AATCC Review

12 July 2025: Received
03 September 2025: Revised
10 September 2025: Accepted
08 October 2025: Available Online

https://aatcc.peerjournals.net/

Original Research Article

Open Access

Dynamic regulation of an *NRT1 isoform* under variable nitrogen, heat stress, elevated CO_2, and their combination

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ABSTRACT

Wheat is highly sensitive to heat stress, and even slight temperature fluctuations during critical growth stages can severely affect its growth, development, and yield. This sensitivity is further influenced by nitrogen availability and atmospheric CO_2 levels. The dynamic regulation of nitrate transporters in wheat under variable nitrogen, heat stress (HS), elevated CO_2 (eCO_2), and their combination plays a crucial role in modulating nitrate transport and plant adaptation, impacting crop productivity under changing environments. In this experiment, four Indian wheat cultivars (RAJ3765, PBW343, BT-Schomburgk, and Dharwad Dry) were subjected to heat stress (42 ± 2 °C), elevated CO_2 (650 ± 30 ppm), and their combination (HS + eCO_2) under three nitrogen regimes: nitrogen-deficient, nitrogen-optimum, and nitrogen-surplus. A putative NRT1 isoform (2476 bp) was cloned from the Dharwad Dry cultivar, sequenced, and submitted to GenBank (Accession no: OQ184870). In silico analysis revealed 12 transmembrane helices and a Major Facilitator Superfamily conserved domain, with an ORF of 1706 bp spanning from 338 to 2044 bp. Phosphorylation sites were enriched for threonine residues, suggesting a regulatory role in nitrate transport affinity. The cloned gene showed the highest homology to the NRT1/PTR FAMILY 6.4 gene of Aegilops tauschii subsp. strangulata. Maximum expression (3-fold upregulation) was observed in Dharwad Dry under elevated CO_2 at the pollination stage in root tissue under N-deficient conditions, while minimum expression was observed under heat stress in PBW343 under N-surplus conditions. Under heat stress conditions, RAJ3765 maintained better NRT1isofrom expression level compared to other cvs. These findings highlight the role of NRT1isoform in nitrogen use efficiency and stress adaptation, providing molecular targets for developing wheat cultivars better suited to future climate scenarios.

Keywords: *NRT1isoform, elevated CO_2, Heat stress, Wheat, Nitrate transporters, Nitrogen.*

1.0. Introduction

Wheat (*Triticum aestivum* L.), a major cereal crop of the Poaceae family, contributes approximately 30% to global food production[1]. Often referred to as the "king of cereals," it serves as a staple food for nearly one-third of the world's population. As the most widely cultivated cereal grain, wheat covers about 17% of the total global agricultural land. It is a primary dietary staple for 35% of the global population, providing a greater share of calories and protein than any other crop[2].

Wheat is continuously exposed to diverse biotic and abiotic stresses that adversely affect its growth and productivity. Among abiotic factors, heat stress (HS) is particularly detrimental, severely impacting physiological and developmental processes and ultimately reducing grain yield [3]. The rising global temperature due to climate change poses a serious threat to agricultural productivity [4]. Terminal heat stress, defined as temperatures exceeding 32°C during the reproductive and grain-filling stages, significantly disrupts wheat growth, leading to pollen sterility, stigma desiccation, reduced fertilisation, malformed seeds, impaired starch synthesis, nutrient imbalance, and enzyme degradation [5].

Atmospheric CO_2 concentration is another major factor influencing crop nitrogen use efficiency (NUE). Since the Industrial Revolution, CO_2 levels have increased by about 45%, from 280 ppm to nearly 410 ppm, with projections suggesting levels of 650–1200 ppm in the coming decades[6]. Elevated CO_2 (eCO_2) increases photosynthetic carbon fixation by 19–46%, enhancing biomass and yield and improving water-use efficiency [7]. However, these benefits are offset by reduced nitrogen content in plant tissues. This decline is attributed to the dilution effect from increased carbon accumulation, reduced nitrogen availability, lower transpiration-driven mass flow, and competition for reductants between nitrate assimilation and photosynthesis [8]. Consequently, wheat grown under eCO_2 often exhibits reduced grain protein content.

Nitrogen (N) is essential for plant growth, forming the core of nucleotides, amino acids, and chlorophyll. In agricultural soils, nitrate (NO_3^-) is the dominant form of available nitrogen, and its uptake and transport are mediated by specialised nitrate transporters[9]. Nitrogen limitation is a key constraint for plant productivity under eCO_2 conditions [10]. There are four major families of nitrate transporters: NPF (NRT1/PTR), NRT2, CLC, and SLAC/SLAH[11].

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DOI: https://doi.org/10.21276/AATCCReview.2025.13.04.721

The NPF family is the largest, comprising eight subfamilies with diverse physiological functions. Key examples include AtNPF4.4/NRT1.13, which regulates branching and flowering; AtNPF4.6, which modulates stomatal opening via ABA transport; and AtNPF6.3/NRT1.1, which influences auxin transport and root architecture[12].

Despite significant progress in understanding wheat responses to eCO_2 and heat stress, the combined effects of these factors on nitrate transporter expression remain poorly understood. Here, we identified a putative nitrate *NRT1 isoform* gene and investigated its expression pattern under differential nitrogen, HS, eCO_2, and (HS + eCO_2) plants at both pollination and grain filling stages in leaf and root tissue.

2.0. Materials and Methods

2.1 Plant materials and stress treatments

Four contrasting wheat cultivars—RAJ3765 (heat-tolerant), PBW343 (heat-sensitive), BT-Schomburgk, and Dharwad Dry (nitrogen-responsive)—were obtained from the Division of Genetics, ICAR–Indian Agricultural Research Institute (IARI), New Delhi. Prior to sowing, seeds were surface-sterilised using 0.25% Bavistin and planted in 144 pots (16 × 16 × 13 cm), each filled with a uniform mixture of perlite, sand, and farmyard manure (FYM). All treatments were maintained in triplicate. Regular manual irrigation was provided, and seedlings were thinned to five plants per pot.

The experiment was arranged with four genotypes exposed to different nitrogen (N) regimes. The N-optimum condition corresponded to 120 kg N ha^{-1}, applied as 0.652 g N per pot in two splits: an initial application of 0.217 g urea per pot, followed one month later (vegetative phase) by 0.435 g urea per pot. Nitrogen-deficient soil was procured from the National Phenomics Facility, ICAR-IARI, New Delhi. For the N-surplus treatment, a 25% higher dose (150 kg N ha^{-1}) was supplied, equivalent to 0.815 g urea per pot provided in two split applications.

Plants were grown in controlled-environment chambers set at 22 ± 2°C during the day and 18 ± 2°C at night, with 80% relative humidity, a photosynthetically active radiation (PAR) level of 250 µmol m^{-2} s^{-1}, an 8-h photoperiod, and an ambient CO_2 concentration of 400 ppm until anthesis at the Nandaji Deshmukh Plant Phenomics Centre, IARI. After reaching the post-anthesis stage (Feekes 11.1), stress treatments were imposed. Heat stress (HS) was applied by raising the chamber temperature to 42 ± 2°C for 2 h, with the temperature increment programmed at 1°C every 10 s in a sinusoidal pattern. Elevated CO_2 (eCO_2) conditions were maintained at 650 ± 30 ppm, and combined stress involved simultaneous exposure to eCO_2 and HS in a specialised growth chamber.

Samples were collected from control and treated plants in triplicate, rapidly frozen in liquid nitrogen, and stored at –80°C until further biochemical and molecular analyses.

2.2 Cloning of NRT1isofrom gene

Total RNA was extracted from leaf tissue of the wheat cultivar RAJ3765 using TRIzol reagent (Invitrogen, UK). First-strand cDNA was synthesised from 1 µg of total RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Gene-specific primers were designed to amplify the putative *NRT1isofrom* (Transcript ID 5003) coding region. Reverse transcription PCR (RT-PCR) was performed to amplify the target gene, and the resulting product was visualised on an agarose gel to confirm the expected amplicon size.

Purified PCR fragments were ligated into the pGEM-Easy vector (Promega) following the manufacturer's protocol. Recombinant plasmids were isolated using the alkaline-lysis method and screened through restriction enzyme digestion to verify successful insertion of the target genes. Confirmed plasmid clones were sequenced using universal T7 and SP6 primers to validate the integrity and identity of the cloned *NRT1isofrom.*

2.3 In-*silico* characterisation of the cloned putative *NRT-1* isoform gene

Nucleotide sequences were initially subjected to homology searches using the BLASTn tool ;https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the NCBI database. The physicochemical properties of proteins were analysed using the ProtParam tool ;https://web.expasy.org/protparam/). The open reading frame (ORF) was identified using ORF finder : https://www.ncbi.nlm.nih.gov/orffinder/). The '; https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) Used to identify conserved domains within in gene. Further analysis was performed to predict subcellular localisation using PSORT software ; https://www.genscript.com/psort.html). The phosphorylation sites of these proteins were predicted using NetPhos3.1 :https://services.healthtech.dtu.dk/services/NetPhos-3.1/). Phylogenetic analysis was done to know the evolutionary relationship of the cloned genes using MEGA 11.0 ; https://www.megasoftware.net/). Finally, protein modelling was performed using Phyre2.2 software ; http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

2.4. Expression analysis of *NRT-1* isoform gene

Total RNA was isolated from fully expanded wheat leaves using the TRIzol™ reagent (Thermo Scientific, USA) following the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop™ spectrophotometer (Thermo Scientific), and RNA integrity was verified on a 1.2% denaturing agarose gel. For cDNA synthesis, 1 µg of high-quality RNA was reverse-transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) with oligo(dT) primers. The resulting cDNA was diluted to a working concentration of 100 ng/µL for downstream quantitative PCR analysis. Gene-specific primers were designed using Primer3 software (Premier Biosoft, USA), and the sequences are provided in Supplementary Table 1. Each RT-qPCR reaction (20 µL) contained SYBR Green ER SuperMix Universal (Invitrogen, UK), gene-specific primers, and 1 µL of diluted cDNA. Reactions were run on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) using the following program: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 15 s. Relative transcript abundance was calculated using the 2^-ΔΔCt method [27], with β-actin (GenBank accession AF282624) serving as the internal reference gene.

2.5. Statistical analysis

All data were subjected to a three-way ANOVA to determine the effect of nitrogen doses, stress treatment, genotype, and their interactions. All data analyses and plotting were performed using suitable packages available in the R software [21]. The effects of the treatments and their interactions were analysed using a linear ANOVA. The significance levels for ANOVA were $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

3.0. Results and discussion

3.1. Cloning and *in-silico* analysis of putative *NRT1 isoform* gene

NRT transcript was amplified from wheat *cv.* Dharwad dry yielded an amplicon of ~2476 bp, which was cloned, sequenced, and submitted to NCBI GenBank (accession no. Oq184870). ORF analysis revealed 14 ORFs, with ORF2 (spanning 338–2044 nucleotides and 1706 bp) being identified as a key coding sequence for a nitrate transporter-related protein (Fig.1). *NRT1isoform* encodes a 568-amino acid polypeptide with an approximate molecular weight of 61 kDa and a theoretical isoelectric point (pI) of 8.42, classifying it as slightly basic. Amino acid composition analysis showed a high abundance of leucine, valine, glycine and cysteine, which contribute to structural stability, whereas threonine and histidine were the least abundant. The total number of negatively charged residues was 48, and positively charged residues totalled 59, aligned with the alkaline pI. Its atomic formula, which contains 24 sulfur atoms, suggests its significant role in protein folding and disulfide bond formation. The instability index was computed to be 25.47, which classifies the protein as stable, with high thermostability suggested by the aliphatic index and a slightly hydrophilic nature, as indicated by the GRAVY score. A conserved domain search placed the protein within the Major Facilitator Superfamily (MFS) (Fig.2). The predicted phosphorylation sites were enriched for threonine residues, suggesting a post-translational regulation (Fig.3). Phylogenetic analysis revealed the highest homology to *Aegilops tauschii subsp. strangulata* NRT1/ PTR FAMILY 6.4 gene (Fig.4). It contains 12 transmembrane helices, which were organised into two distinct six-helix bundles forming the transport channel across the plasma membrane supporting its role in nitrate transport.

3.2. Spatio-temporal expression analysis of the *NRT1* isoform in leaf and root tissues under eCO_2, HS and (HS+ eCO_2)

Spatio-temporal expression profiling of the *NRT1 isoform* in wheat leaf and root tissues showed significant variation ($p \leq 0.05$) among the four cultivars under different nitrogen levels (N-deficient, N-optimum, and N-surplus) and treatments (eCO_2, HS, and (HS + eCO_2)) at both pollination and grain filling stages (Fig. 4 and Fig.5).

We observed increased *NRT1isoform* transcript levels at the pollination stage compared to grain filling were seen across all cultivars, indicating the early reproductive phase's heightened nitrate demand. Similar results were reported by [22], who found higher nitrate transporter expression during wheat spike development, aligning with critical periods of sink formation.

Heat stress leads to pronounced downregulation of *NRT1 isoform* in both leaf and root tissues at both developmental stages across cultivars. For instance, PBW343 exhibited a -1.98-fold decrease under N-deficient conditions at pollination. Similar results were reported by[23], who attributed this to impaired membrane function and signalling, reducing nitrate transporter synthesis and activity. The decrease might be due to heat-induced proteolytic processes, membrane instability, and disruption of transcriptional regulators as reported by[24], who found broad repression of assimilation-related genes in wheat and Arabidopsis exposed to high temperature.

Under elevated CO_2 conditions, all cultivars showed upregulation of NRT1 *isoform*; the upregulation was more at the pollination stage compared to the grain filling stage. i.e., Dharwad dry cv showed 3-fold upregulation of *NRT1* isoform in root tissue under eCO_2 in N-deficient conditions. This finding is consistent with that of –[25], who reported enhanced *nitrate transporter-2* expression under eCO_2 in tobacco. Such induction may be due to increased carbon availability stimulating greater root growth and nitrate assimilation, and enhanced C-N signalling.

Under combined (eCO_2 + HS) conditions, the downregulation of *NRT1 isoform* was lower than heat stress alone, indicating that eCO_2 can partially mitigate the adverse effects of high temperature. eCO_2 buffered stress-induced declines in nitrogen uptake and transporter expression in wheat by sustaining root metabolic activity[26].

The expression level of *NRT1 isoform* varied among the four wheat *cvs*. i.e., Raj3765 maintained higher expression, whereas PBW343 maintained lower expression under heat and combined stress conditions. Similar genotype-dependent responses have been reported by. Highlighting the significance of genetic variability in stress adaptation and nitrogen use efficiency. These differences in expression provide an opportunity for breeding programs to enhance stress tolerance by targeting the nitrate transporter in a particular genotype.

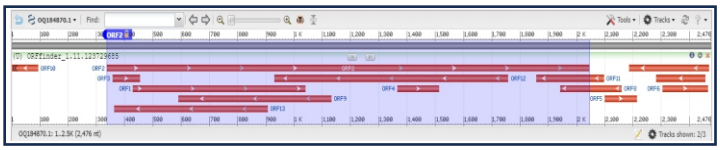

Fig. 1. Predicted of open reading frame of putative *NRT1isoform* gene cloned from wheat cultivar Dharwad dry using ORF Finder tool of NCBI.

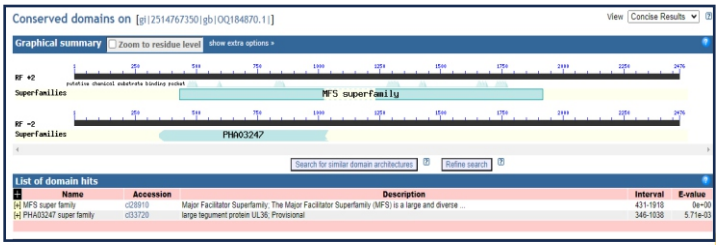

Fig. 2. Predicted of conserved domain of the putative *NRT1 isoform* gene cloned from wheat cultivar Dharwad dry using Conserved Domain Search tool of NCBI.

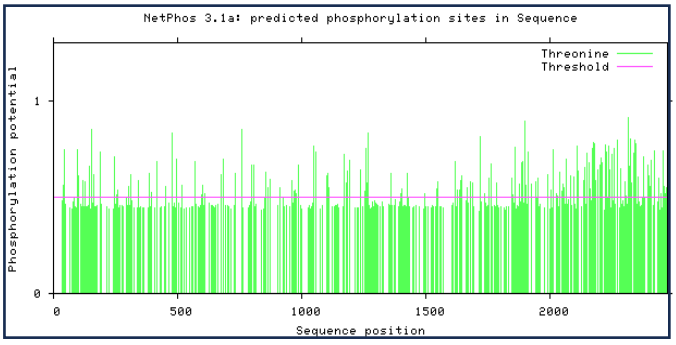

Fig. 3. Predicted the phosphorylation site on the putative *NRT1isoform* gene cloned from wheat cultivar Dharwad dry using NetPhos 3.1.

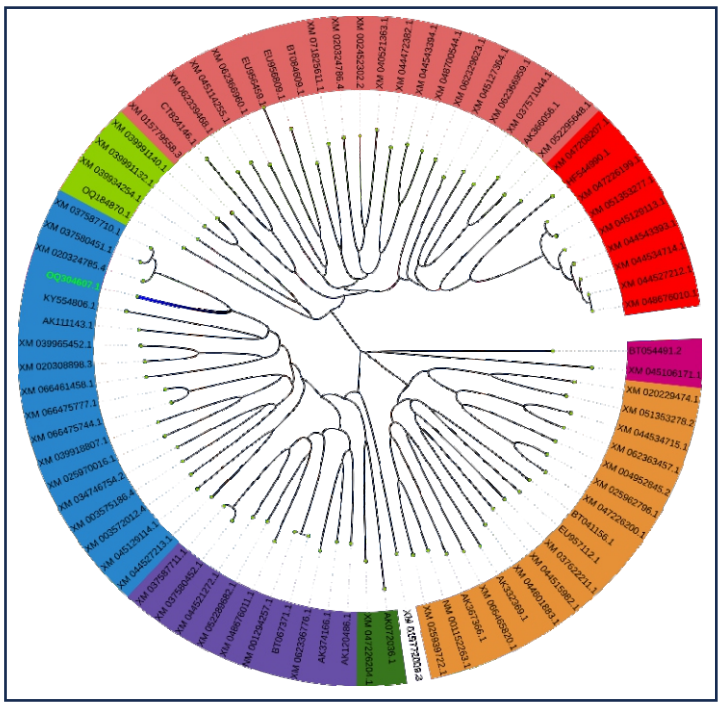

Fig. 4. Phylogenetic tree illustrating the evolutionary relationships between identified nitrate transporters in wheat and other plant species. The tree was constructed using the maximum likelihood method with 1000 bootstrap replicates, implemented in MEGA11 software.

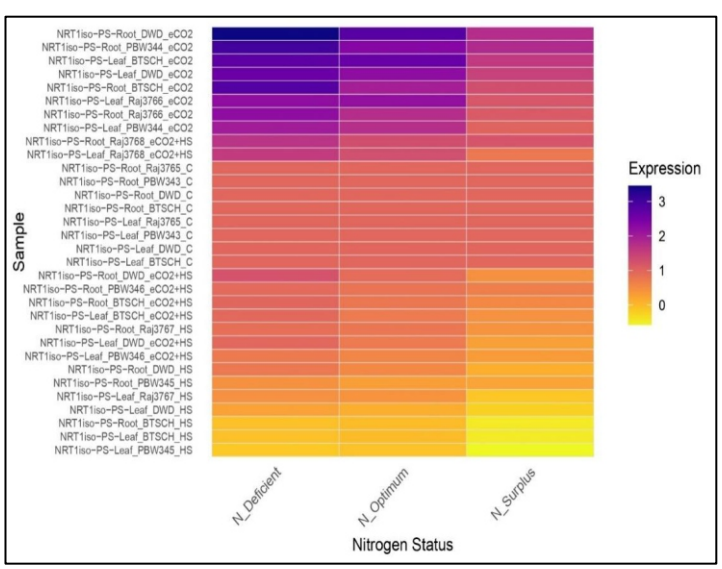

Fig. 5. Heat map depicting the expression pattern of *Nitrate Transporter 1 isoform (NRT1)* gene of four cultivars PBW343, RAJ3765, DWD, and BTSCH of wheat under differential doses of nitrogen (N-Deficient, N-Optimum, N-Surplus) and treatment of eCO2, Heat Stress (HS) and combined stress (eCO2 + HS) at the pollination stage in leaf and root tissue. Vertical bar represents the fold change in expression of the gene with yellow colour (extreme downregulation) and dark blue (extreme upregulation)

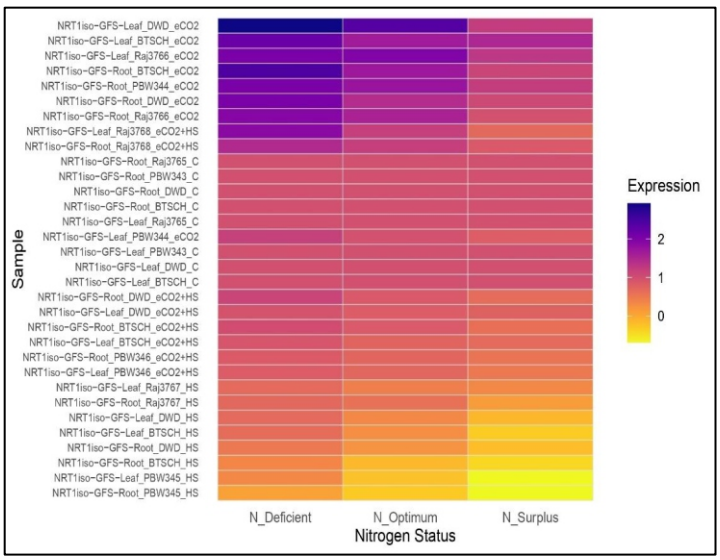

Fig. 6. Heat map depicting the expression pattern of *Nitrate Transporter 1 isoform (NRT1)* gene of four cultivars PBW343, RAJ3765, DWD, and BTSCH of wheat under differential doses of nitrogen (N-Deficient, N-Optimum, N-Surplus) and treatment of eCO2, Heat Stress (HS) and combined stress (eCO2 + HS) at the Grain filling stage in leaf and root tissue. Vertical bar represents the fold change in expression of the gene with yellow colour (extreme downregulation) and dark blue (extreme upregulation).

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