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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

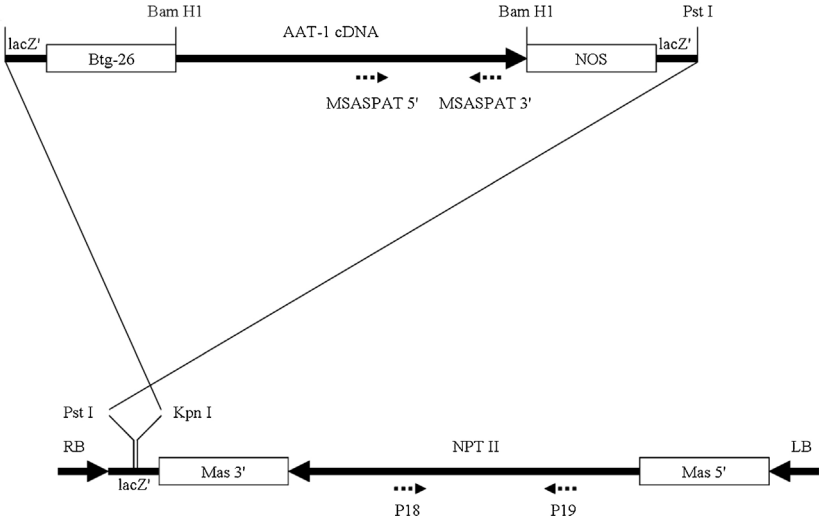

Fig. 1. Organization of genes located between the left and right borders of the binary vector p26gAspATNS47. The locations of primer binding sites used to screen the transgenic lines are indicated by dashed arrows. Primer pairs MSASPAT 5′ and MSASPAT 3′ and P18 and P19 amplify 489 and 657 bp fragments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