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Full Length Article

Expression of Synthetic *Alanine Aminotransferase* Gene Improves Nitrogen Uptake and Response in Transgenic Tobacco

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Abstract

Improvement in nitrogen use efficiency of crop plants has been a major challenge in agricultural research. A barley *alanine aminotransferase* (*HvAlaAT*) gene was codon optimized and got synthesized to achieve optimal expression in tobacco plants. Synthetic *alanine aminotransferase* (co-*AlaAT*) gene was cloned under 2X*CaMV35S* promoter and transformed in tobacco through *Agrobacterium*-mediated method. Out of seven independent transgenic tobacco events, three putative transgenic tobacco lines were selected for evaluation in pots at 0, 75, 100 and 125 kg N/ha nitrogen fertilizer doses applied in three split applications. Molecular analysis of the putative transgenic tobacco plants was conducted through PCR and real-time quantitative PCR. Transgenic tobacco lines evaluated through biochemical and morpho-physiological means showed improvement in N uptake, total free amino acid, crude proteins, *AlaAT* activity, leaf area, stalk diameter, above the ground fresh mass, above ground dry matter and seed weight per plant. All these attributes demonstrated improved nitrogen uptake and its incorporation into biomass as compared to the wild-type. From these findings, it is concluded that co-*AlaAT* gene could be a possible candidate for developing nitrogen responsive and N use efficient crop plants to reduce nitrate contamination in the environment and to minimize the farmer's input in terms of nitrogenous fertilizer application.

Keywords: Biomass; Crop productivity; Gene synthesis; N metabolism; N efficient plants

Introduction

With the advancement in plant molecular biology, synthetic biological approaches are being used routinely to improve crop productivity. Since last 50 years, N fertilizers are being used quite extensively to enhance or to sustain crop yield (McAllister *et al*. 2012). Although N is an essential element but usually it becomes limiting nutrient for plant growth and development due to leaching of nitrate and volatilization process (Kraiser *et al*. 2011; Garnett *et al*. 2015). Only 30–50% of applied N is taken up by plants while the remaining is lost through ammonia volatilization, surface runoff, consumed by soil microbes and by nitrate leaching (Yang *et al*. 2015; Zhu *et al*. 2016).

The excessive use of N fertilizer cause imbalance of other essential nutrients necessary for optimum plant growth, contribute toward greenhouse gas emission, nitrate accumulation in underground water used for drinking and cause serious threats to human health (Ahmed *et al*. 2017). This situation urges the strong demand for the development of nitrogen use efficient crops. Strategies to improve nitrogen use efficiency (NUE) in plants focused mainly upon genetic manipulation for enhancement of N uptake (Araus *et al*. 2016; Chen *et al*. 2017), nitrate translocation, N metabolism (Pena *et al*. 2017) and its regulation. Although, these approaches are generally successful but NUE additionally depends upon N partitioning within plant and efficiency of nitrate or associated amino acid transporters (Mu *et al*. 2015).

During vegetative growth, plants utilize N in three steps: uptake, translocation, and assimilation. Nitrate and ammonium are the major forms of inorganic N present in agricultural soil (Krapp *et al*. 2014). Nitrate is the main form of N consumed by many plant species. When nitrate enters the plant cell, it is reduced to nitrite by Nitrate Reductase (NR). Nitrite is transported further to plastids where it undergoes reduction process and converted to ammonia by nitrite reductase. Ammonia then enters the GOGAT (glutamine-oxoglutarate aminotransferase) cycle and transformed to glutamine and glutamate (Santiago and Tegeder 2016). The amino group of glutamate could then be transferred to various amino acids by different

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aminotransferases (Tegeder 2014). *Alanine aminotransferase* catalyzes the reversible conversion of alanine and 2-oxoglutarate to pyruvate and glutamate. Alanine is an important amino acid for various reasons (Good *et al*. 2007; Shrawat *et al*. 2008) *e.g*., it is usually excreted by N fixing bacteria and then assimilated into plant roots (Zhang *et al*. 2015), suggesting its role in organic N metabolism. Additionally, under certain stresses, alanine has been found to be among the major storage amino acids (Good *et al*. 2007). In most of the cases, 50–75% of applied N to arable lands is utilized by microorganisms or lost through leaching (Yang *et al*. 2015; Zhu *et al*. 2016). So the improvement in plant nitrogen use efficiency through genetic engineering has been the focus of research since last decade. Over-expression of barley *alanine aminotransferase* (*AlaAT*) gene in canola and rice has been demonstrated with improved nitrogen use efficiency as indicated by measured biomass and grain yield under low nitrogen supply (0.5–4.0 m*M*) in growth chambers, hydroponics and field trials (Good *et al*. 2004; Shrawat *et al*. 2008). A nitrogen efficient phenotype was observed in transgenic rice transformed with barley (*HvAlaAT*) gene and assessed physiologically under low, medium and high N supply (Beatty *et al*. 2013). Similarly, transgenic sugarcane, wheat and sorghum expressing *HvAlaAT* gene were developed and assessed under low nitrogen supply, hydroponics and greenhouse experiments respectively, indicated improvement in nitrogen use efficiency (Snyman *et al*. 2015; Pena *et al*. 2017). However, codon optimization of barley *alanine aminotransferase* (*AlaAT*) gene by replacing less frequently predicted codons with most favored codons to achieve optimal transcriptional efficiency and translational ability in tobacco (*Nicotiana tabacum* L.) plants (dicot) is used for the first time to be expressed under *2XCaMV35S* promoter. It was hypothesized that introduction of synthetic *AlaAT* gene could be an effective approach to attain optimal expression level in the targeted plant species.

Materials and Methods

Cloning in binary vector and transformation of tobacco

Barley *alanine aminotransferase* gene (Accession no. Z26322) was codon optimized by replacing codon predicted to be less frequently use in tobacco with more favored codons according to Codon Usage Database (http://www.kazusa.or.jp). Codon optimization was performed by keeping in view a range of factors involved in protein expression, codon adaptability, mRNA structure, various cis-elements in transcription and translation to have optimum transcriptional competence, translational efficiency and protein folding in tobacco (*Nicotian atabacum* L.) plants. The codon optimized (co-*AlaAT*) gene along with the introduction of *Hind*III and *Eco*RI restriction sites was got synthesized commercially from M/S Eurofins Genomics, USA anddelivered in general plasmid vector (*pBluescript*). The *pBluescript* plasmid was restricted with *Hind*III and *Eco*RI restriction enzymes to ligate the codon optimized *alanine aminotransferase* (co-*AlaAT*) gene (1453 bp) into pJIT60 vector under *2XCaMV35S* promoter and *CaMV* terminator. Then *pJIT60* vector having the whole cassette (*2XCaMV35S*- co-*AlaAT*- *CaMV*) was digested with *Kpn*I and *Xho*I hexa cutter enzymes and sub-cloned the complete cassette in a *pGreen0029* binary vector by using the same restriction sites. This construct was transformed into *Agrobacterium tumefaciens* strain 'LBA4404' through electroporation method. Freshly cut tobacco leaf discs were inoculated with *Agrobacterium* culture and incubated on MS0 medium (Murashig and Skoog 1962). After shoot and root development, the regenerated plants were transplanted to the soil in pots and three PCR positive putative transgenic lines were selected to raise T_1, T_2, and T_3 generations.

Evaluation of putative transgenic tobacco lines at different N fertilizer regimes

The PCR positive lines were selected to be tested further during 2017–2018 on the basis of their performance at different nitrogen regimes. The selected lines *i.e*., SAC1, SAC2, and SAC5 were grown in four replicates in pot experiments. Sandy loom soil was weighed to 30 kg/pot of 12' internal diameter x 14' height and two tobacco seedlings were transplanted in each pot. Upon suitable settlement of young seedling, one plant/pot was retained for further experimentation. Different N regimes were created by applying urea as N fertilizer at the rate of 0, 75, 100 and 125 kg N/ha and represented as N0, N75, N100 and N125 respectively. Urea fertilizer was applied in three split applications at transplantation, vegetative growth phase and flowering stage. The other fertilizers *i.e*., phosphorous and potassium were applied ten days after sowing at a rate of 75 kg/ha P_2O_5 and 50 kg/ha K_2O.

Molecular analysis of putative transgenic tobacco lines

DNA was extracted from leaf tissues of putative transgenic tobacco plants by CTAB method (Doyle and Doyle 1987) and tested for codon optimized *alanine aminotransferase* (co-*AlaAT*) gene insertion in tobacco genome by PCR. Gene-specific primers (Table 1) were used to amplify co-*AlaAT* gene with the forward (*AlaAT* AF) and reverse (*AlaAT* BR) primers in a PCR reaction of 50 μL volume containing 100 ng plant DNA, 200 μM MgCl2, 200 nM of each primer and 25 μL Dream Taq Green Mix (Fermentas, U.S.A.). The profile used for PCR reaction consisted of [Initial denaturation temperature 94°C for 4 min; 35 cycles of denaturation, (94°C for 1 min), annealing (55°C for 1 min) and, extension (72°C for 1 min) followed by final extension at 72°C for 10 min]. For expression analysis, RNA was extracted from fully developed 5th leaf from bottom, 1week after second N fertilizer dose, by using Trizol Reagent (Invitrogen, U.S.A.) and cDNA was

synthesized by Revert Aid reverse transcriptase (Fermentas, U.S.A.). Specific forward (*AlaAT* F) and reverse (*AlaAT* R) primers were used to confirm relative expression of co-*AlaAT* gene in transgenic lines through quantitative real time PCR analysis, whileprimer pair of *18SRNA* gene was used as reference control to compare relative expression of co-*AlaAT* gene in different transgenic lines. Real-time PCR was performed for relative quantification of synthetic co-*AlaAT* gene in various transgenic and wild type control lines in 20 μL reaction mixtures containing 50 ng cDNA, 200 μM MgCl2, 200 nM of each primer and SYBR Green qPCR kit (Fermentas, USA) using CFX-96 real-time PCR machine (Biorad, USA.).

Studies on morpho-physiological and biochemical parameters of transgenic plants

Plant height of putative tobacco plants were measured at rapid vegetative growth and at maturity stage by plant height measuring scale. Leaf area of fully developed fifth leaf from bottom was measured through CI-202 Laser Area Meter (CID Bio-Sciences, U.S.A.). Stalk diameter was recorded with a diameter measuring tape. Shoot fresh/dry weights and root dry weights were recorded when plants were harvested and drying plants at 65ºC for 72 h in drying oven. Seed weight per plant of each tobacco line was determined using lab scale analytical balance.

Estimation of the total free amino acids was recorded by following Hamilton and Van Slyke (1943) method. Fresh leaf tissues (1.0 g) were taken 1 week after third N fertilizer dose, sliced in citrate buffer (10 mL; pH 5.0) and incubated at room temperature for 1 h. This mixture was centrifuged at 15,000 rpm for 10 min at 15ºC. The supernatant was taken carefully and used to quantify total free amino acids. One milliliter volume of the eluent was taken in a 20 mL test tube and 1 mL of ninhydrin solution was added. These tubes were kept wrapped in aluminum foil to avoid light contact and heated for 20 minutes at 100°C in a water bath. These tubes were then cooled down to room temperature and 5 mL of the diluent was mixed and incubated again for 15 min at room temperature. The optical density (OD) was measured at 570 nm on a UV-visible spectrophotometer (Hitachi-220, Japan).

The total nitrogen content of transgenic plant material 1 week after third N fertilizer dose was measured by following micro–Kjeldhal's method (Bremner 1965). The tissue samples of tobacco plants were digested with sulfuric acid (5 mL) and transferred in Kjeldhal's tubes before placing them in Kjeldhal's ammonia distillation unit and 5 mL of 40% sodium hydroxide (NaOH) was added to every tube. Boric acid solution (5 mL) was taken in a conical flask with few drops of mixed indicator. When the distillate reached approximately 40 mL, stopped the distillation process and let the distillate cooled down for few minutes and titrated with 0.01 N standard H_2SO_4 till the solution turned pink. A blank control was run during the whole procedure. The values were taken three times for a single sample to rule out the possibility of analytical error.
N content was estimated by using the following formula

$$N\ (\%) = \frac{(V_2\text{-}V_1) \times N \times 0.014 \times 100}{W}$$

Where
V_2: Volume of H_2SO_4 used for sample during titration
V_1: Volume of H_2SO_4 used for blank control during titration
N: Normality of the acid used for titration
N%: Percent nitrogen

After estimation of total nitrogen content, the value of crude protein was calculated by multiplying the obtained data with a conversion factor of 6.25.

Alanine aminotransferase activity was measured 1 week after third N fertilizer dose from freshly harvested leaf tissues of transgenic and wild-type control (as described by McAllister and Good 2015). Harvested leaf samples equal to 5.0 g weight were ground with pestle and mortar by keeping them on ice with extraction buffer, a pinch of sand and Polyvinyl polypyrrolidone (PVPP) was added. The ground samples were centrifuged for 15 min at 15.7 relative centrifugal force. The supernatant from each replicate was column purified through Amicon Filter Column and *AlaAT* activity was assessed in a continuous coupled reaction catalyzed by lactate *dehydrogenase* and *AlaAT*. The change of absorbance due to the formation of NAD^+ from NADH was monitored at 340 nm as reported by Miyashita *et al*. (2007).

Statistical analysis

Three putative transgenic tobacco lines along with wild-type controls were evaluated using CRD split block design with four N treatments (0, 75, 100 and 125 kg N/ha) in four replicates. The data was analyzed by two-way analysis of variance (ANOVA) using MS Excel. The data are means ± SD with $P \leq 0.05$.

Results

Selection of regenerating putative transgenic plantlets

The putative transgenic tobacco (*Nicotiana tabacum* L.) plantlets having co-*AlaAT* gene were selected on media containing kanamycin 100 mg/L. The regeneration potential was found to be 72% while observed transformation efficiency was 49%. T_1-T_3 generations of transgenic tobacco lines were raised in pots under control conditions. Fifty T_3 seeds of homozygous transgenic tobacco lines and wild-type were germinated on MS_0 containing 100 mg/L kanamycin. After a week, it was found that SAC1, SAC2 and SAC5 showed 82, 87 and 78% kanamycin resistant seedlings whereas all wild-type seedlings showed kanamycin sensitivity. Further, genomic DNA was isolated from leaf tissues of putative transgenic as well as non-transgenic (control) plants by CTAB method (Doyle and Doyle 1987).

Table 1: Primers used for PCR detection and real time quantification of co-*AlaAT* gene. *AlaAT*AF/*AlaAT* BR primer pair was used for PCR detection of co-*AlaAT* gene in putative transgenic tobacco plants while *AlaAT* F/*AlaAT* R and *18S* F/*18S* R primer pairs used for of co-*AlaAT* gene quantification in different transgenic tobacco lines

Primer Name	Primer Sequence (5'-3')
*AlaAT*AF	TACTCCGCCTCTATCGCACT
AlaAT BR	CCCTCACTGGAGCTGAAAAG
AlaAT F	TTCTCTCCCCTTTGACGAGA
AlaAT R	TGATCGCATAACGCTAGCAC
18S F	TCTGCCCTATCAACTTTCGATGGTA
18S R	AATTTGCGCGCCTGCTGCCTTCCTT

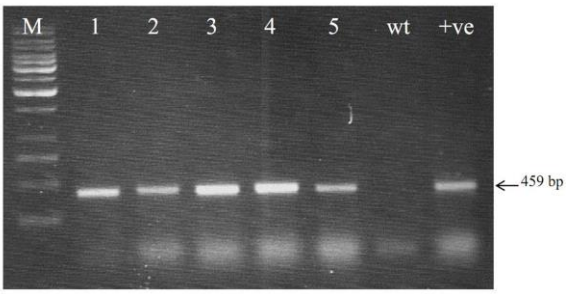

Fig. 1: Agarose gel electrophoresis showing the PCR amplification of 459 bp fragment of co-*AlaAT* gene from five independent transgenic events in tobacco. Lanes 1- 5 show gene amplification from SAC1, SAC2, SAC5, OAC2 and OAC3 transgenic plants (only first three lines described in this study), wt is a non-transformed negative control, +ve show gene amplification from plasmid control while M show 1 kb DNA ladder

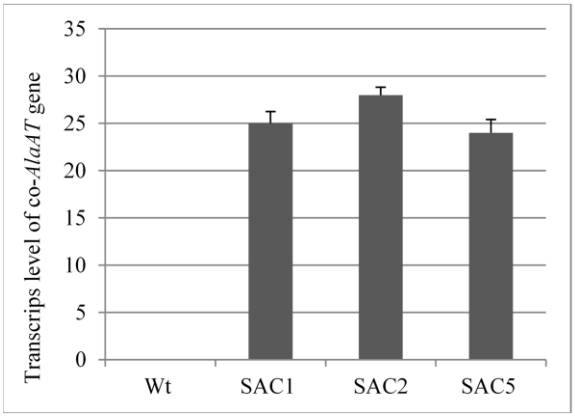

Fig. 2: Relative quantification of co-*AlaAT* gene. Y-axis is representing Real Time PCR data using delta delta Ct (ddCt) method, while X-axis represents average fold change of co-*AlaAT* transcripts level in different transgenic (SAC1, SAC2 and SAC3) lines versus wild-type (Wt) control. No transcripts of co-*AlaAT* gene detected in wild-type tobacco plants

Molecular testing of co-*AlaAT* transgenic tobacco lines

Initial screening of putative transgenic plants was done by amplifying co-*AlaAT* gene specific fragment through PCR. Plasmid DNA was used as a positive (+ve) control while DNA from a non-transformed plant was used as the negative control. Amplification of gene specific 459 bp internal DNA fragment of the co-*AlaAT* gene indicated the successful transformation of this gene in five different transgenic lines (Fig. 1, Lanes 1–5). However, data of only three lines (SAC1, SAC2, and SAC5) were presented in this manuscript. No amplification was observed in the non-transformed control (Wt: wild-type).

RT-PCR detection system (CFX96, Biorad USA) was used to quantify transgene expression in different transgenic tobacco lines. The acquired data was normalized with *18S* housekeeping reference gene. No expression of co-*AlaAT* gene was detected in wild-type (Wt) control, while the transgenic line SAC2 showed highest expression (4X higher expression than SAC1) followed by SAC1 and SAC5 (Fig. 2).

Synthetic co-*AlaAT* gene triggers morpho-physiological alterations in transgenic plants

Selected transgenic tobacco lines were evaluated at 0, 75, 100 and 125 kg N/ha (N0, N75, N100 and N125) fertilizer doses through different molecular, morpho-physiological and biochemical means. Various morpho-physiological attributes like plant height, leaf area, stalk diameter, fresh mass, dry mass and seed weight/plant were recorded and analyzed through two factor ANOVA of Microsoft Excel2010 (Fig. 3A–F). The values are mean of four replicates of tested transgenic lines at different nitrogen regimes. Plant heights of wild type control plants were significantly higher (at $P < 0.001$) than transgenic plants under all nitrogen fertilizer doses at rapid vegetative growth (Fig. 3A). However, upon reaching at maturity stage, plant height of wild type and transgenic plants differ non-significantly. The differences in the leaf area of fully developed (fifth) leaf of transgenic and control (non-transformed) tobacco plants (Fig. 3B and 5) were measured. At different N fertilizer regimes, transgenic plants showed significantly higher leaf area than wild-type control. The leaf area of control plants (Fig. 3B) was significantly lower than that of all transgenic lines at N0 fertilizer application. SAC1 and SAC2 possessed highest leaf area at N0 and N75. However, SAC2 and SAC5 showed more leaf area at N100 and N125 fertilizer dose but increase in leaf area beyond N75 remained non-significant (Fig. 3B).

Stalk diameter of SAC2 and SAC5 were significantly higher from SAC1 and wild-type at N0, N75, and N100 fertilizer dose while the stalk diameter of SAC1 was significantly greater than SAC2 and wild-type at N125 fertilizer level (Fig. 3C). Fresh mass of SAC1 and SAC2 were significantly higher from wild-type and SAC5 at N0 fertilizer regime, however the fresh mass of all three transgenic lines was significantly higher than wild-type control plants at N75 fertilizer dose. At N100 and N125 fertilizer level, SAC1 and SAC2 retained significantly more fresh mass than SAC5 and wild-type control (Fig. 3D).

Dry mass value of SAC2 was statistically higher from SAC1, SAC5 and wild-type control at N0 nitrogen fertilizer

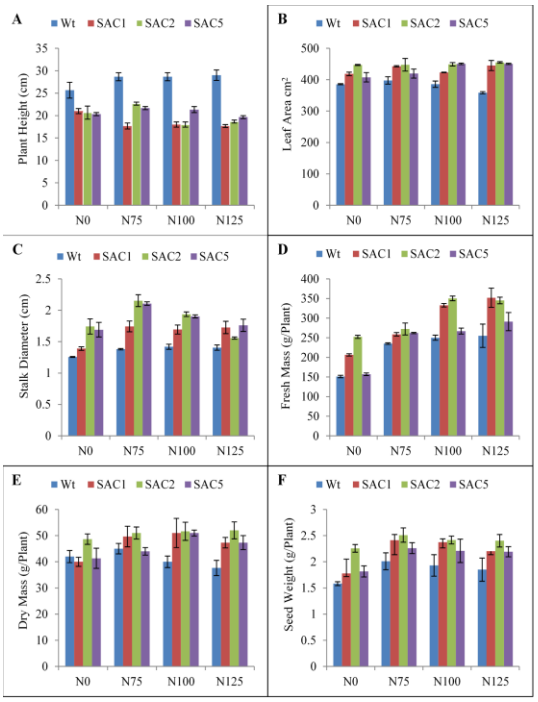

Fig. 3: Morpho-physiological assessment of; **A**. Plant height, **B**. Leaf area (fifth leaf from bottom), **C**. Stalk diameter, **D**. Fresh mass, **E**. Dry mass, **F**. Seed weight of wild-type (Wt) and putative transgenic co-*AlaAT* tobacco lines (SAC1, SAC2 and SAC3) at N0, N75, N100, N125 kg/ha N fertilizer doses

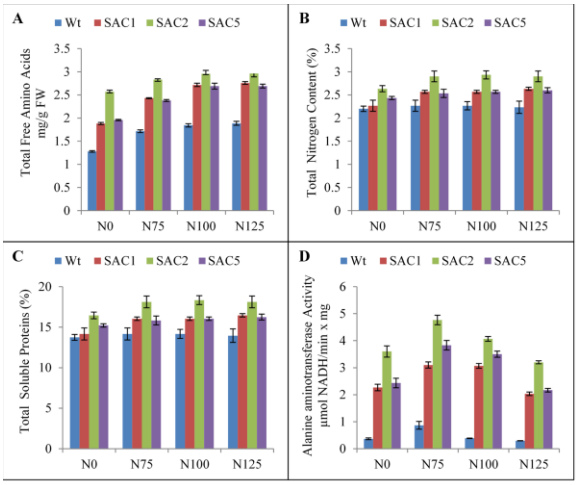

Fig. 4: Biochemical evaluation of; **A**. Total free amino acids, **B**. Total nitrogen content, **C**. Crude proteins, **D**. *AlaAT* activity of wild-type (Wt) and putative transgenic co-*AlaAT* tobacco lines (SAC1, SAC2 and SAC3) at N0, N75, N100, N125 kg/ha N fertilizer doses

dose. Whereas dry mass values of SAC1 and SAC2 were statistically higher than SAC5 and wild-type control at N75. However, dry mass values of all transgenic lines remained significantly higher than wild type at N100 and N125 nitrogen regimes (Fig. 3E). Seed weight of SAC2was statistically higher than SAC1, SAC5 and wild type at N0 fertilizer dose, while seed weight of all transgenic lines were significantly higher from wild-type at N75, N100 and N125 fertilizer environment (Fig. 3F).

Synthetic co-*AlaAT* gene insertion elicit biochemical changes in transgenic plants

Data of different biochemical parameters like total free amino acid, total nitrogen contents, crude protein and alanine aminotransferase activity was measured (Fig. 4A–D). The experiment was repeated three times from year 2015–2017. The total free amino acids of SAC1, SAC2, and SAC5 were significantly higher than wild-type control at N0, N75, N100 and N125 fertilizer dose. However, level of total free amino acids was statistically similar in SAC1 and SAC5 but varied significantly with respect to SAC2 at all fertilizer regimes (Fig. 4A). Total nitrogen content value at N0 fertilizer level was non-significant between wild-type and SAC1 but its values began to increase with the increase of nitrogen fertilizer application. The total nitrogen content of SAC1, SAC2, and SAC5 was significantly higher than wild-type at N75, N100 and N125 fertilizer level. However, SAC2 attained significantly higher nitrogen content than SAC1 and SAC5 at all nitrogen fertilizer applications. Total nitrogen content values reached to a maximum at N75 fertilizer regime in case of SAC2 but varied non-significantly at higher N fertilizer doses (Fig. 4B).

SAC2 and SAC5 varied significantly from each other and had significantly higher crude proteins than SAC1 and wild-type at N0 fertilizer dose. Similarly, SAC1, SAC2 and SAC5 possessed significantly more crude protein than wild-type at N75, N100 and N125 fertilizer level (Fig. 4C). Alanine aminotransferase activity of different transgenic lines was calculated at various nitrogen fertilizer exposure levels. All the transgenic lines possessed significantly higher alanine aminotransferase activity than wild-type control at all fertilizer levels. However, highest *AlaAT* activity was measured in SAC1, SAC2 and SAC5 lines at N75. Then its activity tends to be decreasing at N100 and N125fertilizer doses. Lowest level of alanine aminotransferase activity was observed at N125 nitrogen regime in all transgenic and wild-type lines, even then the level of *AlaAT* activity of all the transgenic lines was significantly higher than wild-type plants at said fertilizer dose (Fig. 4D).

Discussion

In an effort to improve nitrogen use efficiency (NUE) in crop plants, a synthetic codon optimized co-*AlaAT* gene was used in this study. Codon optimized *alanine aminotransferase* (co-*AlaAT*) gene was introduced and overexpressed under *2XCaMV35S* promoter in tobacco (*Nicotiana tabacum* L.). Putative transgenic tobacco (T_3) lines having optimum expression of co-*AlaAT* was used for evaluation under

different N fertilizer regimes. The over-expression of co-*AlaAT* gene and *AlaAT* enzymatic activity resulted in significant increase in leaf area, stalk diameter, fresh and dry masses, root dry weight and seed weight/plant as compared to non-transformed wild-type control under various N fertilizer doses. Pena *et al*. (2017) reported that transformation of *HvAlaAT* gene driven by constitutive and root specific promoter in wheat and sorghum, exhibited higher alanine aminotransferase (Alt) activity. Increased Alt activity influenced boosting of vegetative biomass, tillering and plant height relative to wild type wheat plants under hydroponic environment. However, wild type plants in case of tobacco, were using available N for increasing their heights during rapid vegetative growth stage at different N fertilizer applications. Nevertheless, putative transgenic tobacco plants tend to accumulate and assimilate more N which was depicted in enhanced leaf area, stalk diameter and biomass produce. These results corroborate when *HvAlaAT* gene was expressed under *OsANT1* tissue-specific promoter in rice, wheat and sorghum (Shrawat *et al*. 2008; Pena *et al*. 2017). A comparison of *2XCaMV35S*: co-*AlaAT* tobacco plants indicated that over-expression of co-*AlaAT* gene showed association with the increase in leaf area, stalk diameter, fresh/dry mass produce (Fig. 3A-F) at N0, N75, N100 and N125 nitrogen fertilizer applications. Contrary to higher expression values of inserted gene some time does not result in the desired phenotype, in case of sorghum transformed with *HvAlaAT*, displayed higher alt activity but no or little phenotypic changes were observed (Pena *et al*. 2017). The differences in values for these attributes in transgenic plants were prominent under low level of nitrogen (N75) supply, whereas no or little differences were observed under high nitrogen fertilizer applications (Shrawat *et al*. 2008; Snyman *et al*. 2015). It is indicated that under optimal and overdose of N amendments (N100 and N125), the phenotypic and biochemical differences were minimum (Good *et al*. 2007). Lines SAC1, SAC2 and SAC5 exhibited highest amino acid, total N content, crude proteins and *AlaAT* activity (Fig. 4A-D), which represented direct association with the moderate to the highest expression of co-*AlaAT* gene as investigated in sugarcane expressing *HvAlaA T*gene (Snyman *et al*. 2015). In these transgenic tobacco lines synthetic co-*AlaAT* finally resulted to improve nitrogen uptake, metabolism, assimilation, nutrient use efficiency, phenotypic traits (McAllister and Good 2015; Snyman *et al*. 2015) and biochemical attributes (Beatty *et al*. 2013; Pena *et al*. 2017). It has been demonstrated that *AlaAT* activity is involved in the production of either pyruvate or alanine for maintaining carbon-nitrogen balance (Miyashita *et al*. 2007) and control seed dormancy in the plant kingdom (Sato *et al*. 2016). Pena *et al*. (2017) confirmed that vegetative biomass, grain yield, and nitrogen contents in wheat were enhanced by over-expression of *HvAlaAT* gene under the control of root-specific and constitutive promoter. In this pot experiment, transgenic tobacco plants sustained significantly higher biomass as compared to wild-type control and resulted in 25-30% decrease in nitrogen fertilizer requirement without causing any yield penalty as observed in case of *HvAlaAT* transgenic sugarcane pot experiment (Snyman *et al*. 2015). The results of expressing synthetic co-*AlaAT* in tobacco revealed many similar physiological, molecular and biochemical attributes as observed in transgenic canola, rice, wheat and sorghum (Good *et al*. 2007; Shrawat *et al*. 2008; Pena *et al*, 2017).

The increased fresh and dry biomass was evident from their high values for leaf area, stalk diameter, fresh/dry biomass, seed weight (Fig. 3B-F), root dry mass and less internodal distance (data not shown here) values. It is implicated that reduced internodal distance in SAC2 and SAC5 as compared to wild-type might be due to co-*AlaAT* gene over-expression for speedy translocation of essential nutrients from root to shoot. Root structure and size is an important parameter for ensuring efficient nutrient supply to various parts of shoot system. Transgenic tobacco plants retained densely arranged root system. SAC1, SAC2, and SAC5 possessed bushier, more lateral root proliferation, finer roots and root hairs as compared to wild-type (data not shown). Finer roots are reported to have more surface area to volume ratio as compared to thick root and plants nitrogen uptake is dependent on location, size and number of root hairs (Snyman *et al*. 2015). It has been observed that availability of nitrate may interfere with the location and number of lateral root initiation sites (Malamy and Ryan 2001).

Transgenic tobacco revealed a significant increase in total free amino acid, nitrogen content, crude protein, and alanine aminotransferase activity. An increase in nitrogen flow was observed from Glutamine, Glutamate and Alanine when barley *AlaAT* gene expressed under the root-specific promoter in *Brassica napus* (Good *et al*. 2007). These amino acids are translocated to xylem sap immediately and trigger the plant growth. It is true for transgenic tobacco transformed with the co-*AlaAT* gene. Increase in *alanine aminotransferase* activity significantly increases the amount of total free amino acid, crude protein and total nitrogen content in transgenic lines (Pena *et al*. 2017). In shoot tissues an increase in the level of certain amino acids like Glutamate, Glutamine and Aspartic acid could be utilized as available nitrogen pool for the creation of nitrogen-containing compounds or other amino acids (Shrawat *et al*. 2008). Glutamine (Gln) is proposed to play a central role as a possible signaling molecule and in amino acid metabolism. Synthetic co-*AlaAT* gene could play a vital role in starch synthesis under hypoxic or limited N condition (Yang *et al*. 2015).

SAC1, SAC2 and SAC5 lines confirmed significant improvement in N efficient phenotype when compared with wild-type control. Prominent N efficient phenotype was observed in transgenic lines SAC1 and SAC2 under limited N (N0 and N75) supply, indicating their efficiency to uptake available N while SAC2 and SAC5 were performing better at higher N doses (N100 and N125) indicated their

responsiveness to optimal or overdoses. These results are promising and support the application of transgenic approaches for improvement of nitrogen use efficiency in crop plants which could reduce the fertilizers input cost, increase the profitability in term of yield and decrease the environmental damage or probable risk to human health due to nitrates toxicity (Ahmed *et al*. 2017).

Optimal and efficient use of N fertilizer by plants is important to increase crop yield, to reduce fertilizers application and their hazardous impact on environment. The over-expression of co-*AlaAT* gene enhances the N uptake, translocation and mobilization. It is therefore assumed that synthetic co-*AlaAT* gene could be transformed in major crop plants to enhance their N efficient phenotype. However, N efficient phenotype is a complex and multifactorial trait might be influenced by genetic, environmental and climatic change. Therefore, vigilant precautions should be considered upon evaluation of selected lines. As enhanced *AlaAT* activity exhibited N efficient phenotypes in transgenic wheat lines but Alt activity did not translate to significant phenotypic effects in sorghum when transformed with *HvAlaAT* gene (Pena *et al*. 2017). Therefore, it is assumed that synthetic biology tools could help to achieve desired results in required crop plants.

Conclusion

Alanine aminotransferase (*AlaAT*) is an important metabolic pathway gene for nitrogen acquisition, translocation and metabolization in plants. Over-expression of synthetic codon optimized *alanine aminotransferase* gene under double strength of constitutive (2x*CaMV35S*) promoter in tobacco resulted in various morphological, physiological and biochemical changes in stably transformed plants. It has been envisaged that synthetic *alanine aminotransferase* gene could be used to achieve improvement of crop productivity and yield enhancement under limited N fertilizer application.

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