Pak. J. Bot., 46(5): 1789-1795, 2014.

MECHANISMS OF NITROGEN RE-DISTRIBUTION IN RESPONSE TO ENZYME ACTIVITIES AND THE EFFECTS ON NITROGEN USE EFFICIENCY IN *BRASSICA NAPUS* DURING LATER GROWTH STAGES

ZHENHUA ZHANG[1,2*Δ], WENMING WANG[1Δ], CHUNYUN GUAN[3], QIANG LIU[1,2], XIANGMIN RONG[1,2], YONGLIANG HAN[1,2], JIANGWEI PENG[1,2], QIONG LIAO[1], YING YU[1] AND HAIXING SONG[1,2*]

[1]*College of Resources and Environment Sciences, Hunan Agricultural University, Changsha 410128, China;*
[2]*National Engineering Laboratory for Efficient Utilization of Soil and Fertilizer Resources; College of Resources and Environment, Hunan Agricultural University, Changsha 410128, China;*
[3]*National Center of Oilseed Crops Improvement, Hunan Branch, Changsha 410128, China*
[Δ]*These authors contributed equally to this work*
[*]*Corresponding author's e-mail: zhzh1468@163.com, shx723@163.com, Tel: +86-15873141718*

Abstract

In order to investigate the mechanisms of nitrogen (N) re-distribution in response to proteolytic enzyme (PE), glutamine synthetase (GS) and glutamate synthetase (GOGAT) activities and the effects on N use efficiency (NUE). Two oilseed rape genotypes were grown in sand culture in a greenhouse under normal (15.0 mmol L^{-1} NO_3^-) and limited-N (7.5 mmol L^{-1} NO_3^-) levels. Isotope (^{15}N) labeling and enzyme inhibitors against the PE, GS and GOGAT enzymes were used. We found that, when the two genotypes were subjected to specific inhibitors of PE, GS, and GOGAT, the activities of these enzymes were significantly decreased, resulting in reduced N re-distributed from leaf to grain, as well as reduced NUE. L-glutamine and free amino acid contents in the phloem sap were primarily influenced by PE and GS activities, whereas grain yield was primary regulated by GOGAT activity during the later growth stages. These findings suggest that PE, GS, and GOGAT are key enzymes for the regulation of N re-distribution in plant tissues during later growth stages, with grain yield and NUE of oilseed rape being positively regulated by PE, GS and GOGAT activities.

Key words: Nitrogen (N) redistribution, N use efficiency, Proteolytic enzyme (PE), Glutamine synthetase (GS), Glutamate synthase (GOGAT)

Introduction

Annual consumption of nitrogen (N) fertilizer in China has been estimated to be as high as 25 million tons (pure N) (Shen *et al*., 2003). However, the law of diminishing returns from N fertilizer, i.e., inhibited increase or reduction of crop yield if N fertilizer is excessively applied, is a serious concern (Shen *et al*., 2003; Rahimizadeh *et al*., 2010). Hence, there is a need for better N use efficiency (NUE) through the regulation of plant physiological mechanisms. In recent years, it has become a hot topic in plant nutrition research to improve NUE in plants (Zhang *et al.,* 2010). NUE has several definitions, but is generally defined as an index of production per unit of N taken up (Hirel *et al.,* 2001; Gallais *et al.,* 2004). N is generally absorbed, assimilated, and accumulated in plant tissues during the vegetative growth stage, and can be re-distributed from vegetative to reproductive organs during the reproductive growth stage (Rossato *et al.,* 2001; Andersson *et al.,* 2005; Malagoli *et al.,* 2005). The proportion of N re-distributed from the leaves and stem to the grain is > 60% in wheat (Palta *et al.,* 1995). The activities of proteolytic enzyme (PE), glutamine synthetase (GS), and glutamate synthetase (GOGAT) are positively correlated with the proportion of the re-distributed N during the reproductive growth stage (Hirel *et al.,* 2001; Corruzzi, 2003; Thomas *et al.,* 2007). The activity of nitrate reductase (NR) is induced by NO_3^- (Cookson *et al.,* 2005) and is primarily regulated by phosphorylation (Garcia-Mata *et al.,* 2003). In general, the assimilation of nitrate by means of NO_3^- reduction within the cytoplasm of leaves is adequate to support healthy plant growth (Cookson *et al.,* 2005). N compounds (protein molecules and polypeptides) can be degraded into small organic molecules (amino acid and peptide) by PE, and re-distributed into destination organs through the phloem distribution system during later growth stages, with the small organic molecules being subsequently assimilated into amino acids via the GS/GOGAT assimilation cycle (Lea *et al.,* 2004). Therefore, PE, GS, and GOGAT in plant tissues are all involved in N re-distribution during later growth stages, and NUE can be regulated via these physiological mechanisms (Daniel-Vedele *et al.,* 2010). However, the mechanisms of N re-distribution in response to the activities of PE, GS and GOGAT remain obscured, and effects of PE, GS, and GOGAT activities on the NUE of crop species require further study. Here, two oilseed rape genotypes were cultivated in a greenhouse under normal and N-limited conditions. Isotope (^{15}N) labeling and specific inhibitors of PE, GS, and GOGAT activities were used to investigate the mechanisms of N re-distribution in response to the activities of PE, GS, and GOGAT and the consequent effects on NUE.

Materials and Methods

Plant materials cultivation: The experiments were conducted at the Resources and Environment Department of Hunan Agricultural University in Hunan province P.R. China, which is the primary growing area of oilseed rape in China. Two genotypes of oilseed rape (X-1 and X-2) were used as plant materials. The seeds of oilseed rape were provided by the Hunan Sub-center of

the Improvement Center of the National Oil Crop, and vernalization was required during seed germination. Specific inhibitors of PE, GS and GOGAT activities and an ^{15}N labeling method were used during the experiment. Plants were cultivated in a brown plastic bowl (diameter and height were 30 cm × 30 cm) with sand culture and complete Hoagland nutrients solution; water and dilute hydrochloric acid were used to clean the growth matrix. Two N levels (normal, 15.0 mmol L^{-1} and N-limited, 7.5 mmol L^{-1}, NO_3^-) were applied in this study. The nutrient solution was renewed every 5 days. The composition of Hoagland nutrients solution was: KNO_3 5 mmol L^{-1}, KH_2PO_4 1 mmol L^{-1}, $MgSO_4$ 7 mmol L^{-1}, $Ca(NO_3)_2{\cdot}4H_2O$ 5 mmol L^{-1}, Fe-EDTA 3 mmol L^{-1}, B 0.5 mg L^{-1}, Mn 0.5 mg L^{-1}, Zn 0.05 mg L^{-1}, Cu 0.02 mg L^{-1}, Mo 0.01 mg L^{-1}. $Ca(^{15}NO_3)_2$ and $K^{15}NO_3$ (Shanghai Chemical Engineering Corporation Research Institute) with ^{15}N excess of 20.28% were used as N resources for the labeling treatment. The inhibitors used against PE, GS and GOGAT are cystatin (Weeda *et al.,* 2010), methionine sulfoximine (MSX) (Logusch *et al.,* 1991) and azaserine (Cordoba *et al.,* 2003) respectively. All inhibitor chemicals were purchased from Sigma Aldrich. The inhibitor concentrations used were 0.2 mmol L^{-1} (cystatin), 73 μmol L^{-1} (MSX) and 0.16 mmol L^{-1} (azaserine). PE-H: inhibitor of PE activity treatment, GS-H: inhibitor of GS activity treatment, GOGAT-H: inhibitor of GOGAT activity treatment.

Experiment design: The seeds were germinated on September 22, 2010, transplanted on November 1st, 2010 at a density of one plant per bowl, and harvested on May 10th, 2011. ^{15}N was labeled at the silique stage (April 15-23, 2011, 9 days total) and sampled at the end of the labeling treatment (April 24th, 2011). To distinguish between re-distributed and adsorbed N, the other labeled plants which was ^{15}N labeled at the silique stage (April 15-23, 2011, 9 days total) were transplanted (April 24th, 2011) into sand culture without ^{15}N nutrient and sampled at harvesting stage (May 10th, 2011). A random study design with 3 replications for each treatment was used.

Inhibitor treatments were initiated at silique stage (April 15-23, 2011, 9 days total), with inhibitor solutions sprayed evenly onto leaves (cystatin and MSX) and siliques (azaserine) at 10 am every day during the silique stage. Pure water was evenly sprayed onto leaves and siliques as a control treatment. Samples were taken on April 24, 2011 to measure the activities of PE, GS and GOGAT, as well as the content of free amino acid and L-glutamine in the phloem sap. Sampling at the harvesting stage was done on May 10th, 2011 and the grain yield, dry biomass, and N content of the plant tissues was measured. To precisely calculate total N, fallen leaves were collected during plant senescence.

Enzyme activity measurement: PE was extracted from 0.2g fresh weight (FW) leaves using casein fluorescein isothiocyanate (FITC)-casein as substrate (Sigma) (Kumar *et al.,* 1999). The assay was initiated by adding 300 μL of sample extract to 200 μL of potassium phosphate buffer (50 mmol L^{-1}, pH 6.5) containing 3.7 mm DTT and 500 μg FITC-casein. The reaction was incubated at 37°C in the dark for 90 min and terminated by adding 100 μL 50% (w/v) TCA to each sample. Protein and unreacted FITC-casein were pelleted (1610 g, 22 min, 4°C) and 350μL of the supernatant was transferred to 1 ml Tris buffer (1 mol L^{-1}, pH 8.0). The sample was vortexed and triplicate aliquots of 300μL were added to the wells of a 96-well white polystyrene flat-bottomed medium-binding microtitre plate (Corning Inc.). Emission (520 nm, excitation 500 nm) was measured on a fluorescence spectrophotometer (Varian Cary Eclipse, Varian Inc., Palo Alto, CA, USA). Protease activity was expressed as units' activity: amount of PE for 1 μmol tyrosine synthesis per minute (Weeda *et al.,* 2010).

For determination of GS activity, 0.2 g FW plant material was homogenized in 4 mL, 50 mmol L^{-1} MOPS-buffer (3-[N-Morpholino] propanesulfonic acid, pH 7.0), 1 mmol L^{-1} EDTA (ethylenediaminetetraacetic acid disodium salt), 1 mmol L^{-1} $MgCl_2$, and 1 mmol L^{-1} dithiothreitol at 4°C. Cell debris was removed via centrifugation for 10 min at 30000 ×*g*. Enzyme activity was measured via a transferase assay according to method described by Florencio and Vega (Florencio *et al.,* 1983) with slight modifications, i.e., the final volume used was 1.9 mL and the amount of glutamine was 130 μmoL (Johansson *et al.,* 1986).

Proteins were extracted from 0.2g FW grains and the enzyme activity of NADH-GOGAT was measured as previously described (Groat *et al.,* 1981), protease activity was expressed as unit activity: amount of GOGAT for 1 μmol R-glutamyl hydroxyl deficit acid reduction per minute.

Phloem sap collection and measurement: The oilseed rape plants were cut 4 cm above the ground level; a pre-weighed absorbent cotton ball was attached to the cut surface of above ground and covered with plastic film for 1 h. Next, the collected phloem sap was drawn from the cotton using a syringe.. The volume of the exudates was calculated from the increase in the weight of the cotton (Tang *et al.,* 2012). Free amino acid content was determined using the auto-sampler of an Agilent 1100 Series (Agilent Technologies. Atlanta, Georgia) high performance liquid chromatograph (HPLC) in accordance with the method of (Showler *et al.,* 2007), and the L-glutamine concentration was measured as previously described (Wang *et al.,* 2001).

Sampling and calculation: Whole plant samples were taken from different plant tissues after cleaning and drying. Biomass and grains were dried to a constant weight and measured. Dry samples were ground and sifted for isotope analysis. The concentration of N in the plant was measured using the Kjeldahl method (Kamprath *et al.,* 1982). The physiological parameters were calculated by the following formulas (Table 1):

Total N = plant dry biomass × N content
Harvest index = grain yield / plant dry biomass
NUE = dry biomass per plant / total N per plant

Table 1. Effects of inhibitor treatments on physiological parameters of oilseed rape during later growth stages.

Genotype	Treatment	Grain yield (g plant^{-1})	Dry biomass (g plant^{-1})	Total N (g plant^{-1})	Harvest index	NUE	NUE increased (%)
Normal N level							
X-1	Control	25.2 a	104.6 ab	1.11 b	0.24 a	94.20 a	
	PE-H	22.7 ab	110.9 a	1.53 ab	0.21 a	72.50 b	-23.0
	GS-H	23.7 ab	108.8 a	1.67 a	0.23 a	65.15 b	-30.8
	GOGAT-H	19.0 b	95.2 b	1.69 a	0.20 a	56.33 c	-40.2
X-2	Control	20.2 ab	121.2 a	1.28 a	0.19 b	94.69 a	
	PE-H	19.4 b	111.2 b	1.34 a	0.22 a	82.99 b	-12.4
	GS-H	19.3 b	113.3 ab	1.27 a	0.20 ab	89.18 b	-5.8
	GOGAT-H	17.1 c	97.4 c	1.15 a	0.18 b	84.65 b	-10.6
Limited N level							
X-1	Control	20.0 a	89.0 a	0.84 ab	0.23 a	120.29 a	
	PE-H	20.2 a	84.9 a	0.90 a	0.24 a	94.32 b	-21.6
	GS-H	18.7 a	83.5 a	0.74 b	0.25 a	99.45 b	-17.3
	GOGAT-H	17.0 b	76.4 a	0.72 b	0.22 a	106.04 b	-11.8
X-2	Control	17.8 a	92.1 a	0.77 a	0.19 b	119.55 a	
	PE-H	17.0 a	77.1 a	0.79 a	0.22 a	97.64 c	-18.3
	GS-H	16.2 a	88.5 a	0.80 a	0.20 ab	110.56 b	-7.5
	GOGAT-H	15.5 b	87.7 a	0.85 a	0.18 b	103.22 bc	-13.7

Note: PE-H: 0.2 mmol L^{-1} cystatin as PE inhibitor; GS-H: 73 μmol L^{-1} MSX as GS inhibitor; GOGAT-H: 0.16 mmol L^{-1} azaserine as GOGAT inhibitor. Under each N level, letters following the data indicate significant difference among treatments within a genotype ($p<0.05$)

Samples were pre-processed by semi-micro-determination (Brenna *et al.,* 1998). The excess ^{15}N in each plant tissue was measured using a continuous flow isotope mass spectrograph at the Institute of Genetics, Hebei Academy of Agricultural Sciences, P.R. China. The physiological parameters were calculated by the following formulas (Table 3):

Proportion (%) of redistributed N (absorbed at silique stage) in grain at the harvesting stage = (amount of accumulated ^{15}N in grains at the harvesting stage - accumulated amount of ^{15}N in grains at the end of the labeling treatment) / accumulated amount of ^{15}N per plant at the end of the labeling treatment × 100%

Amount (mg plant^{-1}) of redistributed N (absorbed at silique stage) in grain at the harvesting stage = proportion of re-distributed N in grains × amount of accumulated N per plant at the end of labeling treatment

Proportion (%) of redistributed N (absorbed at silique stage) in leaf at the harvesting stage = (amount of accumulated ^{15}N in leaf at harvesting stage - amount of accumulated ^{15}N in leaf at the end of labeling treatment) / amount of accumulated ^{15}N per plant at the end of labeling treatment × 100%

Amount (mg plant^{-1}) of re-ddistributed N (absorbed at silique stage) in leaf at the harvesting stage = proportion of redistributed N in leaf × amount of accumulated N per plant at the end of the labeling treatment

The partitioning of absorbed N was calculated from the excess ^{15}N in each of the plant tissues combined with the calculated total plant N absorption. This was based on the assumption that unlabeled N from the sand culture was absorbed and assimilated in the different plant tissues in a way similar to that of labeled N.

The response sensitivity of grain yield to enzyme activity is the change in grain yield per unit of enzyme activity (Fig. 1). The response sensitivity of the amount of redistributed N to enzyme activity is the change in amount of redistributed N per unit of enzyme activity (Fig. 2). The response sensitivity of free amino acid content to enzyme activity is the change in free amino acid content per unit of enzyme activity (Fig. 3). The response sensitivity of L-glutamine content to enzyme activity is the change in L-glutamine content per unit of enzyme activity (Fig. 3).

Statistical analysis: Two-way ANOVA and the *t*-test ($p<0.05$) were used to compare data for control and inhibitor treatments using the SPSS (Statistical Product and Service Solutions V13.0, USA) software.

Results

Responses of physiological parameters to inhibitor treatment: The grain yield per plant of inhibitor-treated plants was lower than that of control-treated plants; the grain yield per plant of the GOGAT-H-treated plants were significantly lower than that of control plants, under both the normal and N-limited conditions (Table 1). The NUE of inhibitor-treated plants was significantly lower than that of control plants. Compared with control plants, the NUE of plants subjected to PE-H, GS-H and GOGAT-H treatments decreased by 18.8% (average value of 23.0, 12.4, 21.6, and 18.3%), 15.4% (average value of 30.8, 5.8, 17.3, 7.5%) and 19.1% (average value of 40.2, 10.6, 11.8, and 13.7%), respectively (Table 1). The response sensitivities of grain yield to GOGAT activity were 49.1% and 69.8% higher than that of GS and PE, respectively, and these differences were significant (Fig. 1).

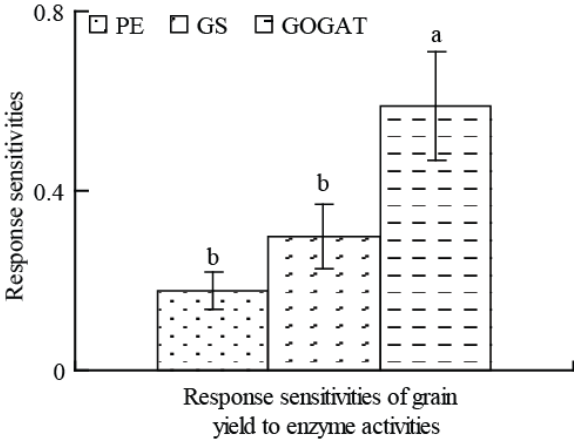


Fig. 1. Response sensitivities of grain yield to changes of PE, GS and GOGAT activities. Error bars indicate SE (n=3), different letters on the graphs indicate that the results are significantly different.

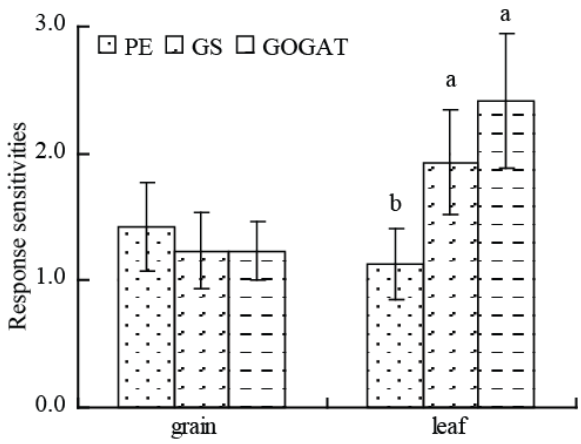
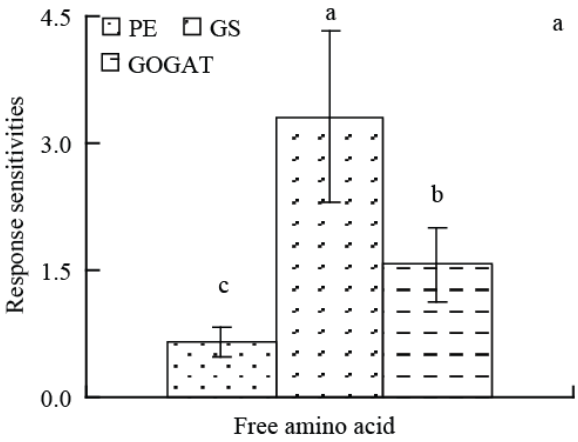


Fig. 2. Response sensitivities of N re-distribution amount to changes of PE, GS and GOGAT activities. Error bars indicate SE (n=3), no letters on the graphs indicate that the results are not significantly different.



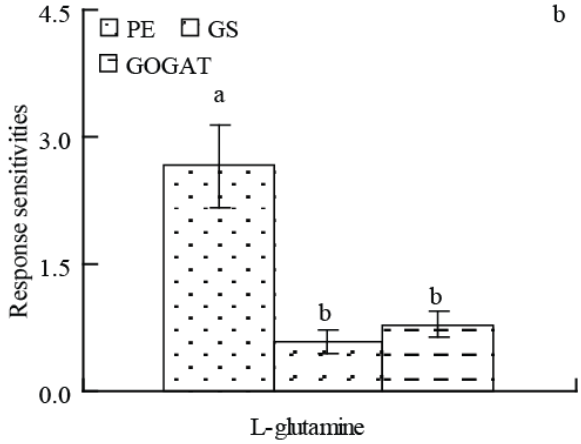


Fig. 3. Response sensitivities of free amino acid content (a) and L-glutamine content (b) to changes of PE, GS and GOGAT activities. Error bars indicate SE (n=3).

Responses of PE, GS and GOGAT activities to inhibitor treatments: PE, GS and GOGAT activities were significantly inhibited by PE-H, GS-H and GOGAT-H treatments respectively, but were not significantly influenced by other inhibitors under the two N application levels. Compared with control plants, PE activities of PE-H treated plants decreased by 19.7% and 28.3%, GS activities of GS-H treated plants decreased by 24.0% and 18.3%, and GOGAT activities of GOGAT-H treated plants decreased by 23.9% and 29.8% at normal and N-limited levels, respectively (Table 2).

Responses of N re-distribution proportion and amount to inhibitor treatments: The proportion of re-distributed N in grains under inhibitor treatments was lower than that of the control treatment (Table 3). Whereas the amount of re-distributed N in grains under inhibitor treatments was significantly lower than that of plants under the control treatment. Compared with controls, the amount of re-distributed N in grain decreased by 28.4% (average value of 44.2, 26.3, 20.5, and 22.7%) under PE-H treatment, decreased by 24.3% (average value of 18.5, 13.6, 39.5, and 25.4%) under GS-H treatment, and decreased by 31.8% (average value of 19.2, 34.8, 51.0, and 22.2%) under GOGAT-H treatment.

However, proportion of re-distributed N and the amount of N in leaves under inhibitor treatments were relatively higher than that in control plants (Table 3). Compared with control treatment, amount of re-distributed N in leaf increased by 34.0% (average value of 25.1, 2.4, 10.3, and 98.0%) under PE-H treatment, increased by 40.3% (average value of 3.4, 43, 22.8, and 91.9%) under GS-H treatment, and increased by 39.3% (average value of 34.3, 7.3, 10.7, and 104.7%) under GOGAT-H treatment.

The response sensitivities of amount of re-distributed N to changes in PE, GS and GOGAT activities in grain were almost identical. However, the response sensitivities of amount of re-distributed N to changes in GS and GOGAT activities in leaf were significantly higher than that of PE (Fig. 2).

Table 2. Effects of inhibitor treatments on activities of PE, GS and GOGAT in oilseed rape during later growth stages.

Enzyme	Treatment	Normal N level		Increased (%)	Limited N level		Increased (%)
		X-1	X-2		X-1	X-2	
PE	Control	9.53 a	6.83 a		15.08 a	9.36 a	
	PE-H	8.13 b	5.00 b	-19.7	12.46 b	5.06 b	-28.3
	GS-H	9.43 a	6.42 a		13.87 ab	8.89 a	
	GOGAT-H	9.53 a	6.80 a		14.11 a	8.17 ab	
GS	Control	4.18 a	2.24 a		5.16 a	3.14 a	
	PE-H	4.03 a	2.62 a		4.67 a	3.55 a	
	GS-H	3.18 b	1.70 b	-24.0	4.29 b	2.49 b	-18.3
	GOGAT-H	3.80 a	2.17 ab		5.29 a	3.49 a	
GOGAT	Control	1.45 a	0.52 a		0.52 a	0.44 a	
	PE-H	1.47 a	0.49 a		0.31 bc	0.43 a	
	GS-H	1.45 a	0.46 a		0.34 b	0.45 a	
	GOGAT-H	1.26 b	0.24 b	-23.9	0.33 c	0.37 b	-29.8

Note: For each enzyme, letters following the data indicate significant difference among treatments within a genotype ($p<0.05$)

Table 3. Effects of inhibitor treatments on re-distribution proportion and amount of N (absorbed at silique stage) in grain and leaf of oilseed rape during later growth stages.

Genotype	Treatment	N redistribution in grain			N redistribution in leaf		
		Proportion (%)	Amount (mg plant ^{1})	Increased (%)	Proportion (%)	Amount (mg plant ^{1})	Increased (%)
Normal N level							
X-1	Control	68	711.4 a		80	34.9 b	
	PE-H	56	396.8 c	-44.2	84	43.7 a	25.1
	GS-H	65	580.0 b	-18.5	86	36.1 ab	3.4
	GOGAT-H	46	575.0 b	-19.2	87	46.9 a	34.3
X-2	Control	53	458.7 a		82	39.5 b	
	PE-H	42	338.1 b	-26.3	84	40.4 b	2.4
	GS-H	49	396.3 b	-13.6	85	56.4 a	43.0
	GOGAT-H	42	298.9 c	-34.8	88	42.6 b	7.3
Limited-N level							
X-1	Control	42	299.4 a		91	10.0 b	
	PE-H	37	237.9 b	-20.5	90	11.0 a	10.3
	GS-H	32	181.0 c	-39.5	91	12.2 a	22.8
	GOGAT-H	28	146.8 d	-51.0	93	11.0 a	10.7
X-2	Control	32	198.0 a		72	3.4 b	
	PE-H	29	153.1 b	-22.7	83	6.8 a	98.0
	GS-H	29	147.7 b	-25.4	89	6.6 a	91.9
	GOGAT-H	29	154.0 b	-22.2	90	7.4 a	104.7

Note: Under each N level, letters following the data indicate significant difference among treatments within a genotype ($p<0.05$)

Table 4. Effects of inhibitor treatments on free amino acid and L-glutamine contents in phloem sap of oilseed rape during later growth stages.

Treatment	L-glutamine		Free amino acid	
	Content (μg mL^{-1})	Increased (%)	Content (μg mL^{-1})	Increased (%)
Control	41.25 b		5.43 b	
PE-H	12.75 d	-69.1	4.51 c	-16.9
GS-H	36.25 c	-12.1	6.28 a	15.7
GOGAT-H	51.25 a	24.2	4.97 c	-8.5

Note: Increased percentage = (content in inhibitor treatment □ content in control) / content in control × 100%. Different letters after content data indicate significant difference among treatments at $p<0.05$

Responses of free amino acid and L-glutamine contents in phloem sap to inhibitor treatments: The L-glutamine and free amino acid contents of plants under the PE-H treatment decreased by 69.1% and 16.9%, respectively. In GS-H-treated plants, the L-glutamine content decreased by 12.1%, but the free amino acid content increased by 15.7%. In GOGAT-H treated plants, the L-glutamine content increased by 24.2%, but the free amino acid content decreased by 8.5% (Table 4).

The response sensitivity of free amino acid content to GS activity was significantly higher than that to PE and GOGAT activity. The response sensitivity of L-glutamine content to PE activity was significantly higher than that to GS and GOGAT activity (Fig. 3).

Discussion

Physiological mechanisms of PE, GS and GOGAT enzymes: Generally, PE is responsible for the degradation of organic compounds into small organic molecules in plant tissues. These small organic molecules can then be re-distributed into other plant organs via the phloem, which is the primary transport system for N re-distribution in plant tissues during later growth stages (Weeda *et al.,* 2010). These small organic molecules are then used to synthesize glutamine by GS (Thomas *et al.,* 2007; Logusch *et al.,* 1991), and the glutamine was synthesized into amino acid (glutamate) by GOGAT (Cordoba *et al.,* 2003). Our findings that PE activity is significantly inhibited by PE-H treatment (Table 2) and that L-glutamine and free amino acid contents in phloem sap of PE-H treated plants are significantly decreased (Table 4) may be attributed to the inefficient degradation of organic compounds into small organic molecules, which could not be transported via the phloem sap (Tables 2, 4). In addition, inhibition of GS activity resulted in decreased L-glutamine content (but increased free amino acid content) in the phloem sap (Tables 2 and 4). The activity of GOGAT was inhibited by the azaserine inhibitor, therefore the amino acid synthesized ability of GOGAT was inhibited by the azaserine inhibitor, and the accumulation amount of amino acid in the phloem sap was decreased (Cordoba *et al.,* 2003). This can also account for why free amino acid content in the phloem sap was significantly decreased and L-glutamine content was significantly increased under GOGAT-H treatment (Table 4). Free amino acid and L-glutamine content in the phloem sap was primary influenced by GS (Fig. 3a) and PE activities (Fig. 3b).

Response mechanisms of N re-distribution ability to PE, GS and GOGAT activities: Crop growth can be generally divided into vegetative and reproductive growth stages (Andersson *et al.,* 2005). N is absorbed, assimilated, and accumulated in plant tissues during the vegetative growth stages and can be subsequently re-distributed from vegetative to reproductive organs during later growth stages (Malagoli *et al.,* 2005). PE, GS, and GOGAT are key enzymes involved in N re-distribution during the later plant growth stages (Thomas *et al.,* 2007). Here we show that, the re-distribution amount of N in grain was significantly decreased, and the re-distribution amount of N in leaf was relatively increased (Table 3), when PE, GS, and GOGAT activities were inhibited (Table 2). We hypothesize that organic compounds cannot be transported into the phloem sap when PE activity is inhibited, and that less N can be re-distributed into the siliques when leaf-GS activity and silique-GOGAT activity are inhibited (Table 2). Our findings suggest that PE, GS, and GOGAT equally contribute to the re-distribution of N in grain during the later plant growth stage (Fig. 2).

Response mechanisms of grain yield and N use efficiency to PE, GS and GOGAT activities: Plant NUE is influenced by the re-distribution of N from vegetative to reproductive organs (Xu *et al.,* 2011), with both root N absorption during later growth stages and N re-distribution contributing to grain yield (Zhang *et al.,* 2010). The re-distribution of N during later growth stages has been identified as one of the key factors for satisfying the N requirements of crop (Andersson *et al.,* 2005); although the soil can supply adequate N after the crop flowering stage, at least 50% of N in grain is re-distributed from the vegetative organs. In this study, NUE was significantly decreased under inhibitor treatments (Table 1), probably due to reduced N re-distribution ability in grain as a result of lower PE, GS and GOGAT activities (Table 3). Grain yield was significantly influenced by GOGAT activity (Fig. 1) and, compared with control, the grain yield of the GOGAT-H treatment was significantly decreased (Table 1).

Conclusion

The L-glutamine and free amino acid contents of phloem sap can be regulated by PE, GS and GOGAT activities, with the phloem sap L-glutamine content being primarily regulated by PE activity and phloem sap free amino acid content primarily regulated by GS activity. When activities of PE, GS and GOGAT were inhibited, more N accumulated in leaf and less N was re-distributed into grain. NUE was significantly decreased when PE, GS or GOGAT activities were inhibited. We propose that N re-distribution ability was significantly decreased by exogenous application of PE, GS and GOGAT inhibitors.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (grant number 31372130, 31101596). National oilseed rape production technology system of China. Open novel science foundation of Hunan province (13K062). FuRong Scholar Program of Hunan Province, P.R.China. The "Twelfth Five-Year" National Science and technology support program (2012BAD15BO4).

References

Andersson, A., E. Johansson and P. Oscarson. 2005. Nitrogen redistribution from the roots in post-anthesis plants of spring wheat. *Plant Soil,* 269: 321-332.

Brenna, T.J.T.N., H.J. Corso, M. Tobias and R.J. Caimi. 1998. High-precision continuous-flow isotope ratio mass spectrometry. *Mass Spectrometry Reviews*, 16: 227-258.

Cookson, S.J., L.E. Williams and A.J. Miller. 2005. Light-dark changes in cytosolic nitrate pools depend on nitrate reductase activity in Arabidopsis leaf cells. *Plant Physiol.*, 138: 1097-1105.

Cordoba, E., S. Shishkova, C. Vance and G. Hernandez. 2003. Antisense inhibition of NADH glutamate synthase impairs carbon/nitrogen assimilation in nodules of alfalfa (*Medicago sativa* L.). *The Plant J.,* 33: 1037-1049.

Corruzzi, G.M. 2003. Primary N-assimilation into aminoacid in Arabidopsis. I. *The Arabidopsis book, Rochville, M.D. American Society Plant Biolog*.

Daniel-Vedele, F., A. Krapp and W.M. Kaiser. 2010. Cellular biology of nitrogen metabolism and signaling. *Plant Cell Monographs,* 17: 145-172.

Florencio, F.J. and J.M. Vega. 1983. Utilization of nitrate, nitrite and ammonium by *Chlamydomonas reinhardii. Planta,* 158: 288-293.

Gallais, A. and B. Hirel. 2004. An approach to the genetics of nitrogen use efficiency in maize. *J. Exp. Bot.,* 55: 295-306.

Garcia-Mata, C. and L. Lamattina. 2003. Abscisic acid, nitric oxide and stomatal closure is nitrate reductase one of the missing links. *Trends Plant Sci.,* 8: 20-26.

Groat, R.G. and C.P. Vance. 1981. Root nodule enzymes of ammonia assimilation in alfalfa (*medicago sativa* L.) developmental patterns and response to applied nitrogen. *Plant Physiol.*, 67: 1198-1203.

Hirel, B., P. Bertin, I. Quilleré, W. Bourdoncle, C. Attagnant, C. Dellay, A. Gouy, S. Cadiou, C. Retailliau, M. Falque and A. Gallais. 2001. Towards a better understanding of the genetic and physiological basis for nitrogen use efficiency in maize. *Plant Physiol.*, 125: 1258-1270.

Johansson, L. and C.M. Larsson. 1986. Effects of the glutamine synthetase inhibitor methionine sulfoximine on CO_2 fixation in *Lema gibba*. *Plant Soil,* 91: 373-376.

Kamprath, E.J., R.H. Moll and M. Rodrigues. 1982. Effects of nitrogen fertilization and recurrent selection on the performance of hybrid populations of corn. *Agron. J.,* 74: 955-958.

Kumar, G.N.M., R.L. Houtz and N.R. Knowles. 1999. Age-induced protein modifications and increased proteolysis in potato seed tubers. *Plant Physiol.*, 119: 89-100.

Lea, P.J. and B.J. Miflin. 2004. Glutamate synthase and synthesis of glutamate in plants. *Plant Physiol. Bioch*., 41: 555-564.

Logusch, E.W., D.M. Walker, J.F. McDonald and J.E. Franz. 1991. Inhibition of plant glutamine synthetases by substituted phosphinothricins. *Plant Physiol.*, 95: 1057-1062.

Malagoli, P., P. Laine, L. Rossato and A. Ourry. 2005. Dynamics of nitrogen uptake and mobilization in field-grown winter oilseed rape (*Brassica napus* L.) from stem extension to harvest. *Annals Bot.,* 95: 853-861.

Palta, J.A. and I.R.P. Fillery. 1995. N application enhances remobilisation and reduces losses of pre-anthesis N in wheat grown on an Duplex soil. *Austr. J. Agri. Research*, 46: 519-531.

Rahimizadeh, M., A. Kashani, A. Zare-Feizabadi, A.R. Koocheki and M. Nassiri-Mahallati. 2010. Nitrogen use efficiency of wheat as affected by preceding crop, application rate of nitrogen and crop residues. *Austr. J. Crop Sci.,* 4: 1835-2707.

Rossato, L. P. Laine and A. Ourry. 2001. Nitrogen storage and remobilization in *Brassica napus* L. during the growth cycle: nitrogen fluxes within the plant and changes in soluble protein patterns. *J. Exp. Bot.,* 52: 1655-1663.

Shen, Q.R., L. Tang and Y.C. Xu. 2003. A review on the behavior of nitrate in vacuoles of plants. *Acta Pedolo. Sin*., 40: 465-470.

Showler, A.T., J.O. Cavazos and P.J. Moran. 2007. Dynamics of free amino acid accumulations in cotton leaves measured on different timelines after irrigation. *Subtropical Plant Sci.*, 59: 38-55.

Tang, Z., X.R. Fan, Q. Li, H.M. Feng, A.J. Miller, Q.R. Shen and G.H. Xu. 2012. Knock down of a rice stellar nitrate transporter alters long distance translocation but not root influx. *Plant Physiol.*, 160: 2052-2063.

Thomas, K.H., H. Bertrand, D. Emmanuel, L. Frédéric and G. Jacques. 2007. In winter wheat (*Triticum aestivum* L.), post-anthesis nitrogen uptake and remobilization to the grain correlates with agronomic traits and nitrogen physiological markers. *Field Crops Research*, 102: 22-32.

Wang, S.H., W.Z. Bai, X. Kang and S.F. Wu. 2001. The quantitative determinations of L-Glutamine in fermented liquor. *J. Hubei Polytechnic Univer.,* 16: 56-58.

Weeda, S.M., G.N.M. Kumar and N.R. Knowles. 2010. Correlative changes in proteases and protease inhibitors during mobilization of protein from potato (*Solanum tuberosum*) seed tubers. *Functional Plant Biol.*, 37: 32-42.

Xu, G.H., X.R. Fan and A.J. Miller. 2011. Plant nitrogen assimilation and use efficiency. *Ann. Review Plant Biol.*, 63:51-53.

Zhang, Z.H., H.X. Song, Q. Liu, X.M. Rong, G.X. Xie, J.W. Peng, Y.P. Zhang, C.Y. Guan and S.Y. Chen. 2010. Nitrogen redistribution characteristics of oilseed rape varieties with different nitrogen-use efficiency during later growth period. *Commu. Soil Sci. Plant Anal.*, 41: 1693-1706.

(Received for publication 7 May 2013)