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IMPROVING NITROGEN USE EFFICIENCY IN WHEAT (*Triticum aestivum* L.) THROUGH TRANSFORMATION OF CODON OPTIMIZED *Alanine aminotransferase* GENE

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Food security and environmental protection have become major challenges under climate change scenario. Considerable efforts have been made to improve nitrogen use efficiency in crop plants. Codon optimization of barley *alanine aminotransferase* (co-*AlaAT*) gene has been made to achieve optimal transcriptional efficiency and translational ability in wheat. Co-*AlaAT* was cloned under the control of constitutive and root specific promoters and transformed into wheat (*Triticum aestivum*; bread wheat). T_2 generation of five putative transgenic wheat lines were analyzed at four different N fertilizer regimes (0, 75, 100 and 125 kg N/ha) in four split applications. Molecular analysis of selected wheat lines was performed through PCR and qPCR. Transgenic wheat lines have demonstrated improvement in nitrogen use efficiency as evaluated through morpho-physiological and biochemical means. Transgenic wheat lines exhibited higher N content, amino acids, soluble proteins, AlaAT activity, flag leaf area, number of tillers, number of spikes, grain yield, nitrogen use efficiency and harvest index under limited N fertilizer dose. All these experiments were conducted in wheat biotechnology lab. at NIBGE, Faisalabad during 2015-2018. It is concluded that codon optimized *alanine aminotransferase* (co-*AlaAT*) gene may prove to be a potential candidate gene to improve N use efficiency in wheat crop for reduction of nitrogenous fertilizer application and to cut short nitrate accumulation in aquatic and terrestrial environment.
Keywords: Crop productivity, gene synthesis, N uptake, nutrient efficiency, spring wheat.

INTRODUCTION

Bread wheat (*Triticum aestivum* L.) having AABBDD genome is widely cultivated and important staple food crop. It is crucial for the rural economy and has a central role in global food security. The increasing world population demands to enhance wheat production by 50% until 2030 (http://iwyp.org).Global cropping pattern mostly depends upon higher N fertilizer applications for yield enhancement (Nazemi and Salvi, 2016). At present higher crop production requires around 60% annual global N (225 Mt) fertilizer input to achieve 100-110% excessive yield by the mid of this century (Ladha *et al*. 2016). However, sustainable wheat production has drastically been affected by various diseases, extreme climatic conditions, reduction in available land area, water scarcity, insect pests, soil fertility and low existing N use efficiency (Ruan and Johnson 1999; Wang *et al.*, 2018).

Several studies in bread wheat indicated the presence of genetic variation with respect to nitrogen use efficiency. Morpho-physiological analyses of 39 winter wheat varieties demonstrated a range of variability i.e. 24-42% regarding nitrogen use efficiency and grain yield at different N regimes. These elite wheat lines also represented significant variation for nitrogen utilization efficiency (Barraclough *et al.*, 2010). Crop improvements have been achieved through advancement in crop management and agronomical practices combined with crop genetic progress either through conventional or GM approaches (Han *et al.*, 2016). These genetic improvements reported to be responsible for 50-60% yield increase and still it is considered an important approach to enhance nitrogen use efficiency and crop productivity. In addition to crop genetic improvements, application of synthetic N, P and K fertilizers took part in crop yield enhancement (Duvick, 2005).

At present, there are two concerns about fertilizer application and crops yield: 1) Further increase in N fertilizer application will result in serious environmental issues and it may not lead to yield enhancement (Cassman *et al.*, 2003; Mulvaney *et al.*, 2009; Vitousek *et al.*, 2009; Wuebbles, 2009; David *et al.*, 2010;Good and Beatty, 2011; Zhang *et al.*, 2012). 2) Crop improvement through classical breeding approaches has reached a plateau. FAO indicated that global cereal crop yield since 1980s slowed down to just 1% annual increase in developing countries and developed countries it is closer to zero (Fischer *et al.*, 2009). Although, the use of N fertilizers was increasing after World War II but

a dramatic increase in their applications were observed since 1960s (Daberkow *et al.,* 2000; Grassini *et al.,* 2013). However, the rate of N fertilizer dosage reached to 512% increase to achieve cereal crop yield around 65% in China between 1980-2010 (Chen *et al.,* 2011; Zhang *et al.,* 2011), resultantly the crop yield has failed to increase to the ratio of inorganic N fertilizers applied leading to severe environmental problems and decrease in nutrient use efficiency (Shen *et al.,* 2013). Achieving higher crops yield while reducing N fertilizer supply (nitrogen uptake/ metabolize efficient crop plants) remained the major challenge for sustainable crop productivity (Cassman *et al.,* 2003; Godfray *et al.,* 2010; Good and Beatty, 2011; Hawkesford, 2012).

Ammonium and nitrate are two forms of inorganic N available in arable soil (Howitt and Udvardi, 2000), however, nitrate is the key form of N taken up and utilized by several plant species. Upon nitrate entry in plant system, it passes through various biochemical processes, which lead its reduction to nitrite and finally to ammonia in plastids by nitrite reductase. Ammonia then passed through glutamine-oxoglutarate aminotransferase (GOGAT) cycle to be converted to glutamate and glutamine (Andrews et al. 2004). Alanine aminotransferase is an important enzyme of GOGAT pathway, which catalyzes the reversible reaction of 2-oxoglutarate and alanine to glutamate and pyruvate (Good *et al.,* 2007). By the catalytic activity of different amino transferases, the amino group of glutamate transferred to various amino acids (Lam *et al.,* 1996). Additionally, alanine served as the major storage amino acid under certain stresses, secreted by various N fixing bacteria and assimilated into plant roots. All these activities of alanine suggest its key role in organic nitrogen metabolism (Good *et al.,* 2007)

The completion of genome sequencing of various crops plant and advances in different genomic tools provide an opportunity to express or alter desired gene for a particular trait (Jaganathan *et al.,* 2018; IWGSC, 2018). In this study, we performed codon optimization of *alanine amino transferase* gene of barley (*HvAlaAT*) by exploiting various bioinformatics tool for better transcriptional efficiency and translational ability in locally adapted wheat cultivar i.e. Galaxy 2013. Codon optimized *alanine aminotransferase* (co-*AlaAT*) got synthesized commercially and cloned under 2X*CaMV35S* and *OsAnt1* (*Oryza sativa antiquitin*) root specific promoters in *pGreen0029* binary vector for its onward transformation through *Agrobacterium tumefaciens*. Five putative transgenic wheat lines of T_2 generation were evaluated at four different N fertilizer regimes and analyzed through various molecular, biochemical and morpho-physiological means.

MATERIALS AND METHODS

***Cloning of co-AlaAT gene in binary vector and wheat transformation*:** Codon optimization of *alanine aminotransferase* gene (Accession no. Z26322) of barley was performed by replacing less frequently predicted codons with most favored codons for optimal gene expression in wheat by utilizing http://www.kazusa.or.jp codon usage database. Codon optimized gene sequence of *alanine aminotransferase* (co-*AlaAT*) was retrieved by considering codon adaptability, optimal protein synthesis, involvement of cis acting elements in transcription and translation, mRNA structure and protein folding to achieve better transcriptional ability and translational efficiency in wheat (*Triticum aestivum* L.). *OsANT1* promoter, codon optimized *alanine aminotransferase* (co-*AlaAT*) gene and *Nos* (termination sequence of *nopaline synthase* gene) terminator along with suitable restriction sites got synthesized from commercial vender (MS Eurofins, USA) in *pBluescript*, a general cloning plasmid vector. This vector was restricted with *Hind*III and *Eco*RI to liberate 1449bp fragment of co-*AlaAT* gene, for onward ligation of said fragment under 2X*CaMV35S* promoter in *pJIT60* vector. The complete (2X*CaMV35S*-co-*AlaAT*-*CaMV* terminator) cassette from *pJIT60* vector was lifted by digesting with *Kpn*I and *Eco*RI restriction enzymes and cloned in *pGreen0029* binary vector by exploiting same restriction sites and named this construct as SAC. Similarly, *pBluescript* plasmid was restricted with *Kpn*I and *Eco*RI digestion enzyme to release *OsANT1*-co-*AlaAT* (2420bp) fragment for onward cloning in *pGreen0029* vector to express co-*AlaAT* gene under root specific promoter (*OsANT1*) and named this vector as OAC. Both constructs (SAC and OAC) were transformed in *AGL1* strain of *Agrobacterium tumefaciens* through electroporation.

Immature embryos (9-14 days post anthesis) of spring wheat var. "Galaxy 2013" were used as explants for transformation. Under aseptic conditions, immature embryos were excised and placed on pre-culture MS0 medium at 22℃ in darkness for 3-4 days before inoculation of *Agrobacterium tumefaciens*. On fourth day, embryos were inoculated with *Agrobacterium tumefaciens* containing SAC and OAC vectors and placed on callus induction medium for 3-4 weeks and weekly sub-cultured on co-cultivation medium. Calli were placed on selection medium for two weeks. In this case kanamycin was used for selection of putative transgenic calli. Rapidly proliferating calli were transferred to regeneration medium. Freshly regenerated plantlets were transferred to jars on root induction medium for 7-10 days or until development of sufficient root system. Putative transgenic plants were shifted to soil in small pots under controlled condition. Seeds were collected from mature wheat plants to raise T_1 generation in pots under normal wheat growing season. T_1 plants were subjected to

molecular testing through PCR and qPCR to check possible segregants and level of transgene expression in various putative transgenic wheat lines. Five homozygous transgenic wheat lines of T_2 generation were selected to test under four different nitrogen fertilizer regimes (0, 75, 100 and 125 kgN/ha) in four separate blocks with complete randomization (RCBD).

***Biochemical and morpho-physiological evaluation of transgenic wheat*:** Total nitrogen content of transgenic wheat plants was measured in leaf tissues by adapting Kjeldhal's methods as described by Bremner (1965). Total free amino acids in leaf tissues were estimated through Hamilton and Van Slyke (1943) method. Amount of soluble or crude proteins were predicted by multiplying the value of total nitrogen content with 6.25 conversion factor. Activity of alanine aminotransferase enzyme was tested in freshly harvested leaf tissues by following McAllister and Good (2014) protocol.

Fully developed flag leaf area of various transgenic and wild type lines was measured through Laser Leaf Area Meter (CI-201, CID Bio-Sciences, USA). Number of tillers and number of spikes were counted at heading and maturity stage of wheat plants. After harvesting, wheat grain yield i.e. 1000 grain weight and grain yield (g/m^2) were recorded. Nitrogen use efficiency of selected wheat lines were calculated as suggested by Moll *et al*.(1982) i.e. grain yield produced/unit area of available nitrogen. Harvest index was determined by the ratio of grain yield of each row/above ground biomass g/row.

RESULTS

***Wheat transformation, molecular testing and field evaluation at different N regimes*:** Putative transgenic wheat events possessing co-*AlaAT* gene were selected on MS medium containing 100mg/L and150mg/L kanamycin. In case of SAC plasmid, 220 calli were placed on kanamycin containing selection medium, 105 calli survived and 60 calli started regeneration. However, in case of OAC plasmid 168 calli were transferred to selection medium, 87 calli survived and 45 calli initiated regeneration on designated medium. Wheat transformation efficiency in locally adapted elite wheat cultivar i.e. Galaxy 2013, was recorded to be 0.8% in case of SAC gene construct and 0.6% in case of OAC gene construct. T_1 generation was raised in pots in normal wheat growing season for seed multiplication and to perform PCR, real time qPCR (Figure 1, 2), leaf dip assay in antibiotic containing medium, different biochemical and various morpho-physiological analysis. T_2 seeds were collected to raise T_2 generation and to test five potential lines (GS11, GS21, GS22, GO13, GO14) at four N fertilizer regimes i.e. 0, 75, 100 and 125 kg N/ha in four split applications at germination, tillering, booting and grain filling stages of wheat plants.

Genomic DNA was extracted by CTAB (Doyle and Doyle, 1987) method. Amplification of 700bp co-*AlaAT* gene fragment indicated the successful integration of co-*AlaAT* gene cassette in wheat genome. SAC plasmid DNA was used as positive indicator during PCR while DNA of wild type (non-transformed) wheat plants were served as negative control. Relative quantification of co-*AlaAT* gene expression in selected wheat lines were performed by utilizing CFX96 Biorad, USA real time PCR detection system by using gene specific and *18S* primers for data normalization. GS22 line found to have higher co-*AlaAT* gene expression as compared to other transgenic wheat lines (Figure 2).

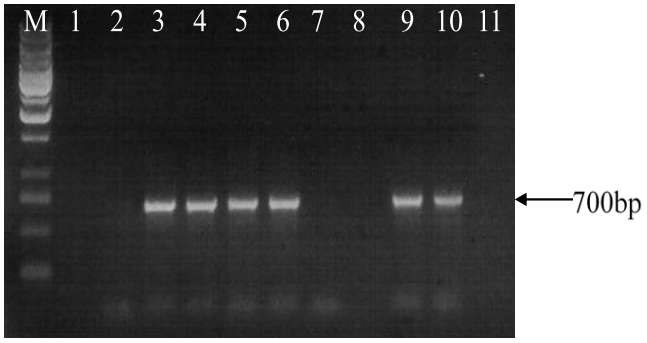

Figure 1. PCR testing of transgenic wheat(co-*AlaAT*) plants (transformed with SAC and OAC constructs) using co-*AlaAT* gene specific primers, Lane M correspond to 1 kbp DNA ladder, Lane 3, 4, 5, 6, 9 and 10 (GS11, GS21, GS22, GO13, GO14) are related to transgenic plants and +ve control. Lane 11 represent wild type. Lane 1, 2, 7 and 8 represent segregant null plants.

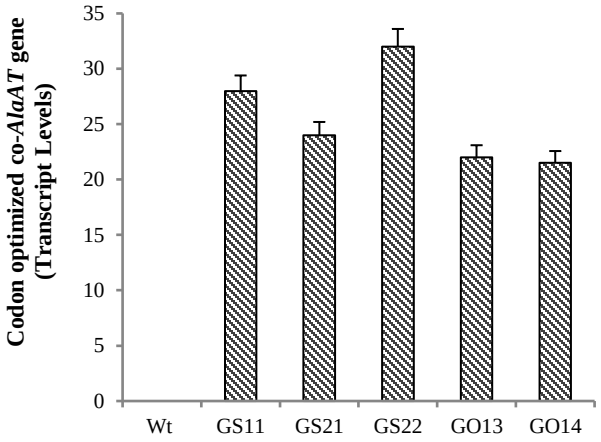

Figure 2. Relative quantification of codon optimized alanine aminotransferase (co-*AlaAT*) gene through real time PCR using delta delta Ct (ddCt) method. Representing average fold change of co-*AlaAT* gene transcripts level of

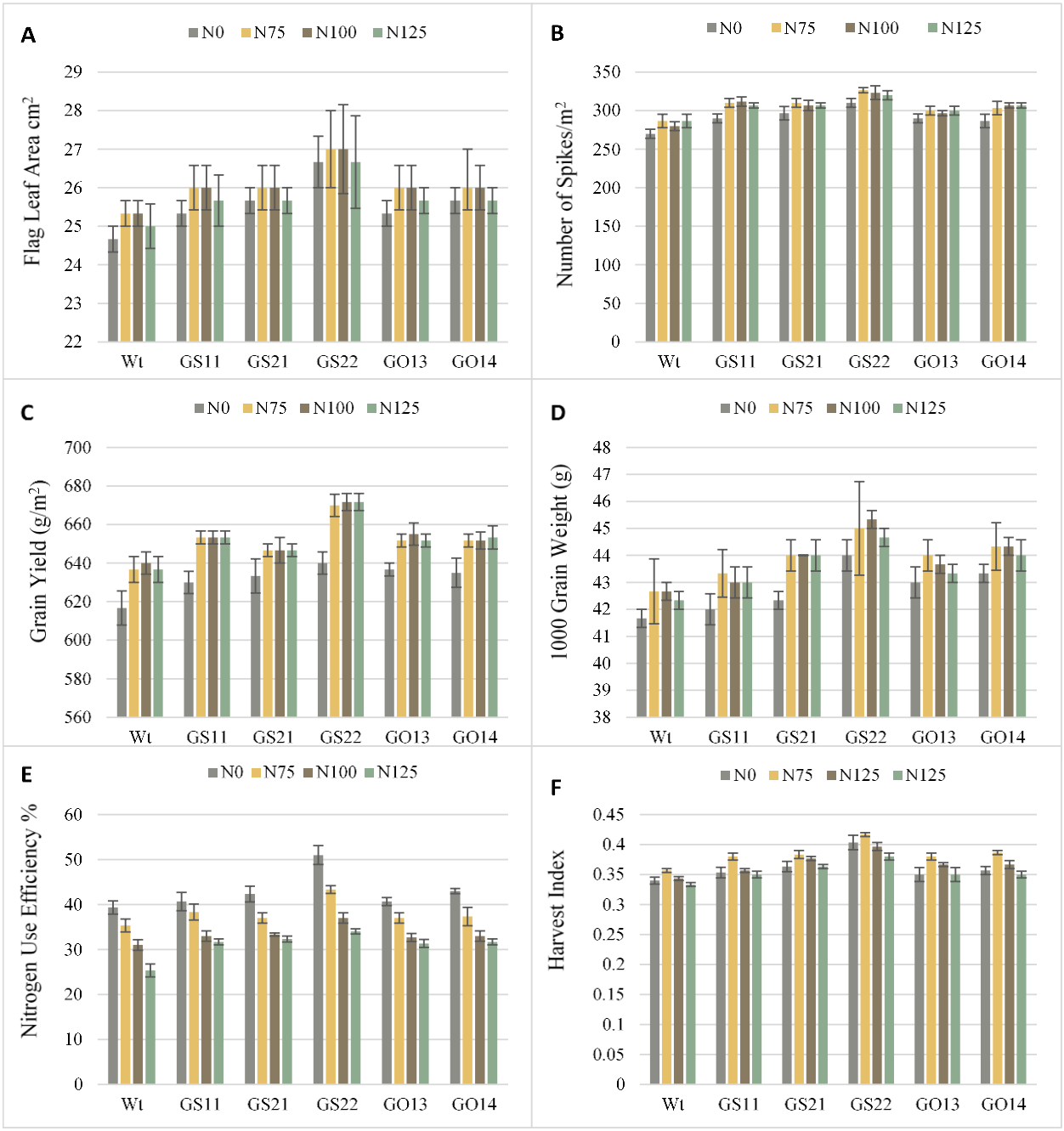

Figure 3. Estimation of; A. Flag leaf area, B. Number of spikes, C. Grain yield, D. 1000 grain weight, E. Nitrogen use efficiency, D. Harvest index of wild type and putative transgenic co-*AlaAT* wheat lines at 0, 75, 100, 125 kg/ha N fertilizer doses.

selected transgenic wheat lines and wild type control plants.

***Co-AlaAT gene elicits morpho-physiological and biochemical responses in wheat*:** Biochemical, morpho-physiological and yield related parameters of putative transgenic wheat plants were studied at 0, 75, 100 and 125 kg N/ha fertilizer regimes and data was analyzed by applying two-way analysis of variance of MS Excel program.

Flag leaf area of transgenic wheat lines was found to be significantly higher as compared to wild type control. However, in all selected lines, no further increase in flag leaf area was observed beyond 75 kg N/ha fertilizer dose, rather

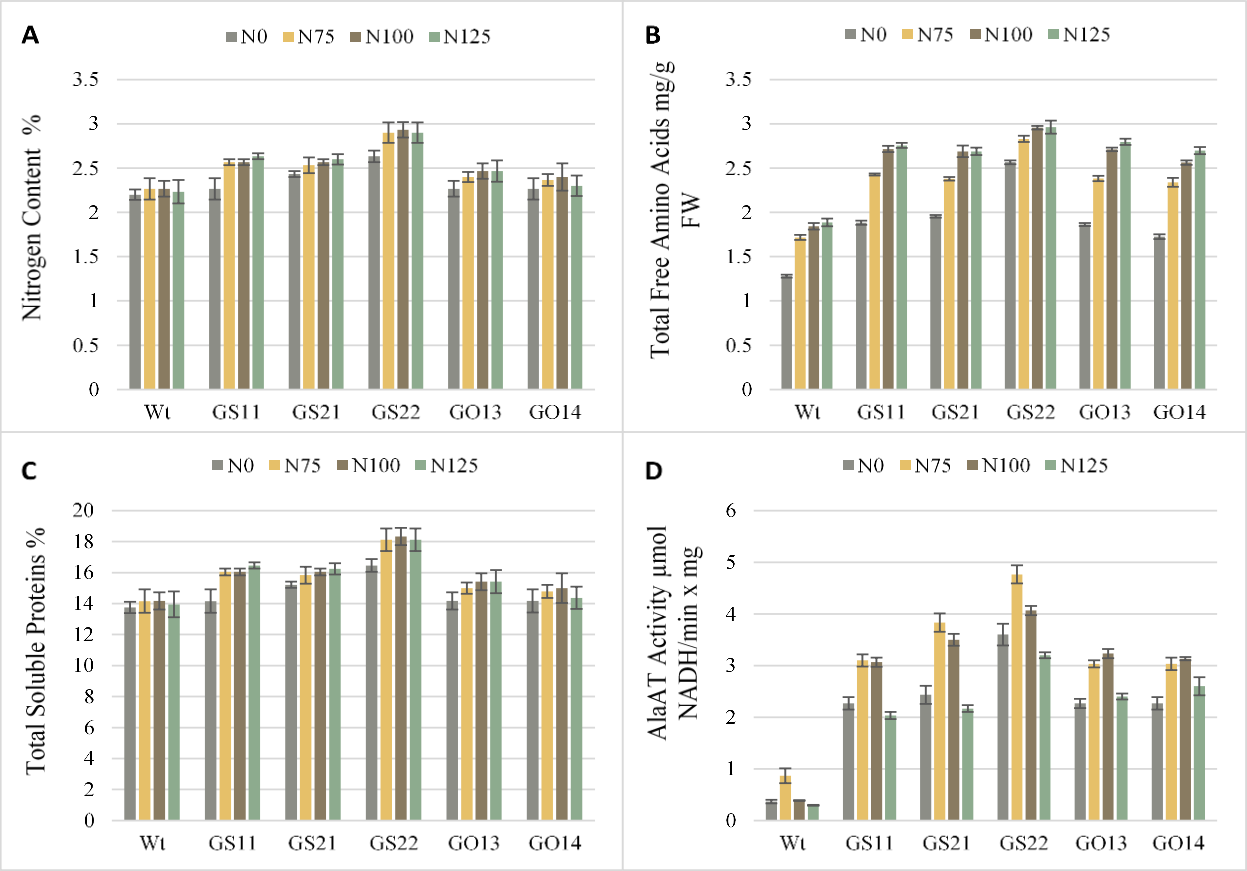

Figure 4. Biochemical analysis of; A. Nitrogen content, B. Total free amino acid, C. Total soluble proteins, D. AlaAT activity of wild type and putative transgenic co-*AlaAT* wheat lines at 0, 75, 100, 125 kg/ha N fertilizer doses

it decreased at 125 kg N/ha (Figure 3A). Similarly, by gradually increasing N fertilizer, total biomass increased with a greater number of tillers and spikes in all lines. Numbers of spikes in transgenic lines were also higher as compared to wild type. Although, at 100 and 125 kg N/ha fertilizer dose, number of spikes either remained same as these were at 75 kg N/ha or their number decreased slightly (Figure 3B). Grain yield (g/m2) and 1000 grain weight significantly increased by application of N fertilizer from 0-75 kg N/ha but in both cases, no further increase in grain yield and 1000 grain were recorded at 100-125 kg N/ha (Figure 3C, D). Based on morpho-physiological and yield data, NUE efficiency and harvest index were calculated. NUE declined significantly with the increase in N fertilizer dose from 0-125 kg N/ha, as per Moll *et al.*(1982) method. However, NUE of transgenic wheat lines were higher as compared to wild type (Figure 3E). Consequently, harvest index of transgenic wheat lines were significantly higher at 0-75 kg N/ha, then it decreased at 100-125 kg N/ha fertilizer dose (Figure 3F).

Biochemically total N content in wild type plants remained same at all N fertilizer regimes. However, in case of all transgenic lines, it increased significantly from 0-75 kg N/ha, then it remained almost unaltered at 100-125 kg N/ha and maximum N content was recorded in GS22 line at 75 kg N/ha fertilizer application (Figure 4A). The amount of total free amino acids raised at 0-100 kg N/ha fertilizer dose in all selected lines but no or little variation was recorded at 100-125 kg N/ha. However, significant increase in amino acids content were noted at 0-75 kg N/ha (Figure 4B). Similarly, total soluble protein values remained unaltered in case of wild type at all N fertilizer doses, nevertheless in all transgenic lines, its content tended to enhance significantly at 0-75 kg N/ha. Higher amounts of total soluble proteins were recorded in GS22 line (Figure 4C). AlaAT enzyme activity was significantly higher in all transgenic lines as compared to wild type under all fertilizer doses, however beyond 75 kg N/ha fertilizer application, it reduced drastically at 100-125 kg N/ha fertilizer regime (Figure 4D).

DISCUSSION

Codon optimized *alanine aminotransferase* (co-*AlaAT*) gene was used in an attempt to improve nitrogen use efficiency in

wheat. Commercially synthesized co-*AlaAT* gene was cloned under 2X*CaMV35S* (constitutive) and *OsANT1* (root specific) promoter, transformed and expressed optimally in wheat variety Galaxy 2013. Stably transformed five transgenic wheat lines (GS11, GS21, GS22, GO13 GO14) were evaluated at four different N fertilizer regimes (0, 75, 100 and 125 kg N/ha). The optimal expression of co-*AlaAT* gene consequently enhanced the flag leaf area, number of spikes, grain yield (g/m^2), 1000 grain weight, nitrogen use efficiency and harvest index in selected transgenic wheat lines. These findings are in agreement with earlier work, when sorghum wheat and rice were transformed with *HvAlaAT* gene either under control of constitutive or root preferred promoters (Pena *et al*., 2017; Shrawat *et al.,* 2008). Wheat plants transformed with co-*AlaAT* demonstrated more flag leaf area (Figure 3A), number of spikes (Figure 3B), grain yield (Figure 3C), harvest index (Figure 3F) and NUE (Figure 3E), associated with enhanced expression of co-*AlaAT* gene i.e. GS22 line (Figure 2) under limiting N supply (75 kg N/ha). Conversely, higher transgene expression does not necessarily result in preferred or superior phenotype (Brauer *et al.,* 2010). The distinction in morpho-physiological attributes of transgenic wheat were more prominent at 75 kg N/ha (lower N regime), however little or no differences were observed at higher N fertilizer application as reported in rice sugarcane, sorghum and wheat (Shrawat *et al*. 2008; Snyman *et al*. 2016; Pena *et al*. 2017). Under optimal and higher N fertilizer regimes, biochemical and physiological difference remained at minimum level as observed in canola plants transformed with barley *AlaAT* gene under *btg* promoter (Good *et al*., 2007). Selected transgenic wheat lines revealed to have highest N content (Figure 4A), AlaAT activity (Figure 4D), free amino acids (Figure 4B) and soluble proteins (Figure 4C) under all N regimes, associated directly with high expression of co-*AlaAT* (Figure 2) gene and consequently improving morpho-physiological characteristics, nitrogen use efficiency (McAllister and Good 2014) and biochemical attributes (Beatty *et al.,* 2013). These findings are in agreement as described earlier in *Brassica napus* expressing *HvAlaAT* cDNA under *btg* promoter (Good *et al.,* 2007).

An increase in N uptake and conversion to Glutamine, Glutamate and Alanine by passing through GS-GOGAT pathway in plants expressing *AlaAT* gene, results in translocation of these amino acids to various metabolic pathways to be utilized for plant growth (Good *et al.,* 2007). Similarly, an increase in AlaAT activity lead to the enhancement of N content, free amino acids and soluble protein (Shrawat *et al.,* 2008) as observed in transgenic wheat lines. In this limited field trial experiment of transgenic wheat expressing co-*AlaAT* under 2X*CaMV35S* and *OsANT1*promoter, significantly higher grain yield and harvest index was observed due to increase in NUE in wheat plants. However, these attributes are more prominent under limited N fertilizer (75 kg N/ha) as compared to non-transgenic control wheat plants demonstrating 20-25% less N fertilizer requirement. Non-significant variation of morpho-physiological, biochemical or yield related parameters were observed at higher N fertilizer (100-125 kg N/ha) application. Additionally, transgenic lines GS11, GS21 and GS22 expressing co-*AlaAT* gene under 2X*CaMV35S* promoter found to be better performing in comparison with transgenic lines GO13 and GO14 expressing co-*AlaAT* gene under control of *OsANT1* promoter. These findings support that higher expression of co-*AlaAT* gene in transgenic lines GS11, GS21 and GS22 were observed where this gene was expressed under 2X*CaMV35S* promoter. The higher expression was possibly due to double strength of *CaMV35S* promoter. Domain A of *CaMV35S*constitutive promoter suggested driving root specific activity in transgenic plants. Barley *AlaAT* gene expression under *OsANT1* promoter in transgenic rice demonstrated significant improvement in NUE, while co-*AlaAT* gene expression under *OsANT1* promoter in transgenic wheat did not reveal as much improvement (Figure 2) in nitrogen uptake, translocation and remobilization as it was reported in rice (Shrawat *et al.*,2008).

Promising morpho-physiological, biochemical and grain yield data led to the improvement of 5-29% NUE at 0 kg N/ha and 3-16.5% NUE at 75 kg N/ha fertilizer dose in various transgenic wheat lines. However, no further improvement in NUE observed at higher N doses in different transgenic lines except as observed in GS22 line, which responded to higher N doses as well. This study may help to reduce fertilizer input, improve profitability by having more grain yield and decrease the associated environmental risks due to nitrate toxicity (Ahmed *et al*., 2017).

***Conclusion*:** Efficient and optimal use of N fertilizer by crop plants is essentially required to increase crop yield, to reduce fertilizers application and their hazardous impact on the environment. The over-expression of co-*AlaAT* gene boosts the N uptake, translocation and its mobilization during various cellular processes. It is therefore assumed that co-*AlaAT* gene could be suitable target to be transformed in major food crop plants to improve their N efficient phenotype.

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