 3' (forward) and 5' ACCCTCTGAC TTGGCGTTCTC 3' (reverse) for nitrate transporter gene (*nrt 2.1*); 5′ ATTCCTTCTCCCCGTC ACC 3′ (forward) and 5′ GCCAGTTCAGCATCGACAAG 3′ (reverse) for NR gene (*nia 2*); 5′ ACGCGGGATCATC TCATAAACCAAA 3′ (forward) and 5′ AAAACATG AAAAGGCCTTT TCAGATC 3′ (reverse) for GS gene (*gs1)*.

Statistical analysis

Data are presented as means with standard errors calculated from five replicates (n=5). A GraphPad InStat (GraphPad Software, San Diego, CA, USA) was used to perform statistical calculations. Samples were compared through analysis of variance (ANOVA) and the Tukey's test. Different letters indicate significant difference at $P<0.05$.

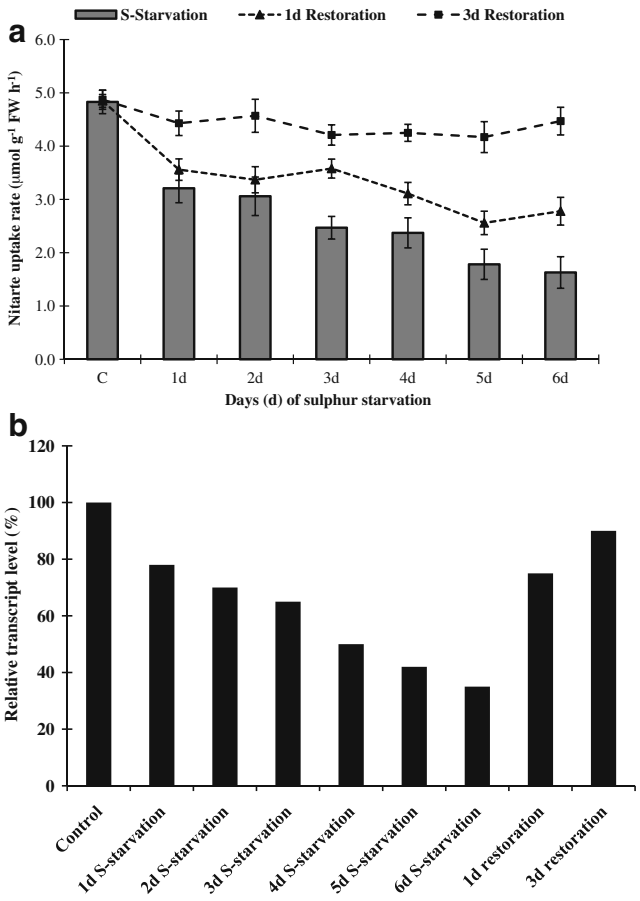

Fig. 1 **a** Nitrate uptake of S-starved and S-restored plants. *Vertical columns* indicate the nitrate uptake of S starved plants. *Lines* indicate nitrate uptake of S-starved plants, which was restored for 1 and 3 days. **b** Transcript level of nitrate transporter gene of S-starved and S-restored plants by quantitative real-time RT-PCR. Data are mean of five replicates

Results

Nitrate uptake rate

A reduction of 34%, 37%, 49%, 51%, 63%, and 66% in the nitrate uptake rate was observed, respectively, in the 1d, 2d, 3d, 4d, 5d, and 6d S-starved plants (Fig. 1a). Transcript level of nitrate transporters as analyzed by real-time RT-PCR also showed reduction in the range of 35–78% in 1d to 6d S-starved plants (Fig. 1b). Re-supply of S to these S-starved plants resulted in restoration of the nitrate uptake rate to normal level on the 3d of restoration of S-supply (Fig. 1a).

Nitrate assimilatory enzymes

Significant changes were reported in the activities of NR and GS by S-starvation (Fig. 2a, c), while the activities of NiR and GOGAT did not show any significant change by S-starvation (Fig. 2b, d). NR activity declined by 15%, 27%, 36%, 43%, 48%, and 50% at the 1d, 2d, 3d, 4d, 5d, and 6d of S starvation (Fig. 2a), whereas GS activity declined by 49%, 55%, 58%, 63%, 65%, and 66% on these days, respectively (Fig. 2c), as compared with the control. Figure 4 shows changes in the transcript level of NR and GS at 1d, 2d, 3d, 4d, 5d and 6d of S-starvation. In the leaves of 1d–6d S-starved rapeseed plants, the NR transcript level was found in the range of 64–78% with reference to control plants. Transcript level for GS decreased more rapidly than for NR in response to S starvation. In the leaves of 1d S-starved plants, GS-transcript level declined by 35%. The corresponding values for 2d, 3d, 4d, 5d, and 6d S-starved plants were 35%, 50%, 52%, 55%, and 60%, respectively (Fig. 4).

The S-starved plants were re-supplied with S for 1d, 3d, 5d, and 7d to investigate the effect of restoration of S-supply on N-assimilatory enzymes. The results are presented in Fig. 3. With the increase in the days of restored S supply to 1d-starved plants, there was a continuous recovery in the activity of NR, when compared with the control. NR activity reached the level of control plant at the 5d of S-restoration (Fig. 3a). Restoration of S supply to 2d, 3d, 4d, 5d, and 6d S-starved plants resulted in continuous recovery of NR activity with increase in the days of S restoration. Complete recovery in the NR activity was observed at the 7d of S-restoration. However, NiR activity was not significantly affected by S-restoration (Fig. 3b). GS activity was successfully recovered, almost within 5 days of restoration of S supply to the S-starved plants (Fig. 3c). Recovery was also observed in the transcript level of NR

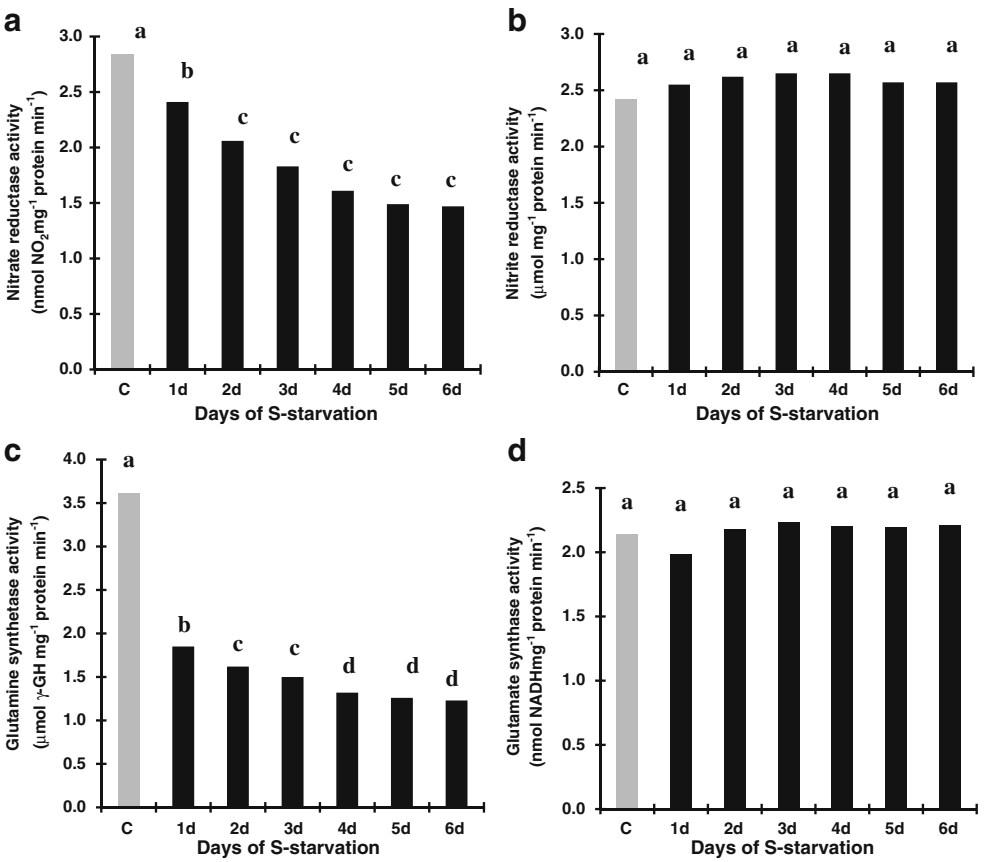

Fig. 2 Effect of duration of S-starvation (1d–6d) on the activity of nitrate reductase (**a**), nitrite reductase (**b**), glutamine synthetase (**c**), and glutamate synthase (**d**) in the leaves of rapeseed. Data are mean of five replicates. The values indicated by similar letters are not significant according to Tukey's test

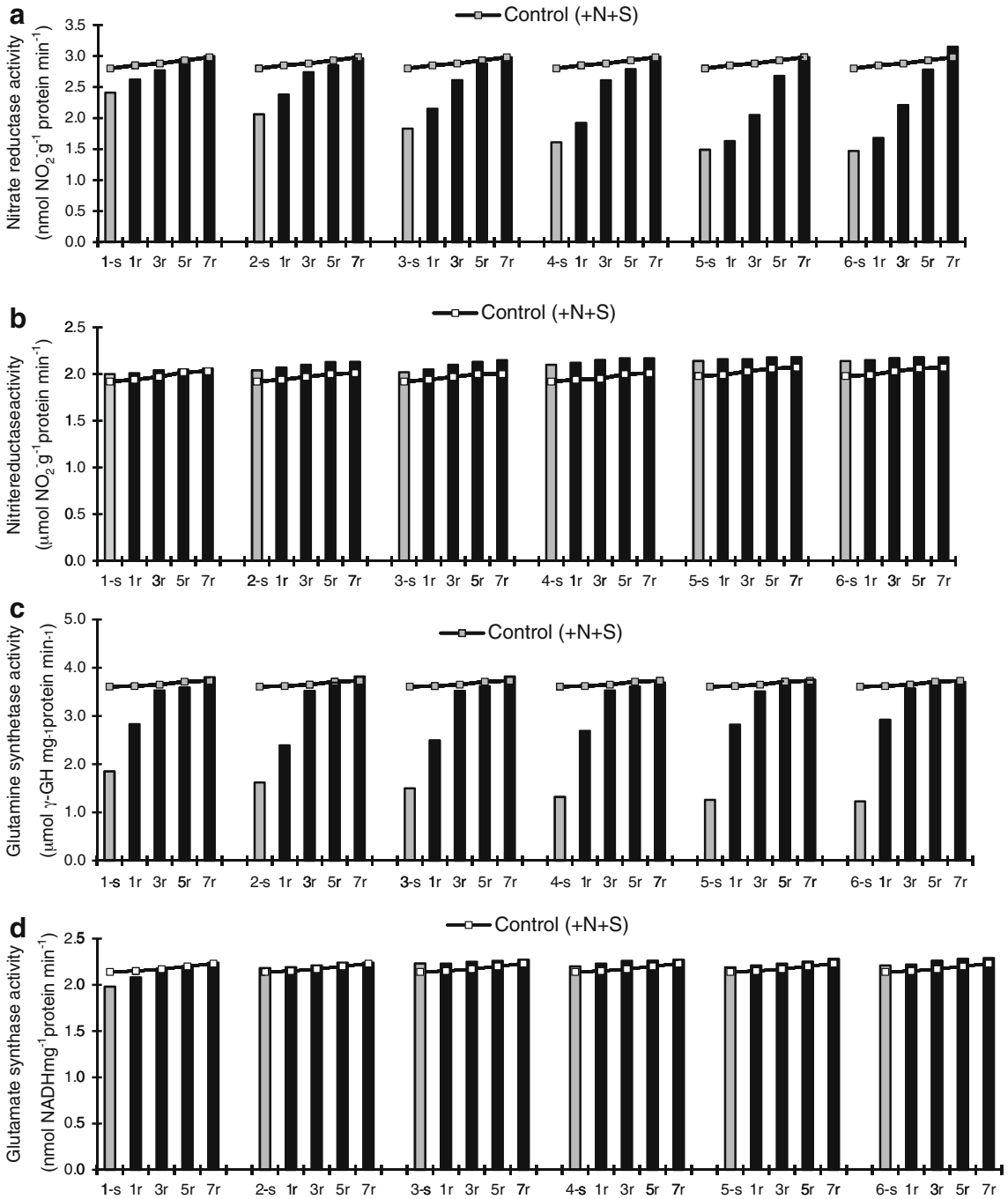

Fig. 3 Effect of restoration of S-supply on the activity of nitrate reductase (**a**), nitrite reductase (**b**), glutamine synthetase (**c**), and glutamate synthase (**d**) in the leaves of 1d–6d S-starved rapeseed plants. Data are mean of five replicates. Treatments of S, indicated on X-axis indicate- 1-s = 1-d S-starved; 2-s = 2-d S-starved; 3-s = 3-d S-starved; 4-s = 4-d S-starved; 5-s = 5-d S-starved; 6-s = 6-d S-starved; 1r = restoration for 1 day; 3r = restoration for 3 day; 5r = restoration for 5 day; 7r = restoration for 7 day. *Line* indicates the data for control plants (grown in complete nutrient solution)

and GS following the restoration of S-supply. It reached the level of control plants at the 5d of S-restoration (Fig. 4). GOGAT activity was unaffected by S-restoration (Fig. 3d). In short, restoration of S-supply to S-starved rapeseed plants recovered the NR and GS activity back to the level of control plants.

Amino acid profile

Increase in the duration of S-starvation increased the contents of arginine (Arg), asparagine (Asn), glutamine (Gln), and serine (Ser) by 47–118%, 8–119%, 38–108%, and 131–262%, respectively, as compared with the control

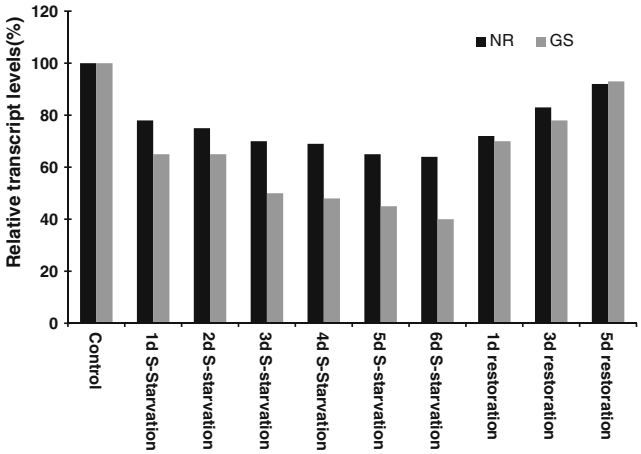

Fig. 4 Transcript level of NR and GS genes of S-starved and S-restored rapeseed plants by quantitative real-time RT-PCR. Data are mean of five replicates

(Table 1). However, the cysteine (Cys) and methionine (Met) contents decreased due to S-starvation. Cysteine content decreased from 51% to 78% over the control, whereas Met content decreased by 43%, 51%, 60%, 62%, 64%, and 66%, as recorded in the 1d- to 6d-starved plants, respectively, in comparison with the control.

Effect of restoration of S-supply to S-starved plants on the contents of Arg, Asn, Cys, Gln, Met, and Ser was measured since these amino acids were mainly affected by S-starvation in our earlier experiment (Fig. 8). All these amino acids were recovered to normal levels following the restoration of S-supply. However, there was a marked difference in the time of recovery. Arginine and Asn content recovered to normal level after the 3d of restoration (Fig. 8a, b), while glutamine content recovered at the 5d of S-restoration (Fig. 8d). The levels of Cys and Met contents were restored to normal the very 1d of S-restoration (Fig. 8c, e). Serine content came back to normal level on the 7d of restoration (Fig. 8f).

Contents of nitrate, sulfate, and OAS

Nitrate content increased with all the treatments in comparison with the control (Fig. 5a). It increased by 14%, 16%, 31%, 32%, 33%, and 32% at 1d–6d of S starvation, respectively. The increase was significant for all the treatments except at days 1 and 2. Sulfate content did not change significantly during the first day of S starvation (Fig. 5b). It declined by 15–31% at 2d–6d of S-starvation. The OAS content also underwent significant enhancement (Fig. 5c). Marked increase of 123% was noted in 1d S-starved plants. With increase in the days of S starvation, there was an increase in the OAS content by 181%, 272%, 315%, 396%, and 410% in the 2d, 3d, 4d, 5d, and 6d S-starved plants, respectively, as compared with the control.

S-starved plants were re-supplied with S for 1d, 3d, 5d, and 7d, and the effect of restoration of S-supply on nitrate and sulfate content was measured (Fig. 6). In the 1d, 2d,

Table 1 Effect of duration of S-starvation (1–6 days) on amino acid profile (ng g^{-1} FW) of 15-day-old rapeseed leaves

Amino acids	Control	Duration of S-starvation (days)					
		1	2	3	4	5	6
Ala	320 a	500 b	580 b	630 b	600 b	570 b	550 b
Arg	400 a	590 b	681 c	805 d	810 d	870 d	872 d
Asp	155 a	175 ab	210 b	282 c	340 d	210 b	167 a
Asn	300 a	410 ab	480 b	525 bc	628 cd	690 d	720 d
Cys	290 a	142 b	100 bc	90 c	83 c	70 c	65 c
Glu	360 a	237 b	235 b	232 b	235 b	237 b	245 b
Gln	1,300 a	1,800 b	2,010 b	2,220 bc	2,460 cd	2,520 cd	2,700 d
Gly	810 a	820 ab	850 b	870 bc	875 bc	880 c	888 c
His	760 a	765 a	780 ab	793 b	800 bc	810 bc	825 c
Ile	310 a	310 a	322 a	328 a	329 a	330 a	331 a
Leu	660 a	660 a	658 a	660 a	655 a	655 a	653 a
Lys	720 a	721 a	725 a	725 a	728 a	730 a	730 a
Met	530 a	300 b	260 c	210 d	200 d	190 d	180 d
Phe	420 a	420 a	418 a	415 a	415 a	412 a	412 a
Pro	230 a	220 a	220 a	215 a	215 a	215 a	212 a
Ser	130 a	400 b	470 b	400 b	360 b	340 b	300 c
Thr	630 a	680 b	690 b	710 b	715 b	720 b	720 b
Tyr	530 a	530 a	530 a	525 a	525 a	525 a	525 a
Val	165 a	152 a	150 a	140 a	135 a	130 a	125 b

Data are mean of five replicates. The values indicated by similar letters are not significant according to Tukey's test

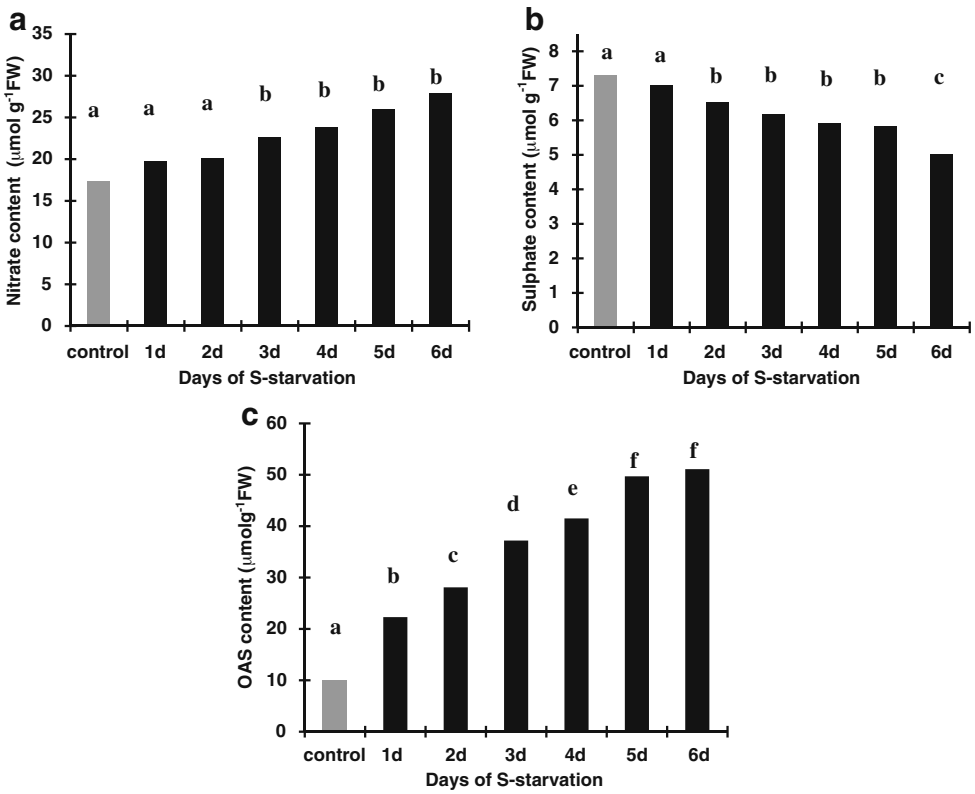

Fig. 5 Effect of duration of S-starvation (1–6 days) on the contents of nitrate (**a**), sulfate (**b**), and OAS (**c**) in the leaves of rapeseed. Data are mean of five replicates. The values indicated by similar letters are not significant according to Tukey's test

and 3d S-starved plants, nitrate content was restored quickly back to normal levels on the third day of S-supply restoration (Fig. 6a). In the 4d, 5d, and 6d S-starved plants, nitrate content declined and reached the level of control at the fifth day of restoration. Restoration of S-supply to S-starved plants resulted in a complete recovery of sulfate content on the third day of restoration (Fig. 6b). Resupply of S was unable to restore the OAS content to the level of control. The maximum recovery of OAS content occurred on the seventh day of restoration (Fig. 6c).

Effect of N-assimilatory metabolites on uptake and assimilation of nitrate

In an earlier experiment, it was found that the levels of arginine, asparagine, cysteine, glutamine, OAS, and methionine changed markedly by various treatments of S supply. To find out the signal metabolite(s), the effect of these metabolites was investigated on the uptake of nitrate and activities of NR and GS. The nitrate uptake rate was reduced by the supply of metabolites (Fig. 7a), the reduction being 20%, 25%, 55%, 40%, 40%, and 60% with Arg, Asn, Cys, Gln, Met, and OAS, respectively. Arg treatment increased the activity of GS by 5%. Interestingly, Asn proved inhibitory for NR activity (21%), but stimulatory for GS activity (5%). Glutamine treatment marginally increased the activities of both these enzymes (Fig. 7b). OAS treatment caused a drastic (80%) decline in NR activity and an increase of 10% in GS activity. Cysteine treatment resulted in 30% and 8% increase, while Met treatment in 25% and 3% increase in the activities of NR and GS, respectively.

Discussion

Plants grown in an environment limiting for a particular nutrient exhibit responses specific for the limiting nutrient and more general responses elicited by a variety of stress conditions. General responses to nutrient limitation include modification of metabolic processes to satisfy the demands of a reduced nutrient environment. In this study, we focused on early adaptive responses to relatively short-term periodic sulfur deficiency. We intended to elucidate the role of OAS in the regulatory mechanism of gene expression profile in the context of S-deficiency stress. Fifteen-day-old rapeseed plants were subjected to S-deficiency, followed by restoration of S-supply and supplementation of metabolites like arginine, asparagine, cysteine, glutamine, OAS, and methionine. Under these growth conditions, the activity and expression of genes encoding nitrate transporters and nitrate assimilatory enzymes changed significantly. Our results showed that S-deprivation significantly reduced the nitrate uptake rate, and with the increase in the duration of S-deprivation, the rate of nitrate uptake declined continuously. Earlier reports in spinach and cultured cells of tobacco have

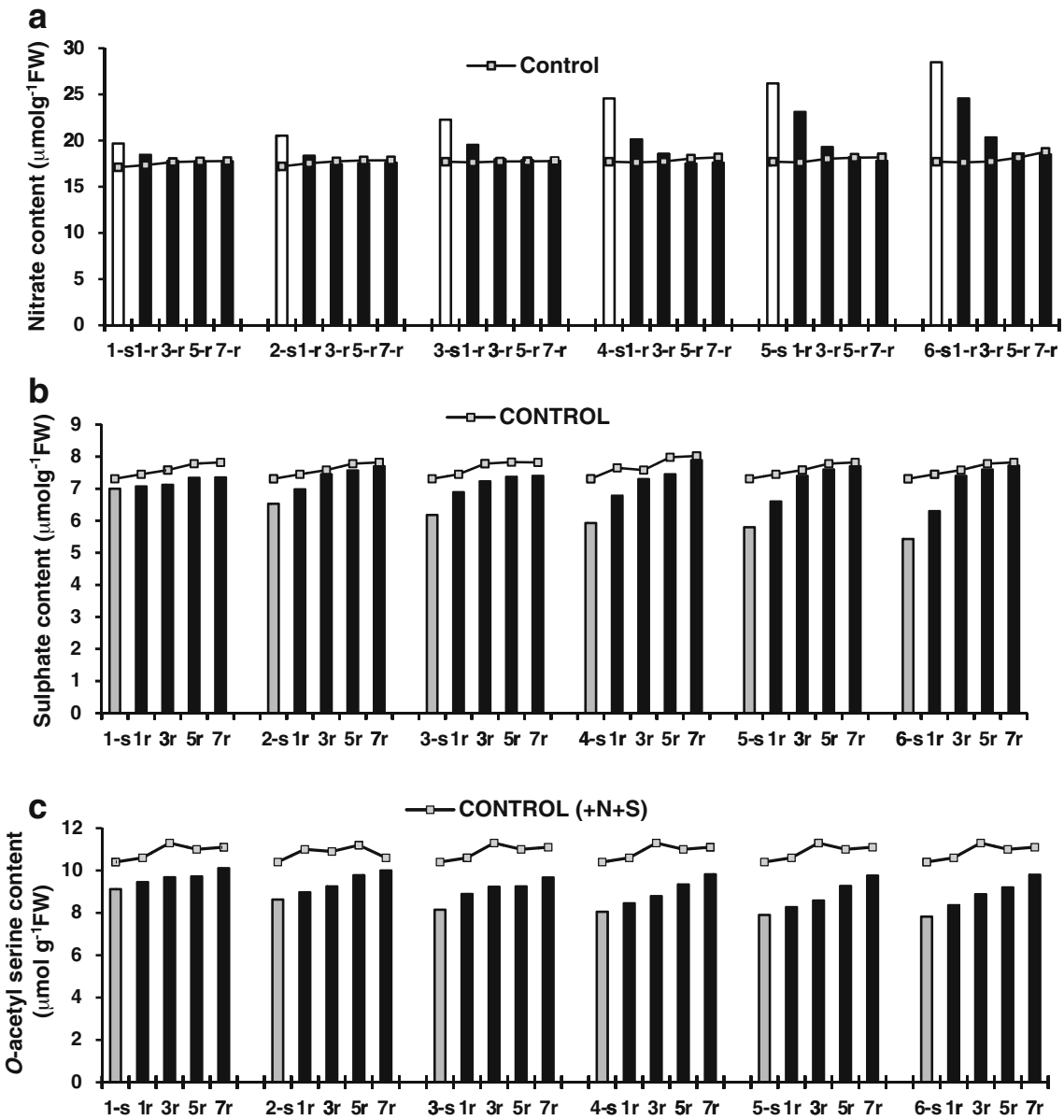

Fig. 6 Effect of restoration of S-supply on the contents of nitrate (**a**), sulfate (**b**), and OAS (**c**) in the leaves of 1–6 days S-starved rapeseed plants. Data are mean of five replicates. Treatments of S, indicated on X-axis are same as explained in Fig. 3. *Line* indicates the data for control plants (grown in complete nutrient solution)

shown that nitrate uptake declined by S-deprivation (Prosser et al. 2001). Of the various metabolites, OAS caused the maximum reduction in the nitrate uptake rate. Prosser et al. (2001) suggested that OAS accumulates under S-deprivation, which could possibly have a feedback inhibition on the uptake and assimilation of N. It could be enunciated that, due to a regulatory interaction between the N and S assimilatory pathways, S-deprivation resulted in decreased formation of sulfide, which thereby reduced cysteine production and increased OAS accumulation (Table 1, Fig. 5c).

There were also apparent sulfur-stress effects on the activity of various enzymes of N-assimilatory pathway. A continuous decline in NR activity and its transcript was observed with increase in the duration of S-deprivation. A decline in NR transcripts in the S-deprived tobacco has been observed earlier (Migge et al. 2000). It is suggested that modulation of NR gene expression possibly represents an integral part of the strategy developed by plants to tolerate S-deficit. Accumulation of nitrate increased with increase in the duration of S-starvation in our study (Fig. 5a). Earlier reports have also shown large accumulation

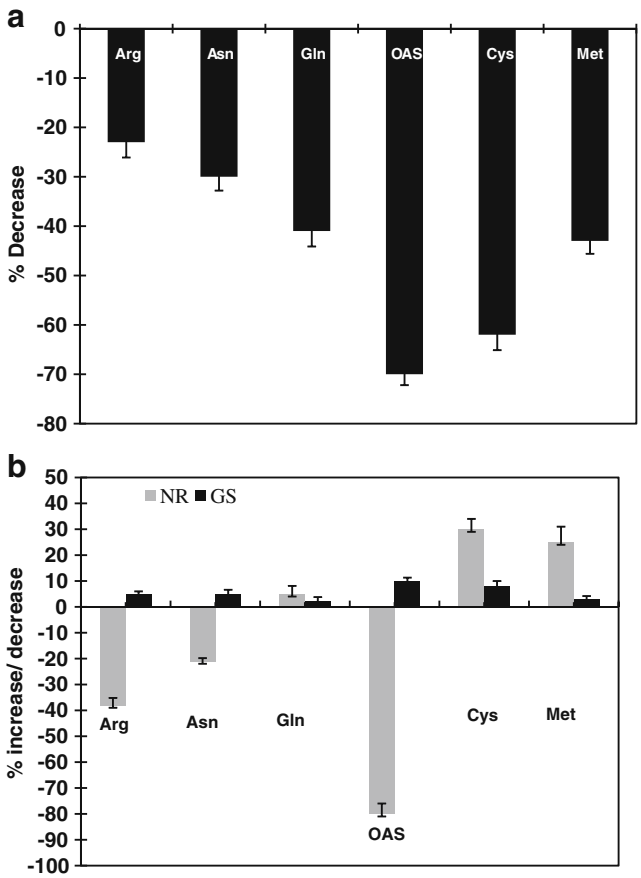

Fig. 7 Effect of various metabolites on nitrate uptake rate (**a**), activities of nitrate reductase and glutamine synthetase (**b**) in the leaves of rapeseed. Data are mean of five replicates and the *vertical bars* indicate standard error ($n=5$). Data are shown as percentage increase/decrease

of nitrate, particularly in the family Brassicaceae, under sub-optimal S-nutrition (McGrath and Zhao 1996; Koralewskaa et al. 2009). Amino acid accumulation during S-deficiency could be involved in the inhibition of NR activity. Our result also demonstrated an increase in the OAS content by many folds under S-deprivation condition. It appears that OAS accumulation under S-limiting conditions reduces NR activity, thereby diminishing NO_3^- assimilation in order to maintain constant levels among organic and S compounds. Restoration of S-supply (Exp. II) resulted in a complete recovery of NR activity in 5 days.

In the S-deprived plant, GS activity and its transcript was reduced continuously. GS transcripts have also been reported at low levels in N-starved maize seedlings (Redinbaugh and Campbell 1993). Restoration of supply of N and S markedly restored the GS activity. Accumulation of Gln, Asn, and Arg in S-starved plants suggests that the decline in GS activity could be due to a feedback regulation by the accumulated amino acids. Studies on *Arabidopsis* (Scheible et al. 2004) and Medicago (Ruffel et al. 2008) have indicated that many genes of NO_3^- assimilation are under feedback repression by downstream N metabolites.

Plant responses to S nutritional stress are mediated by some signals. Concentrations of metabolites, which reportedly act as signals in the pathway under S nutrition, were investigated. Amino acids serve as precursors for a large array of metabolites with multiple functions in plant growth and response to various stresses (Less and Galili 2008). Methionine has been reported to act as a signal of S stress (Chiaiese et al. 2004). Under S-deprived conditions, accumulation of Arg, Asn, and Gln was observed with a decline in the level of sulfur amino acids Cys and Met. This means that S-starvation is associated with down-regulation of sulfur amino acid biosynthesis. Sulfur deficiency induces changes in the biochemical composition including the relative abundance of Arg, Asn, and OAS, in plants (Brunold 1993). Accumulation of Gln and Asn was observed in S-deprived barley roots (Karmoker et al. 1991). Arg increased from 2% to 36% of the total amino acids in response to insufficient S supply in the pot-grown sugar beet (Thomas et al. 2000). According to Amancio et al. (1997), S-deprivation in cultured maize cells caused significant increase in the pool size of Gln. S-deficiency causes accumulation of nitrogenous amino acids in plants.

Since Gln, Asn, and Arg represent important N transport compounds and soluble N reserve substances, accumulation of these three amino acids reflects the removal of surplus reduced N in order to maintain the N and S balance. It was suggested that the internal pools of N metabolites such as amino acids within plants may indicate N status by providing a signal that can regulate N uptake and assimilation by the plant. In an experiment on S-starved *Arabidopsis*, levels of sulfur-containing metabolites such as Cys declined, but Met content remained unchanged in plants (Nikiforova et al. 2005). They also found accumulation of OAS as the Cys precursor, and increasing levels of its upstream metabolites, Ser, and Gly, which reflect rechanneling of assimilated carbon. In some earlier reports, Cys was found to decline under S-stress in oilseed rape and *Arabidopsis* (Lencioni et al. 1997; Nikiforova et al. 2003). However, contrary to this result, Hirai et al. (2003) found no change in Cys content in *Arabidopsis* plants under the same stress. In experiments with barley roots, Karmoker et al. (1991) showed that accumulation of Gln and Asn after S deprivation was not only attributed to effects on protein synthesis, but also to a slower incorporation and/or transport of recently absorbed N, so that the export of reduced N was diminished. As Cys becomes increasingly scarce during S-deprivation, its conversion into cystathionine during Met biosynthesis was strongly impaired.

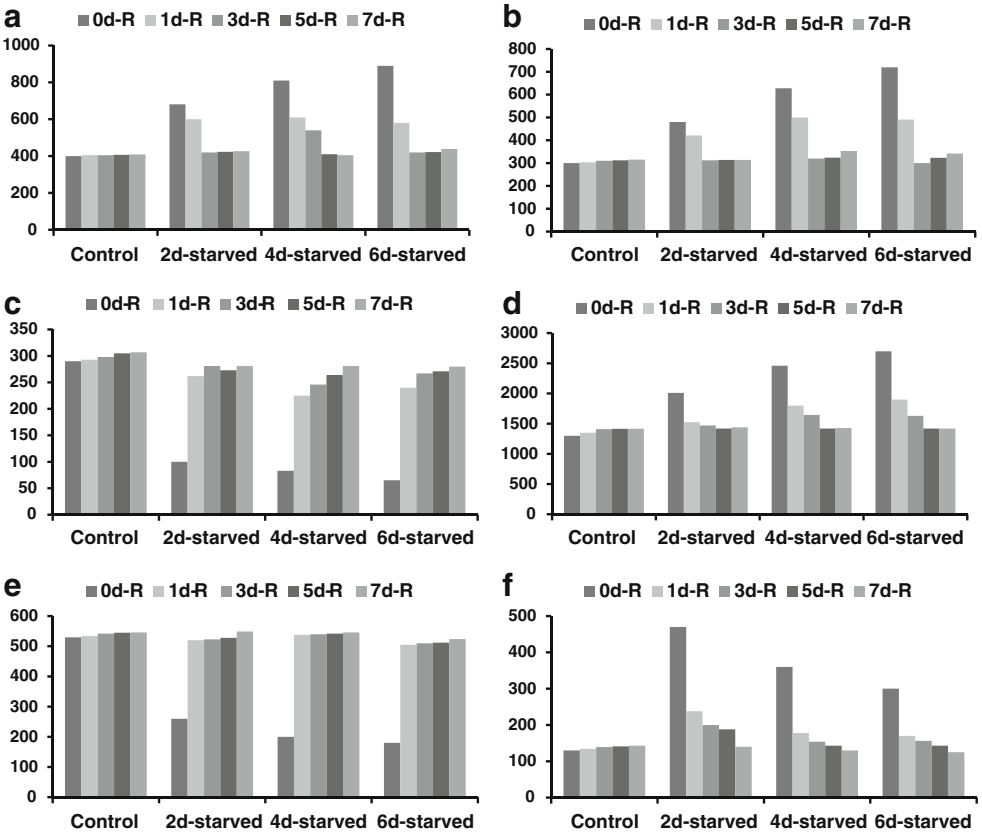

Fig. 8 Effect of duration of restoration of S-supply on the contents of arginine (**a**), asparagine (**b**), cysteine (**c**), glutamine (**d**), methionine (**e**), and serine (**f**) in S-starved leaves of rapeseed. Amino acid contents are expressed as nanograms per gram FW. In the legend, *0d-R*, *1d-R*, *3d-R*, *5d-R*, and *7d-R* refer to the restoration of S-supply for 0, 1, 3, 5, and 7 days, respectively. Control refers to the set of plants grown on complete nutrient medium. Data are mean of five replicates

Therefore, Asn is not used in Met biosynthesis and may then be converted into Asn. This could be a reason for Asn accumulation. Sulfur nutritional deficiency has previously been reported to have a stronger negative effect on free cysteine than on free methionine concentration in pea seeds (Macnicol 1983). A striking result of this study was the consistently increased levels of OAS with increase in the S-stress. In earlier reports also, OAS has been reported to vary with plant sulfur nutrition. OAS was elevated in siliques of *Arabidopsis thaliana* plants grown with high N and low S (Kim et al. 1999). It has been proposed that, during S-starvation, an unknown primary signal stimulates a variation in cytosolic calcium concentration, which results in desensitization of cytoplasmic serine acetyl transferase (SAT) to feedback inhibition by cysteine, leading to over-accumulation of OAS (Saito 2000). Thus, the primary signal could be OAS acting in the metabolic coupling between N and S. The shortage of S-amino acids caused by S-starvation brings in a general reduction in protein synthesis. This points to a delicate poise between protein synthesis and inorganic S supply. S deficiency increases proteolysis and reduction in rate of protein synthesis due to absence of key S-containing amino acids, Cys, and Met. The decline in contents of amino acids due to low S supply is mainly a consequence of the linkage of N and S metabolism at the level of protein synthesis.

From the present study, it is suggested that there is a significant interdependence between nitrogen and sulfur nutrition in *Brassica rapa* L. plants, illustrated clearly by the apparent change in nitrate uptake and activities of N-assimilatory enzymes due to change in S nutrition. Thus, the regulation of N metabolism in plants is very much responsive to nutritional signals. With the change in the S nutrition, the level of many downstream metabolites also changed. OAS turned out as a significant signal metabolite playing a major role in the regulation and coupling of N and S metabolism. The role and interactions of downstream metabolites could be factored into strategies for optimizing N response and nitrogen use efficiency. This may help in developing a nutrient management technology for optimization of crop productivity.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Abrol YP, Raghuram N, Sachdev MS (2007) Agricultural nitrogen use and its environmental implications. IK International, New Delhi

Ahmad A, Abdin MZ (1999) NADH: nitrate reductase and NAD(P)H: Nitrate reductase activities in mustard seedlings. Plant Sci 143:1–8

Ahmad A, Abdin MZ (2000) Photosynthesis and its related physiological variables in the leaves of Brassica genotypes as influenced by sulphur fertilization. Physiol Plant 110:144–149

Ahmad A, Khan I, Anjum NA, Abrol YP, Iqbal M (2005) Role of sulphate transporter systems in sulphate efficiency of mustard genotypes. Plant Sci 169:842–846

Amancio S, Clarkson DT, Diogo E, Lewis M, Santos H (1997) Assimilation of nitrate and ammonium by sulphur deficient *Zea mays* cells. Plant Physiol Biochem 35(1):41–48

Bates B, Kundzewicz ZW, Wu S et al (2008) Climate change and water (Technical Paper of the Intergovernmental Panel on Climate change), IPCC Secretariat. (http://www.ipcc.ch/meetings/session28/doc13.pdf)

Batista K, Monteiro FA (2007) Nitrogen and sulphur in Marandu grass: relationship between supply and concentration in leaf tissues. Sci Agric 64:44–51

Brunold C (1993) Regulatory interactions between sulfate and nitrate assimilation. In: DeKok LJ, Stulen I, Rennenberg H, Brunold C, Rauser WH (eds) Sulphur nutrition and sulphur assimilation in higher plants. SPB Academic Publishing, The Hague, pp 61–75

Buchner P, Stuiver CE, Westerman S et al (2004) Regulation of sulphate uptake and expression of sulfate transporter genes in *Brassica oleracea* as affected by atmospheric H_2S and pedospheric sulphate nutrition. Plant Physiol 136:3396–3408

Campbell WH, Samarrelli J (1978) Purification and kinetics of higher plant NADH: nitrate reductase. Plant Physiol 61:611–616

Chesnin L, Yien CH (1950) Turbidimetric determination of available sulphates. Soil Sci Soc Amer Proc 15:149–151

Chiaiese P, Ohkama-Ohtsu N, Molvig L et al (2004) Sulphur and nitrogen influence the response of chickpea seeds to an added, transgenic sink for organic sulphur. J Exp Bot 55(404):1889–1901

Droux M, Ruffet ML, Douce R, Job D (1998) Interactions between serine acetyltransferase and O-acetylserine (thiol) lyase in higher plants. Structural and kinetic properties of the free and bound enzymes. Eur J Biochem 255:235–245

FAO (2007) Current world fertilizer trends and outlook to 2010/11. Food and Agriculture Organization of the United Nations, Rome

Fowler MW, Jessup W, Sarkissian GS (1974) Glutamate synthetase type activity in higher plants. FEBS Lett 46:340–342

Fujiwara T, Matoh T (2009) Plant Nutrition- roots of life for fundamental biology and better crop production. Plant Cell Physiol 50(1):2–4

Grover HL, Nair TVR, Abrol YP (1978) Nitrogen metabolism of the upper three leaf blades of wheat at different soil nitrogen levels. I: Nitrate reductase activity and content of various nitrogenous constituents. Physiol Plant 42:287–292

Gruber N, Galloway JN (2008) An Earth-system perspective of the global nitrogen cycle. Nature 451:293–296

Gupta SC, Beevers L (1984) Synthesis and degradation of nitrite reductase in pea leaves. Plant Physiol 75:251–252

Hirai MY, Fujiwara T, Awazuhara M, Kimura T, Noji M, Saito K (2003) Global expression profiling of sulfur-starved *Arabidopsis* by DNA macroarray reveals the role of O-acetyl-L-serine as a general regulator of gene expression in response to sulfur nutrition. Plant J 33:651–663

Hunt J, Seymour DJ (1985) Method for measuring nitrate-nitrogen in vegetables using anion-exchange high performance liquid chromatography. Analyst 110:131–133

Ida S, Morita Y (1973) Purification and general properties of spinach leaf nitrite reductase. Plant Cell Physiol 14:661–671

Karmoker JL, Clarkson DT, Saker LR, Rooney JM, Purves JV (1991) Sulphate deprivation depresses the transport of nitrogen to the xylem and the hydraulic conductivity of barley (*Hordeum vulgare* L.) roots. Planta 185:269–278

Kim H, Hirai MY, Hayashi H, Chino M, Naito S, Fujiwara T (1999) Role of O-acetyl-L-serine in the co-ordinated regulation of the expression of a soybean seed storage-protein gene by sulphur and nitrogen nutrition. Planta 209:282–289

Koralewskaa A, Buchnerb P, Stuivera CEC et al (2009) Expression and activity of sulfate transporters and APS reductase in curly kale in response to sulfate deprivation and re-supply. J Plant Physiol 166:168–179

Lencioni L, Ranieri A, Fergola S, Soldatini GF (1997) Photosynthesis and metabolic changes in leaves of rapeseed grown under long-term sulphate deprivation. J Plant Nutr 20:405–415

Less H, Galili G (2008) Principle transcriptional programmes regulating plant amino acids metabolism in response to abiotic stresses. Plant Physiol 147:316–330

Macnicol PK (1983) Differential effect of sulphur deficiency on the composition of the aminoacyl-tRNA and free amino acid pools of the developing pea seed. FEBS Lett 156:55–57

Mcgrath SP, Zhao FJ (1996) Sulphur uptake, yield responses and the interactions between nitrogen and sulphur in winter oilseed rape (*Brassica napus*). J Agri Sci 126:53–62

McNally SF, Hirel B, Gadal P, Mann AF, Stewart GR (1983) Glutamine synthetases of higher plants-Evidence for a specific isoform content related to their possible physiological role and their compartmentation within the leaf. Plant Physiol 72:22–25

Migge A, Bork C, Hell R, Becker TW (2000) Negative regulation of nitrate reductase gene expression by glutamine or asparagine accumulating in leaves of sulphur-deprived tobacco. Planta 211:587–595

Millard P, Sharp GS, Scott NM (1985) The effect of sulphur deficiency on the uptake and incorporation of nitrogen in ryegrass. J Agri Sci 105:501–504

Mohanty B, Fletcher JS (1980) Ammonium influence on nitrogen assimilating enzymes and protein accumulation in suspension cultures of Paul's scarlet rose. Physiol Plant 48:453–459

NAAS (2005) Policy options for efficient nitrogen use. National Academy of Agricultural Sciences, New Delhi

Nikiforova V, Freitag J, Kempa S, Adamik M, Hesse H, Hoefgen R (2003) Transcriptome analysis of sulfur depletion in *Arabidopsis thaliana*: interlacing of biosynthetic pathways provides response specificity. Plant J 33:633–650

Nikiforova VJ, Kopka J, Tolstikov V, Fiehn O, Hopkins L, Hawkesford MJ, Hesse H, Hoefgen R (2005) Systems rebalancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of *Arabidopsis* plants. Plant Physiol 138:304–318

Olivares J, Martin E, Recalde-Martinez L (1983) Effect of nitrogen and sulphur application and seed inoculation with *Rhizobium leguminosarum* on the yield of beans (*Vicia faba*) in field trials. J Agri Sci 100:149–152

Prosser IM, Purves JV, Saker LR, Clarkson DT (2001) Rapid disruption of nitrogen metabolism and nitrate transport in spinach plants deprived of sulphate. J Exp Bot 52:113–121

Redinbaugh MG, Campbell WH (1993) Glutamine synthetase and ferredoxin-dependent glutamate synthase expression in the maize (*Zea mays*) root primary response to nitrate. Plant Physiol 101: l249–l1255

Rhodes D, Rendon GA, Stewart GR (1975) The control of glutamine synthetase level in *Lemna minor* L. Planta 125:201–211

Richardson D, Felgate H, Watmough N, Thomson A, Baggs E (2009) Mitigating release of the potent greenhouse gas N_2O from the nitrogen cycle - could enzymic regulation hold the key? Trends in Biotech 27(7):388–397

Ruffel S, Freixes S, Balzergue S et al (2008) Systemic signaling of the plant nitrogen status triggers specific transcriptome responses depending on the nitrogen source in *Medicago truncatula*. Plant Physiol 146:2020–2035

Saito K (2000) Regulation of sulphate transport and synthesis of sulphur-containing amino acids. Curr Opin Plant Biol 3:188–195

Scheible WR, Morcuende R, Czechowski T et al (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. Plant Physiol 136:2483–2499

Tallec T, Diquelou S, Fauveau C, Bataille MP, Ourry A (2008a) Effects of nitrogen and sulphur gradients on plant competition, N and S use efficiencies and species abundance in a grassland plant mixture. Plant Soil 313:267–282

Tallec T, Diquelou S, Lemauviel S, Cliquet JB, Lesuffleur F, Ourry A (2008b) Nitrogen:sulphur ratio alters competition between *Trifolium repens* and *Lolium perenne* under cutting: Production and competitive abilities. Eur J Agron 29:94–101

Thomas SG, Bilsborrow PE, Hocking TJ, Bennett J (2000) Effect of sulphur deficiency on the growth and metabolism of sugar beet (*Beta vulgaris* cv Druid). J Sci Food Agric 80:2057–2062