Engineering nitrogen use efficiency with alanine aminotransferase

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Abstract: Nitrogen (N) is the most important factor limiting crop productivity worldwide. The ability of plants to acquire N from applied fertilizers is one of the critical steps limiting the efficient use of nitrogen. To improve N use efficiency, genetically modified plants that overexpress alanine aminotransferase (*AlaAT*) were engineered by introducing a barley *AlaAT* cDNA driven by a canola root specific promoter (*btg26*). Compared with wild-type canola, transgenic plants had increased biomass and seed yield both in the laboratory and field under low N conditions, whereas no differences were observed under high N. The transgenics also had increased nitrate influx. These changes resulted in a 40% decrease in the amount of applied nitrogen fertilizer required under field conditions to achieve yields equivalent to wild-type plants.

Key words: alanine aminotransferase (AlaAT), nitrogen use efficiency, *Brassica napus*.

Résumé : L'azote est le plus important facteur limitant la productivité des cultures, au monde. La capacité des plantes à obtenir l'azote à partir des fertilisants appliqués, constitue une étape critique limitant l'efficacité de l'utilisation de l'azote. Afin d'améliorer l'efficacité de l'utilisation de l'azote, les auteurs ont modifié génétiquement des plantes qui surexpriment l'aminotransférase de l'alanine (*AlaAT*), en introduisant un cADN de l'*AlaAT* de l'orge guidé par un promoteur du canola spécifique aux racines (*btg26*). Comparativement au canola de type sauvage, les plantes transgéniques montrent un rendement en biomasse et en graines, au laboratoire aussi bien qu'aux champs, sous de faible apport en azote, alors qu'on observe aucune différence en présence d'apports élevés en azote. Les plantes transgéniques augmentent également leur influx en nitrates. Ces changements conduisent à une diminution de 40 % l'application de fertilisant azoté nécessaire, pour obtenir des rendements équivalents à ceux du type sauvage.

Mots-clés : aminotransférase de l'alanine (*AlaAT*) efficacité de l'utilisation de l'azote, *Brassica napus*.

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Introduction

All crops are dependent on inorganic nitrogenous fertilizers. Global use of nitrogen (N) in 2000 amounted to 87 million metric tonnes and is projected to increase to 236 million metric tonnes by the year 2050 (Tilman et al. 2001). While the ability of plants to capture N from the soil is dependent on a series of variables including soil type, environment, and plant species (Craswell and Godwin 1984; Kaye and Hart 1997; Hodge et al. 2000), in many cases 50%–75% of N applied to agricultural lands is used by microorganisms or lost through leaching (Pilbeam 1996; Hodge et al. 2000). In Canada, N fertilizers are also the largest source of N released into the air, representing significant greenhouse gas emissions (e.g., nitrous oxide; www.nwri.ca). It is important to improve the N use efficiency (NUE) of crop plants for two reasons. First, nitrogenous fertilizers typically account for up to 40% of production costs in crops such as corn and wheat (Bock 1984), and while these costs are substantial for all producers, they are often prohibitive for subsistence farmers. Second, the environmental damage associated with the use of N-based fertilizers is becoming significant (Vitousek et al. 1997). N deposition is also no longer a local problem, with the globalization of N deposition beginning to have significant consequences for terrestrial ecosystems, particularly as these ecosystems become N saturated (Matson et al. 2002). Therefore, increasing the efficiency by which plants are able to assimilate N has the potential to reduce both the environmental and production costs of agriculture.

Primary N utilization during vegetative growth by plants involves three steps: uptake, translocation, and assimilation (Lam et al. 1996). In agricultural soils, nitrate and ammonium are the predominant forms of inorganic N present, and the movement of inorganic N into roots from the soil is mediated by at least two transport systems for each ion (Forde 2000; Howitt and Udvardi 2000). Nitrate is the predominant form of N used by many plant species. Once

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nitrate enters the cell, it is reduced by nitrate reductase (NR) to nitrite. Nitrite is transported to the plastid where it is further reduced to ammonium by nitrite reductase. Ammonium then enters the glutamine synthetase (GS) and glutamate synthase (i.e., glutamine-oxoglutarate aminotransferase, GOGAT) cycle where it is converted to glutamine and glutamate. The glutamate amino group can then be transferred to different amino acids by a suite of aminotransferases (Lam et al. 1996). A variety of compounds can be used for N transport in the xylem such as nitrate, amino acids, and their derivatives, depending on the species and where the primary site of N assimilation occurs (Lea and Ireland 1999).

Most attempts to manipulate N assimilation have involved the early steps in N uptake and metabolism, such as the nitrate transporters, NR and GS. In a recent review, Good et al. (2004) identified a number of different ways to measure NUE and a variety of different genes that have been overexpressed in plants with the goal of increasing the plant's NUE. These studies have met with mixed results, and in most of the experiments the genes were expressed using the constitutive cauliflower 35S promoter. One gene candidate, GS, has been shown in both overexpression studies and gene-mapping studies to be correlated with enhanced growth, under certain conditions (Oliveira et al. 2002). More recent studies on GS in pea, maize, and rice have used both correlative approaches (Hirel et al. 2005; Kichey et al. 2006) and gene knockout and overexpression studies to demonstrate the importance of this enzyme on growth, N accumulation and, in particular, grain filling (Tabuchi et al. 2005; Fei et al. 2006; Martin et al. 2006). Yanagisawa et al. (2004) overexpressed the maize transcription factor Dof1 in *Arabidopsis*. They found that the ectopic expression of Dof1 resulted in a marked increase in amino acid content in the transgenics and significantly, the Dof1 transgenics exhibited improved growth under low N conditions.

In the course of experiments to test the ability of alanine to function as a compatible solute, we noted that transgenic plants expressing *AlaAT* under the control of a stress inducible promoter displayed a N-efficient phenotype. This unexpected observation suggested that the targeted expression of *AlaAT*, which catalyzes the synthesis of alanine and 2-oxoglutarate from pyruvate and glutamate, may have significant impacts on the plant's N metabolism. Alanine is of particular interest for several reasons. First, Waters et al. (1998) have shown that alanine is excreted by N_2-fixing bacteria, followed by its assimilation into the plant root, suggesting that it may have an important role in organic N metabolism. Second, alanine has been shown to be the major storage amino acid under certain stresses (e.g., flooding), perhaps because pyruvate is the only readily available carbon skeleton under anaerobic conditions (Vanlerberghe and Turpin 1990). In the present report, we introduced a barley *AlaAT* cDNA (Muench and Good 1994) that encodes a cytoplasmic form of *AlaAT* in barley under the control of a root-specific promoter (Stroeher et al. 1995) and observed increases in biomass and seed yield of canola plants (*Brassica napus* L.) grown under conditions of low N. These results are of particular interest because of the requirement for a specific, nonconstitutive promoter to express the phenotype and because the phenotype is only displayed under reduced N conditions. Our results also indicate that it may be possible to successfully improve N uptake and utilization by manipulation of a downstream step in N metabolism.

Materials and methods

Plasmids and plant transformations

The alanine aminotransferase cDNA was isolated from barley (*Hordeum vulgare* L.) as previously described (Muench and Good 1994). Primers were used to introduce two *Bam*HI restriction sites into the barley *AlaAT* cDNA clone, one between nucleotides 48 and 53 and the other between nucleotides 1558 and 1563. The 1510 bp fragment was then cloned into the *Bam*HI site of a vector containing a duplicated *CaMV 35S* promoter and a nopaline synthase (*nos*) terminator. The *btg26* promoter was cloned from *Brassica napus* L. as described previously (Stroeher et al. 1995). The *btg26:AlaAT* construct was created by inserting a 300 bp *Kpn*I/*Bam*HI fragment from the *btg26* promoter into the *Kpn*I/*Bam*HI site of the *CaMV35S AlaAT* construct, to replace *CaMV35S*. The two constructs, *CaMV35S:AlaAT* and *btg26:AlaAT*, were subcloned into the binary vector pCGN1547 (McBride and Summerfelt 1990). The same 300 bp *Kpn*I/*Bam*HI *btg26* promoter fragment was cloned into the pBI101 vector, which contains a promoterless GUS/Nos binary vector. All three constructs (including the *btg26:GUS:Nos* construct) were used to transform *B. napus* 'Westar' through *Agrobacterium*-mediated transformation (Moloney et al. 1989). The *CaMV35S:AlaAT* construct was transformed into *Arabidopsis* using vacuum infiltration and has been described previously (Dennis et al. 2000; Miyashita et al. 2007).

Plant material and growth conditions

T_2 seeds from homozygous lines of *B. napus* and a T_3 homozygous *Arabidopsis* line (CaAT10) that was a strong *AlaAT* overexpressor were used for all experimental analyses. The CaAT10 line is described in detail in Miyashita et al. (2007). Homozygosity was determined by ensuring that 100% of the T_2 progeny (a minimum of 50 individuals) from a individual line were kanamycin resistant. For the analysis of different N metabolism genes using either reverse transcriptase – polymerase chain reaction (RT-PCR) or Northern analysis and for the metabolite analysis and nitrate uptake studies, lines 26gA-5 and 26gA-8 were used for all experiments.

Soil-less potting mix system

Seeds from wild-type (WT) and transgenic plants were germinated on filter paper moistened with double distilled water in Petri plates for 24 h at 4 °C, then transferred to custom growth rooms (23 ± 2 °C, 16 h light : 8 h dark, 70% humidity, 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR at canopy). Seedlings were selected for similarity in developmental stage and transplanted into 15 cm pots with a standard soil-less potting mixture (Terra LITE 2000; Metro Mix, Grace Horticultural Products, Ajax, Ont.) containing a slow release fertilizer (40 g Nutricote 14:14:14; Plant Products Co. Ltd., Brampton, Ont.) per 18 L potting mixture). Following planting, each pot was provided with 200 mL of a chelated micronutrient mix ($0.3\ g \cdot L^{-1}$; Plant Products Co. Ltd.) plus 5 $mmol \cdot L^{-1}$ $MgSO_4$

and 2.5 mmol·L^{-1} K_2SO_4. The plants were then fertilized every 2 weeks with 330 mL of a nutrient solution containing 2.5 mmol·L^{-1} KH_2PO_4, micronutrients, and urea at either 5.0 (high N) or 0.5 mmol·L^{-1} (low N).

Hydroponic system

Plants in hydroponic experiments were grown in growth chambers on standard inorganic nutrient solution (Basu et al. 1994), containing 2.0 mmol·L^{1-} N, supplied as ammonium nitrate, for 5 weeks under the growth conditions described above (Basu et al. 1994). The nutrient solution was changed after 2 weeks and then weekly after that. All plants were harvested at full expansion of the fifth leaf, just prior to bolting for both the potted and the hydroponic experiments.

Reverse transcriptase – polymerase chain reaction analysis

All procedures utilizing kits were carried out according to the manufacturers' instructions. Total RNA was extracted using an RNeasy Plant Mini extraction kit (Qiagen Inc., Santa Clarita, Calif.). Contaminating DNA was removed by DNase I treatment (DNA-free™, Ambion Inc., Austin, Tex.), and the absence of DNA was subsequently verified by PCR analysis. RNA samples were quantified, and 50 ng samples were used as templates for the first-strand cDNA synthesis using Superscript III reverse transcriptase and random hexamers (Superscript III first-strand sythesis system for RT-PCR, Invitrogen Corp., Carlsbad, Calif.). PCR reactions for AlaAT were performed using Platinum *Taq* polymerase (Invitrogen) and a PE 9700 thermocycler (Applied Biosystems, Foster City, Calif.) with the following parameters: one cycle at 94 °C for 2 min, forty cycles at 55 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min. The reactions were spiked with 5 μCi (1 Ci = 37 GBq) of [^{32}P]-dCTP (Perkin Elmer, Boston, Mass.), and the linear range of each reaction was determined by analyzing samples from every second cycle in a phosphorimager 455SI (Amersham Biosciences Piscataway, N.J.). For quantification of transcript abundance, the barley AlaAT primers were multiplexed with an *18S rRNA* internal standard (Ambion), and the PCR reactions were terminated at the cycle within the linear range as determined above. To ensure even amplification efficiencies between the target template and the internal standard, the *18S rRNA* primers were mixed with 18S PCR competimers (Ambion) in a ratio of 0.7:19.3. The PCR reactions were resolved by electrophoresis on a 6% denaturing polyacrylamide gel, and the *AlaAT* and *18SrRNA* bands were quantified using a phosphorimager and Image Quant software (Amersham). For the analysis of the N transporters (nitrate and ammonia) and nitrate reductase, total RNA was extracted and RT-PCR was performed as described above. The primer sets used amplified the following genes: *BnNRT1*, *BnNRT2.1*, *BnNRT2.2*, *BnAMT1*, and *HvAlaAT1*. Gene accession numbers and amplification conditions are provided in Table 1.

AlaAT activity and immunodetection

After extraction of leaf and root proteins, native and SDS gels were run using 10 μg of protein, as determined by Bradford analysis, with the exception of the CaAT10 *Arabidopsis* line, where 2 μg of protein were used (because of the high levels of AlaAT activity from the transgene). SDS–PAGE and electroblotting were carried out using precast 4%–15% gradient gels (Bio-Rad, Mississauga, Ont.) as described in Vanlerberghe and Turpin (1990). Electroblotting of the gel to nitrocellulose was performed at 4 °C overnight according to the manufacturer's specifications (Trans-blot transfer cell, Bio-Rad). AlaAT protein was detected with an antiserum raised to purified barley AlaAT-2 (Good and Muench 1993) and a horseradish peroxidase conjugated secondary antibody. Blocking, antibody incubation, and chemiluminescent detection were carried out as recommended by the manufacturer, with an Amersham ECL Western blot detection system (RPN-2109, Amersham Biosciences, Baie d'Urfe, Que.) but using the Roche Diagnostics Western blot blocking reagent (Roche Diagnostics, Laval, Que.), and exposed to X-ray film. We have found that the Roche blocking reagent reduces the background, when compared with the Amersham blocking reagent provided with this kit. AlaAT activity was assayed spectrophotometrically as described previously (Good and Muench 1993; Muench and Good 1994).

In vivo and in vitro GUS activity

Seed was sterilized, germinated, and grown hydroponically in Magenta jars containing 60 mL of standard inorganic medium in sterile conditions, which was replaced weekly. After an additional 2 d, plants were stained for in vivo GUS activity by adding 2 mL of 50 mmol·L^{-1} phosphate buffer (pH 7.5) containing 0.2 mmol·L^{-1} X-gluc (5-bromo-4-chloro-3-indolyl-β-glucuronic acid) and incubated for 24–48 h according to the protocol of Martin et al. (1992). In vitro GUS activity was measured according to Breyne et al. (1993) with minor modifications. The reaction was carried out for 1 h in a microplate on an ELISA reader (SpectraMax Plus, Molecular Devices, Union City, Calif.) at 37 °C.

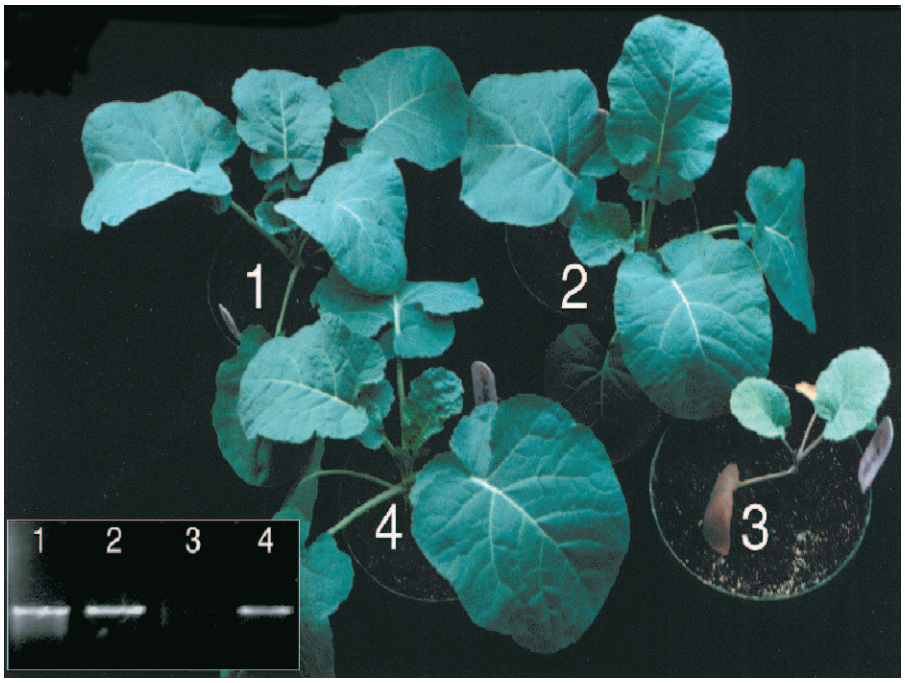
Metabolite analysis

Leaf and root tissues were collected, blotted, weighed, and immediately frozen with liquid nitrogen and ground in 0.6 mol·L^{-1} $HClO_4$ as described in Good and Muench (1993). To collect xylem exudates, the plants were detopped at the collar level. The exudates were collected from the stumps using flexible silicon capillaries, transferred to Eppendorf tubes, and immediately frozen in liquid nitrogen and stored at –80 °C. The exudates were combined with 0.6 mol·L^{-1} $HClO_4$ at a ratio of 1:3. The resulting solutions were neutralized with 2 mol·L^{-1} KOH in 0.4 mol·L^{-1} imidazole buffer. Metabolites including glutamine, glutamate, and alanine were analyzed using coupled spectrophotometric assays, as described previously (Good and Muench 1993) using a Molecular Devices SpectraMax Plus spectrophotometer. Nitrate was determined according to Savidov et al. (1997) using a Molecular Devices SpectraMax Plus microplate reader (SpectraMax Plus, Molecular devices).

$^{13}NO_3^-$ influx

Five-week-old *B. napus* plants (WT and *btg26:AlaAT*, 26gA-5, and 26gA-8) were grown hydroponically on standard inorganic medium minus N containing 0.5 mmol·L^{-1} nitrate as described by Vidmar et al. (2000). $^{13}NO_3^-$ influx

Fig. 1. Expression of the *btg26:AlaAT* transgene confers a N-efficient phenotype in *Brassica napus*. A representative sampling of T_1 segregating transgenic (*btg26:AlaAT*; line 26gA-4) plants under low N conditions. Plants 1, 2, and 4 contain the transgene, plant 3 is the negative sibling as demonstrated by PCR amplification of the transgene using *Hordeum vulgare AlaAT* specific primers (insert, lower left).

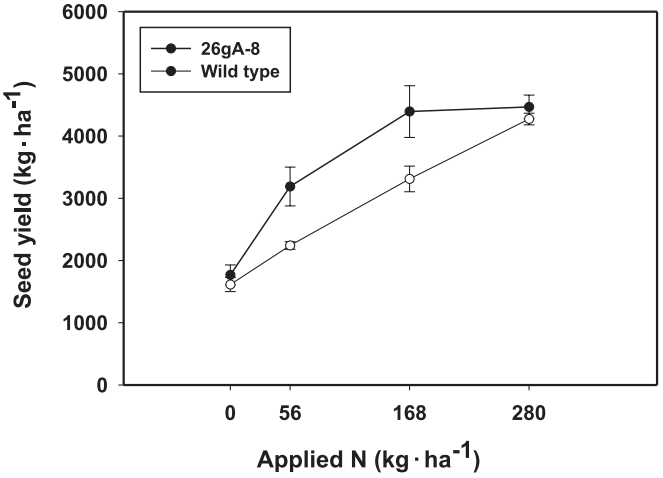


was measured using an influx solution containing 100 μmol·L^{-1} NO_3^- labeled with $^{13}NO_3^-$. Nitrogen-13 (tl = 9.96 min) was produced by proton irradiation of H_2O at the TRIUMF cyclotron on the University of British Columbia UBC campus, purified, and used within 20 min of irradiation as described by Kronzucker et al. (1995). Each of the experimental treatments consisted of five replicates; the experiments were repeated three times for each genotype.

Field trials

Field trials were conducted by Arcadia Biosciences at Brawley, California, USA (32.59° NL; 115.30° WL) in the 2002–2003 crop season, to test the seed yield of 26gA-5 and 26gA-8 transgenic lines and the wild-type (WT) controls at four N levels (0, 56, 168, and 280 kg N·ha^{-1}) supplied with two different N sources: ammonium nitrate and urea. The soil in this area is sandy loam, and the site was irrigated when required; therefore, higher levels of N were added to the soil than would be common in many growing areas. A split plot design with five replications was employed, with each plot consisting of four rows 3.66 m long and 0.67 m apart for a total area of 7.43 m^2. To prevent any potential drift effects of different N application levels, there was a 2 m space between main plots and a 1 m space between subplots. Initial N levels in the soil were determined at an independent laboratory (IAS Labs, Phoenix, Ariz.) for total N, NO_3-N, and NH_4-N, and then fertilizer was applied one time, at the transition period from plant rosette to initial bolting stage. Plots at maturity were windrowed first, and plants were secured in the field for drying for 8–10 d before threshing seeds. Final seed yield and 1000-seed mass of all trial plots from blocks 1–5 were adjusted to a seed moisture of 8% for treatment comparisons. All data were analyzed using the analysis of variance (ANOVA) procedures of SAS for the split-plot experimental design with the four levels of N assigned to the main plots and the three genotypes into subplots, respectively.

Fig. 2. Response of seed yield to different N addition regimes in the field. Trials were conducted in Brawley, California for *btg26:AlaAT* transgenic canola line 26gA-8 and wild-type 'Westar'. Values are the mean ± SE from four replicated plots in a split-plot design. All data were analyzed using the analysis of variance (ANOVA) procedures for split-plot experimental design. Differences were observed for seed yield between genotypes and different N levels at 56 and 168 kg of applied N ($p < 0.01$).



Results

Phenotypic evaluation of transgenic plants

We introduced a barley *AlaAT* cDNA (Muench and Good 1994) driven by a *B. napus* turgor responsive gene promoter

Table 1. Reverse transcriptase – polymerase chain reaction (RT-PCR) primers, amplification conditions, and probes for RT-PCR and Northern analysis of different genes evaluated in the transgenic AlaAT lines.

Gene description	Acc. No.	Left primer	Right primer	Anneal temp.	Product size
BnNRT1	AJ278966	5′ ccacgggtgtttcgattttaaccctct	5′cctttatcgctgctttgtccaatgacc	65 °C	603 bp
BnNRT2.1	AJ293028	5′aaggcaacggttttcaagctcttctcc	5′gatctcatagacaaccggcatgagcaa	65 °C	495 bp
*BnNRT2.2**	AJ293028	5′ actggggaaaatgacacaaagtcac	5′ccgcttgtggagccacctttgccatcgt	65 °C	430 bp
BnAMT1	AF188744	5′tcgcttttgccgtagacaacacgtatc	5′ccatccatcaacagaccagaaccagtg	65 °C	437 bp
HvAlaAT1	Z26322	5′cattgggaacccacaatctc	5′gccgagtacaaggggtactg	65 °C	381 bp
*BnNR1**	D38220	NA (analysis performed using Northern blots)	*Bn18S* (standard; AF513990; 560 bp)	NA	2.9 kb

*The sequence for *BnNRT2.2* is essentially identical to AJ293028 (J. Vidmar and A.G. Good, unpublished data).

Table 2. Leaf area (5th leaf), stem diameter, biomass, and total N content (for shoots only) for four independent transgenic lines and a wild-type (WT) line under high (H) and low (L) N conditions (See Materials and methods).

		5th leaf area		Stem diameter		Shoot FM		Shoot DM		Shoot total N	
Line	N level	(cm^2)	(% WT)	(cm)	(% WT)	(g)	(% WT)	(g)	(% WT)	(%)	(% WT)
26gA-4	H	106±6	93.8	0.7±0.03	100.0	37.0±3	92.3	4.5±1.0	88.2	2.8±0.2	103.7
	L	105±9	147.9	0.7±0.06	116.7	34.1±8*	155.0	4.7±1.0*	130.6	2.7±0.2	122.7
26gA-5	H	102±8	90.3	0.8±0.03	114.3	46.9±6	117.0	6.6±0.9	129.4	2.5±0.1	92.6
	L	118±15	166.2	0.9±0.02*	150.0	43.7±4*	198.6	6.3±1.0*	175.0	2.1±0.1	95.5
26gA-8	H	119±7	105.3	0.6±0.03	85.7	44.3±7	110.5	5.7±1.0	111.8	2.5±0.1	92.6
	L	121±12	170.4	0.8±0.02*	133.3	41.7±8*	189.5	5.3±0.8*	147.2	2.1±0.1	95.5
26gA-18	H	116±7	102.7	0.8±0.03	114.3	42.0±5	104.7	5.4±1.0	105.9	2.6±0.1	96.3
	L	115±9	162.0	0.7±0.02	116.7	40.7±2*	185.0	4.8±0.2*	133.3	2.0±0.1	90.9
Control	H	113±6		0.7±0.04		40.1±5		5.1±1.0		2.7±0.2	
	L	71±9		0.6±0.02		22.0±2		3.6±0.2		2.2±0.1	

Note: Five independent experiments were performed in separate growth chambers ($n = 8$ per experiment); the mean is presented with SD. Significant differences were found for the different genotypes for fresh mass (FM), dry mass (DM), and stem diameter ($p < 0.05$). Fifth leaf area showed significant effects at the genotype × N level. Shoot total N levels differed between N treatment and at the genotype × N level ($p < 0.01$), but not at the genotype level. All statistical analyses were performed using the ANOVA function of SAS 6.12.

*Significantly different ($p < 0.01$) from the control at low N.

(*btg26)* (Stroeher et al. 1995) into canola (*B. napus* 'Westar'), as well as two other constructs, *CaMV35S:AlaAT* and *btg26:GUS*. Preliminary screening of transgenic canola plants expressing *btg26:AlaAT* indicated that under high N conditions, the biomass of transgenic plants did not differ from the WT, whereas under low N conditions, four independent transformed lines demonstrated a biomass increase from 30% to 75% DM (Fig. 1; Table 2). To further characterize this NUE phenotype, four homozygous lines were evaluated under different N regimes using two different experimental systems: soil-less potting mix and a hydroponic culture system (to allow access to root tissue).

In soil-less mix, the transgenic lines produced more biomass under low N conditions than WT plants; however, there was no difference in biomass among the genotypes under high N conditions (Table 2). In the hydroponic system, *btg26:AlaAT* transgenic plants grown on low N showed a 55%–64% increase in total biomass over WT plants (data not shown). In contrast, the *CaMV35S:AlaAT* and *btg26:GUS* lines did not exhibit an increase in biomass and were phenotypically similar to WT plants under either soil-less mix and hydroponic conditions (data not shown).

Field trials were conducted in the winter–spring of 2003 in Brawley, California. Figure 2 illustrates the yield data. At the suboptimal N rate of 56 kg·ha^{-1}, the 26gA-8 transgenic line yielded 3189 ± 312 kg seeds·ha^{-1} (mean ± SE, n = 4), representing a 42.3% increase in seed yield, as compared with WT at 2241 ± 62 kg·ha^{-1}. When the N rate was 168 kg·ha^{-1}, the seed yield of the transgenic line was 4394 ± 416 kg·ha^{-1}, significantly higher than WT at 3311 ± 206 kg·ha^{-1}, representing a 32.7% increase in seed yield compared with WT.

Evaluation of transgene expression

The presence and expression of the transgene was assessed by PCR, RT-PCR, enzyme activity analysis (Table 3), 3), and Western blot analysis (Fig. 3). RT-PCR consistently detected a transcript of the expected size (381 bp), with the level of expression being higher in the roots than shoots (Table 3). The level of AlaAT enzyme activity was also measured in the shoots and roots of the transgenic lines. As was observed with RT-PCR and Western blot analysis, the level of enzyme activity in shoots compared between the transgenic and control lines was not significantly different. However, in root tissue, the level of activity in the transgenics was higher than in the control lines (Table 3). Western blot analysis did not detect the barley AlaAT protein in shoots (Fig. 3*a*), but did detect a high level of the barley AlaAT protein in the roots of the transgenic lines (Fig. 3*b*). Although the AlaAT antibody is a polyclonal Ab, it is concluded here that it preferentially detects the barley AlaAT band, since no clear AlaAT band was detected in the non-transgenic plants (*B. napus* and *Arabidopsis*; Fig. 3) other than the positive control, an *Arabidopsis* transgenic line containing a *CaMV:AlaAT* construct (CaAT10). The obvious increase in the AlaAT band observed in roots (Fig. 3*b*) may

Table 3. Analysis of the four independent *btg26:AlaAT* lines for the presence of the NPTII transgene, *Hordeum vulgare* AlaAT transcript in roots and shoot tissue, the root/shoot ratio of the *H. vulgare* AlaAT transcript, and AlaAT activity (IU·mg protein^{-1}).

					AlaSAT activity	
Line	Tissue	NPT transcript	*H. vulgare* AlaAT transcript	AlaAT R/S ratio	IU·mg·protein^{-1}	WT (%)
26gA-4	R	×	3.80±0.9	6.3	0.332*	123.4
	S	×	0.60±0.4		0.278	101.8
26gA-5	R	×	1.00±1.0	2.7	0.323*	120.1
	S	×	0.37±0.1		0.265	97.1
26gA-8	R	×	7.38±6.7	13.2	0.372*	138.3
	S	×	0.56±0.2		0.289	105.9
26gA-18	R	×	3.40±3.1	5.1	0.381*	141.6
	S	×	0.67±0.4		0.256	93.8
Control	R	ND	ND		0.269	
	S	ND	ND		0.273	

Note: All statistical analyses were performed using the ANOVA function of SAS 6.12. ×, present; ND, not detected. All transcript levels are the average levels of AlaAT transcript to 18S rRNA control ± SD (n = 4).

*Indicates significant differences were found for specific tissues in the different genotypes for AlaAT activity ($p < 0.05$).

either be due to the fact that the AlaAT antibody only detects the barley AlaAT enzyme, whereas AlaAT activity measurements also include the endogenous AlaAT enzymes, or may suggest that the activity of the introduced protein is downregulated.

We investigated the expression of the *btg26* promoter by in vitro and in vivo histochemical analyses of *btg26:GUS* plants (Figs. 4*a*–4*e*). In vitro analysis of GUS activity for 11 independent *btg26:GUS* lines indicated that the average ratio of root/shoot GUS activity was approximately 9:1 with the average levels of GUS activity in roots and shoots being 0.88 versus 0.09 U·mg protein^{-1}, respectively (Fig. 4*e*). In vivo histochemical analysis showed that expression occurs primarily in the roots, and within the roots expression had several distinct features. There was no expression of GUS in the root tip; however, expression was very strong in the cortex of the root and in lateral roots. The lateral roots displayed heavy staining in the root tip, in contrast with the primary roots (Figs. 4*a*–4*c*). In cotyledon and leaf tissue, activity was observed in the vascular tissue; however, no activity was detected in the remainder of the leaf tissue (Fig. 4*d*). Seedlings were also grown on an N-free nutrient solution for 3 d, prior to staining. No noticeable difference was observed in the staining pattern between the high N and N-free conditions.

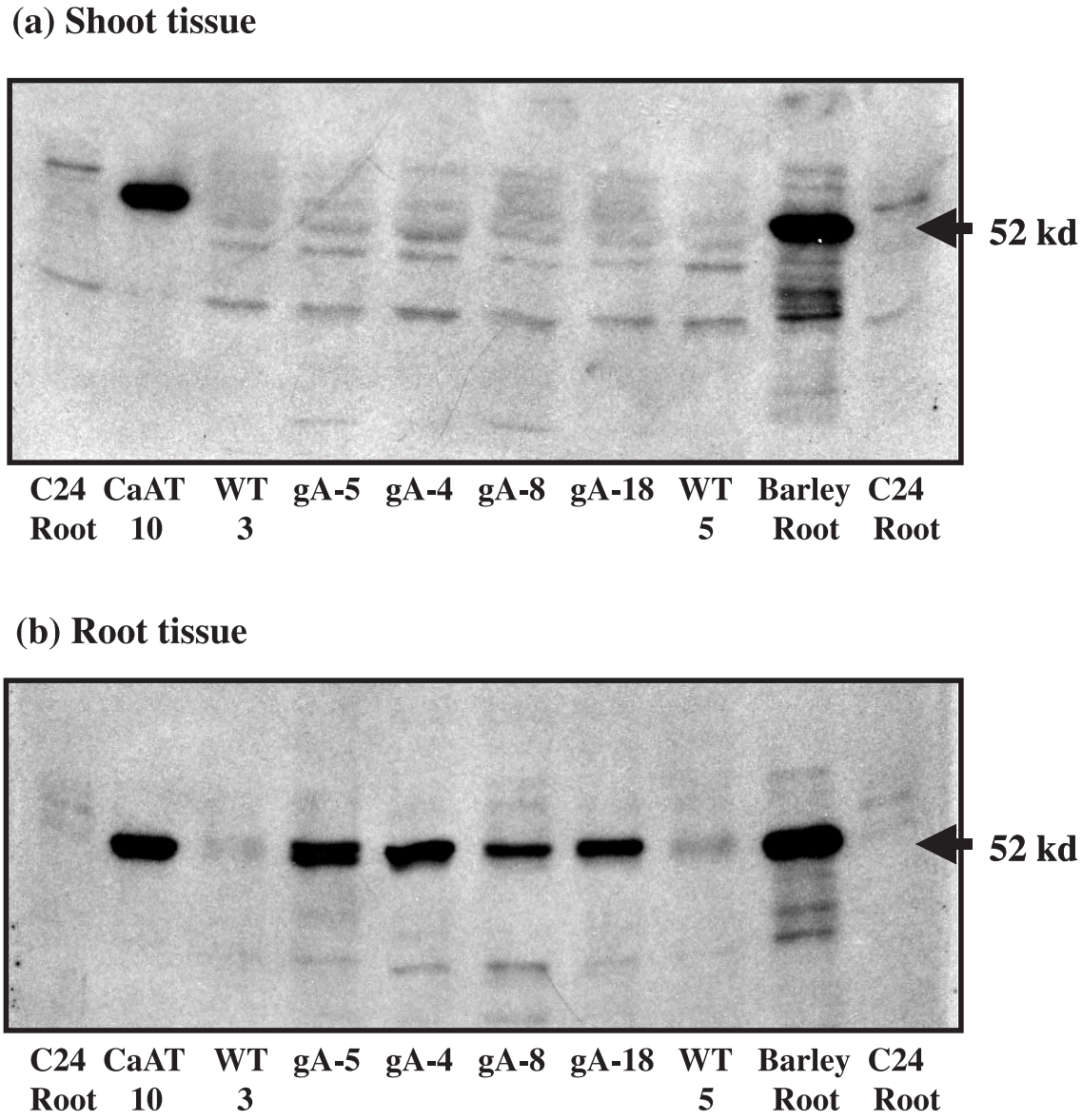
To understand the effect of the transgene on N metabolism, we analyzed hydroponically grown *btg26:AlaAT* and WT plants for free amino acids and nitrate concentration in the roots and shoots (Fig. 5). Glutamine did not differ between roots of the transgenics versus controls; however, there were significantly lower concentrations of glutamine in the shoots of all of the transgenic lines, compared with the nontransgenics (ANOVA; $p < 0.001$). Glutamate concentrations in the roots did not differ between the control and transgenic lines, but glutamate levels were significantly higher in the shoots of the nontransgenics (ANOVA; $p < 0.05$) (Fig. 5*a*). Alanine levels were higher in the roots of transgenic plants (an average of 10.7× higher than in the control line); however, there were no differences in alanine levels in the shoots of the different genotypes (Fig. 5*b*). There were no significant differences in nitrate concentration in the roots or shoots; however, the concentration of nitrate in xylem exudate of the WT plants was higher than observed in the transgenics (Fig. 5*b*).

The observation that glutamine and nitrate levels were lower in the transgenic plants suggested that the transgenic plants might recognize a N deficiency and respond by increasing the rate of N uptake. This hypothesis was tested by measuring nitrate influx into roots, which was shown to increase by 76% in *btg26:AlaAT* plants compared with wild-type plants (Fig. 6). Second, we analyzed transcript abundance of the barley *AlaAT* transgene and other endogenous genes involved in N transport and assimilation, by RT-PCR and Northern analysis. The endogenous genes analyzed included the high-affinity nitrate transporters (*BnNrt2.1*, *BnNrt2.2*), high-affinity ammonium transporter (*BnAmt1*), low-affinity nitrate transporter (*BnNrt1*), and nitrate reductase (*BnNR1*) (See Table 1 for genes and amplification primers). We found that transcript abundance of genes involved in high-affinity nitrate transport (*BnNrt2.1*, *BnNrt2.2*) was upregulated because of the presence of the transgene (3.2 ± 0.2 and 3.9 ± 0.2 fold increases, respectively) in comparison with the 18S rRNA control, whereas the expression of *BnAmt1*, *BnNrt1*, and *BnNR1* was unchanged in comparison with WT plants.

Discussion

Of all the mineral elements required by plants, N is needed in the greatest amount and limits growth and yield most frequently. However, the inability of plants to efficiently absorb and metabolize applied N results in runoff that impacts both terrestrial and aquatic ecosystems and is a major environmental issue (Tilman et al. 2001). Plants are known to vary in their ability to take up inorganic N within and between species. Moreover, under the right environmen-

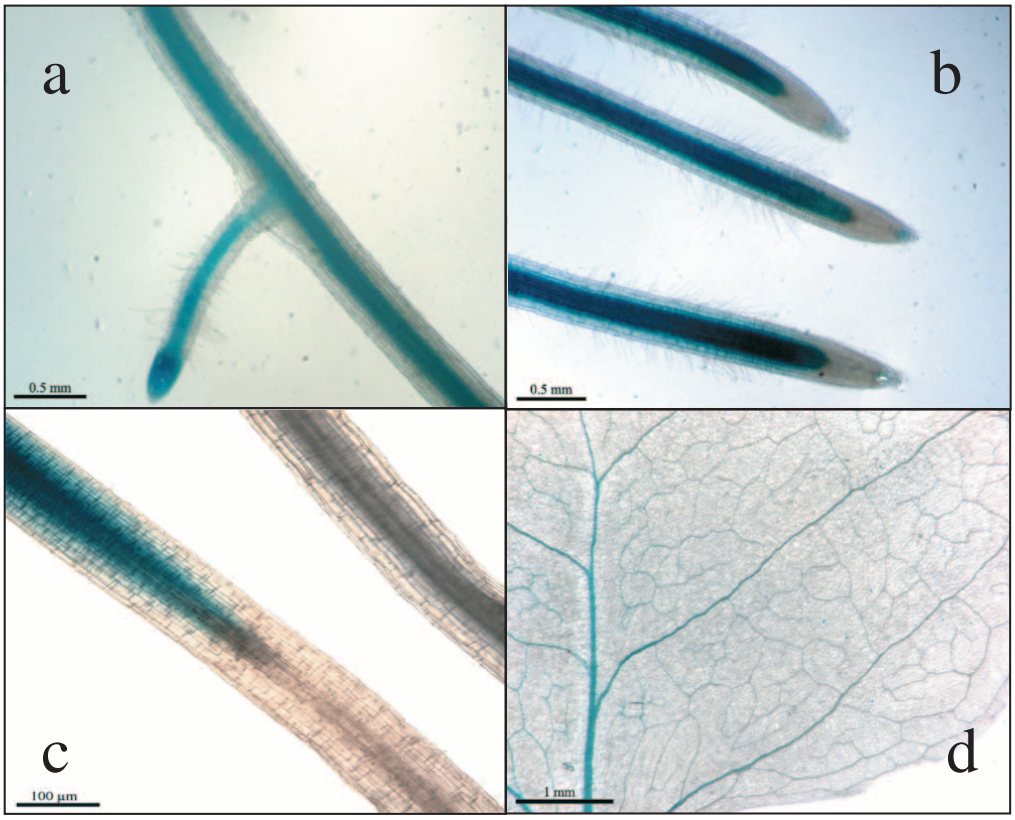
Fig. 3. Expression analysis of the *AlaAT* protein in four *btg26/AlaAT* transgenic canola lines. Immunoblot analysis of either root (A) or leaf (B) total protein extracts was carried out with a barley (*H. vulgare*) AlaAT-2-specific antiserum (Lee et al. 1994). Plants shown were grown in potting mix under high N (in low N conditions, results were similar). The lanes included transgenic lines (gA-4, gA-5, gA-8, gA-18) and WT ('Westar'; 3 and 5 are specific lines), barley root or shoot tissue, *Arabidopsis* (ecotype C24), and CaAT10 (an overexpressing line of *Arabidopsis*) containing the CaMV35S promoter driving the constitutive overexpression of AlaAT.

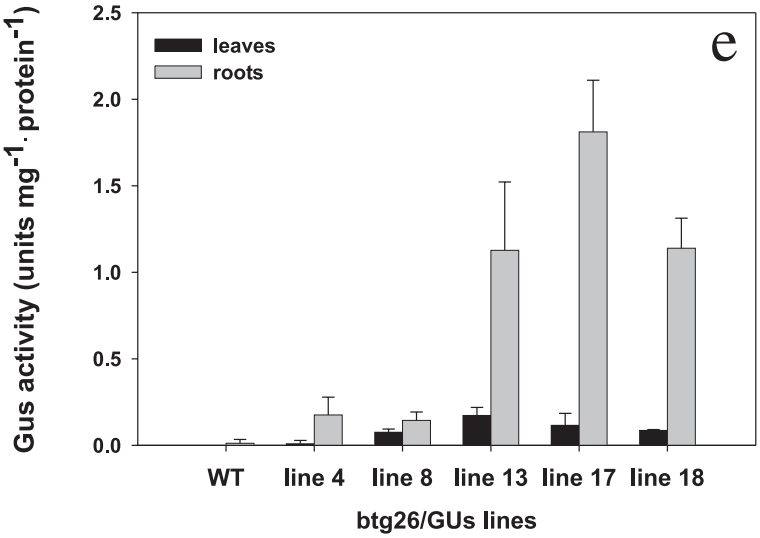


tal situations, notably N starvation, individual plants can significantly increase N uptake and assimilation by activating specific mechanisms for high-affinity N assimilation (Krapp et al. 1998). The current concept of the regulation of N assimilation assumes first that inorganic N is the major source of N for plants, and second that the key steps in regulating N uptake and metabolism occur at the level of nitrate transport and nitrate reduction. However, it is also known that the cycling of amino acids between shoots and roots is one component of the signaling of whole-plant N status, enabling roots to regulate N uptake accordingly (Cooper and Clarkson 1989; Imsande and Touraine 1994; Krapp et al. 1998).

In this report, the tissue-specific expression of *AlaAT* conferred by the *btg26* promoter was critical to display this phenotype. The *btg26* gene is a member of the aldehyde dehydrogenase superfamily, which encodes a protein called antiquitin and appears to function in the regulation of osmotic stress and cellular turgor pressure. It is highly conserved among the plant and animal kingdoms; however, the exact physiological function of antiquitin has not been determined (Lee et al. 1994). We have shown previously that in leaf tissue, the *btg26* gene is induced by drought stress, temperature stress, NaCl, and abscisic acid (Stroeher et al. 1995). This was one of our reasonings in selecting this stress induced promoter to express *AlaAT*. A comparison of the *btg26:AlaAT* plants with wild-type *CaMV35S:AlaAT* and *btg26:GUS* transgenics clearly indicates that expression of

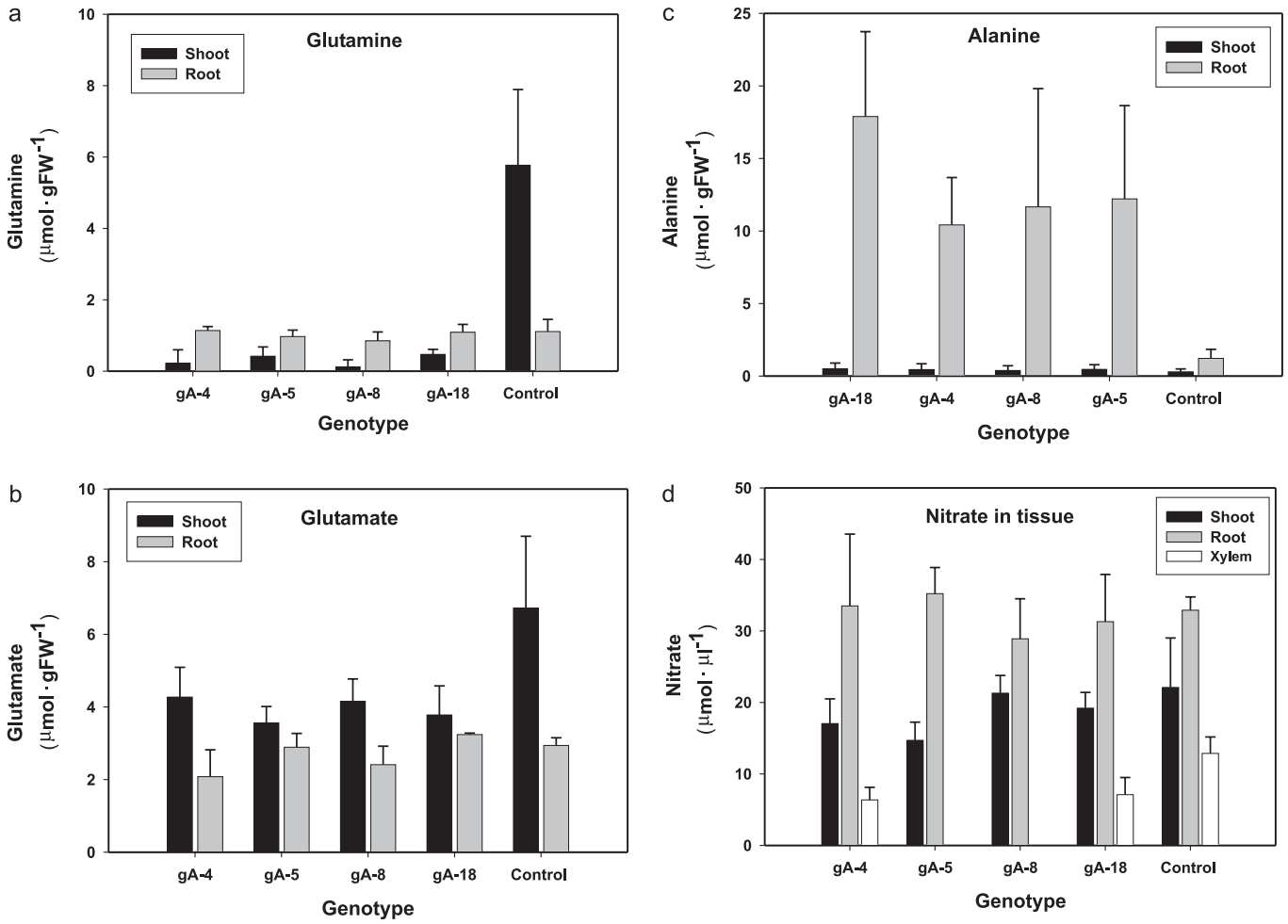
Fig. 4. In vitro and in vivo GUS analysis of *btg26:GUS* transgenic lines. (*a–d*). In vivo GUS levels of several *btg26:GUS* lines. Plants were germinated and grown hydroponically in Magenta jars containing 60 mL of standard inorganic medium in sterile conditions, which was replaced after 2 weeks of growth. After 2 d, plants were stained for in vitro or in vivo GUS activity. In vitro GUS activity was determined by adding 2 mL of 50 mmol·L^{-1} phosphate buffer (pH 7.5) containing 0.2 mmol·L^{-1} X-gluc (5-bromo-4-chloro-3-indolyl-β-glucuronic acid) and incubated for 24–48 h. Root tissue and shoot tissue were viewed and photographed at the magnification indicated. (*a*) Line 18, 16 d, lateral root); (*b*) line 18, 16 d, root tip; (*c*) line 13, 16 d, primary root; (*d*) line 17, 24 d, cotyledon. **(*e*)** In vitro GUS activity was measured according to Breyne et al. (1993) with minor modifications. The reaction was carried out for 1 h in a microplate on an ELISA reader (SpectraMax Plus, Molecular Devices) at 37 °C. Means are presented with standard errors (n = 6).





the *AlaAT* gene by *btg26* is required to show an increase in biomass and yield in the transgenic plants. We believe that the *btg26* promoter regulates this effect by directing expression to the root vascular system, the primary site of N transport. Fei et al. (2006) also found that the promoter used to express the GS15 gene was critical in determining phenotype. The *rolD-GS15* construct resulted in increased total N and increased biomass in two of the transgenic lines,

Fig. 5. Effect of the transgene on endogenous nitrate and amino acid levels. Five-week-old *B. napus* plants (WT) and *btg26:AlaAT*) were grown hydroponically on standard inorganic medium containing 0.5 mmol·L^{-1} nitrate as described in Materials and methods. Plants were harvested, ground in the appropriate extraction buffer, and amino acid levels were measured spectrophotometrially. Means are presented with standard errors (n = 6).

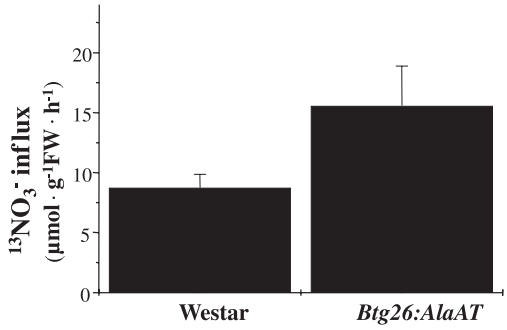


whereas no phenotype was observed in the *CaMV35S-GS15* or *LBC$_3$-GS15* transgenics.

Although alanine is rarely viewed as a key amino acid in N metabolism, it is interesting for several reasons. First, until recently, the model of N transfer from symbiotic nitrogen-fixing bacteria was believed to involve the diffusion and assimilation of ammonia. However, the demonstration that alanine is excreted by nitrogen-fixing bacteria, followed by its assimilation into the plant root suggest it may be a more important role in N metabolism than first realized (Waters et al. 1998). Second, the demonstration that alanine can be a major storage amino acid, under certain stresses, clearly indicates that this amino acid has the potential to be important in N storage and metabolism (Good and Muench 1993).

It has been suggested that the cycling of amino acids between shoots and roots may provide the necessary information regarding whole-plant N status, enabling roots to regulate N uptake accordingly (Lam et al. 1996; Cooper and Clarkson 1989). The results presented here allow us to propose the following model to explain the changes observed in the *btg26:AlaAT* transgenics. An increase in the expression of *AlaAT* in the root tissue results in increased concentrations of N in the form of alanine in the roots. Alanine can then be transported to the shoots, where it provides additional N for plant growth. The observation that glutamine and glutamate levels in the shoots were lower in the transgenics compared with the controls suggests that the transgenic plants may be sensing a reduced N status and responded by increasing both NO_3^- influx and mRNA levels of the high-affinity nitrate transporters. As previously demonstrated in barley, tobacco, and *Arabidopsis*, the transcription of genes encoding high-affinity nitrate transporters is downregulated by amino acids and high N treatments (Cooper and Clarkson 1989; Imsande and Touraine 1994; Krapp et al. 1998). It has also been demonstrated in *Arabidopsis* that genes encoding low-affinity nitrate transporters (*AtNrt1.1* and *AtNrt1.2)* are not responsive to N status of the whole plant (Lejay et al. 1999). These researchers have proposed that removing the negative signals responsible for repression of nitrate transporter genes would increase high-affinity nitrate transport and plant growth. Research is currently underway to determine changes in the flux of specific amino acids in the transgenic plants. Regardless of what the

Fig. 6. $^{13}NO_3^-$ influx in transgenic versus WT *Brassica napus*. Five-week-old *B. napus* plants (control and *btg26:AlaAT*, 26gA-8) were grown hydroponically on standard inorganic medium minus N, containing 0.5 mmol·L^{-1} nitrate as described by Vidmar et al. (2000). $^{13}NO_3^-$ influx was measured using an influx solution containing 100 μmol·L^{-1} labeled with $^{13}NO_3^-$. Each of the treatments consisted of five replicates.



key signal molecule is that indicates the plant's N status (e.g., glutamine, glutamate, or alanine), it is clear that the roots perceive a need for increased N uptake, resulting in the observed increase in N influx, which is then incorporated into key transport amino acids.

In our study, we provide evidence for the enhancement of N use efficiency in canola, but also demonstrate that this can be achieved by the manipulation of metabolic pathways downstream of primary N uptake and metabolism. Since N is the single largest input in modern agriculture, and a major pollutant, it is essential that we begin to find ways to reduce the inadvertent loss of this nutrient into the ecosystem.

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