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Molecular and phenotypic characterization of transgenic wheat and sorghum events expressing the barley alanine aminotransferase

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Abstract

***Main conclusion* — The expression of a barley alanine aminotransferase gene impacts agronomic outcomes in a C3 crop, wheat.**

The use of nitrogen-based fertilizers has become one of the major agronomic inputs in crop production systems. Strategies to enhance nitrogen assimilation and flux in planta are being pursued through the introduction of novel genetic alleles. Here an *Agrobacterium*-mediated approach was employed to introduce the alanine aminotransferase from barley (*Hordeum vulgare*), *HvAlaAT*, into wheat (*Triticum aestivum*) and sorghum (*Sorghum bicolor*), regulated by either constitutive or root preferred promoter elements. Plants harboring the transgenic *HvAlaAT* alleles displayed increased alanine aminotransferase (alt) activity. The enhanced alt activity impacted height, tillering and significantly boosted vegetative biomass relative to controls in wheat evaluated under hydroponic conditions, where the phenotypic outcome across these parameters varied relative to time of year study was conducted. Constitutive expression of *HvAlaAT* translated to elevation in wheat grain yield under field conditions. In sorghum, expression of *HvAlaAT* enhanced enzymatic activity, but no changes in phenotypic outcomes were observed. Taken together these results suggest that positive agronomic outcomes can be achieved through enhanced alt activity in a C3 crop, wheat. However, the variability observed across experiments under greenhouse conditions implies the phenotypic outcomes imparted by the *HvAlaAT* allele in wheat may be impacted by environment.

Keywords: Nitrogen use efficiency, *Agrobacterium tumefaciens,* Abiotic stress, Biotechnology

Introduction

Cereals such as wheat and sorghum account for over 20% of the calories consumed globally. To meet the high demand for these grain crops numerous efforts have focused on understanding their genomes, expanding genetic diversity and enhancing key traits such as nitrogen use efficiency to

maximize production, while mitigating the environmental footprint (Brenchley et al. 2012). Agriculture productivity relies substantially on nitrogen-based fertilizers. Along with the imminent increase in global population, the total fertilizer demand is estimated to increase by threefold by 2050 (Nations 2009). Enhancing nitrogen use efficiency of crops will be essential to ensure food security for the increasing global population, in a sustainable fashion.

Plants acquire nitrogen from the soil primarily as nitrate or ammonium via multiple specific transporters, into the root system (He et al. 2015; Krapp et al. 2014; Miller et al. 2007; Xu et al. 2012). The ammonium derived from nitrate reduction, or via direct uptake is assimilated into glutamine or glutamate by the GS/GOGAT cycle, the primary route for inorganic nitrogen assimilation (Lea and Miflin 2003). These amino acids serve as a source for the synthesis of other amino acids, nucleic acids, proteins, polyamines and alkaloids (Suzuki and Knaff 2005). Enhancing nitrogen use efficiency (NUE) is being pursued genetically through various breeding, and biotechnology approaches (Fischer et al. 2013; Han et al. 2015; Li et al. 2016; Masclaux-Daubresse et al. 2010; McAllister et al. 2012; Xu et al. 2012). Most of the biotechnology strategies have focused around the introduction of transgenic alleles targeting primary nitrogen metabolism. However, results obtained across species have been inconsistent with regard to NUE phenotypic outcomes (Xu et al. 2012). The metabolic network coordination between carbon and nitrogen can influence NUE. Exploiting these linkage strategies that addressed repletion of N metabolism with carbon skeletons such as 2-oxoglutarate and oxaloacetate from the TCA cycle have been promising as a strategy to boost mineral use efficiencies (Good et al. 2007; He et al. 2015; Kurai et al. 2011; McAllister and Good 2015; Shrawat et al. 2008; Yanagisawa et al. 2004).

Alanine aminotransferase (*AlaAT*) is a ubiquitous enzyme that may influence nitrogen assimilation and remobilization in planta (McAllister and Good 2015). The transamination between alanine and 2-oxoglutarate by alt results in the production of glutamate and pyruvate in a reversible manner, providing dual functions in carbon and nitrogen metabolism (Kendziorek et al. 2012). *AlaAT* has been implicated in many processes during plant development and as a stress responsive element (Diab and Limami 2016; Duff et al. 2012; Good and Crosby 1989; Kendziorek et al. 2012; Liepman and Olsen 2003; Miyashita et al. 2007; Muench and Good 1994; Rocha et al. 2010a, b; Yang et al. 2015). In addition to participating in general amino acid metabolism, alt, serves as a carbon shuttle, which mobilizes pyruvate from the mesophyll to the bundle sheath cells in C_4 plants (Son and Sugiyama 1992). In barley, an alt encoded by *Qsd1* controls seed dormancy, and manipulation of the gene can lead to reduced dormancy period in the grain (Sato et al. 2016). In addition, under hypoxic stress plants use alanine as a major storage amino acid, which can be easily converted to glutamate and pyruvate by *AlaAT* upon normalization of aerobic conditions (Diab and Limami 2016).

Modulating alt activity, as a target to enhance NUE, was demonstrated when a cytosolic *AlaAT* from barley (*HvAlaAT*) was introduced in canola (*Brassica napus*). Here an *AlaAT* cassette controlled by the root preferred, *Btg26*, promoter, was introduced and derived transgenic events exhibited increases in biomass and seed yield in N-limiting conditions, under controlled environment. This translated to approximately 40% of the fertilizer input to maintain growth and yield under field environments. The phenotype outcomes observed were attributed to the root-preferred expression of *HvAlaAT* leading to changes in metabolite concentrations and alterations in nitrate flux (Good et al. 2007). The *HvAlaAT* has also been shown to alter plant development through NUE changes in rice (Shrawat et al. 2008). Mirroring the canola study, a root-preferred *HvAlaAT* cassette, regulated by the *OsAnt1* promoter was introduced into rice. Phenotyping of the derived transgenic events revealed, boosts in biomass and yield under sufficient nitrogen conditions and changes in key metabolites and total nitrogen content (Beatty et al. 2009, 2013; Shrawat et al. 2008). The outcomes observed in rice were recently translated to sugarcane, wherein the data revealed improved growth

and higher NUE in events grown under limiting N conditions, under a controlled setting (Snyman et al. 2015).

The study communicated herein discusses the phenotypic outcomes observed when the *HvAlaAT* (Muench and Good 1994) is expressed in wheat and sorghum, under control of either a constitutive UBI4 promoter from sugarcane or a root preferred promoter, *OsANT1* from the rice *aldehyde dehydrogenase 1* gene (Beatty et al. 2009; Shrawat et al. 2008).

Materials and methods

Vector construction and generation of transgenic plants

Two constructs were designed that harbor codon-optimized version of the *HvAlaAT* cDNA from barley, *Hordeum vulgare* (Muench and Good 1994) (GenBank Z26322, 1446 bp) (GenScript, Piscataway, NJ, USA). Each expression cassette carried the *HvAlaAT* downstream of either the sugarcane (*Saccharum officinarum*) UBI4 promoter (Wei et al. 2003) for constitutive expression, or the rice (*Oryza sativa*) *OsAnt1* promoter (Shrawat et al. 2008) for tissue-specific expression. Each cassette was terminated with the T35s polyAAA signal and cloned into the binary plasmid pPZP212 (Hajdukiewicz et al. 1994). The designed plasmids referred to as pPTN1031 and pPTN1040, respectively, carried a *neomycin phosphotransferase II* (*NPTII*) cassette for plant selection. The binary vectors were mobilized via triparental mating into the *Agrobacterium tumefaciens* strain C58C1/pMP90 (Koncz and Schell 1986) and the NTL_4/ pKPSF2 (Luo et al. 2001) for wheat (*Triticum aestivum*) and sorghum (*Sorghum bicolor*) transformation, respectively. The spring wheat genotype CB037 was used for transformation following the protocol outlined by Clemente and Mitra, 2004. The sorghum grain genotype TX430 was used for transformation as previously described by Howe et al. (2006a).

Selection of transgenic plants

Expression of NPTII was monitored by an ELISA kit (Agdia® Cat# PSP73000/0480) to select T_0 and T_1 transformants. Plants from subsequent generations were selected based on the amplification of a fragment containing a region of the *HvAlaAT* cDNA through PCR analysis. Following genomic DNA isolation (Springer 2010), 100 ng DNA, 0.5 µM of each primer (Online Resource 4), and 1X GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) were mixed and the genetic fragments amplified using the PCR conditions: 95 °C for 2 min, 35 cycles of 95 °C for 20 s, 51 °C (pPTN1031) or 54 °C (pPTN1040) for 30 s and 72 °C for 1 min, and a final extension of 72 °C for 10 min. Electrophoresis on a 0.8% agarose gel was used to separate the PCR products.

Molecular characterization of transgenic plants

Southern blot hybridization was performed to monitor transgenic allele integration. Young leaves were frozen in liquid nitrogen genomic DNA was isolated following the procedures described by Dellaporta et al. (1983). The restriction enzymes *Eco*RI and *Nco*I were selected based on a single recognition site, to digest 15 µg of genomic DNA from transgenic events bearing the pPTN1031 and the pPTN1040 expression cassettes, respectively. Genomic DNA from wild type genotypes were used as negative control. Expression of *HvAlaAT* was monitored by northern blot hybridization. RNA from either young leaves or roots was isolated with TRIzol® reagent (Invitrogen cat. # 10296-028) according to Chomczynski (1993). The quality and concentration of RNA was determined with a NanoDrop® ND 1000 Spectrophotometer (Thermo Fisher, Wilmington, DE, USA). Fifteen µg of

digested DNA and 15 µg or RNA were separated by electrophoresis in 1% agarose gels for Southern and northern blot hybridizations, respectively. The samples were blotted and cross-linked using a Stratalinker® UV Crosslinker 1800 (Stratagene, La Jolla, CA, USA) to a nylon membrane (Bio-Rad cat #162-0196, Hercules, CA, USA). Probes were generated by random prime labeling (Prime-It II Cat # 300385, Stratagene, La Jolla, CA, USA). Hybridization parameters, with a dCT32P labeled probes of the *HvAlaAT* (504 bp) were conducted as described by Eckert et al. (2006).

Alanine aminotransferase enzyme assay

Alanine aminotransferase assays were performed following the protocol described by Shrawat et al. (2008). Approximately 150 mg of leaf or root tissue was ground in liquid nitrogen and homogenized in an extraction buffer containing 100 mM Tris HCl pH 7.8, 5 mM EDTA, 1 mM DTT, 10 mM cysteine, 0.1 mM PMSF and 5 µM leupeptin. The alt activity was measured in an assay buffer containing 100 mM Tris HCl pH 8.0, 10 mM α-ketoglutarate, 0.28 mM NADH, 1.2 U ml^{-1} lactate dehydrogenase and 70 mM Ala. The reaction was initiated upon the addition of Ala. The oxidation of NADH was measured at 340 nm. Enzyme activity is expressed as AlaAT activity µmol min^{-1} mg^{-1} protein. Leaf soluble protein concentration was determined at 595 nm according to Bradford (1976).

Hydroponic system

The performance of transgenic wheat expressing *HvAlaAT* was evaluated under a hydroponic system. The independent events identified as pPTN1031-NN591-1-3-1, pPTN1031- NN591-4-5-1, and pPTN1031-NN591-4-4-1 bearing the UBI4/*HvAlaAT* cassette, and the pPTN1040-SS529-4-1-1, pPTN1040-NN572-1-1-1, and pPTN1040-NN589-2-2-1 carrying the *OsAnt1/HvAlaAT* construct were selected for this study. Positive transgenic plants were identified based on PCR analysis as previously described and only lineages carrying the transgene were selected for the study. Plants were grown in 1:1 sand:fine vermiculite. One week after germination, two nitrogen treatments were supplied to the selected transgenic events and the wild type during 4 weeks. The N treatments, 15 mM NO_3 and 0.3 mM NO_3 in a modified Hoagland solution (Hoagland and Arnon 1950) were replaced three times per week. Leaf chlorophyll was monitored at anthesis in the two most expanded leaves with a Minolta SPAD meter. Chlorophyll concentrations were estimated using the SPAD readings in the exponential equation: µmol /m^2 = $10^{(M0.265)}$, r^2 = 0.94 (Markwell et al. 1995). Physiological measurements were collected upon termination of the experiment.

Field evaluations

The subset of transgenic events carrying homozygous alleles and control plants selected for the hydroponic trials, were evaluated under field conditions in Mead, NE, USA, during Spring–Summer 2015. Homozygous plants were treated with two N rates: a moderate N rate: 95 kg ha−1, and a low N: 53.8 kg ha^{-1}. A total of 600 plants, distributed across six rows, were used per plot (3 m × 3 m). Three plots per N rate and genotype combination were evaluated in this study. The experiment was arranged as a split plot design with nitrogen rate as a whole plot and six independent events and a wild type randomized as a CRD within whole plots. Evaluations of transgene expression, enzymatic activity and chlorophyll were measured at anthesis as previously described. Photosynthetic capacity was also determined at anthesis with a LICOR LI-6400XT Portable Photosynthesis System (LI-COR, Lincoln, NE, USA). Light was supplied using 6400-02 LED lights at 1500 µmol m^{-2} s^{-1}, 55 ± 5 relative humidity and 400 ppm of CO_2. Biomass measurements were determined at anthesis by harvesting 0.3 m of row of plants per plot, and at physiological maturity using 1 m of row of plants per plot.

The aboveground tissue was collected and dried at 100 °C during 2 days. Height was determined at physiological maturity. Yield per plot and 100 seed weight were also ascertained.

Soil -less potting system for sorghum

Independent events designated as pPTN1031-ZG120-2-2a, pPTN1031-ZG120-4-8a, pPTN1040-ZG151-1-24a, and pPTN1040-ZG158-2-3a (SUA1, SUA2, SAA1, SAA2, respectively) were selected for a phenotypic characterization. Positive transgenic plants were identified based on PCR analysis as previously described and only T4 and T5 lineages carrying the transgene were selected for the study. Plants were grown in Metro Mix 200. Three N treatments (15 mM NH4NO3, 3 mM NH4NO3 and 0.3 mM NH_4NO_3) formulated in a modified Hoagland solution (Hoagland and Arnon 1950), were evaluated. The treatment was started 1 week after planting and supplied three times per week until maturity. Leaf chlorophyll was monitored at anthesis in the two most expanded leaves as previously described. Physiological measurements were collected upon termination of the experiment.

Statistical analyses

The wheat hydroponics and field trials were evaluated as split plot designs with two N treatments as whole plots and seven genotypes as subplots. The sorghum soil-less system was evaluated as a CRD. The data from these evaluations were analyzed using analysis of variance (ANOVA) procedures of SAS® 9.3 software (SAS Institute Inc., Cary, NC 27513-2414, USA). Statistical differences for alt activity were determined with t-tests using GraphPad Prism 6 software (La Jolla, CA 92037, USA).

Results

Generation of transgenic wheat

A synthetic version of the *HvAlaAT* cDNA was introduced in spring wheat (CB037) under the control of either the constitutive polyubiquitin promoter (UBI4) from sugarcane (Wei et al. 2003), or the rice tissue-specific promoter (*OsAnt1*) (Shrawat et al. 2008). The binary vectors assembled were designated pPTN1031 and pPTN1040 for the UBI4/*HvAlaAT* and *OsAnt1/HvAlaAT* constructs, respectively (Fig. 1a, b). A total of 33 UBI4/*HvAlaAT* and 15 *OsAnt1/HvAlaAT* independent events were generated via *Agrobacterium*-mediated transformation (Clemente and Mitra 2004). The transgenic events were selected by monitoring *NPTII* through an ELISA assay, followed by the transgenic allele integration patterns and transgene expression of *HvAlaAT* via Southern and northern blot hybridization, respectively.

Molecular characterization of transgenic wheat

A subset of the transgenic events generated was selected for further characterizations. These are identified as pPTN1031- NN591-1-3-1, pPTN1031-NN591-4-5-1, and pPTN1031- NN-591-4-4-1 carrying the UBI4/*HvAlaAT* cassette are referred to as UA1, UA2 and UA3, respectively. The transgenic events selected for the *OsAnt1/HvAlaAT* construct are pPTN1040-SS529-4-1-1, pPTN1040-NN572-1-1-1, and pPTN1040-NN589-2-2-1, hereafter abbreviated as AA1, AA2, and AA3, respectively. Southern blot hybridizations indicated an integration of 1–3 transgenic alleles in the selected wheat events (Fig. 2a). Northern blot hybridizations detected the accumulation of *HvAlaAT* transcripts in leaves of the *UBI4/HvAlaAT* events and in roots of the *OsAnt1/HvAlaAT*

events (Fig. 2b, c). The transcript accumulation was also detected in leaves on the transgenic events bearing *HvAlaAT* under the control of the *OsAnt1* promoter (Fig. 2c), albeit at low levels. The enzymatic activity of alt was assessed in a continuous assay following the NADH oxidation at 340 nm. The assays were conducted at anthesis using the flag leaf and root tissue for the selected *UBI4/HvAlaAT* and *OsAnt1/HvAlaAT* events, respectively. All the transgenic events displayed a significant increase in enzymatic activity when compared to the wild type (Fig. 2e, f). The UBI4/*HvAlaAT* events exhibited an increase in alt activity up to 7.3-fold, while the *OsAnt1/HvAlaAT* events showed up to 1.6-fold increase in alt activity. The transcript accumulation detected in leaves of the *OsAnt1/HvAlaAT* events also promoted between 55 and 68% increases in alt activity relative to the controls in leaf tissue.

Phenotypic impact of HvAlaAT expression in wheat under hydroponic conditions

The effects of expression of *HvAlaAT* on growth and development of the selected transgenic wheat events were evaluated hydroponically. Plants were grown for 5 weeks under two nitrogen regimes, either a Hoagland solution containing 15 mM NO_3, categorized as optimal nitrogen conditions, or a modified Hoagland solution containing 0.3 mM NO_3 for limiting nitrogen. The combined data across three independent experiments indicated that expression of *HvAlaAT* under the control of either UBI4 or *OsAnt1* alters growth in transgenic wheat. The data revealed an impact in total dry biomass, driven by enhancement in shoot production rather than in root biomass (Table 1). Five out of six events exhibited a significant increase in total and shoot biomass under both N treatments. The expression of *HvAlaAT* also triggered up to a 13% gain in height in all the transgenic events, and increased tillering in a subset in plants grown under 15 and 0.3 mM NO_3 (Table 2). Modulation in chlorophyll content was also observed, but this parameter fluctuated across events evaluated. Under the hydroponic conditions used herein, there were no differences in plant phenotype associated with the promoter used to control the expression of *HvAlaAT*.

While the combined data indicated that *HvAlaAT* has the capacity to alter growth in wheat leading to improved agronomic outcomes, phenotypic variation, among some parameters measured, across the hydroponics experiments was detected, suggestive of a 'seasonal' component (including daylight hours and the temperature differences recorded). Hydroponics experiments conducted during spring 2013 and winter 2015 displayed similar results as those from the combined data. The greenhouse conditions were 23.8 °C and 13/10 h photoperiod for spring and 23.3 °C and 10/14 photoperiod for winter. However, a trial conducted during summer 2013 revealed up to 30% reduction in chlorophyll content across transgenic events. Despite this detrimental effect, plants had the capacity to maintain similar biomass accumulation to the wild type. The average temperature recorded was 25.5 °C and the actual photoperiod was 14/10 h.

Phenotypic impact of the HvAlaAT expression in wheat under field conditions

A field trial was conducted during spring–summer 2015 in Mead, NE (USA). A split plot design with N rate as a whole plot and genotype as a split plot in a CRD was utilized in this trial. Six homozygous transgenic events (UA1, UA2, UA3, AA1, AA2, AA3) and WT were randomized in six plots for a moderate N rate (94.1 kg ha^{-1}) or a low N rate (53.8 kg ha^{-1}). Each plot contained 600 plants distributed in six rows with 100 plants each. Results from this trial indicated that most of the transgenic events have the capacity to accumulate more biomass, ascertained at anthesis, relative to WT, with no relationship to N treatment (Fig. 3a). However, biomass at maturity remained unaltered, with the exception of a significant reduction in event UA1, grown under low N regime (Fig. 3b). Significant enhancement of grain harvest per plot and harvest index was observed in

events UA1 and UA3 (Fig. 3c, d). Both the UA1 and UA3 events exhibited significant increase in yield, on a per plot basis, under both N treatments (Fig. 3c). Mirroring the data gathered under hydroponics, the height of the transgenic events was increased across N rates in comparison to the WT (Fig. 4a). Most of the events with no increases in seed plot weights exhibited a decrease in 100 seed weight (Fig. 4b). The expression of *HvAlaAT* did not manifest in alterations in the photosynthetic capacity of the transgenic plants, under the field study (Online Resource 1).

Effects of the HvAlaAT expression in sorghum

The expression cassettes harboring the UBI4/*HvAlaAT* and *OsAnt1/HvAlaAT* (Fig. 1a, b) were introduced in the sorghum grain genotype TX430 via *Agrobacterium*-mediated gene transfer (Howe et al. 2006b). A total of 11 independent events were generated from pPTN1031 and 30 from pPTN1040. Preliminary evaluations were conducted to identify transgenic events monitoring for the expression of the selectable marker via ELISA assays, visualizing transgenic allele integration patterns and corresponding transcript accumulation. The events designated as SUA1, SUA2, SAA1, and SAA2 identified as pPTN1031-ZG120-2-2a, pPTN1031-ZG120-4-8a, pPTN1040-ZG151-1-24a and pPTN1040-ZG158-2-3a, respectively, were selected for further characterizations. The wild type was designated SWT, to distinguish it from the wheat wild type (WT). The vetted events carried between one and two transgenic alleles, based on Southern blot hybridizations (Online Resource 2a). A strong transcript accumulation was observed in leaves of the selected sorghum events, with no obvious changes in gene expression across constructs (Online Resource 2b), suggestive that the specificity of the *OsAnt1* regulator element does not translate to sorghum. Given the observed relative comparative transcript accumulation in leaves across the events, alt activity was measured from leaf extracts in all the selected transgenic events. The enzymatic activity was increased between 2.4- and 3.5-fold in the four independent events (Online Resource 2c). To evaluate the impact of *HvAlaAT* expression, three independent studies were conducted in plants grown in a soil matrix until maturity under three nitrogen regimes: 15 mM NH4NO3, 3 mM NH4NO3, and 0.3 mM NH_4NO_3. Means tabulated from the derived datasets from these experiments were statistically separated within an N treatment or across N levels depending on ANOVA results for interaction of N by event (Online Resource 3). The analyses of the datasets revealed no correlation with presence of the transgenic allele and positive outcomes in the parameters measured (Online Resource 3). However, modulation in height across events and nitrogen treatments was evident (Online Resource 3).

Discussion

Transgenic alleles with *HvAlaAT* cassettes were introduced into wheat and sorghum to determine if this strategy has the potential to boost nitrogen use efficiency in these crops, in a similar manner observed in canola (Good et al. 2007) and rice (Shrawat et al. 2008).

Expression of *HvAlaAT* in wheat and sorghum translated to enhanced enzymatic activity in both crops. The *OsAnt1* promoter (Fig. 1b) displayed a more root preferred expression pattern in wheat, rather than specificity (Fig. 2). In sorghum, however, both UBI4 and *OsAnt1* regulatory elements had more constitutive expression patterns (Online Resource 2), reflecting the lack of translation from the C3 crop the *OsAnt1* promoter was isolated from, rice, wherein it was classified as having root epidermal cell specificity (Shrawat et al. 2008).

The *OsAnt1* encodes for an antiquitin gene (GenBank AF323586), a member of the aldehyde dehydrogenase family (ALDH), with homology to the canola *Bgt26* gene (Gen-Bank S77096) (Stroeher et al. 1995). Antiquitin is a highly conserved protein across eukaryotes, and has

functionality associated with regulation of cellular turgor pressure and osmotic stress (Guerrero et al. 1990). Members of the ALDH family have been implicated to abiotic stress responses including drought, cold and heat (Nakashima et al. 2014). In wheat, expression profiles of the *TraeALDH7B1-5A,* which encodes for *aldehyde dehydrogenase* gene, indicated that three homologous genes are induced in leaves, roots and spikelets under drought and salt stress (Chen et al. 2015). Similarly, the betaine aldehyde dehydrogenase (*BADH*) in sorghum, which also contributes to salt and drought stress, is strongly expressed in leaves (Wood et al. 1996). Profiling of GUS expression, regulated by *OsAnt1* and bgt26 promoters, indicated a specificity towards primary roots, lateral roots and root hairs in rice and canola, respectively (Good et al. 2007; Shrawat et al. 2008). However, in silico analysis of the expression profile of *OsAnt1* (AK060757) indicated that the *OsAnt1* expression is stronger in leaves, callus and seeds with no expression in the roots (Shrawat et al. 2008). Additional evaluations conducted in rice expressing *HvAlaAT* under the *OsAnt1* indicated the activity of alt was increased in both roots and in shoots, albeit to a lesser extent in the latter (Beatty et al. 2013). In sugarcane, AlaAT expression was detected in both shoots and root in transgenic plants carrying an *OsAnt1/HvAlaAT* cassette (Snyman et al. 2015). The transcript accumulation and enzymatic analyses conducted in this study indicated that the *OsAnt1* promoter does not confer root-specific expression in either wheat or sorghum, with more constitutive pattern in the latter.

Expression of *HvAlaAT* in wheat can boost some agronomic parameters under high and low N conditions. This capacity was observed in wheat events expressing *HvAlaAT* regulated by both promoters, under a controlled environment (Table 1). While under field conditions only constitutive expression promoted increases in harvested grain plot weights (Fig. 3); in contrast, transgenic sorghum only exhibited a boost in height in isolated events while the overall phenotype remained unaltered. Moreover, under both controlled and field environments, the positive phenotypic outcomes were not consistent across the three independent constitutive wheat transgenic events evaluated. Wherein event UA2, with the exception of dry biomass at anthesis, under moderate N (Fig. 3), no statistical differences were observed, relative to the corresponding control. Importantly, the lack of an observed positive outcome over most of the phenotypes measured in event UA2, under both controlled and field settings, did track with transcript level (Fig. 2b) or enzymatic activity of alt (Fig. 2e) monitored in the respective transgenic events. Hence, enhancing alt activity in wheat, does not consistently translate to a benefit.

The combined data from three hydroponic experiments indicated that *HvAlaAT* promotes accumulation of shoot and total biomass in wheat. However, a seasonal component was observed across the wheat hydroponics experiments, with changes in the effects of *HvAlaAT* in plant growth and development depending on time of year experiment was set up. The hydroponic trial conducted in spring was under an average temperature of 23.8 °C and a photoperiod of 13/11 h (day/night). Plants grown under these conditions exhibited a more profound boost in biomass phenotype in the transgenic events relative to control plants, as compared to a summer trial wherein average temperature was 25.5 °C and a 14/10 h photoperiod. A potential explanation to the seasonal variation may lie in the observed significant decrease in chlorophyll content in the summer trial, which theoretically mitigated the positive outcomes associated with *HvAlaAT* expression observed spring trial. Interestingly, the transgenic events maintained the capacity to produce similar amounts of biomass in relation to the control plants even with a 30% reduction in chlorophyll content. Hence, *HvAlaAT*, may provide some protection of yield under heat stress, an abiotic stress that can severely impact yield in wheat (Porter and Semenov 2005).

It has been shown that latitude position, which affects temperature, photoperiod, and radiation intensities, may limit development in spring wheat, influencing various agronomic parameters including biomass, tiller number, grain yield and kernel weight (Fischer 2011; Peltonen-Sainio et al. 2009). A 2 °C above optimal temperature can alter physiological and biochemical processes that

lead to significant yield reductions of up to 50% in wheat (Semenov and Shewry 2011), while periods with less than 14 h of daylight may limit development resulting in lower biomass and yield (Villegas et al. 2016). These observations might explain the seasonal variation observed in the phenotypes outcomes of the transgenic wheat hydroponics studies.

Nitrogen is an essential nutrient for plant growth and development. The overuse of nitrogen-based fertilizers and their environmental impact call for the development of crops that utilize nutrient resources more efficiently. The manipulation of genes involved in N uptake, assimilation, remobilization, signaling, regulation of N, C/N balance and senescence have shown promising results to achieve NUE crops. However, NUE is a complex trait governed by multiple genetic and environmental factors. Therefore, careful considerations should be taken when selecting novel genes, promoters and the environmental conditions by which plants are evaluated. The efforts communicated herein highlight the capacity of *HvAlaAT* to impact some growth parameters including height and shoot biomass accumulation, along with increases in plot grain weight in wheat. However, the large variation observed across experiments, and lack of consistent phenotypic outcomes across the respective transgenic events indicate that introducing *HvAlaAT* alleles into wheat germplasm as a means to promote nitrogen use efficiency may not result in an adequate outcome that satisfies the expected future demands for the crop. Similarly in sorghum, the modest phenotypic changes observed in the studies conducted under controlled environments (Online resources), triggered by enhanced alt activity, is suggestive that this single transgene approach will not translate to an enhanced NUE phenotype in sorghum. Hence, a more thoughtful strategy that incorporates genetic approach that targets N from assimilation from soil through in planta flux to storage reserves will likely be required to ensure a stable, impactful NUE phenotype. The synthetic biology tools are in hand for the design of such an approach in crop plants; however, a key limitation in such genetic designs coming to fruition lies in the availability of a diversified suite of cell and tissue-specific regulatory elements.

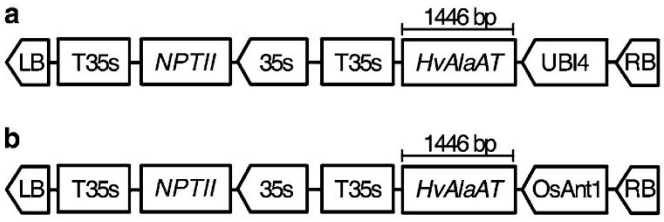

Fig. 1. Expression cassettes harboring an *HvAlaAT*. **a)** Illustration of the expression cassette pPTN1031 (UBI4/*HvAlaAT*) harboring a synthetic version of the alanine aminotransferase coding region from barley under the control of the UBI4 promoter. **b)** pPTN1040 (*OsAnt1/ HvAlaAT*) expression cassette containing a synthetic *HvAlaAT* coding region under the rice *OsAnt1* promoter; both expression cassettes were terminated with a 35s poly A terminator signal and carry the *NPTII* gene for plant selection. Diagram from *right* to *left*: RB, right border; UBI4, *S. officinarum* ubiquitin promoter or *OsAnt1*, *O. sativa* antiquitin promoter; *HvAlaAT*, alanine aminotransferase coding region from *H. vulgare* (1446 bp); T35s, CaMV terminator 35s poly A; 35sP, CaMV 35s promoter; *NPTII*, neomycin phosphotransferase II; T35s, CaMV terminator 35s poly A; LB, left border. Detailed plasmid maps along with vector sequences areamong the Supplementary Materials.

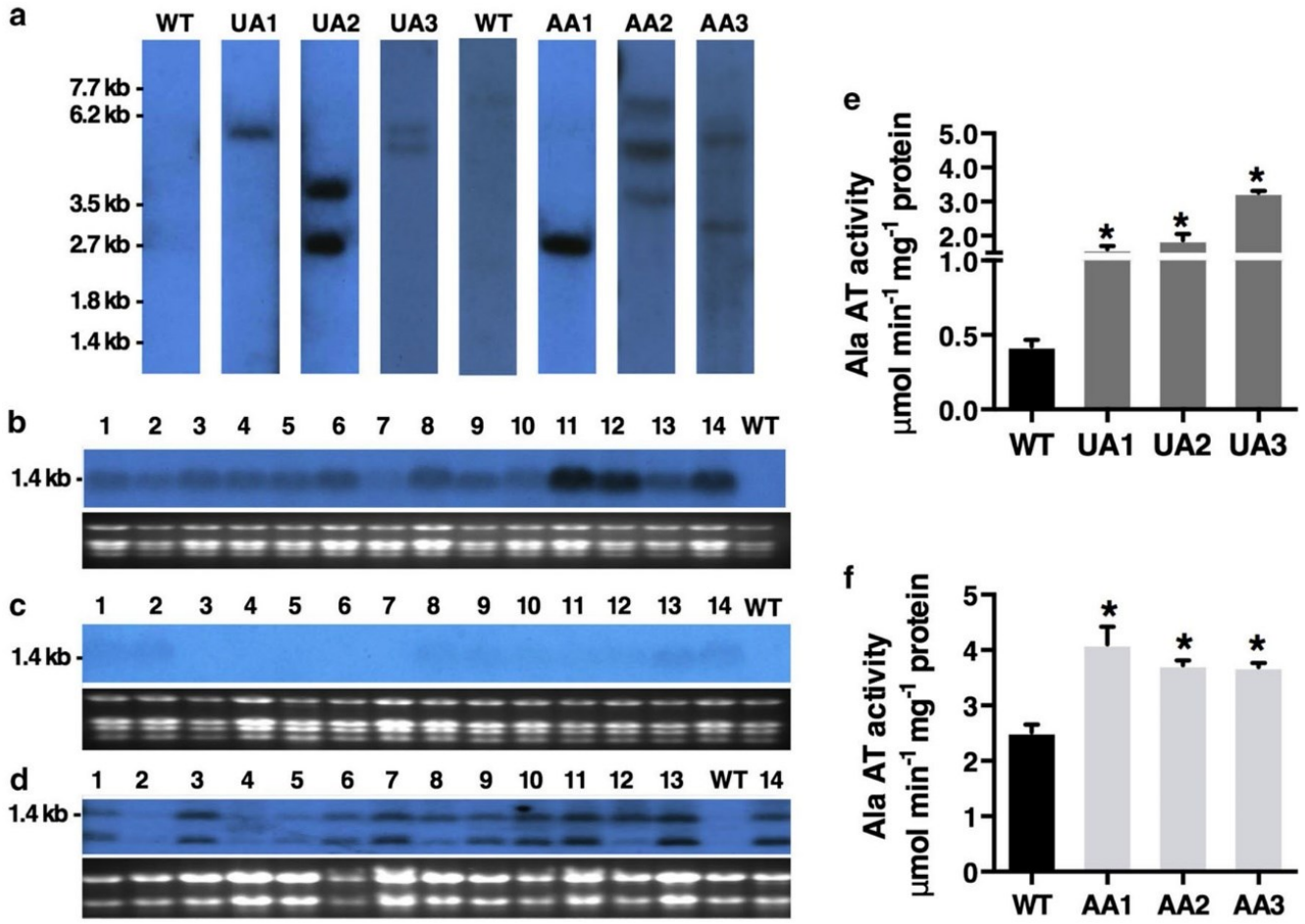

Fig. 2. Molecular characterization of transgenic wheat carrying *HvAlaAT*. **a)** Transgenic allele integration patterns monitored by Southern blot hybridization. Molecular weight (kb) on the *left*, wheat CB037 control plants (WT), three independent events (UA1, UA2, UA3) bearing the pPTN1031 (UBI4/*HvAlaAT*), wheat CB037 control plants (WT) and three independent events (AA1, AA2, AA3) carrying the pPTN1040 (*OsAnt1/HvAlaAT*). The Southern blots for the vetted events were conducted at different times; hence, the figure is shown in separated blots. **b)** Gene expression monitored via northern blot hybridization from leaf tissue of wheat expressing UBI4/*HvAlaAT*: UA1: *lane 9*, *10*; UA2: *lane 13*, *14*; UA3: *lane 11*, *12*; control: WT; remaining lanes represent other independent events evaluated, but not phenotyped. **c)** Transgene expression monitored via northern blot hybridization from leaf tissue on wheat expressing *OsAnt1/HvAlaAT*: AA1: *lane 13, 14*; AA2: *lane 3, 4*; AA3: *lane 9, 10*; control: WT; remaining lanes represent other independent events evaluated, but not phenotyped. **d)** Transgene expression monitored via northern blot hybridization from root tissue of wheat expressing *OsAnt1/HvAlaAT*: AA1: *lane 13, 14*; AA2: *lane 3, 4*; AA3: *lane 9, 10*; control: WT; remaining lanes represent other independent events evaluated, but not phenotyped. The Southern and northern blots were hybridized with a fragment of the *HvAlaAT* gene (504 bp). **e)** Enzymatic activity of AlaAT measured spectrophotometrically from leaf tissue at anthesis of wheat control and UBI4/*HvAlaAT* events. **f)** Enzymatic activity of AlaAT measured spectrophotometrically from root tissue at anthesis of wheat control and *OsAnt1/HvAlaAT* events. Data expressed as mean ± SE (n = 3). *Asterisks* indicate significant differences from the control ($p < 0.05$).

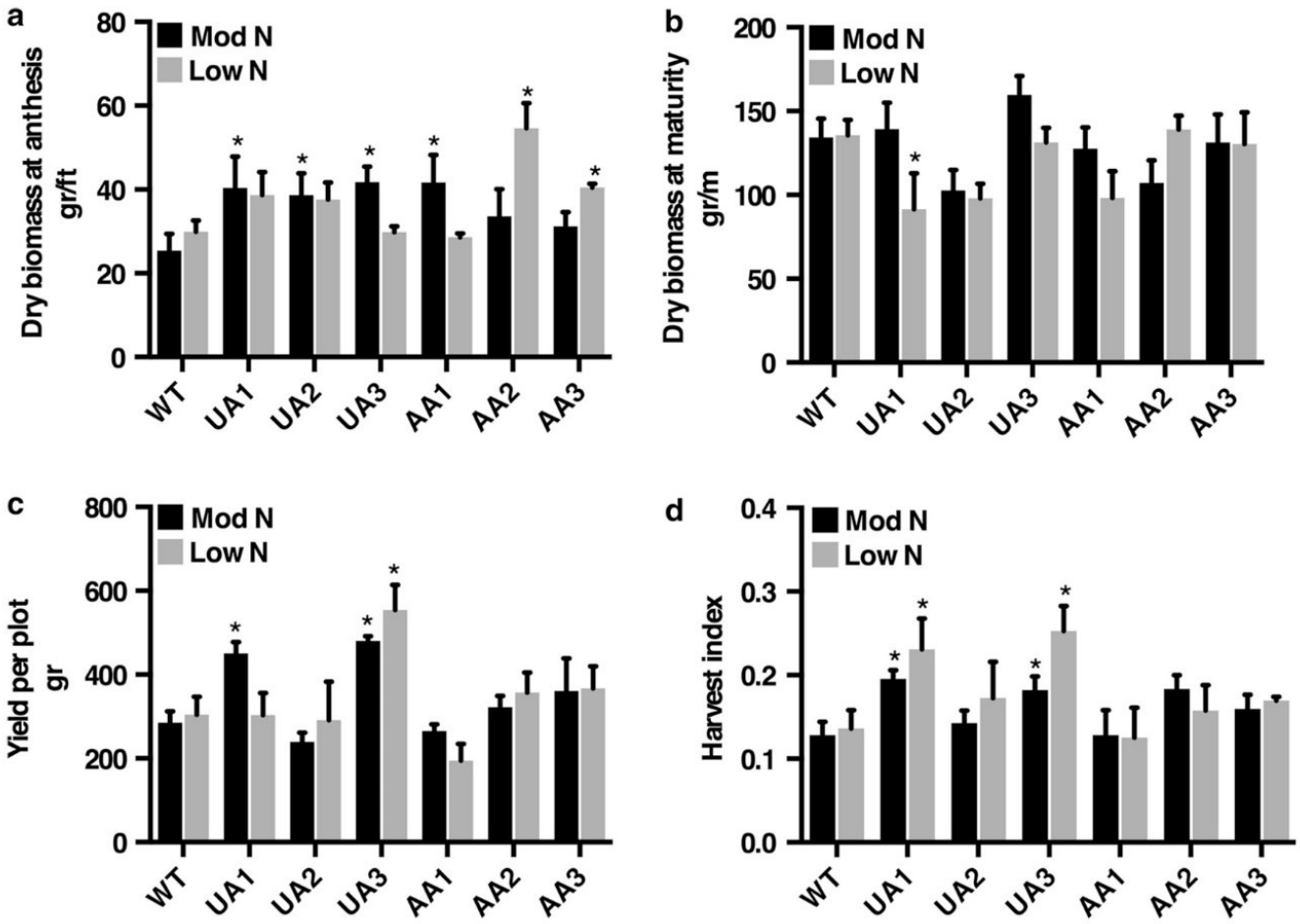

Fig. 3. Biomass analysis of wheat plants expressing *HvAlaAT* under field conditions. **a)** Dry biomass at anthesis, **b)** dry biomass at physiological maturity, **c)** yield per plot, and **d)** harvest index of wheat CB037 control plants (WT), three independent events (UA1, UA2, UA3) bearing the pPTN1031 (UBI4/*HvAlaAT*), and three independent events (AA1, AA2, AA3) carrying the pPTN1040 (*OsAnt1/ HvAlaAT*). Plants were grown in the field under moderate nitrogen: 95.3 kg ha^{-1} N (Mod N), and a low nitrogen: 53.8 kg ha^{-1} (Low N). Data expressed as mean ± SE of three plots. *Asterisks* indicate statistical difference to the control plants ($p < 0.05$).

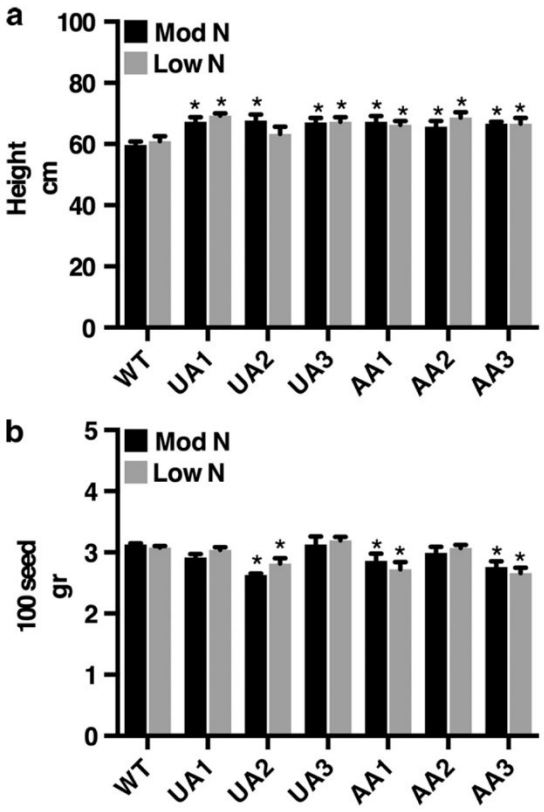

Fig. 4. Physiological components of wheat plants expressing *HvAlaAT* under field conditions. **a)** Height, and **b)** 100 seed weight of wheat CB037 control plants (WT), three independent events (UA1, UA2, UA3) bearing the pPTN1031 (UBI4/*HvAlaAT*), and three independent events (AA1, AA2, AA3) carrying the pPTN1040 (*OsAnt1/ HvAlaAT*). Plants were grown in the field under moderate nitrogen: 95.3 kg ha^{-1} N (Mod N), and a low nitrogen: 53.8 kg ha^{-1} (Low N). Data expressed as mean ± SE of three plots. *Asterisks* indicate statistical difference to the control plants ($p < 0.05$).

Table 1. Biomass analysis of *HvAlaAT* wheat events

Component	N trt (mM N)	WT (M ± SE)	UA1 (M ± SE)	UA2 (M ± SE)	UA3 (M ± SE)	AA1 (M ± SE)	AA2 (M ± SE)	AA3 (M ± SE)
Total biomass	15	3.3 ± 1.4c	4.3 ± 2a	3.5 ± 1.6bc	4.5 ± 2.4a	4.0 ± 2.1a	4.7 ± 2.4a	4.0 ± 1.6ab
(g)	0.3	2.5 ± 0.8c	2.7 ± 0.9a	2.5 ± 1.1bc	3.2 ± 1.1a	3.3 ± 1.4a	2.8 ± 0.9a	2.9 ± 1ab
Shoot biomass	15	2.8 ± 1.3c	3.7 ± 1.7ab	3.0 ± 1.4bc	3.9 ± 2.1a	3.6 ±2a	4.0 ± 2.1a	3.5 ± 1.5a
(g)	0.3	2.0 ± 0.7c	2.1 ± 0.7ab	2.1 ± 0.9bc	2.4 ± 0.9a	2.6 ± 1.1a	2.2 ± 0.8a	2.4 ± 0.9a
Root biomass	15	0.5 ± 0.1bc	0.6 ± 0.3ab	0.5 ± 0.2bc	0.6 ± 0.2ab	0.4 ± 0.1c	0.7 ± 0.3a	0.5 ± 0.1abc
(g)	0.3	0.5 ± 0.2c	0.6 ± 0.2bc	0.4 ± 0.1c	0.8 ± 0.2a	0.7 ± 0.3ab	0.6 ± 0.2bc	0.5 ± 0.1c

Total dry biomass, dry shoot biomass and dry root biomass of control plants (WT); three *UBI4/HvAlaAT* transgenic events (UA1, UA2, UA3); and three *OsAnt1/HvAlaAT* transgenic events (AA1, AA2, AA3). Plants were grown hydroponically for 5 weeks under 15 mM NO_3 or 0.3 mM NO_3. Data expressed as mean ± standard error of three independent experiments. Different letters indicate statistical differences ($p < 0.05$) according to analysis of variance.

Table 2. Physiological components of *HvAlaAT* wheat events

Component	N trt (mM N)	WT (M ± SE)	UA1 (M ± SE)	UA2 (M ± SE)	UA3 (M ± SE)	AA1 (M ± SE)	AA2 (M ± SE)	AA3 (M ± SE)
Height (cm)	15	53 ± 13c	57 ± 13b	59 ± 12ab	56 ± 13ab	59 ± 15ab	59 ± 14ab	61 ± 13c
	0.3	53 ± 11c	57 ± 12b	59 ± 14ab	59 ± 12ab	57 ± 11ab	57 ± 12ab	59 ± 11c
Tillers (number)	15	2.8 ± 0.4d	4.1 ± 0.5a	3.2 ± 0.4 cd	3.4 ± 0.3ab	3.2 ± 0.5bc	3.3 ± 0.4bcd	3.1 ± 0.3bcd
	0.3	2.3 ± 0.5d	2.7 ± 0.4a	2.2 ± 0.5 cd	2.8 ± 0.2ab	2.7 ± 0.7bc	2.4 ± 0.2bcd	2.8 ± 0.3bcd
Chlorophyll	15	563 ± 22b	485 ± 47c	564 ± 32b	556 ± 54b	485 ± 32c	628 ± 53a	614 ± 40a
(µmol m^{-2})	0.3	507 ± 22a	437 ± 28c	481 ± 6abc	476 ± 33abc	462 ± 27bc	512 ± 22a	486 ± 23ab

Height, tillers and chlorophyll of control plants (WT); three *UBI4/HvAlaAT* transgenic events (UA1, UA2, UA3); and three *OsAnt1/HvAlaAT* transgenic events (AA1, AA2, AA3). Plants were grown hydroponically for 5 weeks under 15 mM NO_3 or 0.3 mM NO_3. Data expressed as mean ± standard error of three independent experiments. Different letters indicate statistical differences ($p < 0.05$) according to analysis of variance.

Author contribution statement — PAP conducted research, data analyses. TQ conducted research. SS, ZG, and NN generated the transgenic events and contributed to molecular analyses. PAP, MS, and TC wrote the article. MS, ID, and TC supervised project activities.

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Molecular and phenotypic characterizations on transgenic wheat and sorghum events expressing the barley alanine aminotransferase gene.

Planta

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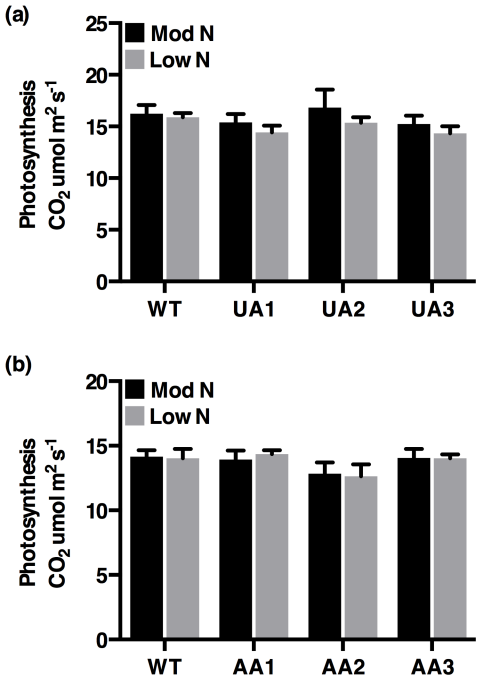
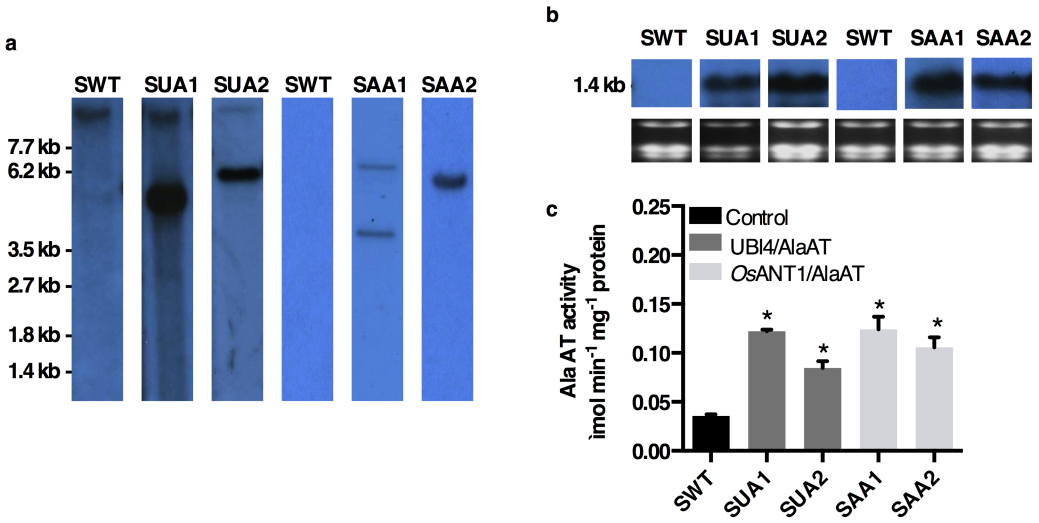


Online Resource 1 Photosynthetic capacity of wheat plants overexpressing *HvAlaAT* under field conditions. (a) Photosynthesis measurements in wheat CB037 control plants (WT), three independent events (UA1, UA2, UA3) bearing the pPTN1031 (UBI4/*HvAlaAT*), and (b) Photosynthesis measurements in wheat CB037 control plants (WT), and three independent events (AA1, AA2, AA3) carrying the pPTN1040 (*OsAnt1/HvAlaAT*). Plants were grown in the field under moderate nitrogen: 95.3 kg ha^{-1} N (Mod N), and a low nitrogen: 53.8 kg ha^{-1} (Low N). Data expressed as mean ± SE of 3 plots. Asterisks indicate statistical difference to the control plants ($p<0.05$).



Online Resource 2 Molecular characterization of transgenic sorghum carrying *HvAlaAT*.

(a) Transgenic allele integration patterns monitored by southern blot hybridization, and **(b)** gene expression monitored *via* Northern blot hybridization in transgenic sorghum on top. RNA on bottom. Molecular weight (kb) on the left, sorghum TX430 control plants (SWT), two independent events (SUA1, SUA2) bearing the pPTN1031 (UBI4/*HvAlaAT*), sorghum TX430 control plants (SWT), and two independent events (SAA1, SAA2) carrying the pPTN1040 (*OsAnt1/HvAlaAT*). The southern and northern blots were hybridized with a fragment of the *HvAlaAT* gene (504 bp). **(c)** Enzymatic activity of AlaAT measured spectrophotometrically from leaf tissue at anthesis of sorghum control and transgenic plants. Data expressed as mean ± SE (n=3). Asterisks indicate significant differences from the control ($p<0.05$).

Online Resource 3 Biomass analysis of sorghum events.

Component	N Trt (mM N)	SWT (M ± SE)	SUA1 (M ± SE)	SUA2 (M ± SE)	SAA1 (M ± SE)	SAA2 (M ± SE)	Anova	
Total biomass (gr)	**15**	77 ± 3.9	92 ± 2.8	81 ± 1.4	68 ± 3	86 ± 1	T	ns
	3	80 ± 4.1	92 ± 2.7	76 ± 2.1	81 ± 3	77 ± 2.2	E	s
	0.3	80 ± 1.1	83 ± 1.4	70 ± 3.2	70 ± 5.2	69 ± 2.8	T*E	ns
	Mean	79 ± 1.9^{b}	**89 ± 1.6^{a}**	76 ± 1.6^{b}	73 ± 2^{b}	77 ± 1.5^{b}		
Grain yield (gr)	**15**	34 ± 3.2	41 ± 1.5	31 ± 1.8	23 ± 1.3	35 ± 0.9	T	ns
	3	36 ± 1.7	45 ± 2.9	31 ± 0.9	35 ± 0.7	31 ± 0.5	E	s
	0.3	35 ± 0.6	37 ± 1.7	29 ± 1.8	27 ± 3	28 ± 2.4	T*E	ns
	Mean	35 ± 1.3^{b}	**41 ± 1.2^{a}**	30 ± 0.9^{bc}	28 ± 1^{c}	32 ± 0.8^{bc}		
Shoot biomass (gr)	**15**	42 ± 0.9	51 ± 4.2	50 ± 2.3	45 ± 2.8	51 ± 0.9	T	ns
	3	44 ± 1.6	47 ± 1.5	45 ± 1.4	45 ± 3.9	46 ± 1.1	E	ns
	0.3	45 ± 0.8	46 ± 0.4	41 ± 1.2	43 ± 2.2	41 ± 0.4	T*E	ns
	Mean	44 ± 0.6	48 ± 1.6	45 ± 0.9	44 ± 1.7	46 ± 0.8		
Height (cm)	**15**	110 ± 1.8^{a}	110 ± 2.9^{a}	**99 ± 3.2^{b}**	110 ± 2.5^{a}	100 ± 3.4^{ab}	T	ns
	3	100 ± 4.2^{c}	108 ± 0.4^{bc}	100 ± 1.3^{c}	**126 ± 0.4^{a}**	**111 ± 0.4^{b}**	E	s
	0.3	117 ± 0.2^{ab}	110 ± 0^{abc}	**99 ± 0.9^{c}**	119 ± 1.6^{bc}	107 ± 0.9^{bc}	T*E	s
	Mean	109 ± 1.6	109 ± 1.1	99 ± 1.1	118 ± 1.1	106 ± 1.4		
Tillers (Number)	**15**	1.7 ± 0.3	1.8 ± 0.3	1.6 ± 0.4	1.7 ± 0.1	1.7 ± 0	T	s
	3	1.5 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1 .1± 0.1	E	ns
	0.3	1 ± 0	1.6 ± 0	1.1 ± 0.1	1.3 ± 0.2	1 ± 0	T*E	ns
	Mean	1.4 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1		

Total dry biomass above ground, grain yield, shoot dry biomass, height and tillers of sorghum control plants (SWT); two UBI4/*HvAlaAT* transgenic events (SUA1, SUA2); and two *OsAnt1*/*HvAlaAT* transgenic events (SAA1, SAA2). Plants were grown in pots until maturity under three nitrogen treatments: 15 mM, 3 mM and 2 mM NH4NO3. Data expressed as mean ± standard error of three independent experiments. Analysis of variance T: N treatment, E: Event, T*E: N trt* Event. s: significant, ns: not significant. Different and bold letters indicate statistical differences ($p<0.05$)

Online Resource 4 Primer sequences used for selection of transgenic plants.

Id	Orientation	Primer (5'-3')	Annealing (°C)
pPTN1031	Fwd	TGTGTTCAACAGTCAGTTTTTG	51
	Rev	AGGAAGATGTCGTACGCGTTAG	
pPTN1040	Fwd	TGACCTTTCTGGACTTCACCAA	54
	Rev	AGGAAGATGTCGTACGCGTTAG	