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The impact on nitrogen-efficient phenotypes when aspartate aminotransferase is expressed tissue-specifically in *Brassica napus*

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ABSTRACT

Background: Aspartate aminotransferase (AAT) catalyzes a reversible transamination reaction, producing aspartate and 2-oxoglutarate from glutamate and oxaloacetate, in various cellular plant compartments. Previous work in our lab had shown that a similar aminotransferase enzyme, alanine aminotransferase (AlaAT), produced nitrogen use efficient (NUE) phenotypes when over-expressed in canola (*Brassica napus*) under the salt-stress inducible promoter, *btg-26*. Given the similarities between these two enzymes and their roles in plant metabolism, it was hypothesized that over-expression of AAT could also produce an NUE phenotype in canola.

Results: Transgenic *Brassica napus* lines over-expressing *AAT* from *Medicago sativa* were produced and analyzed for NUE phenotypes under both high and low nitrogen conditions. While several lines showed promising increases in biomass under the various fertilizer regimes, these alterations could not be reliably replicated and increases in expression of the transgene detected via RT-PCR did not translate into significant increases in AAT activity in plant tissues.

Conclusions: Transgenic *Brassica napus* lines over-expressing *AAT* do not display NUE phenotypes similar to those plants over-expressing *AlaAT*. Although this work produced a negative result, it is important to compare the NUE phenotype produced by over-expression of *AlaAT* and *AAT*, and differences in metabolism between *AAT* vs *AlaAT* over-expressing lines which may be used to deduce changes in plant N metabolism important for NUE in cereal crops.

1. Introduction

Since the advent of the Green Revolution in the 1960s, agriculture has benefited from high-yielding, semi-dwarf species of cereal crops, with increased harvest index [1–3] and nitrogen (N) responsiveness [4–6]. While this has allowed for increased food production to feed a growing world population, the increases in applied N fertilizers have had considerable negative impacts on the environment [3], including stratospheric ozone depletion, global warming and algal blooms [7–9]. Alterations in climate, due to both natural and anthropogenic factors, have also resulted in alterations in the chemical and physical properties of soils, and thus, have impacted plant breeding programs and research surrounding macro- and micro-nutrient usage in agriculture [10].

Over the last decade, a major focus has been placed on creating cereal crops with increased nitrogen use efficiency (NUE). Various definitions and calculations of NUE exist, with the most basic measuring increases in total biomass or grain weight relative to N input (i.e. NUE = Sw ÷ N, where Sw equals the shoot dry weight and N is the nitrogen content of the shoots; NUE = Gw ÷ Ns, where Gw equals the grain weight and Ns is the nitrogen supplied (g per plant)) [6,11]. At the time of this study, various components of N metabolism in plants had been altered using transgenic approaches in the hope of increasing NUE, including: high affinity nitrate transporters [12,13], nitrate reductase (NR) [14,15], nitrite reductase (NiR) [16], glutamate dehydrogenase (GDH) [17], glutamine synthetase (GS) [18–20], glutamate synthase (GOGAT) [21,22] and asparagine synthetase (AS) [23]. Alterations in these components of N metabolism were reported to affect overall N metabolism and N uptake and/or biomass, however few reported of potential increases in NUE. Since then, further study and alteration of genes and proteins involved in primary N metabolism in plants has shown little promise in terms of producing NUE phenotypes, while modifications to genes and proteins involved in other facets of N assimilation, such as alanine

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aminotransferase (AlaAT) [24,25] and Dof1 [26,27], have shown impacts on NUE and warrant much further study. (For a full review see McAllister et al. [28].)

When this study was carried out, previous work in our lab indicated that tissue-specific over-expression of barley (*Hordeum vulgare*) alanine aminotransferase (*HvAlaAT*) in *Brassica* napus resulted in improved NUE. Driven by a tissue-specific promoter, *btg-26* [29], over-expression of *HvAlaAT* resulted in increased biomass and seed yield relative to control plants under various N regimes [29]. These results proved interesting, as AlaAT is not involved in primary nitrogen metabolism in plants, but had been shown to be intimately involved in plant hypoxic response [30]. Based on the AlaAT over-expression results in *B. napus*, the question arose of whether other aminotransferase enzymes would also show NUE phenotypes in plants.

Aspartate aminotransferase (AAT) catalyzes a reversible transamination reaction, in the presence of the coenzyme pyridoxal-5′-phosphate (PLP), producing aspartate and 2-oxoglutarate from glutamate and oxaloacetate, and vice versa [31]. In plants, multiple isoenzymes of AAT carry out this reaction in distinct subcellular compartments, including the mitochondria, plastid, chloroplast and the cytosol [32–34]. While the mitochondrial, plastid and chloroplastic isozymes have shown to be involved in shuttling reducing equivalents between subcellular organelles, the cytosolic isozyme has shown to serve a non-redundant role in primary N metabolism [33,35]. The amino acids aspartate, asparagine, glutamate and glutamine compromise 70% of the free amino acids in plants and are the main transport molecules for N within the plant [35,36]. During daylight hours, cytosolic AAT has been reported to synthesize the bulk of aspartate within the plant [33,35]; aspartate can then be utilized by the plant as a means of transporting N. During the night, when C skeletons are limited, AS can utilize these reserves of aspartate for substrate, producing asparagine. Asparagine is then used by the plant to shuttle N instead of a aspartate, as this compound is deemed a more efficient transporter of N due to its high N:C ratio (2:4) [33,35,37].

It was hypothesized that, similar to the over-expression of *AlaAT*, targeted over-expression of *AAT* would result in an NUE phenotype in *B. napus*. Although these two enzymes primarily utilize different substrates for their subsequent reactions, it was thought that similar NUE responses would be observed for a number of reasons. First, both utilize substrates that are key intermediates in both carbon and nitrogen metabolism (i.e. 2-oxoglutarate, aspartate and glutamate). Second, both enzymes directly impact concentrations of both glutamate and 2-oxoglutarate, both of which are internal signals of cell nitrogen status [38–40]. Third, both have been shown to be cytoplasmically and subcellularly localized [33]. Finally, while AAT and AlaAT catalyze different primary reactions, many transaminase enzymes, including AAT, have shown to carry out several transaminase reactions given the correct environment and substrates [41,42].

To test this, *AAT* was transformed into *B. napus* and over-expressed in a tissue-specific pattern using the osmotic stress-inducible promoter, *btg-26*. Transgenic, homozygous T_3 plants were analyzed for expression of the transgene, presence of transgenic protein activity and preliminary NUE phenotypes such as alterations in dry weight of roots and shoots, as observed in the AlaAT-NUE canola plants [43]. However, despite evidence of transgene expression, consistent detectable increases in AAT activity were not detected in the transgenic lines studied, and only one line out of 13 showed a putative NUE phenotype, accumulating higher root and shoot biomass than control plants. This increase in biomass however could not be replicated in follow-up experiments. Due to the promising results observed in the AlaAT studies, but not in those utilizing AAT, our lab went on to study over-expression of *AlaAT* in other cereal crops [24,44,45], as well as analyzing the effect various promoters and AlaAT enzymes variants have on the observed NUE phenotypes [46,47]. This study provides useful information and insight into NUE in cereal crops, and differences in metabolism between *AAT* vs *AlaAT* over-expressing lines could be used to deduce changes in plant N metabolism important for NUE in cereal crops.

2. Materials and methods

2.1. Vector construction and B. napus transformation

A 1270 bp region of a cytosolic aspartate aminotransferase (*AAT-1*) cDNA cloned from *Medicago sativa* by Udvardi and Kahn [48] was amplified by PCR: 5′-CCGCTCGAGATGTCTGATTCCGTCTTCGCTCA-3′ and 5′-CCGCTCGAGTCACGGGGATGAATTGATAA-3′. The primers were designed to introduce an Xho1 restriction site at both the 5′ and 3′ ends of the PCR fragment. Klenow, dTTP and dCTP were used to partially fill in the Xho1 sticky ends, creating BamH1 compatible ends on either side of the 1270 bp AAT-1 PCR product. This product was cloned into p25

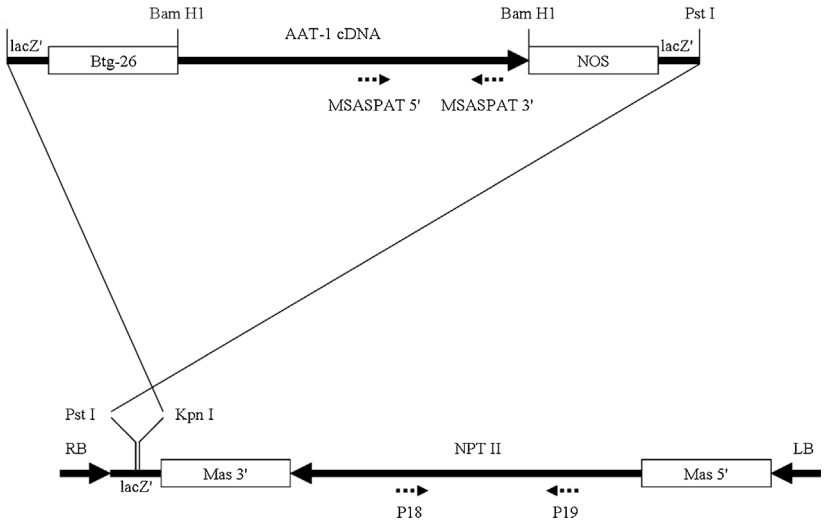

Fig. 1. Organization of genes located between the left and right borders of the binary vector p26gAspATNS47. The locations of primer binding sites used to screen the transgenic lines are indicated by dashed arrows. Primer pairs MSASPAT 5′ and MSASPAT 3′ and P18 and P19 amplify 489 and 657 bp fragments respectively.

(obtained from Maurice Moloney, University of Calgary), which was derived from a pUC19 vector by inserting a duplicated CaMV 35S promoter and NOS terminator, linked by a BamH1, Xba1 and Pvu1 polylinker, between the Kpn1 and Pst1 cut sites of pUC19 [49]. The duplicated CaMV 35S promoter of p25 was replaced with a 300 bp fragment from the 5′ promoter region of *btg-26*, prior to insertion of the *AAT-1* product. Clones containing *AAT-1* inserted in the sense orientation were carried forward and subcloned into pCGN1547 [50] using the Kpn1 and Pst1 cut sites, to create the binary vector p26gAspATNS47 (Fig. 1).

The construct p26gAspATNS47 was transformed into *Agrobacterium tumefaciens* strain LBA4404. The resulting transformed Agrobacterium was used to transform a double haploid line of *Brassica napus* cv Westar (N-o-1), following the protocol of Moloney et al. [51]. Putative T_0 transformants were screened by PCR using primers specific to a portion of the neomycin phosphotransferase selectable marker gene (*NPT II)*, contained within the borders of the transgene: P18 5′-CGCTCAGAA-GAACTCGTCAAGAA-3′ and P19 5′-TTTGTCAAGACCGACTGTCC-3′ (Fig. 1).

2.2. Selection of transgenic lines for characterization

Thirty-nine putative T_0 transgenic lines were selfed to advance the lines to the next generation. PCR amplification of the selectable marker *NPT II* on individual T_1 plants indicated that 37 of the lines contained a portion of the transgene in this generation; the two lines not containing the transgene were excluded from further study.

T_1 plants were also used to identify lines with single or multiple transgene insertion events. The transgene was expected to segregate in T_1 siblings in the Mendelian ratio of 3:1. All T_1 descendants ($n = 19-20$) from each of the independent T_0 insertions lines where analyzed by PCR for presence of the transgene as described previously, and the ratio of insert-positive to insert-negative plants was compared using the chi-squared statistic ($X^2_{0.05,1} = 3.841$). Seventeen single insertion lines were identified; 11 of these lines were chosen to carry forward to the T_3 generation for further characterization. This screen also identified negative siblings (nulls) of each single insertion line; these null siblings were also carried forward to the T_3 generation when enough viable seed was available and if a null sibling was produced (Table 1); null siblings were used as control plants, in addition to wildtype background line N-o-1, in activity assays and RT-PCR. Two additional lines were carried forward to the T_3 generation (6W-4a#5 and 6W-14a#1) even though they did not produce enough T_1 seed to analyze segregation for number of insertional events; both lines showed to be homozygous at the T_2 stage via PCR. T_2 progeny from all 13 lines carried forward were screened for homozygosity via PCR of the 3′ end of the *AAT-1* cDNA insertion: MSASPAT-5′ 5′-AACATGGGTCTTTATGGTGAACGTG-3′ and MSASPAT-3′ 5′-TCACGGGGATGAATTGATAACAAAC-3′ (Fig. 1), and further screened for presence of the transgene and homozygosity by germinating T_3 seeds on $^1/_2$ MS + $Kan_{(150)}$ (kanamycin, 150 μg ml^{-1}) and scoring plants for cotyledon colour: bleached (null or control), partially bleached or not bleached (homozygous for transgene). Based on the results of both the *AAT-1* cDNA PCR and growth of plants on selection media, nine homozygous lines were chosen for further analysis and characterization, with six of the lines being further chosen for phenotypic growth assays (Table 1). Seed from both T_2 and T_3 generations was extremely limiting in some lines; for this reason not all lines were tested in all experimental circumstances. In addition, we were not able to propagate negative siblings for all lines (i.e. 6W-4a) which limited there usage in this study. Finally, due to limitations in growth space only a subset of lines were chosen for biomass analysis and hydroponic growth.

2.3. Nomenclature

All plants/lines in this experiment were given identification numbers. All lines begin with '6W', which denotes the transformation experiment ('6') and the variety, Westar ('W'), of *B. napus* utilized for all transformations. The control line is also derived from Westar, but is denoted 'N-o-1'. The number immediately following 'W' refers to the individual T_0 shoots from separate and distinct calli. The subsequent letter differentiates separate T_0 shoots originating from the same callus. (These shoots may or may not represent independent insertions of the transgene.) The T_1 generation of the plants is designated by the symbol '#', followed by a number between 1 and 20. Subsequent T_2 and T_3 generations are labelled after the T_1 nomenclature.

2.4. Plant growth conditions

Plants for seed increases or line establishment were grown under 16 h days, at a relative humidity of 60%, an average light intensity of 240 μmol photon m^{-2} s and 20 °C day temperatures and 16 °C night temperatures. One seed was sown per pot, in pre-wetted substrate (Terra-Lite 2000, Metro-Mix 220 growing media) with the pots held in trays. Plants were watered three times per week, and fertilized with all-purpose fertilizer (Plant Products, 20-20-20 Plant—Prod) as recommended by the manufacturer. Prior to anthesis, individual plants were covered with clear plastic

Table 1
Overview of transgenic *AAT* lines utilized in this study and their null siblings.

Transgenic line	Number of transgene positive to negative T_2 plants	Kan^R seedlings (%)	Null sibling	Kan^R seedlings (%)
6W-4a#5	12–0	90 (9/10)	*	*
6W-6a#3	12–0	nd	6W-6a#4	nd
6W-8c#11	12–0	100 (15/15)	6W-8c#10	25 (4/16)
6W-9a#4	12–0	70 (7/10)	6W-9a#12	0 (0/13)
6W-10a#19	12–0	nd	6W-10a#20	nd
6W-11b#2	12–0	94 (15/16)	6W-11b#11	nd
6W-12d#19	12–0	82 (9/11)	6W-12d#20	0 (0/17)
6W-13c#18	12–0	100 (18/18)	6W-13c#12	0 (0/17)
6W-14b#9	12–0	nd	6W-14b#15	16 (3/19)

Identification of T_2 individuals homozygous for the transgene was determined by analyzing the ratio of T_2 siblings that contained an amplifiable portion of the transgene (transgene positive). T_3 progeny were analyzed for resistance to kanamycin (Kan^R) by plating 15–20 seeds on $^1/_2$ MS + $Kan_{(150)}$ and scoring for alterations in colour of plants two weeks after plating. Bolded lines are those used for phenotypic growth analysis. "*" indicates that a corresponding negative sibling does not exist because a null T_1 plant did not segregate out in the T_1 progeny. "nd" indicates that resistance to kanamycin was not determined due to limited T_3 seed resources.

pollination bags to ensure self-fertilization. As plants reached maturity, water and fertilizer were slowly reduced, plants were dried, and seed was collected.

Plants for biomass measurements were grown in six inch pots filled with wet vermiculite; each pot contained 2–3 seeds from the T_2 generation. Plants were grown in chambers for five to six weeks with conditions as described above, but with the following modifications. Water was supplied as plants needed. Beginning 2–3 weeks after potting, each pot received 200 μL of 20 mM FeEDTA once a week, 6 mL of fertilizer solution (152 mM $MgSO_4{\cdot}7H_2O$, 14.53 mM $K_2HPO_4{\cdot}2H_2O$, 274.87 mM KH_2PO_4, 694 nM H_3BO_3, 104 nM $MnSO_4{\cdot}4H_2O$, 11.5 nN $ZnSO_4{\cdot}7H_2O$, 16.5 nM $CuSO_4{\cdot}5H_2O$ and 1.25 nM $Na_2MOO_4{\cdot}2H_2O$) twice a week, and 9 mL of the fertilizer solution once a week. The fertilizer solution also contained urea at one of two concentrations: high N fertilizer contained 356 mM urea, low N fertilizer contained 120 mM urea. The seedlings were culled before beginning the fertilizer and FeEDTA treatments so that each pot contained one plant of similar size. Fourteen plants from each line were chosen at two weeks after potting and randomly divided into either the high or low N fertilizer group. Plants surviving the treatment were harvested at the end of five or six weeks, separating the root and shoot portions of each plant. Fresh weights of both roots and shoots of each plant, were recorded. Roots and shoots were dried at 65 °C for 3–4 days and dry tissue weights were recorded. The largest and smallest plants from each line under each fertilizer regime, determined by dry biomass, were removed from further analysis to reduce the impact of outliers on the data. Two separate growth trials were conducted consecutively in this manner, named 'Experiment 1' and 'Experiment 2', respectively.

2.5. Hydroponics

Ten to twelve seeds from the bulked T_3 generation of individual transgenic lines were surface-sterilized in 20% bleach solution for 15–20 min, rinsed several times with sterile water and plated on germination medium (1 mM $MgSO_4{\cdot}7H_2O$, 0.5 mM K_2SO_4, 2 mM $CaCl_2$, 0.05 mM $K_2HPO_4{\cdot}2H_2O$, 0.95 mM KH_2PO_4, 20.82 nM H_3BO_3, 3.12 nM $MnSO_4{\cdot}4H_2O$, 0.345 nM $ZnSO_4{\cdot}7H_2O$, 0.495 nM $CuSO_4{\cdot}5H_2O$, 0.045 nM $Na_2MOO_4{\cdot}2H_2O$, 10 nM FeEDTA and 1.25 g per 500 mL Phytagar (Gibco, Cat. No. 10675-023)) at pH 5.2, under sterile conditions. After five days, seedlings were transferred to a previously sterilized 20 L aquaria, which had blacked-out glass. Aquaria contained 18 L of nutrient solution (2 mM $NaNO_3$, 0.25 mM NH_4NO_3, 1 mM $MgSO_4{\cdot}7H_2O$, 0.5 mM K_2SO_4, 2 mM $CaCl_2$, 0.05 mM $K_2HPO_4{\cdot}2H_2O$, 0.95 mM KH_2PO_4, 41.64 nM H_3BO_3, 6.24 nM $MnSO_4{\cdot}4H_2O$, 0.69 nM $ZnSO_4{\cdot}7H_2O$, 0.99 nM $CuSO_4{\cdot}5H_2O$, 0.09 nM $Na_2MOO_4{\cdot}2H_2O$, 20 nM FeEDTA at pH 5.2–5.5), based on Long Ashton nutrient solution [52]. The nutrient solution pH was monitored daily, and adjusted appropriately with 1 M HCl or 1 M NaOH. Gently aeration was provided to the aquaria throughout plant growth. Photoperiod, humidity, light intensity and temperature were as described above for growth of plants in potted mixes. During soil trials, urea was used as the N source because it had shown to be a preferred form of N for *B. napus* in previous growth chamber studies by our lab. However, because urea is toxic to plants when applied in a hydroponic environment, the N source was changed to a mix of nitrate and ammonium for hydroponic assays.

Plants for aspartate aminotransferase activity assays and gene expression were grown with transgenic lines, their negative siblings and control plants (N-o-1) in the same aquarium, with two aquaria for each line. Plants were randomly assigned a location within each tank. One aquarium was treated with 100 mM NaCl in a stepwise manner as described below, while the other was not. Five days prior to harvest 25 mM NaCl was added to the aquaria in order to induce the expression of the osmotic stress promoter, *btg-26* [49]. Four days prior to harvest, another 25 mM NaCl was added to aquaria, and finally, at two days prior to harvest, 50 mM NaCl was added to the aquaria. Three plants/line were harvested three weeks post-germination, at the fourth or fifth leaf stage. The newest root growth, and the fourth leaf were harvested from each plant. Tissue for protein analysis and enzyme activity was stored on ice until it could be processed the same day. Tissue to be used for verification of the transgene insertion and gene expression studies was flash-frozen and stored at −80 °C until further analysis.

2.6. Extraction of RNA and total protein

An RNeasy Plant Mini Kit (Qiagen, Cat. No. 74904) was used to isolate total RNA from 100 mg of either root or shoot tissue (described in Section 2.5). Extractions were carried out as per the manufacturer's protocol. DNA contamination was removed using Ambion's DNA-Free Kit (Ambion, Cat. No. 1906). RNA was stored at −20 °C.

Total protein was harvested from both root and shoot tissues (described in Section 2.5). Tissues were ground on ice with mortar and pestle and extraction buffer (0.5 M EDTA, 0.1 mM DTT (dithiothreitol), 10 mM cysteine, 0.1 mM PMSF (phenylmethanesulfonyl fluoride), 5 μM leupeptin and 100 mM Tris-HCl pH 7.8), in a ratio of 3:1 (buffer:tissue, μL:μg), with a pinch of sand and PPVP (poly(vinylpolypyrrolidone)). The resultant slurry was centrifuged at 13000 rpm for 15 min and the supernatant stored on ice. (Modified from Ismond et al. [53].)

2.7. RT-PCR

First strand cDNA synthesis was carried out using SuperScript II Reverse Transcriptase (Invitrogen, Cat. No. 18064-022) using primers to prime *AAT-1* (contained within the transgene) and *B. napus actin* mRNA (Genebank Accession# AF111812), respectively: 5′-TCACGGGGATGAATTGATAACAAAC-3′ and 5′-TAGCCGTCTCCAGCTCTTGC-3′. SYBR® Green (Life Technologies) was used to detect PCR product for quantification. Primers (synthesized by IDT) for RT-PCR detection were as follows: *AAT* (5′-AACATGGGTCTTTATGGTGAACGTG-3′ and 5′-TCACGGGGATGAATTGATAACAAAC-3′) and *B. napus actin* (5′-GGTCGTCCTAGGCACACTGG-3′ and 5′-TAGCCGTCTCCAGCTCTTGC-3′). To control for the presence of cDNA contamination, a second round of RT-PCR was done, as described above, but withholding the reverse transcriptase enzyme during first strand cDNA synthesis.

2.8. Quantification of soluble protein and aspartate aminotransferase activity

The concentration of total soluble protein was measured using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Cat. No. 500-0006). Protein fractions, extracted as per the above protocol, were assayed according to the manufacturer's instructions using a microplate spectrophotometer (SpectroMAX Plus, Molecular Devices, Sunnyvale, CA). Bovine serum albumin (BSA) was used as a protein standard. All samples were assayed in triplicate, and the average recorded for each sample.

Aspartate aminotransferase activity was determined using a slightly revised protocol described by Rej and Horder [54]. The protocol was modified to reduce the total assay volume from 2.4 mL to 150 μL allowing for the use of a microplate spectrophotometer (SpectroMAX Plus, Molecular Devices, Sunnyvale, CA). Ten microliters of a diluted aliquot of the total protein isolate was added to 120 μL of assay buffer (240 mM aspartate, 100 mM Tris-HCl pH 7.8, 0.11 mM PLP, 0.16 mM NADH, 1.0 U mL^{-1} malate dehydrogenase (Sigma, Cat. No. M2634) and 0.455 U mL^{-1} lactate

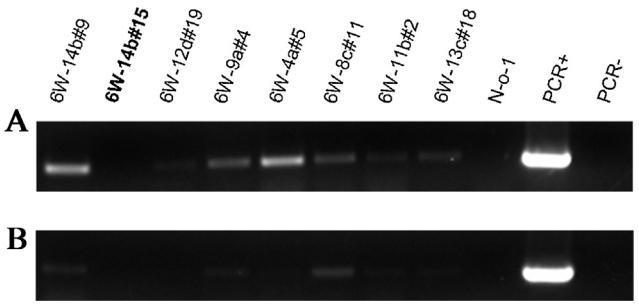

Fig. 2. Presence of transgenic *AAT* transcripts in roots (A) and shoots (B) of independent transgenic lines. Thirty cycles of RT-PCR was used to amplify *AAT* transgene transcripts from 2.5 μL of total RNA extracted from induced, hydroponically grown plants. Primers specific for the *AAT* transgene were used to synthesis the first strand cDNA and to amplify the cDNA template. 6W-14b#15, indicated in bold, is the negative sibling of 6W-14b#9. N-o-1 is the double haploid line derived from *B. napus* cv Westar. The template for the PCR+ reaction was the binary vector. No cDNA template was included in the PCR- sample.

dehydrogenase (Sigma, Cat. No. L2518)). Change in absorbance at 339 nm was monitored for five min to determine background activity levels. Twenty microliters of 2-oxoglutarate was then added to each sample to determine enzyme activity levels, and change in absorbance was again monitored at 339 nm for five min. Change in absorbance between enzyme activity levels and background activity levels was used to determine overall AAT activity in a sample. Three technical replicates were performed for each sample; the average between technical replicates was used to determine mean activity of that sample.

2.9. Statistical analysis

Mean dry root and shoot biomass was compared between transgenic lines in duplicate experiments using SPSS 11.5 statistical software. Differences in biomass, both root and shoot, between the various transgenic lines and the control plants under both high and low N fertilizer conditions were analyzed via ANOVA ($P < 0.05$, $\alpha = 0.05$). Significant differences between the mean dry root and shoot biomass of transgenic lines in relation to fertilizer regime was determined using a Tukey's multiple comparisons test ($P < 0.05$, $\alpha = 0.05$). SPSS 11.5 statistical software was also used to compare enzyme activity between transgenic lines and their null siblings. Differences in enzyme activity between the transgenic line and its negative sibling were determined by an unpaired two-tailed Student's *t*-test ($P > 0.05$, df = 2, n = 3).

3. Results and discussion

3.1. Expression of transgenic AAT driven by the btg-26 promoter in B. napus

Expression of the *AAT* transgene in the transgenic lines was determined using RT-PCR. Three plants per line were analyzed for expression of the transgene using transgene specific primers for both cDNA synthesis and PCR amplification. Transcripts from the transgenic *AAT* were detected in a number of the transgenic lines. Transcript was detected in the roots of four of the lines studied: 6W-14b#9, 6W-9a#4, 6W-4a#5 and 6W-8c#11, and in the shoots of two of the lines studied: 6W-14b#9 and 6W-8c#11(Fig. 2). Higher relative levels of transgene expression were observed in the roots of both 6W-14b#9 and 6W-4a#5 when compared to the other *AAT*-expressing lines.

In an attempt to increase the clarity of these RT-PCR results, the number of RT-PCR reaction cycles was increased to >30. Unfortunately, while increasing the number of PCR cycles resulted in positive expression results in the shoots of all lines, this also resulted in false positives, as evidenced by product in the null sibling (6W-14b#15) (data not shown). This is not surprising given that a high degree of sequence homology exists between known cytosolic *AAT* genes [33,55,56], and indicates that our primers were most likely priming the native gene as well as the transgene under these conditions, but not under lesser rounds of PCR cycles.

3.2. Expression of transgenic AAT protein in B. napus

To determine if the cytosolic *AAT* transgene increased AAT activity in either the shoots or the roots of plants, hydroponic studies were performed on the transgenic lines and their null (negative) siblings (Table 2). Plants were either exposed or not to salt (inducing and non-inducing conditions, respectively), harvested, and tested for increased AAT activity in both shoots and roots. Statistical significance was determined between the transgenic line and its negative sibling by an unpaired two-tailed Student's *t*-test ($P > 0.05$, df = 2, n = 3).

Three lines showed significant differences between the AAT-activity of the transgenic and null siblings; lines 6W-6a (#4/#3) and 6W-9a(#4/#12) showed significant differences under

Table 2
Aspartate aminotransferase enzyme activity in induced and non-induced roots and shoots of hydroponically grown T_3 plants.

Line		Activity (μmol NADH/min* mg soluble protein)			
		Roots		Shoots	
		Non-inducing	Inducing	Non-inducing	Inducing
6W-6a	Transgenic (#3)	800.74 ± 37.61	1106.77 ± 38.33	**274.70±3.69**	277.44 ± 6.26
	Negative Sibling (#4)	828.91 ± 8.90	1169.60 ± 40.25	**256.58±3.44**	273.38 ± 5.06
6W-8c	Transgenic (#11)	766.86 ± 62.70	538.08 ± 265.3	224.96 ± 6.95	**334.67±28.17**
	Negative Sibling (#10)	855.04 ± 41.42	191.02 ± 34.55	230.30 ± 1.06	**452.35±25.59**
6W-9a	Transgenic (#4)	**873.16±15.17**	913.47 ± 76.93	506.21 ± 11.88	243.20 ± 16.52
	Negative Sibling (#12)	**1041.08±52.43**	1026.35 ± 40.60	444.95 ± 32.87	274.76 ± 19.82
6W-11b	Transgenic (#2)	712.44 ± 25.82	1045.47 ± 113.62	225.64 ± 4.70	231. 64 ± 5.49
	Negative Sibling (#11)	789.78 ± 69.64	1083.52 ± 32.80	227.46 ± 5.72	232.20 ± 5.95
6W-12d	Transgenic (#19)	794.84 ± 8.62	759.82 ± 14.29	339.99 ± 9.01	228.13 ± 7.14
	Negative Sibling (#20)	776.05 ± 77.18	782.46 ± 16.41	332.58 ± 24.12	215.41 ± 5.87
6W-13c	Transgenic (#18)	961.97 ± 59.64	995.60 ± 28.35	295.67 ± 23.04	282.07 ± 17.61
	Negative Sibling (#12)	954.00 ± 75.48	936.66 ± 252.36	319.28 ± 15.60	326.89 ± 71.44
6W-14b	Transgenic (#19)	757.83 ± 33.22	874.62 ± 252.36	234.73 ± 7.19	269.97 ± 127.81
	Negative Sibling (#15)	772.46 ± 8.54	946.78 ± 119.54	216.70 ± 7.54	331.89 ± 29.08

Mean activity from three replicates plus or minus the standard error is shown. The addition of 100 mM NaCl to the liquid medium was used to induce expression of the transgene. Values in bold indicate that enzyme activity differed significantly between the transgenic line and its negative sibling as determined by an unpaired two-tailed Student's *t*-test ($P > 0.05$, df = 2, n = 3).

non-inducing conditions and 6W-8c(#10/#11) showed significant differences under inducing conditions (Table 2). However, none of these lines showed differences in both sets of conditions. Moreover, both 6W-8c and 6W-9a showed significantly higher levels of AAT-specific activity in the null sibling, with only line 6W-6a showing specific activity increases in the *AAT*-expressing plants. The reasons for increased expression in the null siblings are unknown, but could be a by-product of transformation, or simply a line that had slightly elevated endogenous AAT activity. 6W-14b#9, which showed significant increases in biomass in growth chamber Experiment 1 (Section 3.3), did not appear to increase or decrease AAT activity relative to its null sibling.

Other studies over-expressing *AAT* have documented increases in AAT activity in various tissues, however these studies utilized the constitutive promoter CaMV 35S [57–59], indicating that higher levels of expression may be required to detect reliable, quantifiable differences in AAT activity in plants. Originally, it was decided that *AAT-1* would be expressed via the *btg-26* promoter of *B. napus*, because of its previous use in driving expression of *AlaAT* in NUE canola, as well as its ability to be induced by salt stress [49]. During the course of this work, it was discovered that the 300 bp promoter region of *btg-26* used in this study, as well as the *AlaAT* canola studies, was expressed tissue-specifically in the roots [29]. This was an interesting discovery, given the previous NUE phenotypes observed when over-expressing *AlaAT*, and lead to the hypothesis that tissue-specific over-expression of *AlaAT* is required to produce NUE phenotypes. However, we were unable to explain why this tissue-specific expression results in plant NUE phenotypes as a result of over-expression of one aminotransferase (*AlaAT*) but not another (*AAT*). And, even now, several years later, the exact mechanism and molecular changes resulting in NUE phenotypes when *AlaAT* is tissue-specifically over-expressed are still not fully understood. It is possible that there is a form of post-translational modification occurring to AAT when expressed in the roots that is not evident with constitutively expressed *AAT*.

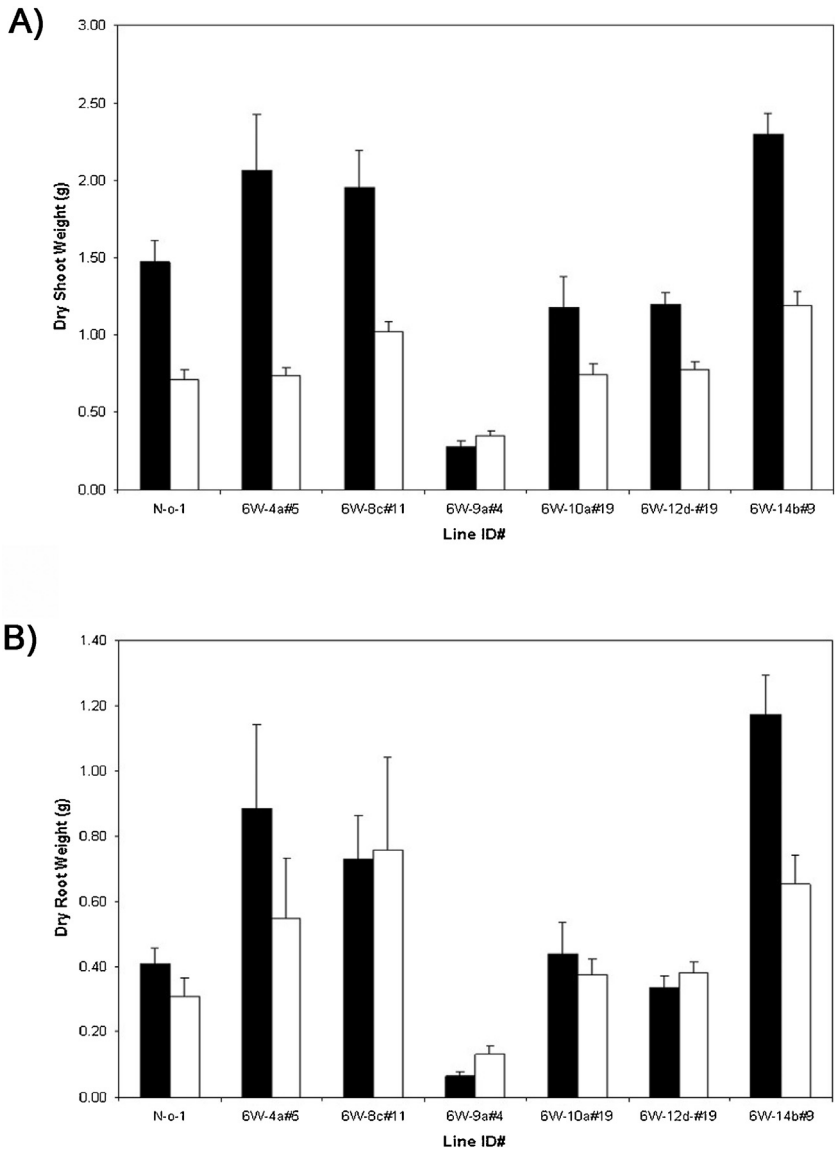

Fig. 3. Mean biomass measurements of dry shoots and roots of independent transgenic lines fertilized with a high or low nitrogen fertilizer in Experiment 1. Dry weight of shoots (A) and roots (B) was determined at two N concentrations: high (black bars) and low (white bars). Fertilizer treatments began two weeks after planting and tissues were harvested four weeks after fertilizer treatments began. Values are the average of three to five T_2 plants. The largest and smallest shoots, per line, determined by mass, where excluded from the average. Error bars are standard error.

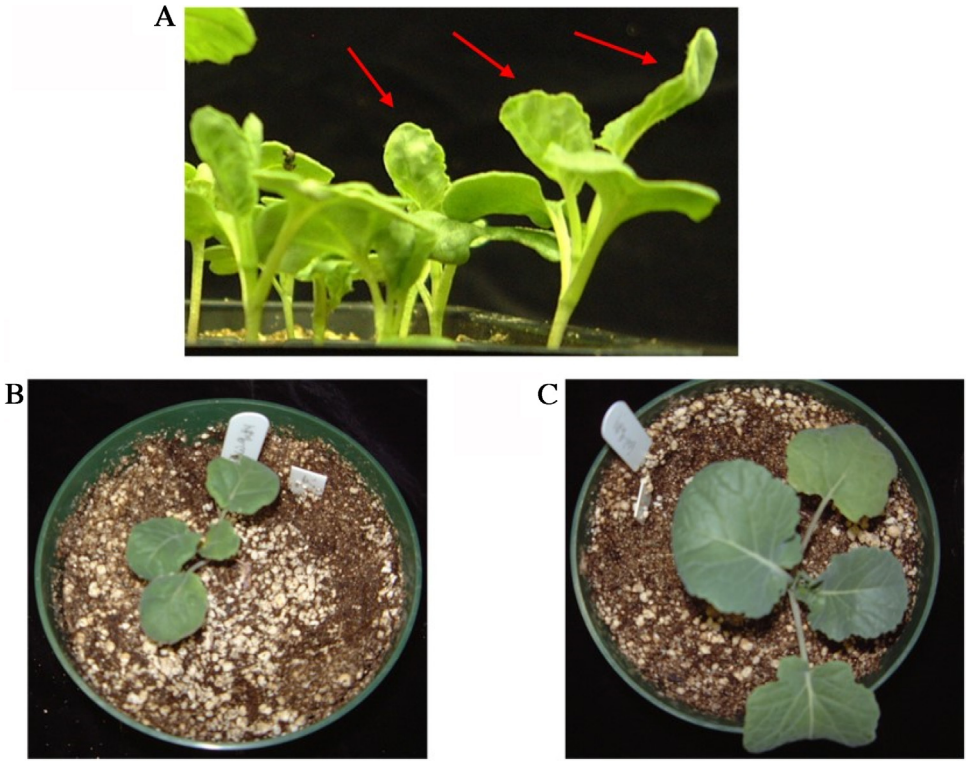

Fig. 4. Aberrant phenotype of T_3 individuals from line 6W-9a#4. (A) The mutant leaf morphology in true leaves (red arrows). (B) The stunted phenotype of a T_3 individual from line 6W-9a#4 compared to an individual from a corresponding negative sibling of the same age (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. *AAT over-expression does not result in NUE phenotypes in* B. napus

Growth chamber experiments were used to assess the effect of over-expression of *AAT*, from *Medicago sativa*, on NUE phenotypes, specifically alterations in dry weight of shoots and roots. Previous studies over-expressing the *AlaAT* gene from barley (*Hordeum volgare*) in canola had resulted in increased plant biomass under low N conditions. Also observed were increases in seed yield [29]. Due to these previous results, variations in biomass, under either high or low N, were chosen as an initial benchmark phenotype for overall alterations in plant NUE. For this study, *AAT* from *M. sativa* and not *H. vulgare* was used based on the availability of a gene clone. Two replicate experiments were conducted to fully assess changes in plant biomass and to verify that any observed changes were real. Experiments were limited by growth chamber space, amount of seed and availability of a null (negative) sibling. Any promising alterations in biomass, whether increases or decreases in either N treatment, were to be followed-up with other NUE related analysis, including assessment of seed yield, assessment of alterations in key N metabolites (i.e. aspartate, glutamate and glutamine) and assessment of N-uptake.

Six transgenic lines were analyzed in experiment one: lines 6W-4a#5, 6W-8c#11, 6W-9a#4, 6W-10a#19, 6W-12d#19 and 6W-14b#9. Of the six lines originally screened, only 6W-14b#9 showed significant increases in both root and shoot biomass in Experiment 1 (Fig. 3). Mean dry shoot weight of these plants, fertilized with high and low N fertilizer regimes respectively, was significantly increased at 1.6 and 1.7 times greater than control plants (N-o-1) (Fig. 3A). Mean dry root weight of this line, under the high N fertilizer regime, was also significantly increased at 2.9 times that of the control line. Mean dry root weight of plants from this line was also increased under low N conditions, but not significantly as determined via ANOVA, at 2.1 times that of control plants (Fig. 3B). The remaining five lines screened had similar or slightly reduced mean dry shoot and root biomass when compared to the N-o-1 plants. Results from 6W-4a#5, 6W-8c#11, 6W-10a#19 and 6W-12d#19 were not significantly different than the controls under either N fertilizer treatment (Fig. 3). Interestingly, plants from line 6W-9a#4 had significant decreases in dry weight of shoots in both the high and the low N treatments, and significant reductions in root biomass under the high N treatment (Fig. 3). Furthermore, plants from this line exhibited an aberrant shoot phenotype (Fig. 4). Individuals from this line were also stunted and had poor seed set when compared to N-o-1 and the negative sibling of this line (6W-9a#12) (Fig. 4). The reasons for this aberrant phenotype are unclear, and were not observed in any of the other lines at any point in the study. It is speculated that point of transgene insertion may have played a key role in the production of this phenotype.

To further analyze the biomass of line 6W-14b#9, a second set of growth chamber experiments was conducted (Experiment 2). Also included in this study were lines 6W-4a#5 and 6W-8c#11. The experimental design was identical to that of Experiment 1, with one exception: fertilizer treatments began one week earlier. Mean dry weights of shoots and roots from all plant lines were analyzed as before. Unfortunately, the increases in dry biomass of line 6W-14b#9 were not observed, and therefore could not be replicated. The dry shoot and root weights in both N treatments were comparable to the control line. Lines 6W-4a#5 and 6W-8c#11 had similar dry shoot weights in both low and high N conditions, and similar root weights under high N conditions. Interestingly, under the low N treatment, both lines were observed to have significant decreases in biomass relative to the control (results not shown).

Although fertilization was started one week earlier than Experiment 1, it is unlikely that this resulted in loss of our biomass phenotype in line 6W-14b#9. Increasing nutrient

availability early in plant growth was expected to increase biomass and overall seed yield, the latter of which was the reason for altering fertilizer regime originally. Therefore, it can be inferred that the alterations in plant biomass were due to factors unrelated to changes in fertilizer regime, but that these factors did not result in a phenotype that was either reproducible or consistent in nature. Considering only one line out of the six tested showed promising increases in biomass, it is still unclear whether these alterations were due to over-expression of the transgene, *AAT*, or the result of epigenetic transgenerational changes associated with generation of transgenic plants [60,61].

4. Conclusions

The work described here indicates that over-expression of *AAT* in canola does not produce a discernable increase in plant biomass under differing N fertilizer regimes, and that over-expression of this gene by the promoter *btg-26* does not result in reliably altered AAT activity in roots of plants, regardless of transgene expression levels. Thus, the over-expression of this enzyme does not produce NUE phenotypes similar to those produced by over-expression of *AlaAT* in canola [43] as hypothesized. Because NUE phenotypes were not observed in plants, and because any perceived alterations were not reliably reproducible, further work on *AAT* as it pertains to plant NUE was not carried out. Interestingly, research done since this study was conducted has shown concurrence with our results, and indicated that over-expression of *AAT* does not increase plant biomass [57–59,62], but instead, impacts amino acid content of tissues, specifically seeds, when using a constitutive promoter, in both Arabidopsis and rice [57,58]. This phenotype was also shown to be isozyme specific, with not all AAT enzymes resulting in amino acid content changes when over-expressed [58]. To date, no specific alterations in N-uptake, N-assimilation or N-mobilization in plants have been documented as a result of alterations in the expression of this enzyme. Given the many temporal, spatial and enzymatic similarities between AAT and AlaAT an in-depth analysis of the different transcriptional and metabolic changes that occur as a result of over-expressing each of these enzymes, may shed light on why *AlaAT* produces an NUE phenotype, and how plants can be further engineered to take advantage of these properties.

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