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HARNESSING *TaNAC2-5A* FOR ECO-EFFICIENT WHEAT CULTIVATION: A DUAL ANALYSIS OF ROOT TRAITS AND NITRATE ASSIMILATION

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Abstract

Low nitrogen-use efficiency (NUE) of agricultural crops frequently necessitates excessive application of fertilizers, increasing both economic costs and environmental damage. Enhancing NUE in major food crops, particularly wheat, is therefore a central objective for sustainable farming systems. This study investigated the contribution of the transcription factor *TaNAC2-5A* to improve nitrate acquisition and root system development in wheat (*Triticum aestivum*). *TaNAC2-5A* is part of the NAC family and regulates plant growth and responses to stress, particularly limited nitrate availability. We assessed T_4 *TaNAC2-5A* overexpressing wheat lines developed in local cultivar FSD 2008 in two light conditions, low spectrum and full spectrum, and under two low nitrate levels, 0.2 and 1.0 mM in hydroponic systems. Molecular confirmation of the transgene integration in T_4 generation plants was done by PCR. Nitrate content was measured using the Cataldo colorimetric assay. The chlorophyll and leaf nitrogen was measured with the help of portable SPAD meter. Root architecture was studied using rhizo-scanning, and root parameters including surface area, root length, volume, and diameter were determined by WinRhizo image analysis system. Total nitrogen and chlorophyll measurements were analyzed using One-way ANOVA followed by Dunnett's post hoc test while Two-way ANOVA with Tukey's HSD was used to test the effects of nitrate and light. Transgenic wheat plants showed higher levels of chlorophyll content (100 to 300 % increase), total nitrogen content (45 % increase), leaf nitrate (30 to 70 % increase), and 1000 grain weight (up to 17%). Overall, overexpression of *TaNAC2-5A* improved nitrogen use efficiency (NUE) and root development of wheat by allowing plants to absorb and process nitrogen more efficiently which ultimately offered a practical approach for breeding programs targeting sustainable production and global food security.

Key words: Nitrogen use efficiency (NUE); Wheat nitrate uptake; *TaNAC2-5A* transcription factor; *Triticum aestivum*; Root development; Nitrate assimilation; Sustainable agriculture

Introduction

Wheat is one of the most important staple food crops in the world, serving as a primary source of human diet as it fulfills approximately 20% of the daily caloric intake of the population and about 25% of protein requirements (Erenstein *et al*., 2022). The world's average area for wheat cultivation is around 220 million hectares, reflecting its importance in ensuring food availability. Whereas, the estimated production of wheat is projected to reach nearly 808 million metric tons in the periods of 2025-2026 (Anon., 2025). Simultaneously, the estimated world population is anticipated to reach nearly 10 billion in the year 2050. Therefore, food production must increase by approximately 50% to meet the growing demand and ensure food security (Anon., 2017; Anon., 2022). However, the productivity of the cereal crops is being largely restricted due to the scarcity of water resources, and heavy reliance on nitrogen fertilizers to maintain the high yields. Shockingly, only 50% of that applied Nitrogen is taken up by the plants while rest escapes into the environment causing nitrate leaching, soil acidification, and emission of greenhouse gases like nitrous oxide (N_2O), which is approximately 300 times stronger than carbon dioxide in global warming (Govindasamy *et al*., 2023; Anon., 2025).

Climate change further aggravates these issues. In South Asia, wheat yield losses are attributable to increasing average temperatures, drought frequency, and extreme weather events. Recent projections predict a 16% reduction in wheat yields in South Asia, with losses of 35% to be expected in droughts years (Anon., 2023; Anon., 2024). Flooding and soil erosion wash away the arable land and the critical soil nutrients needed to sustain wheat production. Drought, salinity, and insufficient light are the major forms of abiotic stresses which slow the nitrogen uptake efficiency (NUpE) and adversely affect photosynthesis and root development (He *et al*., 2014; Zhang *et al*., 2023). Therefore, increasing the NUE in wheat is crucial to the long-term viability of the farming system for sustainable higher yield, which may also help to reduce environmental damage and input cost to the farmer, while assuring food security.

Nitrogen use efficiency in plants depends on two main factors. The first is how well nitrate is taken up from the soil (nitrate uptake efficiency, NUpE) and secondly, how effectively it is used within the plant (nitrate utilization efficiency, NUtE). Both processes are determined by root architecture, certain enzymes, and the nitrogen assimilation cycle, specifically the glutamine synthetase/glutamate synthase cycle (GS/GOGAT cycle, Wang *et al*., 2015). During the nitrate absorption, two categories of transporters are involved, high-affinity transport systems (HATS) and low-affinity transport systems (LATS) (Yin *et al*., 2007). These transporters are regulated by soil nitrate concentrations. Among the key transporters, NRT1.1 and NRT1.2 are important for promoting the uptake of nitrate and root development through auxin signaling (Vidal *et al*.,

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2020). Genomic studies in wheat have identified genes and transcription factors involved in these pathways, including *TaNAC2-5A* which is a nitrogen responsive transcription factor gene. Nitrate uptake, assimilation, and root architecture can be enhanced through overexpressing the *TaNAC2-5A* transcription factor. It is also known to activate important nitrate transporters, and assimilation enzymes e.g., nitrate reductase and glutamine synthetase (GS), which enhance the biomass of roots, promote the development of lateral roots, and increase the overall yield of grains (He *et al*., 2015; Lv *et al*., 2020; Bian *et al*., 2025).

In terms of optimizing NUE, another important element is light and its relationship with nitrogen metabolism. Light controls the expression of the nitrate transporter genes, the process of photosynthesis, and the production of chlorophyll. Full-spectrum light (5500-6500K) improves photosynthetic efficiency and nitrate assimilation, while light at 2500K diminishes nitrate assimilation. For instance, the transgenic wheat lines overexpressing *TaNAC2-5A* maintain higher chlorophyll levels and maintain greater resilience to low light than the non-transgenic lines (He *et al*., 2015; Zhou *et al*., 2021).

Enhancing NUE is now a breeding goal for wheat, with many genes and traits being identified through the process of QTL or genome-wide association analysis. Genes such as *TaNRT2.4* or *TaNPF* demonstrate tissue-specific regulation according to nitrogen status and enable breeders to focus on target specific uptake or transport mechanisms (Sigalas *et al*., 2024). Genes coding for assimilation proteins such as *TaGOGAT*, responsive to limited nitrogen convert nitrate to ammonium or amino acids (Li *et al*., 2024). Root system architecture also plays a major role in nitrate acquisition. Genotypes with deep and flexible root systems, particularly wild wheat introgression lines, perform better than modern shallow-rooted cultivars under low-nitrate conditions (Govta *et al*., 2025). *TaNAC2-5A* contributes to favorable root traits by enhancing lateral root development and deeper rooting, which increases nitrate uptake. Environmental stresses, including drought, salinity, temperature extremes, and circadian regulation, reduce nitrate absorption. Approaches such as calcium nitrate application and precise regulation of genes involved in circadian control, including LUX clock protein pathways, provide viable strategies to maintain nitrogen efficiency under stress.

Apart from regulating the uptake and assimilation of nitrogen, *TaNAC2-5A* has been acknowledged to influence further downstream biochemical pathways defining the stress metabolic status of plants in nutrient-limited conditions. Availability of nitrogen determines the accumulation of biochemical constituents such as soluble sugars, free amino acids, proteins, various forms of chlorophyll, and certain antioxidant enzymes, all of which are needed for cellular homeostasis and growth under limited nitrate conditions. NAC transcription factors are involved in carbon-nitrogen coordination by integrating nitrogen assimilation with the light-dependent carbon fixation and primary metabolic pathways. Many NAC genes have been overexpressed in various crops to improve the amino acid composition, total soluble proteins and chlorophyll in nitrogen-deficient or stressed conditions (He *et al*., 2015). In case of wheat, activation of nitrate reductase and GS/GOGAT enzymes by *TaNAC2-5A* is expected to enhance ammonium assimilation efficiency, supporting the production of nitrogen-rich metabolites and maintaining growth even when nitrate availability remains limited.

TaNAC2-5A has also been linked to improvements in key agronomic traits that directly influence crop productivity under nitrogen-limited conditions. Studies in wheat and related cereal crops show that elevated expression of NAC transcription factors enhances root system size, lateral root density, and root depth, traits that improve nitrogen foraging efficiency in low-input environments. Increased root biomass associated with *TaNAC2-5A* activity supports greater nitrate uptake, which in turn promotes improved tiller formation, shoot biomass accumulation, and canopy development. Literature reports also associate *TaNAC2-5A* overexpression with higher chlorophyll retention and delayed leaf senescence, traits that extend the photosynthetically active period and support assimilate supply during grain filling. Collectively, these effects translate into measurable yield-related gains, including increased grain number per plant, improved grain weight, and more stable yield performance under low nitrogen availability. Thus, highlighting *TaNAC2-5A* as a promising target for agronomic improvement in wheat breeding programs.

In addition, nitrogen assimilation results in enhanced synthesis of proline, phenolics and flavonoids which act as osmo-protectants. The NAC overexpression lines have been reported to have increased antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and peroxidases (POD). The NAC overexpression lines show reduced oxidative damage under abiotic stress (Lv *et al*., 2020).

This study reports the nitrate uptake in leaves and roots of control and transgenic wheat over expressing *TaNAC2-5A* transcription factor in local wheat variety FSD-2008 under different environmental conditions. The experiments were performed both under the effect of low spectrum (2500 K) and high spectrum (5000 – 6500 K) light under three different potassium nitrate levels (0.2 mM, 1 mM and 35 mM). Additionally, the effect of nitrate levels on root architecture and photosynthetic capacity based on chlorophyll content was also determined.

Materials and Methods

Growth of plants: T_4 generation of *TaNAC2-5A* transgenic wheat lines, namely NAC2-1(L2) 27A, NAC2-4(L2) N2, NAC2-4(L6) N5, NAC2-4(L5) N6, and NAC2-4(L22) N9 (Azhar, 2025), were used in this study along with the wild-type cultivar FSD-2008. Grains were sprouted in germination trays under uniform, controlled conditions. After a two-week growth period, seedlings exhibiting uniform growth were selected and transferred to hydroponic culture systems. Two separate hydroponic setups were established with 0.2 mM and 1.0 mM nitrate supply, both representing nitrogen-limited conditions, to allow clearer detection of subtle differences in nitrate uptake and plant performance between control and transgenic plants, which are often masked under high nitrate availability. Plant growth was carried out at a controlled temperature of 22°C, with relative humidity maintained in the range of 60–70%.

Plants were grown under two distinct light regimes using separate hydroponic batches. In the first batch the plants were grown under full-spectrum light (5500–6500 K), simulating natural daylight conditions, provided by KingBrite P55-240W LED fixtures installed in 2 × 2 ft grow tents at 20 inches above the plant canopy. A long photo period of 22 h light followed by 2 h dark was maintained throughout the study in the speed breeding facility to accelerate growth and generation turnover (Watson *et al*., 2018). In the second batch, plants were grown under low-spectrum warm light conditions at 2500 K, while all other experimental parameters were kept identical to those of the full-spectrum treatment. Nutrient solutions in both hydroponic systems were renewed on a weekly basis to maintain consistent nutrient availability. Continuous aeration of the nutrient solution was provided using a Hailea ACO-308 air compressor operating at a flow rate of 45 L min^{-1}. To suppress fungal and algal growth, 60 mL of a 20% hydrogen peroxide solution was applied periodically during the experimental period.

Screening and expression analysis of transgenics: *TaNAC2-5A* transgenics contained the T-DNA including both *TaNAC2-5A* and *bar* gene cassettes (Fig. 1). To identify and analyze transgene expressions, primers targeting each gene cassette were employed. Total genomic DNA was isolated from *TaNAC2-5A* transgenic plants using the cetyltrimethylammonium bromide (CTAB) extraction protocol (Murray and Thompson, 1980 - missing). The transgenic plants were confirmed by PCR amplification of the bar gene using *bar* gene cassette specific primer pair, shown in Table S1, expected to amplify 990 bp fragment. For PCR amplification, 2 µL of genomic DNA from each sample was used. DNA from non-transgenic plants served as a negative control, ensuring that no transgene was present, while plasmid DNA of pSB219-NAC2 vector served as a positive control, validating the expected amplicon size. Primer nucleotide sequences are listed in Table S1, and the PCR reaction setup and profile is given in Tables S2 and S3, respectively.

To examine the transgene-specific expression of the *TaNAC2-5A* gene in the transgenic lines, total RNA from the transgenics was isolated and treated with DNase I to remove any DNA contamination. The cDNA was synthesized by reverse transcription using oligo dT primer. The cDNA was used as a template for RT-PCR amplification of the expressed transgene using the forward primer (NAC2qRTF3) designed on the *TaNAC2-5A* gene and the reverse primer (c3UTRCMV) on the 3' UTR of CaMV terminator (Table S4). Table S5 details the RT-PCR reaction setup, and Table S6 displays the PCR profile.

Nitrate quantification (Cataldo method): After three weeks of growth in nutrient solution, nitrate content of the fresh leaves of T_4 *TaNAC2-5A* transgenics and the non-transgenic parent control (FSD-2008) were determined in triplicate, using the colorimetric approach described by Cataldo *et al*., (1975).

Nitrate standards ranging from 0 to 200 µg/mL NO_3^--N in increments of 20 µg/mL were generated from a 1000 µg/mL stock solution to construct a standard curve. The standard curve was analyzed using linear regression, producing slope and intercept values with a coefficient of determination (R^2) of 0.992. Nitrate concentrations of the samples were determined by applying the calibration equation, y = 0.0021x + 0.023.

Chlorophyll and leaf nitrogen content: A handheld SPAD meter (Brightway, China) was employed to non-destructively measure the leaf chlorophyll and relative nitrogen content in both control and *TaNAC2-5A* transgenic plants grown under 0.2 mM and 1.0 mM nitrate, chosen as nitrogen-limited conditions to reveal subtle differences in plant performance, under full-spectrum light. Readings were recorded after 4 and 8 weeks of transferring into the nutrient medium. Chlorophyll and nitrogen readings were collected at three different locations on the leaf, and the average value of the readings was calculated for each plant. The SPAD measurements were then used to calculate the Chlorophyll Content Index (CCI) and to estimate chlorophyll concentration (µmol/m^2) according to the equations outlined by Parry *et al*., (2014).

$$\mathbf{CCI = 1.0 + 0.00119 * SPAD^{2.67}}$$

$$\mathbf{Chlorophyll\ concentration\ (\mu mol\ m^{-2}) = -84 + 79 * (CCI)^{0.60}}$$

This data helped to determine the correlation between nitrate availability and light treatment with the physiological status of non-transgenic control and *TaNAC2-5A* transgenic plants.

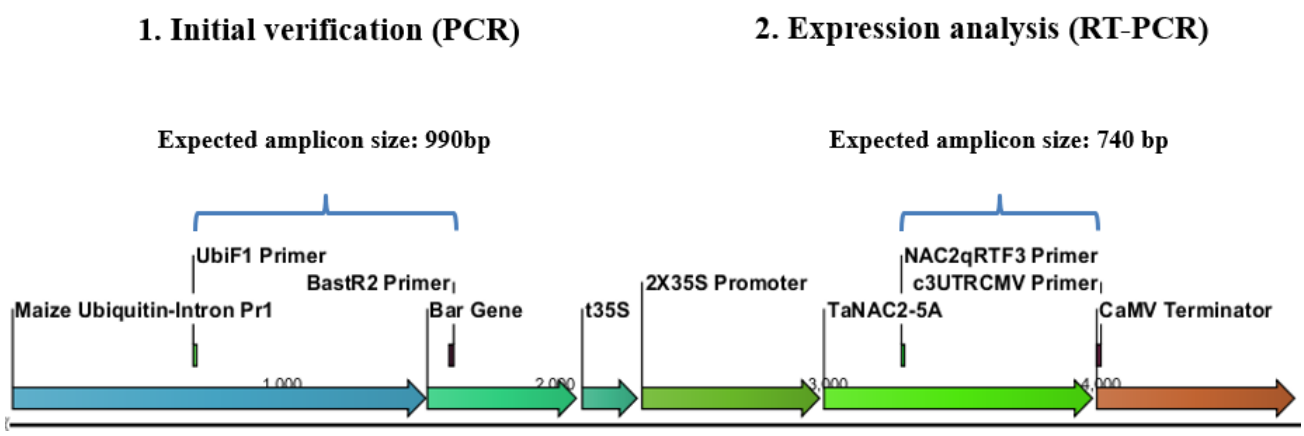

Fig. 1. T-DNA carrying 2 expression cassettes (*bar* gene and *TaNAC2-5A*): The bar gene is controlled by maize ubiquitin promoter and 35S ter while *TaNAC2-5A* is regulated by 2X35S promoter and CaMV ter. Integration of the *bar* gene in transgenic plants was confirmed by PCR amplification of a 990 bp fragment. Expression of *TaNAC2-5A* was evaluated using RT-PCR, which amplified a 740 bp fragment corresponding to the transgene.

Table S1. Primers used for PCR based verification of *TaNAC2*-5A transgenics.

No.	Primer name	Sequence of Primer 5' to 3'	Tm (ºC)
1.	UbiF1	5'ACGGCACGGCATCTCTGTC 3'	62
2.	BastR2	5' TGACCGTGCTTGTCTCGATGTAG 3'	74

Table S2. Composition of PCR mix for the amplification of *bar* gene.

Reagents	Concentration	Quantity used
Taq DNA Polymerase	10U/µL	0.5 µL
Taq buffer	10X	2.5 µL (1X final)
$MgCl_2$	50mM	1 µL (2 mM final)
dNTPs	2.5mM	1 µL (100 µM final)
Forward Primer (UbiF1)	25ng/µL	1 µL
Reverse Primer (BastR2)	25ng/µL	1 µL
Nuclease-free water	-	16 µL
Template (Chromosomal DNA)	100-200 ng/µL	2 µL
Total		**25 µL**

Table S3. PCR profile used for the amplification of *bar* gene from *TaNAC2-5A* transgenics.

Steps	Temperature (°C)	Time (mins/sec)	No. of cycles
Initial denaturation	94	4 min	1
Denaturation	94	45 sec	
Annealing	62	1 min	40
Extension	72	1 min	
Final Extension	72	10 min	1

Table S4. Primers used for the expression analysis of *TaNAC2*-5A transgenics.

No.	Primer name	Sequence of Primer 5' to 3'	Tm (ºC)
1.	NAC2qRTF3	TTCGCCCGTGGTCATTACA	51
2.	c3UTRCMV	GAGAGAGACTGGTGATTTCAGCA	55

Table S5. Composition of reaction mixture for synthesis of cDNA.

Reagents	Concentration	Quantity used
Nuclease-free water	-	10 µL
Oligo dT primer	100µM (5µM final)	1 µL
Reaction buffer	5X (1X final)	4 µL
RiboLock (RNAse Inhibitor)	20 units	1 µL
dNTP Mix	10mM (0.5mM final)	2 µL
Reverse Transcriptase	200U/µL	1 µL
Template (total RNA)	100-200 ng/µL	1 µL
Total		**20 µL**

Table S6. RT-PCR profile used for cDNA synthesis.

Steps	Temperature (°C)	Time (mins/sec)	No. of cycles
cDNA synthesis	42	60 min	1
Termination of cDNA synthesis	70	5 min	1

Table S7. Leaf nitrate concentrations of control and *TaNAC2-5A* transgenic plant lines.

Transgenic lines	Leaf nitrate concentration (mg/L)	
	0.2 mM Nitrate	1 mM Nitrate
	Low spectrum/Warm light (2500 K)	
FSD-2008	35.03 ± 3.06	281.53 ± 3.89
NAC2-4(L6) N5	44.2 ± 3.89	356.86 ± 7.33**
NAC2-4(L5) N6	76.36 ± 1.67**	368.2 ± 5.88**
NAC2-4(L22) N9	54.70 ± 3.82**	322.02 ± 12.16*
NAC2-4(L2) N2	52.86 ± 1.72*	328.7 ± 7.64*
NAC2-1(L2) 27A	75.53 ± 3.64**	353.36 ± 15.14**
	Full spectrum/Sunlight (5500-6500K)	
FSD-2008	40.53 ± 0.99	91.2 ± 2.39
NAC2-4(L6) N5	49.03 ± 2.87**	99.53 ± 3.59*
NAC2-4(L5) N6	43.86 ± 1.19	106.03 ± 1.98**
NAC2-4(L22) N9	54.70 ± 3.89**	107.86 ± 2.44**
NAC2-4(L2) N2	44.86 ± 1.80*	105.7 ± 4.05**
NAC2-1(L2) 27A	57.2 ± 1.26**	97.7 ± 1.91

Asterisks denote p-values. $p<0.05$ (*), and $p<0.01$ (**) indicate that the mean ± SE of three independent replicates of transgenic lines are significant as compared to control FSD-2008. Two-way ANOVA and Tukey HSD test were applied

Root architecture analysis: To examine root architecture and morphology of transgenic lines, seeds from best performing lines were germinated and allowed to grow for two weeks before being transferred to hydroponic systems. Three separate chambers, each containing 30 L of nutrient solution with differing nitrate concentrations, as 1 mM nitrate in the first chamber, 5 mM nitrate in the second, and Hoagland's solution containing 35 mM nitrate in the third, were prepared to provide a gradient of nitrogen availability, allowing assessment of root architectural responses in control and *TaNAC2-5A* transgenic wheat plants under low, moderate, and high nitrate conditions. Plants were grown under direct full-spectrum light (5500–6500 K) in growth chambers kept at 22 ± 1 °C, with the nutrient solution pH maintained between 6.5 and 7. Five weeks post-transfer, PCR-verified plants from three selected transgenic lines (NAC2-4(L2) N2, NAC2-4(L5) N6, and NAC2-4(L22) N9) were proceeded for root analysis using rhizo-scanning. The roots were gently detached from the plants, washed thoroughly with distilled water, briefly stained for one minute using a 0.1 g/L methyl violet solution, and scanned in grey scale mode. Key root traits, including total volume (cm^3), root length (cm), average diameter (mm), surface area (cm^2), and projected area (cm^2), were computed using Epson WinRhizo software.

Biochemical profiling of T_4 seeds: Quality of T_4 grains of *TaNAC2-5A* transgenic wheat lines along with parent control FSD-2008 was measured by conducting different biochemical tests including protein content, starch content, falling number, dry and wet gluten, ash content, test weight, gluten index and product score for Chapati at Ayyub Research institute, Faisalabad. These are critical parameter to check the end-use quality as these parameters influence the nutritional value, baking performance and dough strength. Assessing these traits was essential because *TaNAC2-5A* is linked with stress tolerance and may alter the metabolic processes while impacting the grain composition and functional properties.

Agronomic traits evaluation: Agronomic traits, including 1000-grain weight, number of tillers (spikes) per plant, average spike length, and were recorded at physiological maturity manually using a randomized complete block design (RCBD). Each transgenic line and its non-transgenic control (FSD-2008) were grown in four independent replicates, with 50 plants per replicate. Field plots were established at the Forman Christian College research fields, each measuring 6.5 ft × 8.5 ft, with a plant-to-plant spacing of 18 cm and row-to-row spacing of 60 cm, maintaining an average density of 50 plants per plot. Mean values and standard deviations were calculated from biological replicates, and statistical significance was determined relative to the control. Percentage increase in grain weight was calculated to quantify yield improvement in transgenic lines across generations.

Statistical analysis: Data analyses were carried out using the R statistical environment (version 4.5.1; R Core Team, 2025). The influence of light regime and nitrate supply on leaf nitrate accumulation was examined using a two-factor variance analysis (two-way ANOVA). Separate ANOVA

models were fitted for each treatment combination, including full-spectrum light (5500–6500 K) and low-spectrum light (2500 K), as well as nitrate concentrations of 0.2 mM and 1 mM. Post hoc pairwise differences between treatments were evaluated using Tukey's Honest Significant Difference (HSD) test with a significance threshold set at $\alpha = 0.05$, using the *emmeans* and *stats* packages. To assess relative nitrogen and chlorophyll levels, variation among the transgenic lines was analyzed using one-way ANOVA, with subsequent comparisons to the control using Dunnett's multiple comparison test via the multcomp package. This approach was used to determine the statistical significance of transgenic lines relative to the wild-type control (FSD-2008). Graphical representation of treatment effects and interactions was generated using the *ggplot2* package.

Results

Screening and expression analysis of transgenics: DNA extracted from both the *TaNAC2-5A* transgenics and control plants using CTAB method was used in PCR based identification of transgenic plants. Presence of T-DNA was confirmed by amplification of bar gene which produced a fragment of 990 bps (Fig. 2a).

After confirming the presence of T-DNA, expression analysis of *TaNAC2-5A* gene was done by RT-PCR using forward primer (NAC2qRTF3) designed on *TaNAC2-5A* gene and reverse primer (c3UTRCMV) designed on CaMV terminator. A product of 740 bps was amplified confirming the expression of transgene (Fig. 2b).

Nitrate estimation using Cataldo method: Leaf nitrate content in both transgenic and control plants were determined by relating sample absorbance values to a standard curve generated from known nitrate standards. A strong linear correlation was observed between absorbance and nitrate concentration in the standard curve, yielding an R^2 value of 0.9956 (Fig. 3). Absorbance values for all leaf samples were recorded using the Cataldo colorimetric assay. Nitrate concentrations in control and transgenic samples were subsequently calculated by interpolating their absorbance values against the corresponding standard curve.

Concentrations of leaf Nitrate in T_4 generation *TaNAC2-5A* transgenics along with FSD-2008 under different light and nitrate treatments are summarized in Table S7. Under low-spectrum light, all transgenic lines accumulated markedly higher nitrate levels than the FSD-2008 control at both nitrate treatments (Fig. 4). Under full-spectrum light, the transgenic lines retained higher nitrate levels than FSD-2008, although the magnitude of these differences was reduced (Fig. 5).

Leaf samples were normalized by tissue weight to reduce variation caused by differences in sample size, morphology, and water content. At the time of sampling, fresh leaf weights were recorded and used for normalization. Table S8 reports nitrate concentration expressed per gram of tissue (mg L^{-1} g^{-1}) for both *TaNAC2-5A* transgenics and control FSD-2008 plants. Across all tested conditions, the transgenic lines consistently showed higher nitrate levels per unit tissue weight than the FSD-2008 control.

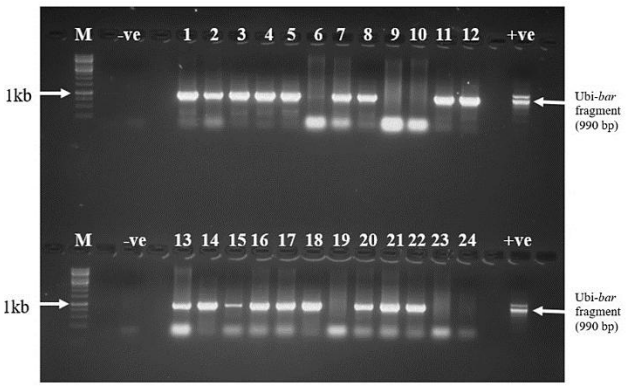

Fig. 2a. PCR-based screening of T_4 *TaNAC2-5A* transgenics. Lane M contains the 1 kb DNA marker. Lane -ve represents the wild-type plant (FSD-2008), used as a negative control, whereas lane +ve represents the positive control derived from PCR amplification of the pSB219-NAC2 plasmid harboring the *TaNAC2-5A* expression construct. PCR products from individual plants of different transgenic lines are shown across subsequent lanes: lanes 1–5 correspond to amplification from line NAC2-1(L2) 27A; lanes 6–9 represent line NAC2-4(L2) N2; lanes 10–14 include NAC2-4(L6) N5 line; lanes 15–19 show NAC2-4(L5) N6 line; and lanes 20–24 display amplification from line NAC2-4(L22) N9. Fragment of 990 bp verified successful T-DNA integration in the transgenic plants.

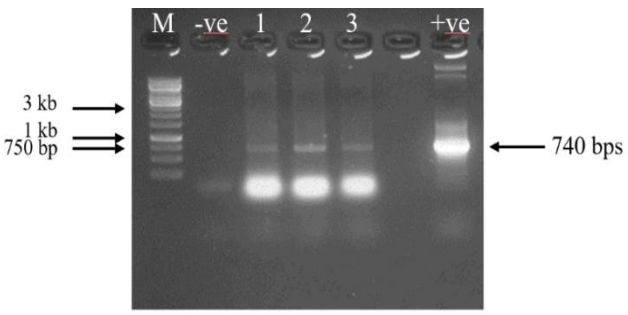

Fig. 2b. Expression analysis of transgenics through RT-PCR using *TaNAC2-5A* cassette-specific primers. Lane M contains the 1 kb DNA marker. -ve represents the PCR amplification from non-transgenic parent control. Lanes 1–3 display a 740 bp PCR amplicon corresponding to *TaNAC2-5A* transcript detection in transgenic plants. Lane +ve is the positive control which represents amplification obtained from the +ve control, pSB219-NAC2 vector harboring transgene.

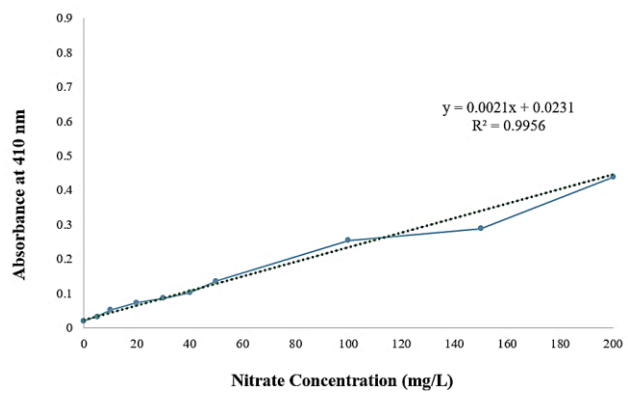

Fig. 3. Standard curve prepared from known standards to determine the leaf nitrate concentrations. X-axis represents the concentrations of known standards (mgL^{-1}) while Y-axis shows the absorbance at 410 nm.

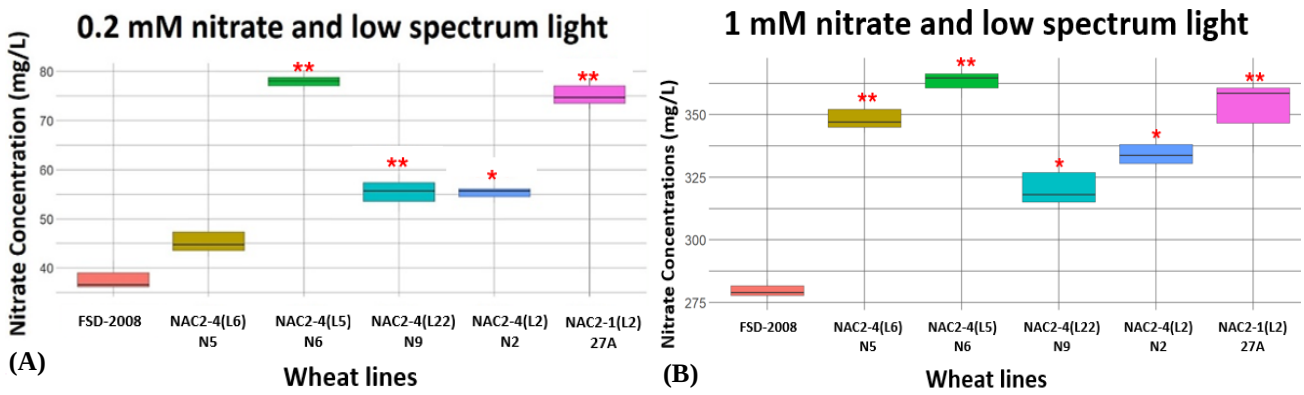

Fig. 4. Nitrate concentrations in leaf tissues of *TaNAC2-5A* transgenic wheat lines (NAC2-4(L6) N5, NAC2-4(L5) N6, NAC2-4(L22) N9, NAC2-4(L2) N2 and NAC2-1(L2) 27A) along with FSD-2008 under low-spectrum light at (A) 0.2 mM nitrate and (B) 1.0 mM nitrate supply. Significant differences in nitrate content are indicated by asterisks, with * representing $p<0.05$, and ** $p<0.01$.

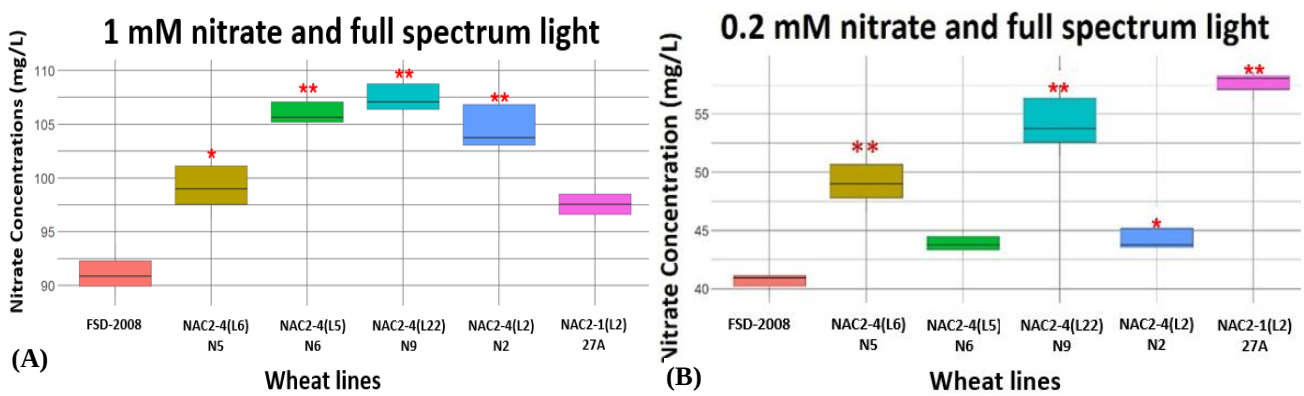

Fig. 5. Nitrate concentrations in leaf tissues of *TaNAC2-5A* transgenics along with FSD-2008 under Full-spectrum light at (A) 0.2 mM nitrate and (B) 1.0 mM nitrate supply. Significant differences in nitrate content are indicated by asterisks, with * representing $p<0.05$, and ** $p<0.01$.

The results are illustrated in two separate panels (Figs. 6A and 6B) to clearly represent the different response patterns under the applied treatments. Figure 6A shows how changes in light intensity affected leaf nitrate concentration of transgenics and control plants under constant nitrate supply. Shifting from low-spectrum light to full-spectrum light under 0.2 mM nitrate caused a marked increase in tissue nitrate concentration (g^{-1}) in transgenics as well as control plants, as indicated by the orange and blue bars. In contrast, at 1.0 mM nitrate, the same increase in light intensity resulted in lower nitrate levels per unit tissue across all genotypes, shown by the green and brown bars.

Raising the external nitrate concentration from 0.2 mM to 1.0 mM led to clear increases in leaf nitrate content per unit tissue weight in both the control and *TaNAC2-5A* transgenic lines (Fig. 6B). This response was evident under both lighting regimes, including low-spectrum warm light at 2500 K (orange and blue bars) and full-spectrum light at 5500–6500 K (green and brown bars).

Leaf chlorophyll content: Chlorophyll levels in control and *TaNAC2-5A* transgenic plants were measured at 4 and 8 weeks of growth using a SPAD meter, and the SPAD readings were converted into chlorophyll concentrations expressed as µmol m^{-2}. The resulting values are summarized in Table S9. At the 4-week stage, higher nitrate supply led to increased chlorophyll content in both the transgenic lines and the control plants (Fig.7A). By 8 weeks, the trend reversed, with all genotypes showing reduced chlorophyll levels at the higher nitrate concentration (Fig. 7B).

Changes in chlorophyll over time revealed a distinct pattern. Under low nitrate supply (0.2 mM), plants initially suffered nitrogen stress and resulted in low chlorophyll content at week 4, however, the plants slowly adapted to the stress with slow growth and by week 8 leaves were greener than the early growth stage. Under 1mM nitrate supply higher nitrogen was available to the plants, which showed greener leaves but entered maturity earlier and by 8th week the leaves started turning yellow. These morphological changes point to the developmental and physiological changes in the plants due to nitrogen stress and are shown in Fig. 8.

At 4-week stage, plants supplied with 1.0 mM nitrate showed visibly greener and healthier leaves compared with those grown under 0.2 mM nitrate. By 8 weeks, this pattern had shifted, as plants receiving 0.2 mM nitrate retained greener foliage, while those under 1.0 mM nitrate showed signs of earlier maturity associated with spikelet formation (Fig. 9).

Leaf nitrogen content: Nitrogen content in the transgenic and control plants was assessed using a SPAD meter (Brightway, China), and the results are summarized in Table S10. Across all treatments, the *TaNAC2-5A* transgenic lines showed higher nitrogen levels than the FSD-2008 control. Increase in nitrate availability from 0.2 mM to 1.0 mM led to higher nitrogen content in both control and transgenic plants (Fig. 10).

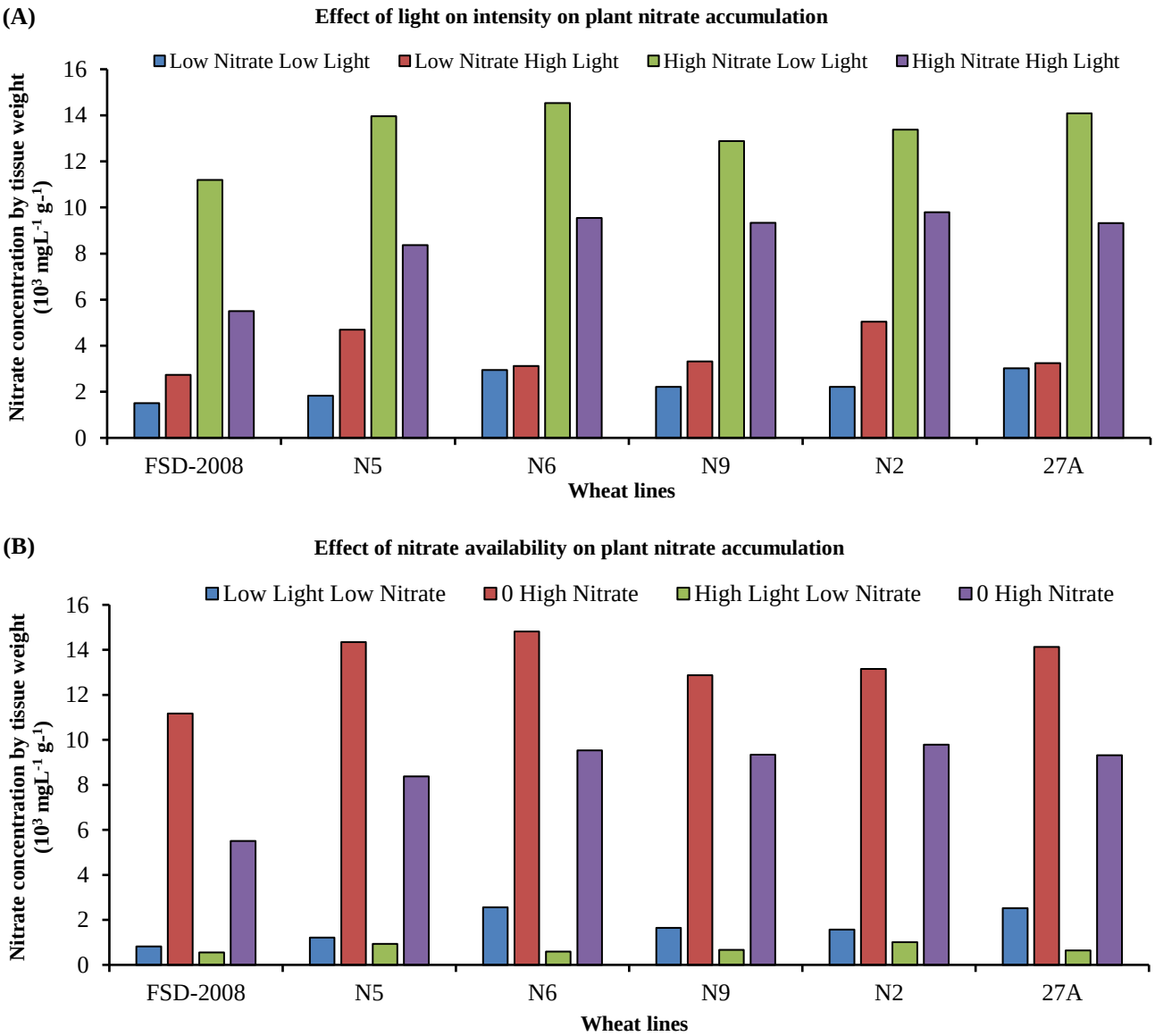

Fig. 6. Effect of light intensity and nitrate availability on nitrate accumulation in leaves. (A) shows the impact of different light intensities, while (B) depicts the effect of altered nitrate supply on leaf nitrate levels

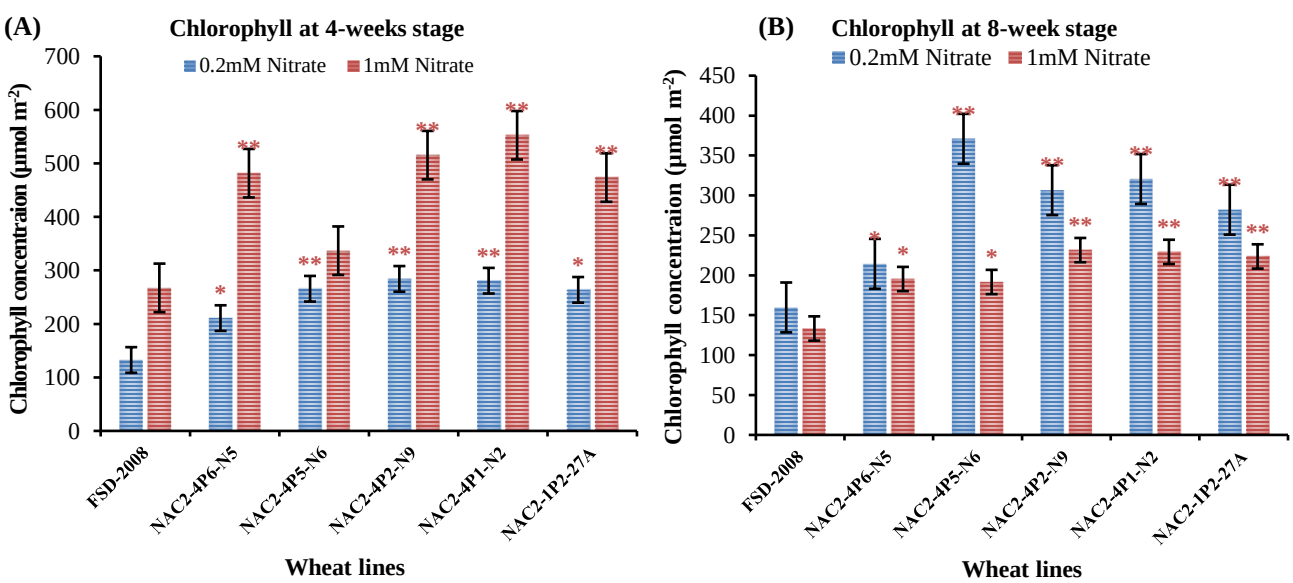

Fig. 7. Chlorophyll content (µmol m^{-2}) measured at 4 and 8 weeks after transfer. Statistical significance is indicated as $p<0.05$ (*), and $p<0.01$ (**).

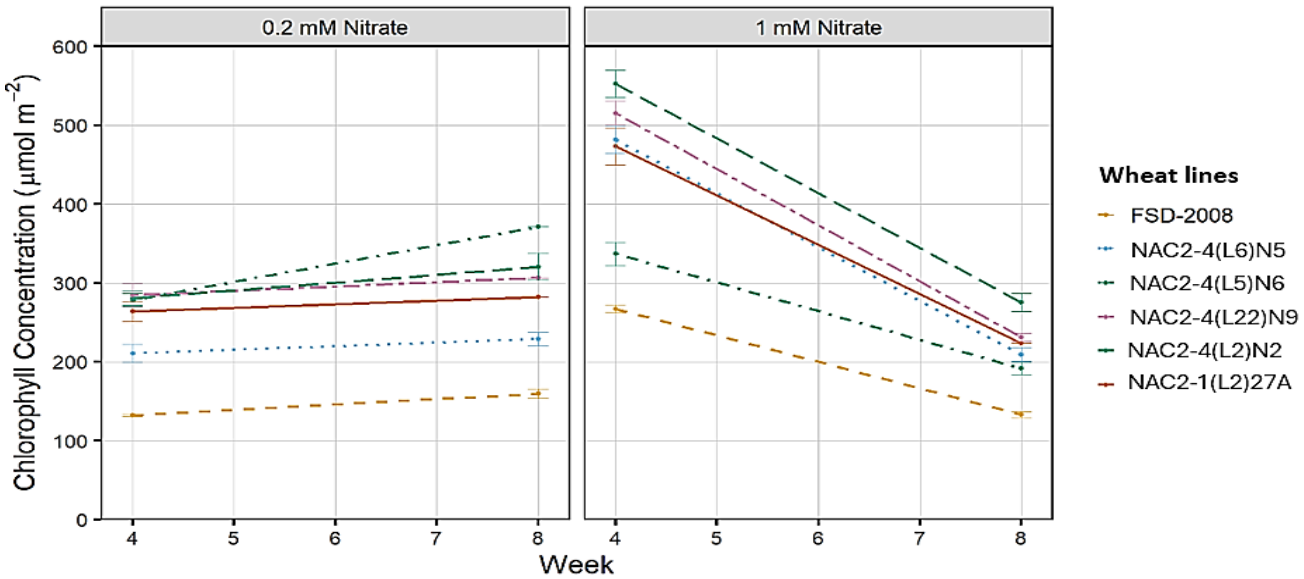

Fig. 8. Changes in chlorophyll content from weeks 4 to 8. Transgenic lines maintained higher chlorophyll levels than the control under both conditions, with a gradual increase under low nitrate and a decline over time under high nitrate. Error bars indicate mean ± standard error.

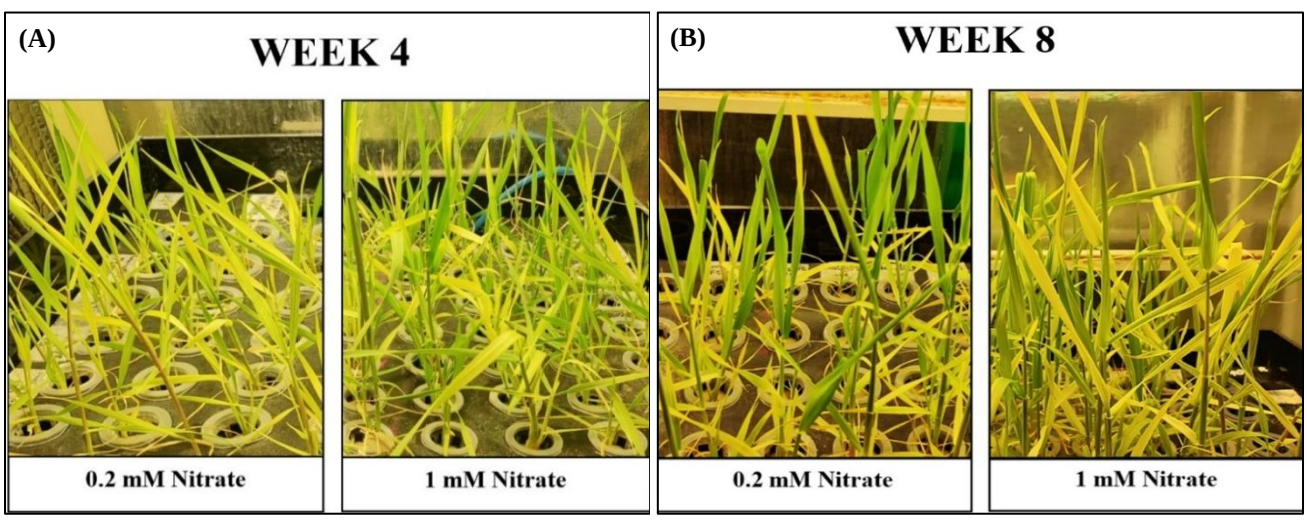

Fig. 9. Visual comparison of plants at 2 stages. (A) represents plants at four-week stage while (B) shows plants at eight-week stage. Plants under 0.2 mM nitrate show early stress but stabilize by week 8, whereas 1.0 mM nitrate plants are greener initially and begin yellowing at week 8 due to early maturity.

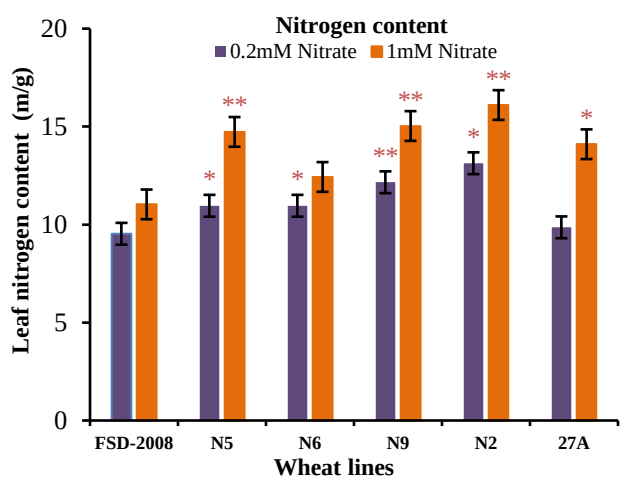

Fig. 10. Total leaf nitrogen. Transgenic lines indicated higher nitrogen content than the parent control FSD-2008 in both nitrate supplies. Asterisks denote p-values, $p<0.05$ (*), and $p<0.01$ (**).

Rhizoscanning: Root architecture of the T_4 *TaNAC2-5A* transgenic lines was analyzed using WinRhizo software. Both control and transgenic plants were scanned at multiple developmental stages to monitor root growth. At 3 weeks, the transgenic lines showed longer roots with more lateral branching, along with higher root volume, average diameter, projected area, and surface area compared with the FSD-2008 control. Increasing nitrate availability further promoted root development in all plants (Fig. 11).

Table S11 summarizes root architecture parameters for both control and *TaNAC2-5A* transgenic lines at 1, 5, and 35-mM nitrate. Root scans of three transgenic lines (NAC2-4(L2) N2, NAC2-4(L5) N6, and NAC2-4(L22) N9 alongside the FSD-2008 control under these nitrate levels are presented in Fig. 12. Across all nitrate conditions, transgenic plants exhibited greater root length, enhanced branching, and improved proliferation. Higher nitrate availability further promoted root development in both control and transgenic plants.

Table S8. Nitrate concentration per gram tissue weight (mg. L^{-1} per gram tissue weight) of control and transgenics.

Transgenic lines	Nitrate concentration (mg/L) per gram tissue weight ($\times 10^3$)			
	0.2 mM Nitrate		1 mM Nitrate	
	Low spectrum light	Full spectrum light	Low spectrum light	Full spectrum light
FSD-2008	1.51	2.73	11.19	5.50
NAC2-4(L6) N5	1.83	4.69	13.96	8.37
NAC2-4(L5) N6	2.95	3.12	14.53	9.54
NAC2-4(L22) N9	2.21	3.32	12.88	9.34
NAC2-4(L2) N2	2.21	5.04	13.38	9.79
NAC2-1(L2) 27A	3.02	3.24	14.08	9.32

Table S9. SPAD readings and chlorophyll content of control and transgenic lines.

Transgenic lines	SPAD Reading	Chlorophyll content (µmol m^{-2})	SPAD Reading	Chlorophyll content (µmol m^{-2})
	4 Weeks stage			
	0.2 mM Nitrate		1 mM Nitrate	
FSD-2008	21.63 ± 0.21	132.55 ± 5.71	30.59 ± 0.54	267.28 ± 9.02
NAC2-4(L6) N5	27.09 ± 1.27	210.80 ± 19.61*	41.92 ± 1.47	481.60 ± 30.53**
NAC2-4(L5) N6	31.26 ± 0.80	265.64 ± 13.74**	34.52 ± 1.40	336.75 ± 25.62
NAC2-4(L22) N9	31.56 ± 1.53	284.09 ± 26.12**	43.53 ± 1.21	515.32 ± 25.60**
NAC2-4(L2) N2	31.37 ± 0.94	280.65 ± 16.25**	45.25 ± 1.36	552.47 ± 29.77**
NAC2-1(L2) 27A	30.36 ± 1.31	263.61 ± 21.84*	41.52 ± 1.99	473.45 ± 40.73**
Transgenic lines	8 Weeks stage			
	0.2 mM Nitrate		1 mM Nitrate	
FSD-2008	23.63 ± 0.76	159.67 ± 10.64	21.68 ± 0.51	133.28 ± 6.61
NAC2-4(L6) N5	28.24 ± 0.89	214.29 ± 14.12*	27.01 ± 0.96	195.24 ± 014.79*
NAC2-4(L5) N6	36.37 ± 1.45	370.92 ± 19.21**	25.83 ± 0.91	191.53 ± 13.60*
NAC2-4(L22) N9	34.84 ± 0.71	306.57 ± 12.97**	28.41 ± 0.58	231.47 ± 9.24**
NAC2-4(L2) N2	33.63 ± 1.63	320.56 ± 29.51**	31.07 ± 1.15	229.24 ± 19.67**
NAC2-1(L2) 27A	34.52 ± 0.75	282.13 ± 13.73**	35.24 ± 0.41	223.52 ± 7.60**

The significance of the chlorophyll content was tested values using Dunnett's test. Asterisks denote p-values. $p<0.05$ (*), and $p<0.01$ (**) indicate that the mean ± SE of three biological replicates of transgenic lines are significant as compared to control FSD-2008

Table S10. Leaf nitrogen content of control and *TaNAC2-5A* transgenics.

Transgenics	Leaf nitrogen content (mg/g)	
	0.2 mM Nitrate	1 mM Nitrate
FSD-2008	9.53 ± 0.49	11.03 ± 1.62
NAC2-4(L6) N5	10.96 ± 0.31*	14.73 ± 0.40**
NAC2-4(L5) N6	10.96 ± 0.35*	12.43 ± 1.22
NAC2-4(L22) N9	12.16 ± 0.81**	15.03 ± 1.36**
NAC2-4(L2) N2	13.13 ± 0.65*	16.10 ± 1.11**
NAC2-1(L2) 27A	9.86 ± 0.64	14.10 ± 0.80*

Each value represents the mean ± standard error (SE) of three biological replicates. Statistical significance between control and transgenics was determined using two-way ANOVA and Tukey HSD test conducted in R. Asterisks denote p-values, $p<0.05$ (*), and $p<0.01$ (**)

Control and *TaNAC2-5A* transgenic plants grown under 0.2 mM and 1.0 mM nitrate for nitrate measurement assays were also monitored for root growth at different developmental stages. Root architecture at eight weeks is shown in Fig. 13. All transgenic lines displayed greater root length and biomass compared with their corresponding control plants.

Biochemical assessment: Variation of nutritional attributes between different transgenic wheat lines and parent control (FSD-2008) is shown in table S12. Biochemical analysis of T_4 seeds revealed clear nutritional advantages of *TaNAC2-5A* transgenic lines over FSD-2008. Some of the significant results are as follows.

Protein content: In terms of protein content, three transgenic lines showed improved protein level from which NAC2-4(L5) N6 and NAC2-4(L22) N9 showed significantly higher protein content of 14.74% and 13.93%, compared with the parent control FSD-2008 (13.10%) (Fig. 14).

Starch content: Starch content remained almost stable across different transgenic lines and control (FSD-2008) except line NAC2-4(L2) N2 which was slightly elevated from FSD-2008 (Fig. 15).

Chapatti score: Most importantly, the product score for Chapatti was improved for most of the *TaNAC2-5A* transgenic lines as compared to the parent control FSD-2008 showing that enhanced nitrogen assimilation improved the end use product quality (Fig. 16).

Agronomic data: All the T_4 generation *TaNAC2-5A* transgenic lines exhibited improved agronomic traits compared with the control cultivar FSD-2008 shown in table 1. Line NAC2-4(L2) N2 produced 47 ± 1 tillers per plant and showed a 12.45% increase in grain weight, whereas NAC2-1(L2) 27A demonstrated enhanced spike length (13.4 ± 0.5 cm), the highest 1000-grain weight (38.95 ± 0.4 g), and the maximum increase in grain weight (16.27%). Line NAC2-4(L5) N6 recorded the highest tiller number (48 ± 2), followed by NAC2-4(L22) N9 and NAC2-4(L6) N5, which also showed significant increases in spike length and grain weight relative to the control. Overall, NAC2-1(L2)27A outperformed other lines in grain weight–related traits, while NAC2-4 lines consistently exhibited superior tillering capacity in the T_4 generation (Table 1).

Table S11. Rhizoscanning data of selected *TaNAC2-5A* transgenic lines.

Transgenic lines	Root length (cm)	Surface area (cm^2)	Avg diameter (mm)	Volume (cm^3)	Projected area (cm^2)
			1 mM Nitrate		
FSD-200	11.17	1.41	0.40	0.01	0.45
NAC2-4(L2) N2	22.19	2.50	0.36	0.02	0.79
NAC2-4(L5) N6	22.10	2.47	0.36	0.02	0.78
NAC2-4(L22) N9	22.03	2.22	0.32	0.02	0.71
			5 mM Nitrate		
FSD-2008	24.77	2.69	0.35	0.02	0.85
NAC2-4(L2) N2	24.55	3.49	0.45	0.04	1.11
NAC2-4(L5) N6	19.05	2.28	0.38	0.02	0.72
NAC2-4(L22) N9	51.89	5.59	0.40	0.06	2.09
			35 mM Nitrate		
FSD-2008	49.61	5.56	0.36	0.05	1.77
NAC2-4(L2) N2	34.41	4.04	0.37	0.04	1.28
NAC2-4(L5) N6	57.09	7.64	0.43	0.08	2.43
NAC2-4(L22) N9	60.38	8.07	0.43	0.09	2.57

Table S12. Biochemical analysis of selected *TaNAC2-5A* transgenic lines.

Sr. No.	Parameters	FSD-2008	NAC2-4(L2) N2	NAC2-4(L5) N6	NAC2-4(L22) N9	NAC2-1(L2) 27A
1.	Protein content (%)	13.1	12.7	14.74	13.93	13.28
2.	Starch content (%)	57.08	57.32	56.13	56.57	56.91
3.	Dry Gluten (%)	7.82	8.32	8.19	8.26	8.48
4.	Wet Gluten (%)	25.29	26.12	26.25	26	27.22
5.	Gluten Index	91	79	93	88	86
6.	Falling Number (sec)	324	321	314	321	305
7.	Ash content (%)	1.91	1.93	1.86	1.9	1.9
8.	Test Weight (kg/hl)	72.92	71.75	70.9	74.5	72.7
9.	Product Score out of 100 (Chapatti)	73	78	76	75	68

Table 1. Agronomic trait of T_4 *TaNAC2-5A* transgenic lines.

Control / Transgenic line	No. of tillers / Spikes per plant	Average spike length (cm)	1000-grain weight (g)	Increase in grain weight (%)
FSD-2008	28 ± 2	11.5 ± 0.3	33 ± 1.0	-
NAC2-1(L2) 27A	45 ± 2 *	13.4 ± 0.5 **	38.95 ± 0.4 **	16.27
NAC2-4(L2) N2	47 ± 1 **	13.0 ± 0.3*	37 ± 0.6 **	12.45
NAC2-4 (L6) N5	43 ± 1 *	13.0 ± 0.5 *	36.7 ± 0.3 *	11.2
NAC2-4 (L5) N6	48 ± 2 **	13.0 ± 0.3 *	37.35 ± 0.4 *	13.18
NAC2-4 (L22) N9	45 ± 1 *	12.8 ± 0.8* *	36.9 ± 0.7 *	11.8

Asterisks signify the mean values ±SE are significant from the wild-type controls at p-value≤ 0.05 (*), and p-value ≤0.01 (**)

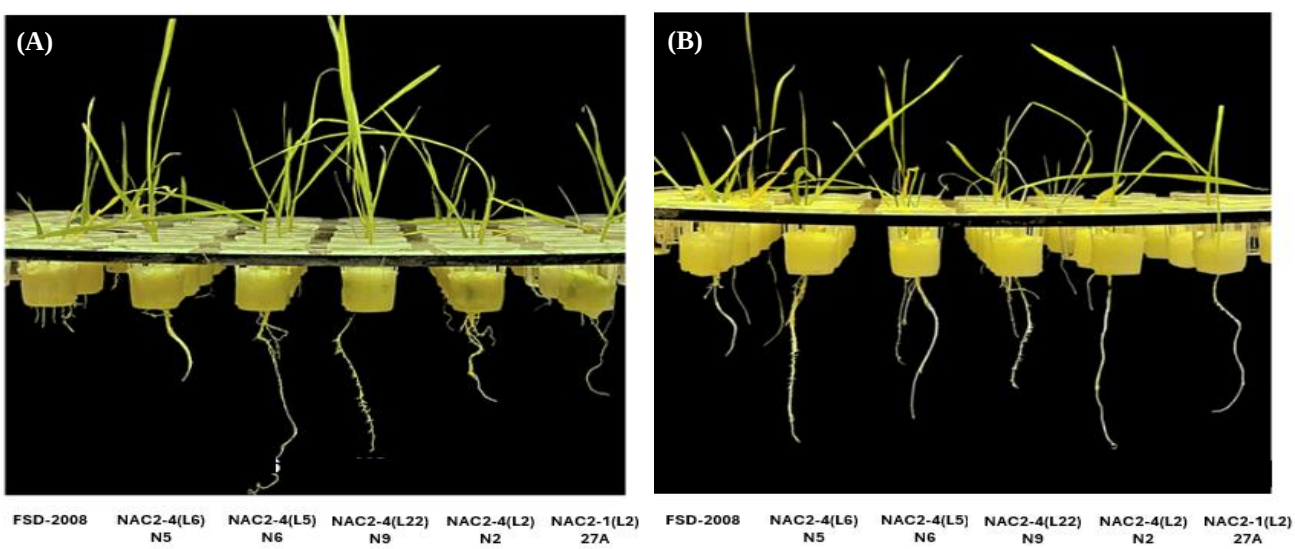

Fig. 11. Visual representation of roots of 3-week-old plants. Root systems of plants grown under (A) 1 mM nitrate and (B) 35 mM nitrate are shown, with transgenic plants displaying longer roots and more extensive branching compared with the control.

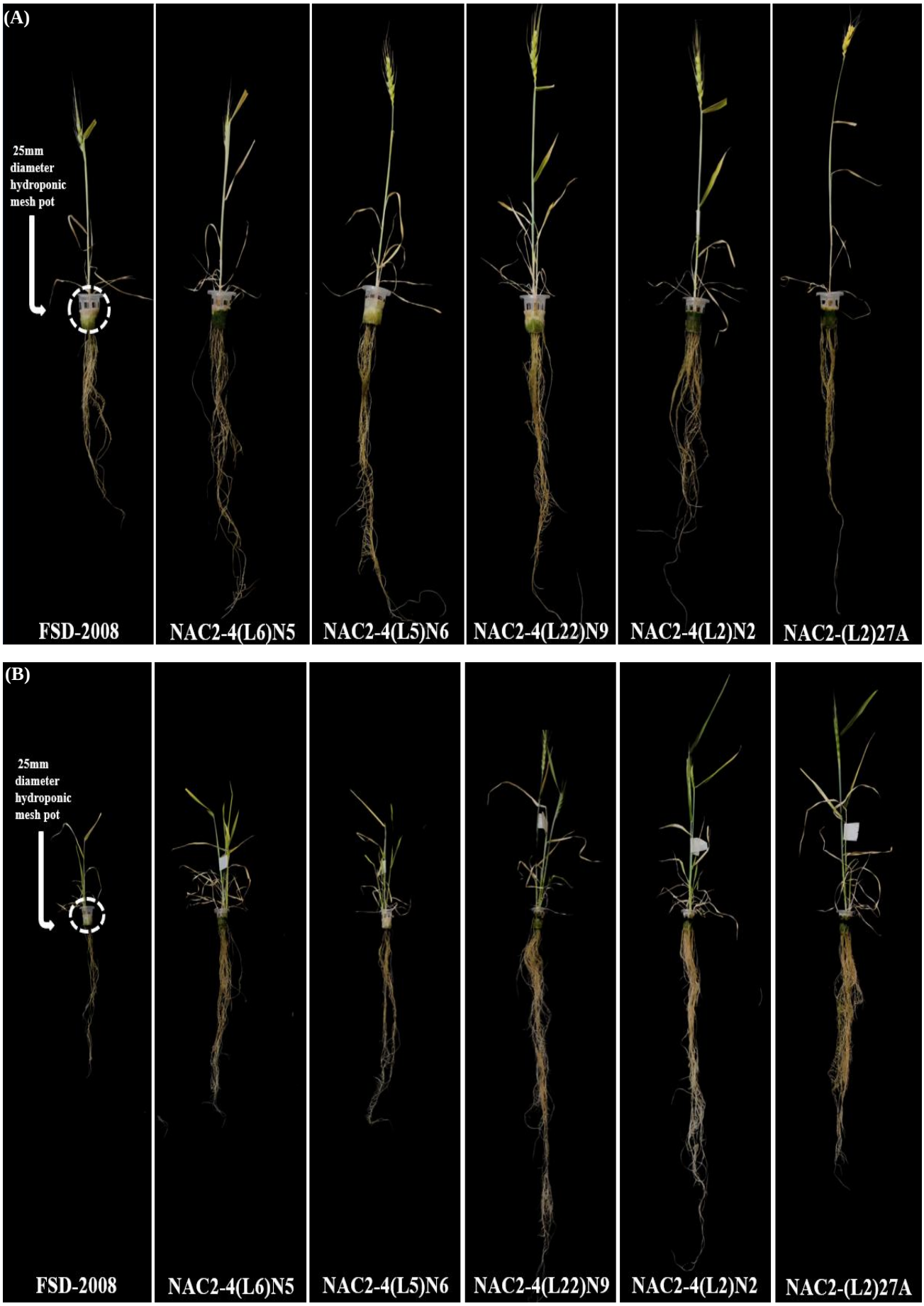

Fig. 13. Root architecture of 8-week-old plants under varying nitrate conditions. (A) represents plant roots at 0.2 mM nitrate availability while (B) shows plants roots at 1 mM of nitrate supply.

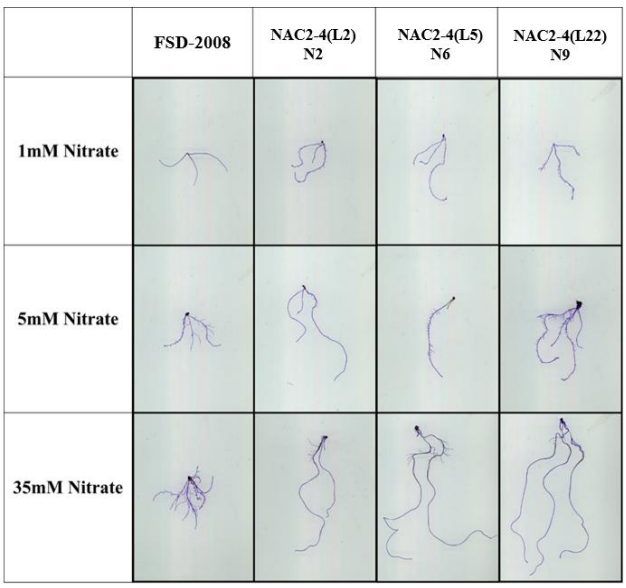

Fig. 12. Root scanning of 3-weeks old plants of control and selected transgenic lines. (A) represents root scans under 1 mM nitrate concentration. (B) represents root scans under 5 mM nitrate. (C) shows root scans under 35 mM nitrate conditions.

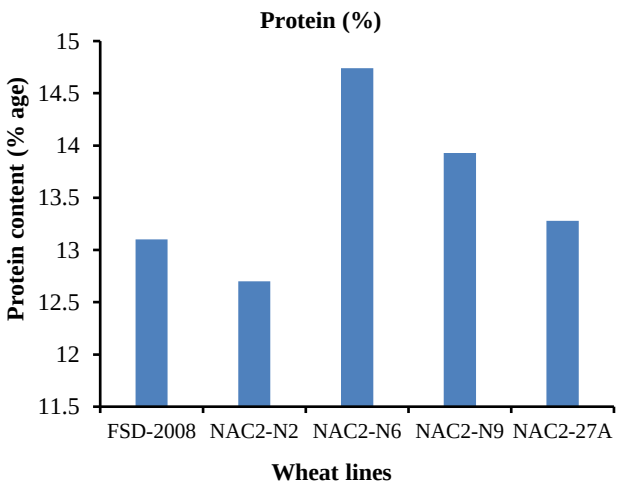

Fig. 14. Protein content of *TaNAC2-5A* transgenic lines along with parent control FSD-2008.

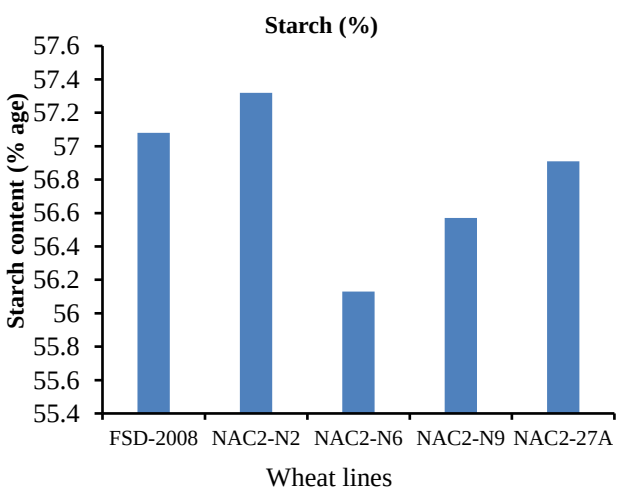

Fig. 15. Starch content in *TaNAC2-5A* transgenic lines and control parent (FSD-2008).

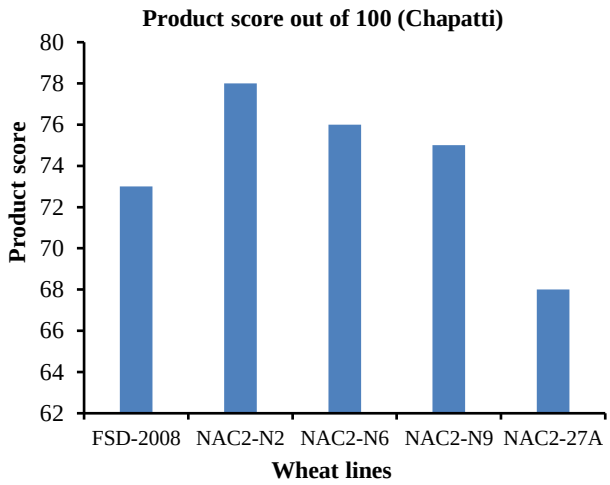

Fig. 16. Product (Chapatti) quality score of control FSD-2008 and *TaNAC2-5A* transgenic lines.

Discussion

Among the cereals, wheat (*Triticum aestivum*) stands at the third place in human consumption. It is a source for one fifth of the caloric values and proteins over the globe (Anon., 2017; Anon., 2023). It is a high nitrogen demanding crop. Nitrogen based fertilizers represent a major investment by the farmer and act as significant contributor to the cost of wheat production. About 50% of the nitrogen fertilizer is lost upon decomposition into nitrous oxide due to slow and inefficient uptake of nitrate by wheat. Therefore, nitrogen uptake efficient wheat varieties can not only decrease the production cost but can also improve the wheat production, thus contributing to food security. The nitrate transporter protein families, NRT1 and NRT2, are responsible for nitrate uptake from the soil in wheat. The transcription factor *TaNAC2-5A* has common motifs in several genes in wheat coding for NRT1 and NRT2 family proteins. Thus, the expression of this transcription factor at a higher level than natural can enhance the uptake of nitrate by the plant from soil (He *et al*., 2015; Li *et al*., 2020). Additionally, these reports claim that this transcription factor can not only improve root development but also increase the chlorophyll level in leaves that can substantially increase the photosynthate and thus the grain yield. This transcription factor was, therefore, expressed constitutively in local wheat variety (FSD-2008), and the results were tested in the soil and contained environment under different light conditions. The study quantified leaf nitrate levels, chlorophyll accumulation, nitrogen assimilation, and root system traits across two nitrate concentrations (0.2 mM and 1.0 mM) and two light environments (low-spectrum light at 2500 K and full-spectrum light at 5500–6500 K). Prior to physiological assessments, integration of the transgene at each generation was verified by PCR analysis, and the expression was confirmed using RT-PCR.

The *TaNAC2-5A* overexpressing lines accumulated higher nitrate levels in the leaves as compared to wild type cultivar (FSD-2008) at both nitrate concentrations. At low (0.2 mM) nitrate level and low spectrum light, the plant leaves contained 35.0 mg/L nitrate. Whereas the NAC2-4(L5) N6 and NAC2-4(L22) N9 transgenic lines had 76.3

mg/L and 54.7 mg/L leaf nitrate content, respectively. The same pattern was detected at 1.0 mM nitrate level and low spectrum light. However, the transgenic lines at both conditions exhibited a higher level of leaf nitrate as compared to controls. The higher nitrate level in transgenic plants may be due to the over production of NRT2.1 and NRT2.5 transporters due to the regulatory effect of *TaNAC2-5A* that increases the nitrate flux from roots to shoots (He *et al*., 2015). The NAC2-4(L5) N6 line accumulated 76.30 mg/L leaf nitrate at 0.2 mM potassium nitrate level, which increased to 368.36 mg/L leaf nitrate at 1.0 mM supply level under low-spectrum light. Higher leaf nitrate concentrations at 1.0 mM potassium nitrate supply when compared with 0.2 mM supply clearly indicated enhanced transcriptional responsiveness of these lines to external nitrate availability. The results concluded that *TaNAC2-5A* enhanced nitrate uptake under increasing nitrogen supply through the upregulation of transporters such as TaNRT2.1-6B and TaNPF7.1-6D as reported by He *et al*., 2015.

Light quality exerted a pronounced effect on nitrate accumulation in leaves. At 0.2 mM nitrate, increasing light color temperature from 2500 K to 5500 K nearly doubled nitrate concentrations in both control and transgenic plants. For instance, nitrate levels in FSD-2008 rose from 1.516×10^3 to 2.737×10^3 mg L^{-1} g^{-1}, while the NAC2-4(L6) N5 line increased from 1.827×10^3 to 4.687×10^3 mg L^{-1} g^{-1}. This response may be explained by the suppression of COP1 activity under full-spectrum light, allowing the accumulation of the HY5 transcription factor (HY5). HY5 translocates from shoots to roots, activating high-affinity nitrate transporter genes such as NRT2.1 in roots (Osterlund *et al*., 2000; Lillo *et al*., 2008; An *et al*., 2017; Li *et al*., 2024). *TaNAC2-5A* overexpressing transgenic lines showed a stronger response (2.5-fold) compared with the control (1.8-fold), reflecting the combined action of HY5 and *TaNAC2-5A* on nitrate transporter gene expression. Conversely, under 1.0 mM nitrate, higher light intensity resulted in decreased leaf nitrate content, likely due to accelerated nitrate reduction and assimilation, as enhanced photosynthesis under high light supplies additional ATP, NADPH, and carbon skeletons required for nitrate reduction by nitrate reductase (NR) and nitrite reductase (NiR) (Lillo *et al*., 2008; Fu *et al*., 2017).

The result of the root architecture analysis revealed that the transgenic lines overexpressing *TaNAC2-5A* showed increased root length when nitrate supply was limited (0.2 mM and 1.0 mM). The transgenic line NAC2-4(L2) N2 showed a 99% increase in root length, a 77% increase in root surface area, and a 57% increase in projected area. The transgenic lines showed reduced root diameter of 10-20%. These lines showed higher root length to biomass ratios indicating a higher nitrate scavenging ability. At moderate nitrate availability (5 mM) the transgenic lines exhibited the same trend as at 0.2 mM and 1 mM nitrate levels. The line NAC2-4(L22) N9 showed more than two-fold increase in root length (52.3 vs. 25.0 cm). Additionally, the root surface area was found to be 102% greater with 65% higher root volume compared to the control plants. Although the differences were less at 35 mM nitrate supply, the transgenic plants still maintained superior root systems. Similarly, line NAC2-4(L22) N9 displayed about 20% longer roots, with roughly 53% more surface area and 70% greater root volume compared to the control. These results support the earlier evidence that low nitrate levels result in expanded root systems have the potential to uptake more nitrate under low nitrate conditions as these roots spread out greater in the soil and can access scattered nitrogen. *TaNAC2-5A* directly activates nitrate transporter and assimilation genes, which raise the plant's internal N status and triggers root foraging responses. The net effect is longer, more highly branched and deeper roots. *TaNAC2-5A* thus sits at a key nexus connecting nitrate signaling to root development. It upregulates target genes that fuel root growth and likely communicates with hormone and root-patterning pathways (auxin, CKX, LBDs). Identifying additional direct targets in wheat (e.g. auxin or cell cycle regulators) will further clarify how *TaNAC2-5A* shapes root length, branching and depth.

The increased capacity for nitrate uptake in transgenic lines was accompanied by elevated nitrogen content and chlorophyll accumulation. Across both nitrate treatments, leaf nitrogen levels were significantly higher in *TaNAC2-5A* lines than in FSD-2008. Under 0.2 mM nitrate, NAC2-4(L2) N2 accumulated 13.13 mg/g leaf nitrogen compared with 9.53 mg/g in control, while at 1.0 mM nitrate, nitrogen reached 16.10 mg/g versus 11.03 mg/g in FSD-2008. Chlorophyll measurements followed a similar pattern: transgenic plants consistently maintained higher chlorophyll levels than the control at both nitrate concentrations and at both four and eight-week growth stages. The elevated chlorophyll content reflects more efficient nitrogen assimilation and enhanced photosynthetic capacity. Higher chlorophyll abundance in transgenic plants indicates a more effective conversion of absorbed nitrate into organic nitrogen compounds (Yang *et al*., 2025). This response is supported by the regulatory function of *TaNAC2-5A*, which activates genes involved in nitrate uptake and assimilation, including *TaNRT2.1*, *TaGS2*, and *TaGOGAT* (He *et al*., 2015; Li *et al*., 2020).

The temporal trends in the accumulation of chlorophyll indicated divergence between nitrate treatment applied. When exposed to 0.2 mM nitrate, the chlorophyll content in the control and transgenic plants increased significantly from week 4 to week 8, most probably because of the slower growth rates and the resultant delayed development and the activation of stay-green mechanisms. For instance, in NAC2-4(L5) N6 line, chlorophyll content rose from 265.6 to 449.8 µmol/m² from 4th to 8th week respectively. Previous studies have reported that adaptive responses to nitrogen limitation, like increased nitrogen use efficiency and delayed senescence, enable continued chlorophyll synthesis, even under limited nitrogen (Wu *et al*., 2019; Wen *et al*., 2019). On the other hand, under 1.0 mM of nitrate supply, plants displayed a rapid growth at four-week stage that was later followed by a decline in the chlorophyll content. In line NAC2-4(L5) N6, the chlorophyll content dropped from 336.8 µmol/m² to 191.5 µmol/m² by the 8th week. As plants advance in development, nitrogen accumulated in chloroplasts is remobilized to support newly developing tissues or reproductive sinks, leading to chlorophyll degradation. This pattern reflects accelerated senescence driven by early nitrogen abundance and increased sink demand, consistent with established models of nitrogen remobilization and leaf aging (Masclaux-Daubresse *et al*., 2008; Bucher *et al*., 2020).

Of all the evaluated transgenic lines in this study, NAC2-4(L2) N2 and NAC2-4(L22) N9 showed the most consistent and significant improvements. These lines showed the highest leaf nitrogen, chlorophyll contents and developed the longest roots at different nitrate concentrations. NAC2-4(L5) N6 showed particularly high chlorophyll content at 0.2 mM nitrate and at low-spectrum light conditions accumulated large pools of nitrate. Intermediate performance was recorded for NAC2-4(L6) N5, while NAC2-1(L2)27A behaved as similar to wild type. This variation among transgenic lines is most likely due to the differences in transgene insertion sites in the chromosomal DNA or the levels of expression. However, each transgenic line outperformed the control for one or more evaluated parameters, underscoring the functional impact of *TaNAC2-5A* overexpression. Under a nitrate supply of 1.0 mM, the *TaNAC2-5A* overexpressing lines exhibited a 30–70% elevation in leaf nitrate content, a two-to-four-fold increase in chlorophyll levels, and nearly two-fold greater root length and root surface area relative to the control. A key strength of this work is the deliberate use of two low nitrate concentrations (0.2 and 1.0 mM), rather than higher or near-optimal nitrogen levels. Under standard Hoagland conditions, plants are typically supplied with ~35 mM nitrate, where physiological responses often reach saturation and subtle regulatory effects of transcription factors may remain masked. In contrast, under low nitrate availability, even modest improvements in uptake, assimilation, or root foraging capacity can result in pronounced and measurable phenotypic differences. By maintaining nitrate at suboptimal levels, these responses highlight the impact of *TaNAC2-5A* to enhance nitrate uptake and assimilation, thus strengthening root exploration capacity.

Conclusion

This study provides strong evidence that overexpression of *TaNAC2-5A* significantly improves nitrogen use efficiency and root system architecture in wheat while lowering fertilizer requirements. The transgenic lines developed deeper and more highly branched root systems, which are particularly advantageous under nitrate-limited conditions. Reduced reliance on nitrogen fertilizers can decrease production costs, limit nitrate leaching, and support sustainable yield maintenance. Overall, *TaNAC2-5A* transgenic wheat lines represent a promising strategy for enhancing wheat productivity and quality while advancing environmentally sustainable agricultural practices.

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