

Functional characterization of the *GhNRT2.1e* gene reveals its significant role in improving nitrogen use efficiency in *Gossypium hirsutum*

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ABSTRACT

Background: Nitrate is the primary type of nitrogen available to plants, which is absorbed and transported by nitrate transporter 2 (NRT2) at low nitrate conditions.

Methods: Genome-wide identification of *NRT2* genes in *G. hirsutum* was performed. Gene expression patterns were revealed using RNA-seq and qRT-PCR. Gene functions were characterized using overexpression in *A. thaliana* and silencing in *G. hirsutum*. Protein interactions were verified by yeast two-hybrid and luciferase complementation imaging (LCI) assays.

Results: We identified 14, 14, seven, and seven *NRT2* proteins in *G. hirsutum*, *G. barbadense*, *G. raimondii*, and *G. arboreum*. Most *NRT2* proteins were predicted in the plasma membrane. The *NRT2* genes were classified into four distinct groups through evolutionary relationships, with members of the same group similar in conserved motifs and gene structure. The promoter regions of *NRT2* genes included many elements related to growth regulation, phytohormones, and abiotic stresses. Tissue expression pattern results revealed that most *GhNRT2* genes were specifically expressed in roots. Under low nitrate conditions, *GhNRT2* genes exhibited different expression levels, with *GhNRT2.1e* being the most up-regulated. *Arabidopsis* plants overexpressing *GhNRT2.1e* exhibited increased biomass, nitrogen and nitrate accumulation, nitrogen uptake and utilization efficiency, nitrogen-metabolizing enzyme activity, and amino acid content under low nitrate conditions. In addition, *GhNRT2.1e*-silenced plants exhibited suppressed nitrate uptake and accumulation, hampered plant growth, affected nitrogen metabolism processes, and reduced tolerance to low nitrate. The results showed that *GhNRT2.1e* could promote nitrate uptake and transport under low nitrate conditions, thus effectively increasing nitrogen use efficiency (NUE). We found that *GhNRT2.1e* interacts with *GhNAR2.1* by yeast two-hybrid and LCI assays.

Discussion: Our research lays the foundation to increase NUE and cultivate new cotton varieties with efficient nitrogen use.

Submitted 7 December 2022

Accepted 10 March 2023

Published 28 March 2023

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Academic editor

Sushil Kumar

Additional Information and
Declarations can be found on
page 24

DOI 10.7717/peerj.15152

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OPEN ACCESS

Subjects Agricultural Science, Bioinformatics, Genomics, Molecular Biology, Plant Science

Keywords NRT2, NUE, Gene family, Expression pattern, High-affinity nitrate transporter, *Gossypium hirsutum*

INTRODUCTION

As an essential nutrient element, nitrogen (N) participates in the composition of nucleic acids, amino acids, chlorophyll, and many secondary metabolites (Akbulak, Filiz & Çetin, 2022). In agricultural production, nitrogen fertilizers are often over-applied to increase yields. However, less nitrogen fertilizer is absorbed by plants, and the rest is retained in soil or leaches into groundwater, causing increase in production costs, waste resources, and environmental problems (Zhou et al., 2022). Therefore, preventing the over-application of nitrogen fertilizer and increasing nitrogen use efficiency (NUE) is significant in protecting the environment and enhancing yield.

There are three forms of nitrogen available to plants including nitrate (NO_3^-), ammonium (NH_4^+), and organic nitrogen, among which NO_3^- is the most important form absorbed and utilized by plants (Ren, Zhou & Zhou, 2020). Nitrate absorption and transportation are primarily based on nitrate transporters (NRTs) (You et al., 2022). To accommodate the different nitrate concentrations in soil, plants have evolved low-affinity transport system (LATS) and high-affinity transport system (HATS) (Li et al., 2007; Lérán et al., 2014). LATS mainly consists of nitrate transporter 1/peptide transporter (NRT1/PTR), while nitrate transporter 2 (NRT2) is part of HATS (Dechorgnat et al., 2011).

NRT2 genes are essential for improving NUE through efficient uptake and transport of nitrate. NRT2 genes have been investigated in *A. thaliana* (Orsel, Krapp & Daniel-Vedele, 2002), rice (Cai et al., 2008), barley (Trueman, Richardson & Forde, 1996), soybean (Amarasinghe et al., 1998), and maize (Lupini et al., 2016). In *Arabidopsis*, seven NRT2 members (AtNRT2.1-AtNRT2.7) were identified. AtNRT2.1 is primarily expressed in roots and significantly affects nitrate uptake (Wirth et al., 2007). Interestingly, elevated expression of AtNRT2.2 compensated for partial loss of nitrate uptake when AtNRT2.1 lost its ability to absorb nitrate (Li et al., 2007). AtNRT2.4 is primarily concerned with nitrate absorption in roots and transportation in the phloem (Kiba et al., 2012). AtNRT2.5 is expressed in leaves and roots, and its expression levels increase with the increase in nitrogen starvation (Lezhneva et al., 2014). AtNRT2.7 regulates nitrate storage in seeds (Chopin et al., 2007). Most NRT2 proteins depend on nitrate assimilation related (NAR2) proteins, and in *Arabidopsis*, most AtNRT2 proteins require binding to AtNAR2.1, whereas AtNRT2.7 can be transported alone (Kotur et al., 2012). A similar situation was demonstrated in rice, where OsNRT2.1, OsNRT2.2, and OsNRT2.3a are required to interact with OsNAR2.1, whereas OsNRT2.3b and OsNRT2.4 can absorb and transport nitrate alone (Wei et al., 2018).

As a cash crop, the growth and yield of cotton are regulated by nitrogen. Therefore, we can effectively improve cotton's nitrogen uptake and utilization efficiency by mining nitrogen-efficient genes through molecular techniques (Magwanga et al., 2019). These essential genes will help us to maintain cotton yield while reducing nitrogen fertilizer input. In this research, we identified *GhNRT2* genes and determined their evolutionary relationships, physicochemical properties, chromosomal location, gene duplication,

collinearity relationship, conserved motifs, gene structure and cis-acting elements. After low nitrogen treatment, we observed the expression pattern of *GhNRT2* genes and found that *GhNRT2.1e* was the most up-regulated gene. We characterized the *GhNRT2.1e* functions by overexpression in *A. thaliana* and silencing in *G. hirsutum*. Furthermore, we validated the interaction between *GhNRT2.1e* and *GhNAR2.1* by yeast two-hybrid and LCI assays. This study lays a foundation for increasing NUE and breeding new cotton cultivars with high nitrogen using efficiency.

MATERIALS AND METHODS

Plant materials and treatments

Cotton material used in the experiment was *G. hirsutum* L. TM-1. The seeds were germinated on moist filter paper until two cotyledons appeared. The seedlings continued to grow in the hydroponic conditions. Growing conditions were 16 h light at 28 °C and 8 h dark at 25 °C with 60% relative humidity. We changed the Hoagland nutrient solution every 5 days. We switched to the nitrogen-free nutrient solution at the three-leaf stage and continued for 1 week. The NO₃⁻ concentration in Hoagland's nutrient solution was then adjusted to 0.25 mM for low nitrogen treatment. Root tissues were collected at 0, 1, 3, 6, 12, 24, and 48 h after treatment and used for RNA extraction. The *Arabidopsis* ecotypes Columbia-0 and *Nicotiana benthamiana* were grown in nutrient soil and vermiculite under light conditions for 16 h at 22 °C and 8 h of dark conditions at 18 °C with 60% relative humidity.

Identification of *NRT2* genes in cotton

To research *NRT2* family members in cotton, CottonGEN (<http://www.cottongen.org/>) was used to download genome files of *G. barbadense* (ZJU_v1.1), *G. hirsutum* (ZJU_v2.1), *G. raimondii* (JGI_v1.0), and *G. arboreum* (CRI_v1.0) (Yu et al., 2014). The protein sequences of AtNRT2 in *Arabidopsis* were downloaded from TAIR (<https://www.arabidopsis.org/>) (Berardini et al., 2015). We used AtNRT2 proteins as query sequences to identify NRT2 proteins in four cotton species by BlastP (Chen et al., 2020). All identified NRT2 proteins were subjected to domain analysis by Pfam (<http://pfam.xfam.org/>) (El-Gebali et al., 2019). We referred to the nomenclature of the rapeseed *NRT2* genes for naming the cotton *NRT2* genes (Tong et al., 2020). The physicochemical properties of NRT2 proteins including amino acid length, molecular weight (MW) and isoelectric point (pI) were analyzed by ExPASy-ProtParam (<http://web.expasy.org/protparam/>) (Wilkins et al., 1999). The subcellular localization of NRT2 proteins was predicted using WOLF-PSORT (<https://wolfpsort.hgc.jp/>) (Horton et al., 2007). The transmembrane helices of *GhNRT2* proteins were predicted by TMHMM 2.0 online server (<https://services.healthtech.dtu.dk/service.php?TMHMM-2.0/>) (Krogh et al., 2001).

Phylogenetic analysis, chromosomal location and synteny analysis of *NRT2* genes

The evolutionary relationships of NRT2 proteins were indicated by phylogenetic analysis. Multiple sequence alignment of NRT2 proteins in *A. thaliana*, *G. hirsutum*, *G. raimondii*,

G. arboreum, and *G. barbadense* was performed through ClustalW (Larkin et al., 2007). The phylogenetic tree was constructed using the Neighbor-Joining (NJ) method in MEGA 7.0 software and drawn by iTOL (<https://itol.embl.de/>) (Kumar, Stecher & Tamura, 2016; Letunic & Bork, 2021). Chromosome location of *NRT2* genes was acquired from the gff3 file downloaded by CottonGen (<https://www.cottongen.org/>), and TBtools was used to visualize the distribution of *NRT2* genes on chromosomes (Chen et al., 2020). The synteny relationship of *NRT2* genes was investigated using MCScanX (Wang et al., 2012). To investigate the selection pressure between homologous gene pairs, we used TBtools to calculate nonsynonymous substitution (Ka) and synonymous substitution (Ks) rates for duplicated gene pairs (Chen et al., 2020).

Gene structure, conserved motif and cis-acting elements analysis

The structure of *GhNRT2* genes were investigated by GSDS (<http://gsds.gao-lab.org/>) (Hu et al., 2015). The conserved motifs of GhNRT2 proteins were predicted by MEME (<http://meme-suite.org/tools/meme>) (Bailey et al., 2015). The 2,000 bp upstream sequences of the initiation codons of *GhNRT2* genes were extracted, and predicted cis-acting elements using the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Lescot et al., 2002).

Analysis of expression patterns of *GhNRT2* genes

To analyze the expression patterns of *GhNRT2* genes in different tissues, we downloaded RNA-seq data (accession number: PRJNA490626) of roots, stems, leaves, petals, receptacles, sepals, bracts, ovules, and fibers of *G. hirsutum* TM-1 from the Cotton Omics Database (<http://cotton.zju.edu.cn>) (Hu et al., 2019). The expression levels of *GhNRT2* genes were visualized by TBtools based on log₂ (FPKM+1) values (Chen et al., 2020). To validate the results of RNA-seq, we collected roots, stems, and leaves tissues from TM-1 plants at the three-leaf stage for RNA extraction.

RNA extraction and qRT-PCR analysis

We obtained total RNA by RNAprep Pure Plant kit (Tiangen, Beijing, China) and measured the quantity and quality of RNA samples by spectrophotometer. The RNA was reverse transcribed into cDNA using StarScript II First-strand cDNA Synthesis kit (GenStar, Beijing, China). The quality of the cDNA was examined by PCR using the *GhActin* gene as an internal gene, and better quality cDNA was used for qRT-PCR. The qRT-PCR was performed using fluorescence quantitative kit 2×RealStar Green Fast Mixture with ROX II (GenStar, Beijing, China), and the *GhActin* gene was used as an internal gene. The experiment was performed with three replicates. We calculated the relative expression levels of genes according to the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak, 2008). For the accuracy of the results, we also selected the *GhHis3* gene as the internal gene for verification. The results using *GhHis3* as an internal control were similar to those of *GhActin*, indicating the accuracy of the results and the availability of both internal reference genes for qRT-PCR analysis. Specific primers for all genes were designed by NCBI Primer-blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table S1).

Overexpression vector construction and transformation

To characterize the function of *GhNRT2.1e*, we amplified the *GhNRT2.1e* gene using primers PRI101-GhNRT2.1e-F (SalI) and PRI101-GhNRT2.1e-R (BamHI) (Table S1). The PCR products were inserted into the pRI101 vector containing the Cauliflower mosaic virus 35S promoter forming an overexpression recombinant. The 35S: *GhNRT2.1e* overexpression recombinant was transformed to wild-type *A. thaliana* (Colombia-0) through *Agrobacterium tumefaciens* flower soaking method. A total of 50 mg/L kanamycin was added to the MS medium for screening transformed T1 and T2 generations (Clough & Bent, 1998). We extracted RNA and genomic DNA from transgenic *A. thaliana* and used *AtActin* as an internal gene to test the quality of cDNA and genomic DNA. We performed PCR analysis of genomic DNA from transgenic *A. thaliana* using primers 35S: PRI101-F and PRI101-GhNRT2.1e-R (BamHI). After PCR and qRT-PCR analysis in the T2 generation, the highly expressed T3 generation was used for phenotypic research. *A. thaliana* grown in vermiculite were provided with 0.25 and 2.5 mM of nitrate, creating a nitrogen deficiency and normal supply. Plants were grown for 6 weeks by irrigating with a nutrient solution once a week.

Virus-induced gene silencing

We performed virus-induced gene silencing (VIGS) using tobacco rattle virus (TRV) to further investigate the function of *GhNRT2.1e* (Gao et al., 2011). A sequence fragment of the *GhNRT2.1e* gene was amplified using specific primers VIGS-GhNRT2.1e-F (EcoRI) and VIGS-GhNRT2.1e-R (BamHI) and inserted into the TRV2 vector to form TRV2:GhNRT2.1e (Table S1). Negative controls were wild-type and TRV2:00, while the effectiveness of the vector was tested by phytoene desaturase (PDS). Cotton seedlings were used for injection when both cotyledons were fully expanded. The NO_3^- concentration was adjusted to 0.25 and 2.5 mM to provide plants with nitrogen deficiency and normal supply. Two weeks after plant growth, RNA from TRV2:GhNRT2.1e, TRV2:00, and WT was extracted, and the silencing efficiency of *GhNRT2.1e* was determined by qRT-PCR.

Plant physiological and biochemical evaluation

We performed physiological and biochemical evaluations of *Arabidopsis* and VIGS plants. Plant samples were dried, and the dry weight was measured using a balance. The relative chlorophyll contents were measured with the SPAD analyzer. The Kjeldahl method measured the total nitrogen contents (Singh et al., 2020). The salicylic acid method measured the nitrate content (Zhao & Wang, 2017). Iqbal et al. (2020a) method calculated total nitrogen accumulation, nitrogen utilization efficiency (NUtE), and nitrogen uptake efficiency (NUPe). Nitrate reductase (NR), glutamine synthetase (GS), and glutamate synthase (GOGAT) activities, as well as amino acid content were measured by assay kits (Solarbio, Beijing, China). In addition, we measured the activity of antioxidants and oxidants: superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and malondialdehyde (MDA) by detection kits (Solarbio, Beijing, China).

Yeast two-hybrid assay

In *Arabidopsis*, the interaction between AtNRT2.1 and AtNAR2.1 has been verified. We used the protein sequence of AtNAR2.1 to verify whether this interaction exists in *G. hirsutum*. Finally, we selected the homolog of AtNAR2.1 and named it as GhNAR2.1. The interaction between GhNRT2.1e and GhNAR2.1 was verified by yeast two-hybrid assays. The specific primers pPR3-N-GhNRT2.1e-F (BamHI)/pPR3-N-GhNRT2.1e-R (EcoRI) and pBT3-C-GhNAR2.1-F (XbaI)/pBT3-C-GhNAR2.1-R (NcoI) were used to amplify the *GhNRT2.1e* and *GhNAR2.1* coding sequences and inserted them into the pPR3-N and pBT3-C vectors to form the pPR3-N-GhNRT2.1e and pBT3-C-GhNAR2.1 constructs (Table S1). The two constructs were transferred into yeast NMY51, the transformants formed were grown on an SD-LW medium, and positive transformants were selected for serial dilution (10^0 , 10^{-1} , 10^{-2} , 10^{-3}) (Liu et al., 2014). The different yeast dilutions were cultured on SD-LW and SD-AHLW media at 30 °C.

Luciferase complementation imaging assay

To verify whether GhNRT2.1e and GhNAR2.1 interact *in vivo*, we tested by LCI assay. The coding sequences of *GhNRT2.1e* and *GhNAR2.1* were amplified using the specific primers nLUC-GhNRT2.1e-F (BamHI)/nLUC-GhNRT2.1e-R (SalI) and cLUC-GhNAR2.1-F (BamHI)/cLUC-GhNAR2.1-R (SalI), and they were inserted into the nLUC and cLUC vectors to form the nLUC-GhNRT2.1e and cLUC-GhNAR2.1 constructs. The *Agrobacterium* inoculum containing the constructs was mixed in equal amounts and injected into the tobacco (Xie et al., 2020). Tobacco was grown under dark conditions for 24 h, followed by 2 days in normal environments. The undersides of the leaves were coated with 1 mM Luciferin sodium salt, and after 10 min of dark treatment, fluorescent pictures were obtained with a CCD imaging device (Li et al., 2021).

RESULTS

Identification of *NRT2* genes in cotton

We identified 14, 14, seven, and seven proteins in *G. hirsutum*, *G. barbadense*, *G. raimondii*, and *G. arboreum* using seven AtNRT2 proteins as query sequences, which were named according their homologs in *A. thaliana*. We further analyzed the physicochemical properties of NRT2 proteins (Table S2). In *G. hirsutum*, the protein lengths ranged from 341aa (GhNRT2.1f) to 542aa (GhNRT2.4a and GhNRT2.4b). The isoelectric point ranged from 8.04 (GhNRT2.7a) to 9.51 (GhNRT2.1b), indicating that all GhNRT2 proteins are basic proteins. The subcellular localization of most GhNRT2 proteins was predicted at the plasma membrane, while GhNRT2.7a and GhNRT2.7b were localized in the vacuole. The difference in subcellular localization results suggested that GhNRT2 proteins may perform transport functions at different locations. The GhNRT2 proteins have seven to 12 transmembrane helices, further supporting the predicted subcellular localization results.

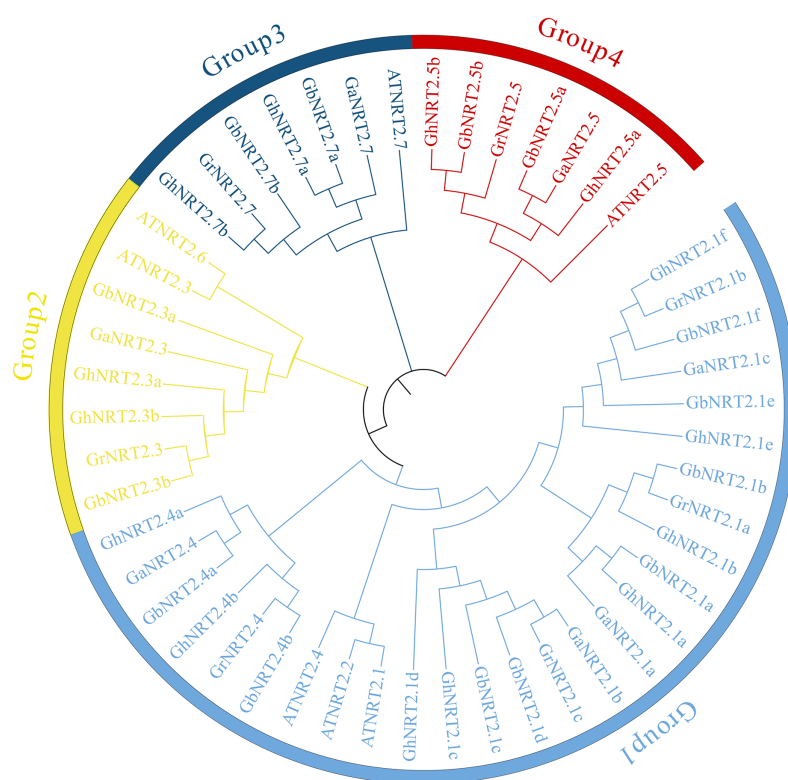


Figure 1 Phylogenetic analysis of NRT2 proteins. The phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA 7.0 software. Different groups were marked with different colors. AT, *A. thaliana*; Gr, *G. raimondii*; Ga, *G. arboreum*; Gh, *G. hirsutum* and Gb, *G. barbadense*.

Full-size DOI: 10.7717/peerj.15152/fig-1

Phylogenetic analysis of NRT2 proteins in cotton

The phylogenetic tree of NRT2 proteins was constructed by the NJ method in MEGA7.0 software (Fig. 1). The NRT2 genes were divided into four groups, named Group 1 to Group 4. Group 1 consisted of *GhNRT2.1s*, *GhNRT2.4s*, and their homologous genes. Group 2 was composed of *GhNRT2.3s* and their homologous genes. *GhNRT2.7s* and their homologs were located in group 3. *GhNRT2.5s* and their homologs were found in group 4. *AtNRT2.2* and *AtNRT2.6* were located in groups 1 and 2, respectively, but their homologous genes were not identified in cotton. The NRT2 proteins of the *At* subgenome of allotetraploid cotton share high homology with those from *G. arboreum*. The NRT2 proteins of the *Dt* subgenome share a high degree of homology with those of *G. raimondii*. The results further verified that the allotetraploid cotton was produced by recombining two diploid species of cotton.

Chromosomal location of *GhNRT2* genes

The distribution of *GhNRT2* genes on chromosomes was mapped through gene location information (Fig. 2). In *G. hirsutum*, fourteen *GhNRT2* genes were distributed on twelve chromosomes. Such as chromosomes A05, A07, A08, A09, A12, D05, D07, D08, D09, and D12 each contained one *GhNRT2* gene, while chromosomes A03 and D03 each contained two genes.

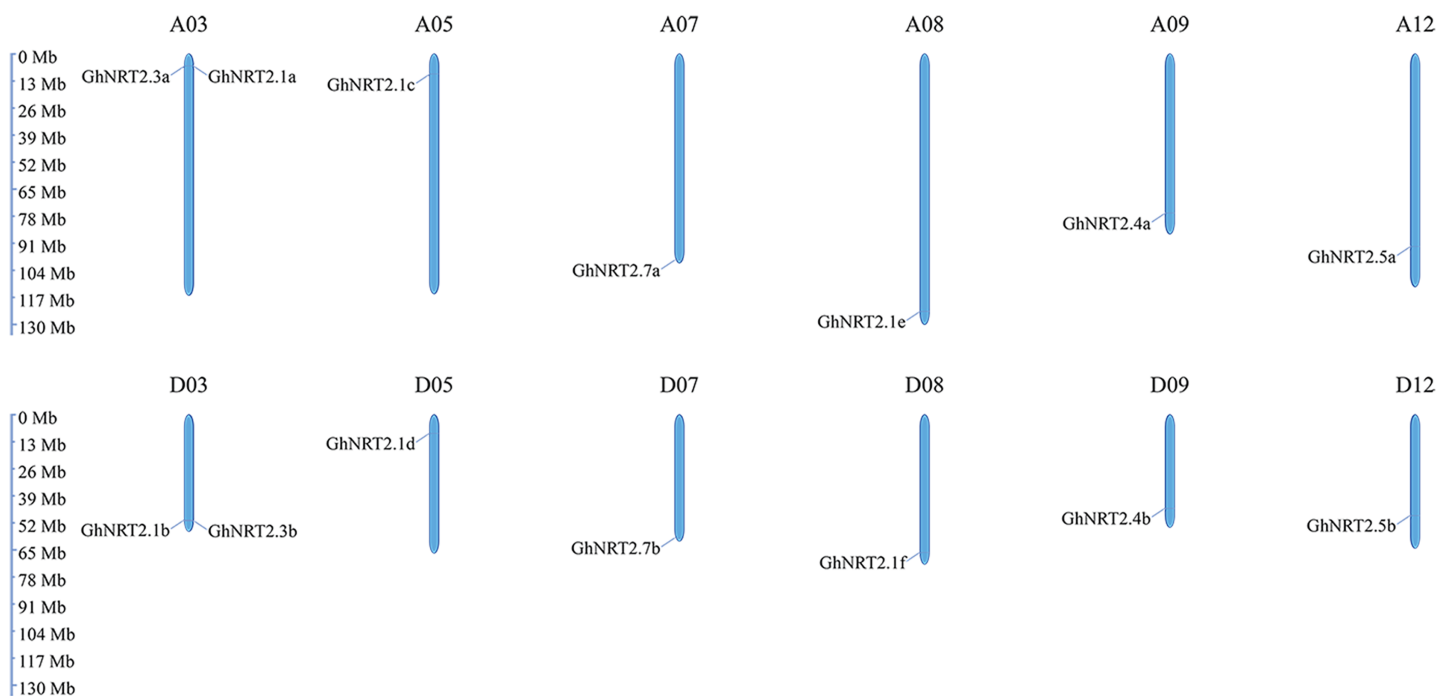


Figure 2 Chromosomal location of *GhNRT2* genes in *G. hirsutum*. The chromosomal locations of the *GhNRT2* genes were mapped by TTools software. The black lines indicate the location of the genes on the chromosomes. The scale bar indicates the chromosome length in the base pair (bp). [Full-size !\[\]\(5fd6ef84f97f42d7f8b34275f1b65312_img.jpg\) DOI: 10.7717/peerj.15152/fig-2](https://doi.org/10.7717/peerj.15152/fig-2)

Gene duplication and synteny analysis of *NRT2* gene in cotton

We performed gene duplication and syntenic relationship analysis in *NRT2* genes and identified two tandem repeats in *G. hirsutum*, *GhNRT2.1a/GhNRT2.3a* and *GhNRT2.1b/GhNRT2.3b*. In addition, we identified 22 segmental duplications and 119 WGD for the *GhNRT2* genes (Table S3). Synteny analysis revealed a high collinearity relationship between *NRT2* genes. We identified 22, 56, 30, 39, 36, 29, and 27 paralogous or orthologous gene pairs from seven combinations (Gh-Gh, Gh-Gb, Gb-Gb, Gb-Gr, Gh-Gr, Gb-Ga, and Gh-Ga), respectively (Fig. 3). In addition, only *GhNRT2.7a/GbNRT2.7a* had a Ka/Ks ratio >1, indicating that positive selection was experienced. In contrast, the Ka/Ks of other gene pairs were <1, demonstrating that almost all *NRT2* genes underwent purifying selection (Table S4).

Gene structure and conserved motif of *GhNRT2* genes

To further study the structural diversity of *GhNRT2* genes, we analyzed their exon-intron structure. Structural analysis of *GhNRT2* genes revealed that *GhNRT2.1s* and *GhNRT2.4s* contained three exons and two introns, while *GhNRT2.3s*, *GhNRT2.5s*, and *GhNRT2.7s* all contained only two exons and one intron (Fig. 4C). Furthermore, we found that genes in the same group have similar structures, probably because the *GhNRT2* genes are highly conserved in structure. The analysis of the gene structure also provided additional evidence for the evolutionary relationship of *NRT2* genes. We identified conserved motifs in *GhNRT2* proteins by MEME. We found that almost all *GhNRT2* proteins have motifs 1, 2,

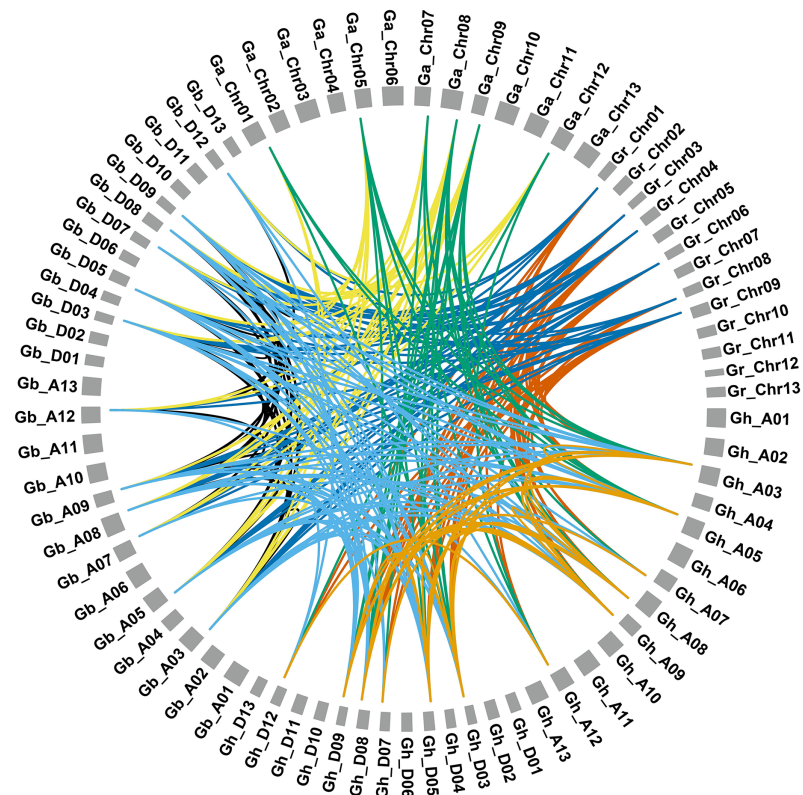


Figure 3 Syntenic relationship of *NRT2* genes in *G. hirsutum*, *G. barbadense*, *G. arboreum*, and *G. raimondii*. The syntenic relationships of the *NRT2* genes were mapped by the MCScanX program in TBtools software. The lines represented by various colors indicate the syntenic regions around the *NRT2* genes. Gr, *G. raimondii*; Ga, *G. arboreum*; Gh, *G. hirsutum* and Gb, *G. barbadense*.

Full-size DOI: 10.7717/peerj.15152/fig-3

3, 4, 5, 6, 7, 8, and 10, suggesting that these proteins are highly conserved and have similar functions. GhNRT2.7a and GhNRT2.7b did not contain motif 9, and GhNRT2.1f was missing in motifs 7, 8, 9, and 10, so we speculated that these proteins might have specific functions (Fig. 4B). To verify the sequence characteristics of GhNRT2 proteins, we performed multiple sequence alignments of GhNRT2 protein sequences by DNAMAN (Fig. 4D). All GhNRT2 proteins contained MFS and NNP motifs, demonstrating that they belong to the MFS and NNP families.

Cis-acting element analysis of *GhNRT2* genes

The promoter cis-acting elements of *GhNRT2* genes were examined by PlantCARE software to elucidate potential regulatory mechanisms (Fig. 5). We classified the examined cis-acting elements into three categories based on their biological functions (Table S5). The first category was phytohormone-related elements, of which MeJA, GA, auxin, SA, and ABA contained 30, 18, 10, 9, and 27, respectively. The second category was stress response elements. We found the highest number of anaerobic induction elements with 34. There was only one anoxic specific inducibility element, and it was present in the promoter region of *GhNRT2.3b*. The third category was growth-related elements. The number of

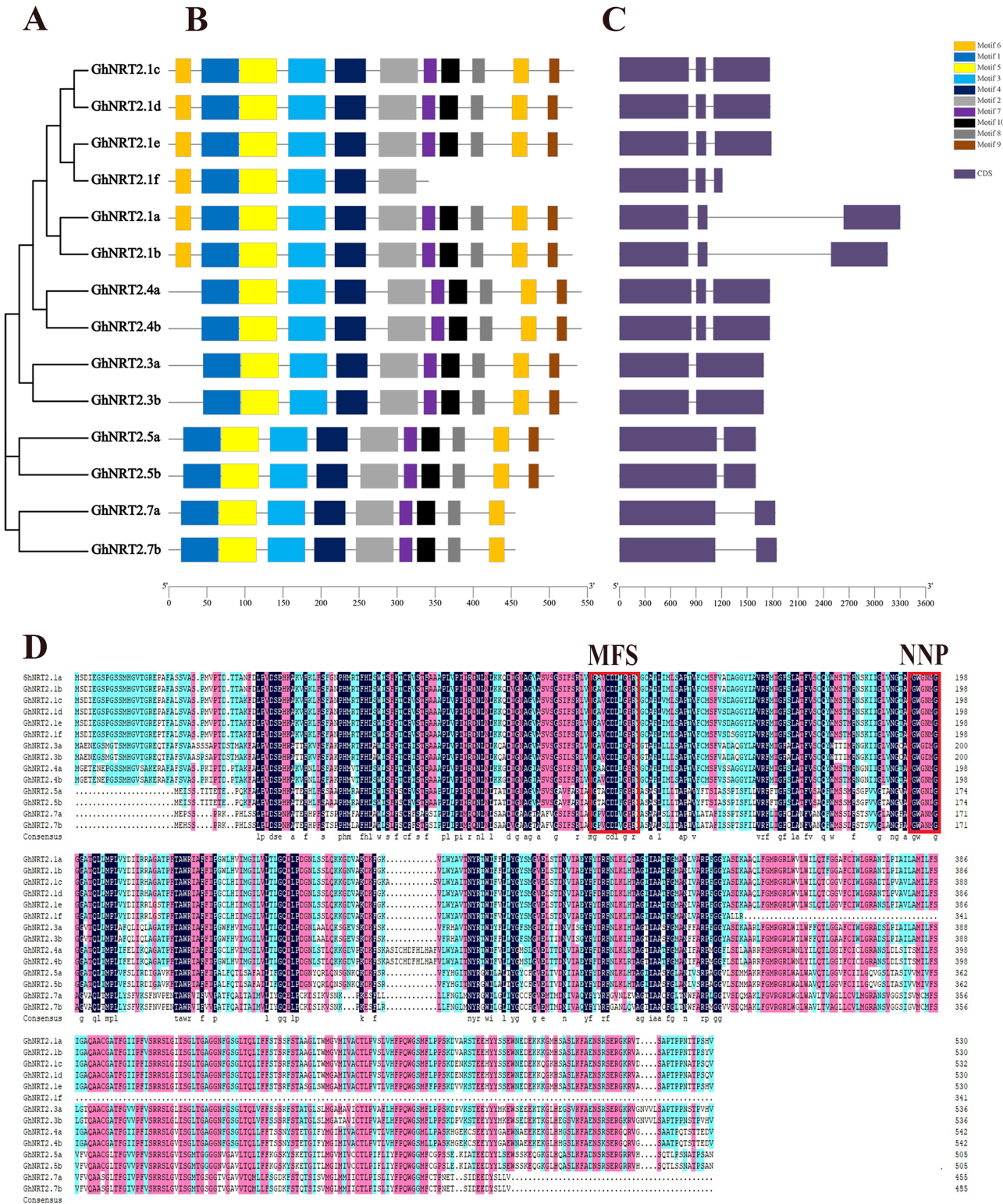


Figure 4 Phylogenetic relationship, conserved motifs, gene structure, and multiple sequence alignment of GhNRT2 proteins. (A) Phylogenetic analysis of GhNRT2 proteins. (B) Conserved motifs of GhNRT2 proteins. The MEME software identified the conserved motifs. Different colored boxes represented different motifs. (C) Exon-intron structures of the *GhNRT2* genes. Gene structure was analyzed by GSDS software. (D) Multiple sequence alignment of GhNRT2 proteins. Amino acid sequence alignment was performed by DNAMAN software. MFS motifs and NNP motifs were boxed in red. Full-size [DOI: 10.7717/peerj.15152/fig-4](https://doi.org/10.7717/peerj.15152/fig-4)

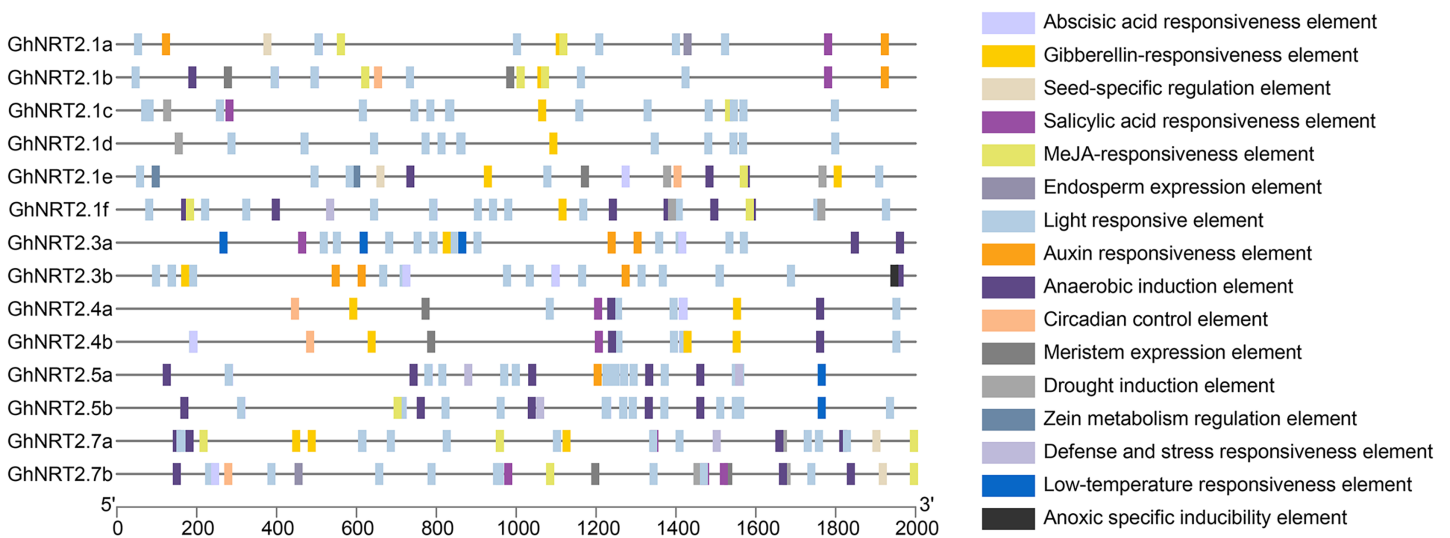


Figure 5 Predicted cis-acting elements in the promoters of *GhNRT2* genes. The cis-acting elements were predicted from the PlantCARE database. Different colored boxes represented different cis-acting elements. [Full-size !\[\]\(ba1b80118482ccef74a5d718ca4d7242_img.jpg\) DOI: 10.7717/peerj.15152/fig-5](https://doi.org/10.7717/peerj.15152/fig-5)

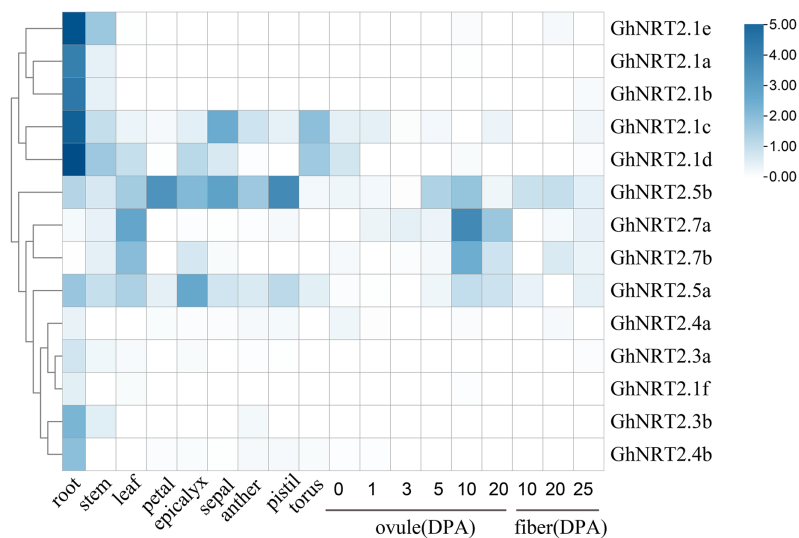


Figure 6 RNA-seq data heat map of *GhNRT2* genes expression in eleven different tissues. The differences in expression of the *GhNRT2* genes are shown in different colors. DPA, days post anthesis. [Full-size !\[\]\(ab8f7a9d25e63edc6ae9f62ddaa1d31c_img.jpg\) DOI: 10.7717/peerj.15152/fig-6](https://doi.org/10.7717/peerj.15152/fig-6)

meristem expression elements was the most abundant, containing seven. Based on the results, it is hypothesized that *GhNRT2* genes may participate in plant growth, phytohormone regulation, and abiotic stress.

Tissue-specific expression pattern analysis of *GhNRT2* genes

The tissue expression pattern of *GhNRT2* genes were revealed using RNA-seq data (accession number: [PRJNA490626](https://www.ncbi.nlm.nih.gov/PRJNA490626)) (Fig. 6). We found that the expression levels of *GhNRT2.1f*, *GhNRT2.3a*, and *GhNRT2.4a* were not detected in all tissues (FPKM < 1).

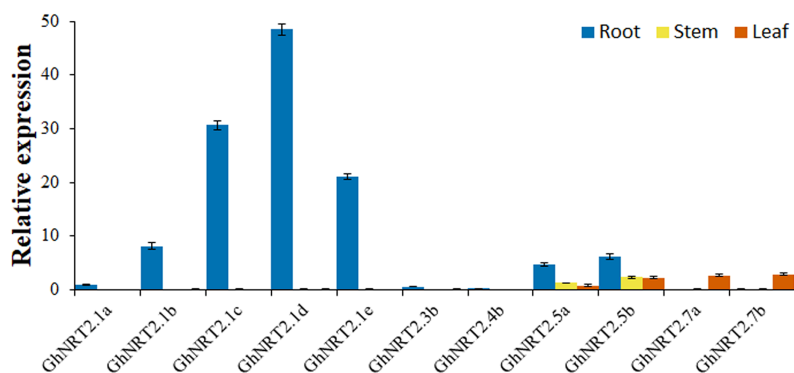


Figure 7 The relative expression levels of *GhNRT2* genes in root, stem, and leaf were determined by qRT-PCR. *GhNRT2.1a* expression in roots was set to 1 and used as a control to reflect the relative expression of the *GhNRT2* genes in roots, stems, and leaves. The *GhActin* gene was used as the internal reference gene. Full-size [DOI: 10.7717/peerj.15152/fig-7](https://doi.org/10.7717/peerj.15152/fig-7)

The expression levels of *GhNRT2.1a-e* were extremely high in roots. *GhNRT2.3b* and *GhNRT2.4b* were expressed only in the roots, but their expression levels were low. *GhNRT2.5a* and *GhNRT2.5b* had high expression levels in several tissues, with *GhNRT2.5a* primarily expressed in epicalyx, root, and leaf, and *GhNRT2.5b* had high levels in petal, pistil, and sepal. The expression levels of *GhNRT2.7a* and *GhNRT2.7b* were higher in leaves and ovules. To validate the RNA-seq results, we analyzed the expression patterns of *GhNRT2* genes in root, stem, and leaf by qRT-PCR (Fig. 7). We designed specific primers for each *GhNRT2* gene to detect accurate gene expression levels. We found similar results of qRT-PCR and RNA-seq analysis, further validating the expression profile of *GhNRT2* genes. The *GhNRT2* genes were highly expressed in roots, suggesting that they may function in nitrate uptake. The qRT-PCR assay showed that *GhNRT2.5a* and *GhNRT2.5b* had the highest expression in roots. Combining the results of RNA-seq and qRT-PCR, we found that *GhNRT2.5a* and *GhNRT2.5b* may be expressed in roots and aerial parts, and their expression patterns were similar to those of *AtNRT2.5* (Lezhneva et al., 2014). *GhNRT2.7a* and *GhNRT2.7b* showed high expression in leaves and ovules, and they may have potential functions in nitrate storage.

Expression patterns of *GhNRT2* genes at low nitrate concentrations

After 1 week of nitrogen-free treatment, cotton seedlings were resupplied with 0.25 mM NO_3^- to investigate the expression pattern of *GhNRT2* genes at low nitrate concentrations. We found that *GhNRT2.7a* and *GhNRT2.7b* were barely expressed in the roots by tissue-specific analysis. Therefore, we selected nine *GhNRT2* genes with high or specific expression in the roots and analyzed their expression levels at low nitrate concentrations by qRT-PCR (Fig. 8). The *GhNRT2* genes exhibited different expression levels after NO_3^- resupply. Interestingly, only the *GhNRT2.1(a-e)* genes were significantly up-regulated and peaked at 3 or 6 h before gradually decreasing. However, the expression levels of *GhNRT2.4b*, *GhNRT2.5a* and *GhNRT2.5b* genes were reduced after NO_3^- resupply, and the reduction was more pronounced for *GhNRT2.5a* and *GhNRT2.5b*. *GhNRT2.3b* may not be sensitive to nitrate resupply and is weakly upregulated. It may be because the *GhNRT2.1*

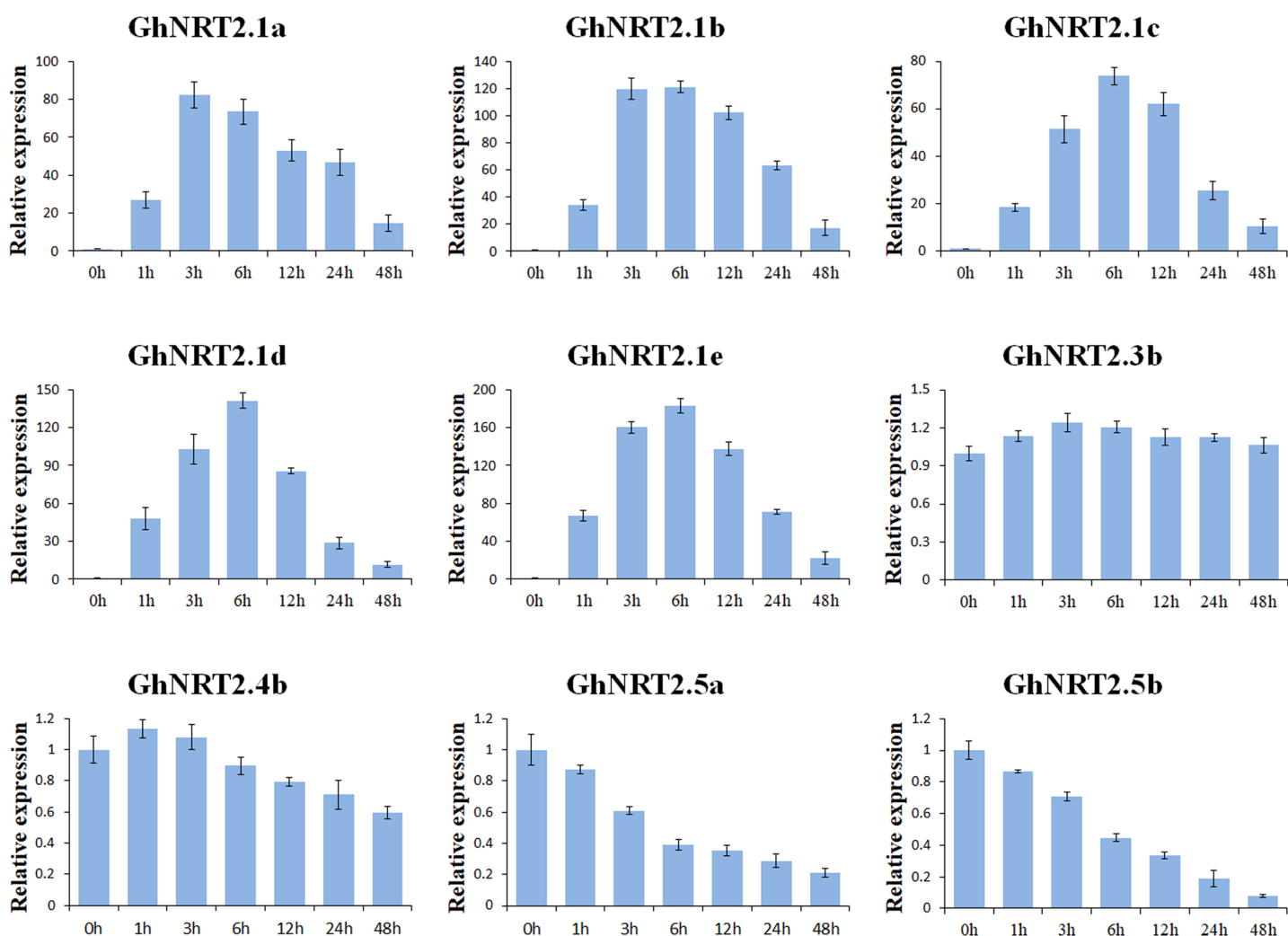


Figure 8 The relative expression levels of *GhNRT2* genes were analyzed by qRT-PCR under low nitrate conditions. Cotton seedlings were grown in a nitrogen-free nutrient solution for 1 week and then resupplied with 0.25 mM NO_3^- . Root tissues were collected at 0, 1, 3, 6, 12, 24, and 48 h after treatment, respectively. The *GhActin* gene was used as the internal control. [Full-size !\[\]\(b345a1c4255362eec3746050dd71ccac_img.jpg\) DOI: 10.7717/peerj.15152/fig-8](https://doi.org/10.7717/peerj.15152/fig-8)

(a-e) proteins belong to the inducible high-affinity transport system (iHATS) which is induced by nitrate supply. In contrast, GhNRT2.4b, GhNRT2.5a and GhNRT2.5b proteins belong to constitutive high-affinity transport systems (cHATS), which are active in plants not supplied with nitrate (Lezhneva et al., 2014). The *GhNRT2.1e* showed the most significant up-regulation of all genes, and its expression level reached the highest at 6 h, which was about 180-fold increase compared to 0 h. *GhNRT2.1e* may be a critical gene to promote the absorption and transport of nitrate in cotton.

Phenotypic, physiological, and biochemical evaluation of *GhNRT2.1e*-overexpressed *Arabidopsis* plants

To elucidate the function of *GhNRT2.1e*, we cloned the coding sequence of *GhNRT2.1e* into the pRI101 vector containing the Cauliflower mosaic virus 35S promoter, and overexpressed it in *Arabidopsis*. We extracted RNA and genomic DNA from T2 generation

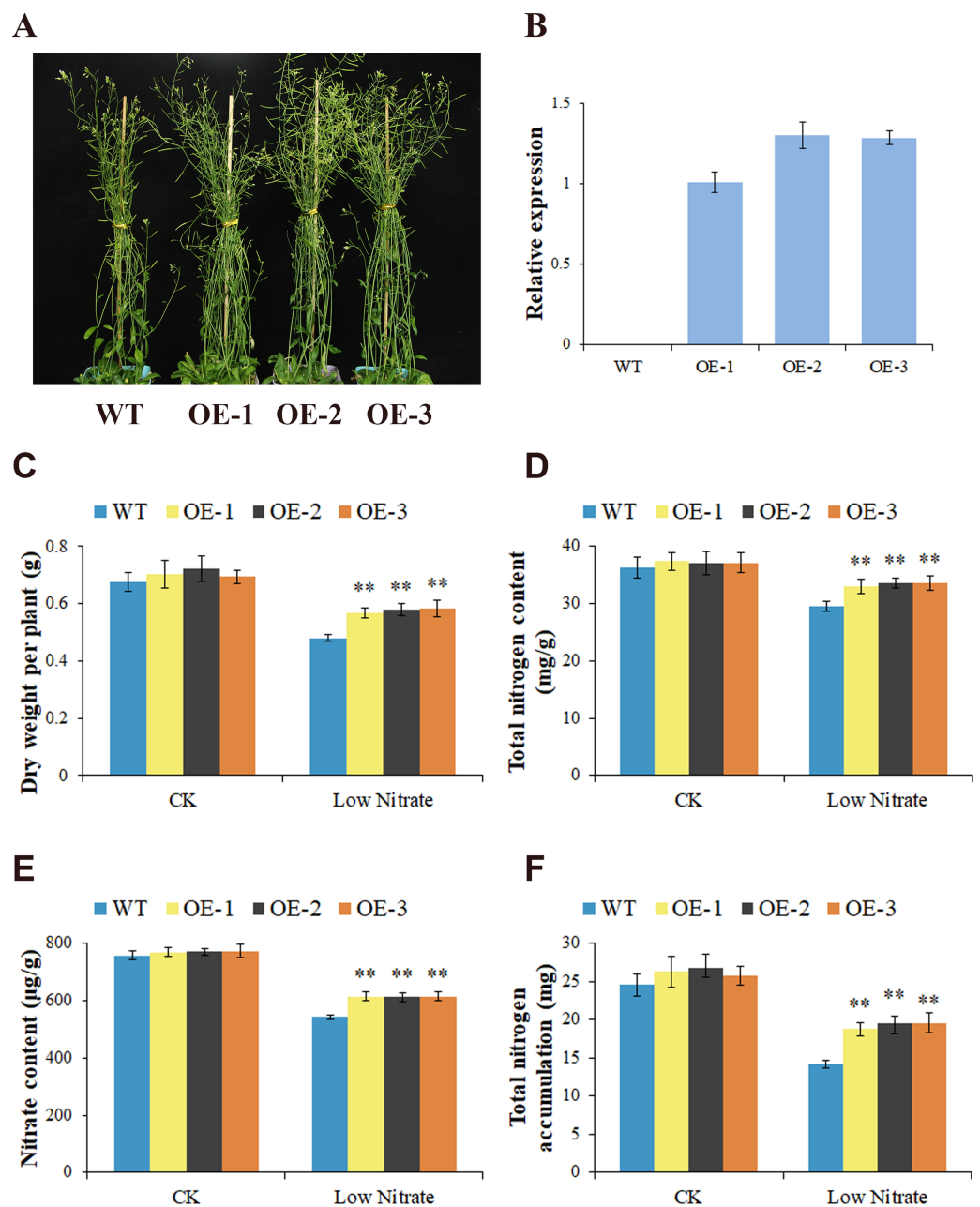


Figure 9 Phenotypic observation and identification of *GhNRT2.1e*-overexpressed *Arabidopsis*.

(A) Phenotypes of wild-type (WT) and overexpression lines grown under 0.25 mM nitrate conditions. Each small pot contained three plants. (B) The relative expression levels of *GhNRT2.1e* in wild-type (WT) and three T2 generations overexpressed lines were analyzed by qRT-PCR under normal nitrate conditions. The *AtActin* gene was used as the internal control. Quantitative determination of biomass (C), total nitrogen content (D), nitrate content (E), and total nitrogen accumulation (F) of WT and overexpression lines. The values are means \pm standard deviation (SD) of ten replicates. Student's *t*-test was used to analyze the significance of differences. ** $P < 0.01$ indicates significant differences between WT and overexpression lines. "CK" represents a nutrient solution with a nitrate concentration of 2.5 mM; "Low Nitrate" represents a nutrient solution with a nitrate concentration of 0.25 mM; "WT" represents the wild type; "OE" represents overexpression line.

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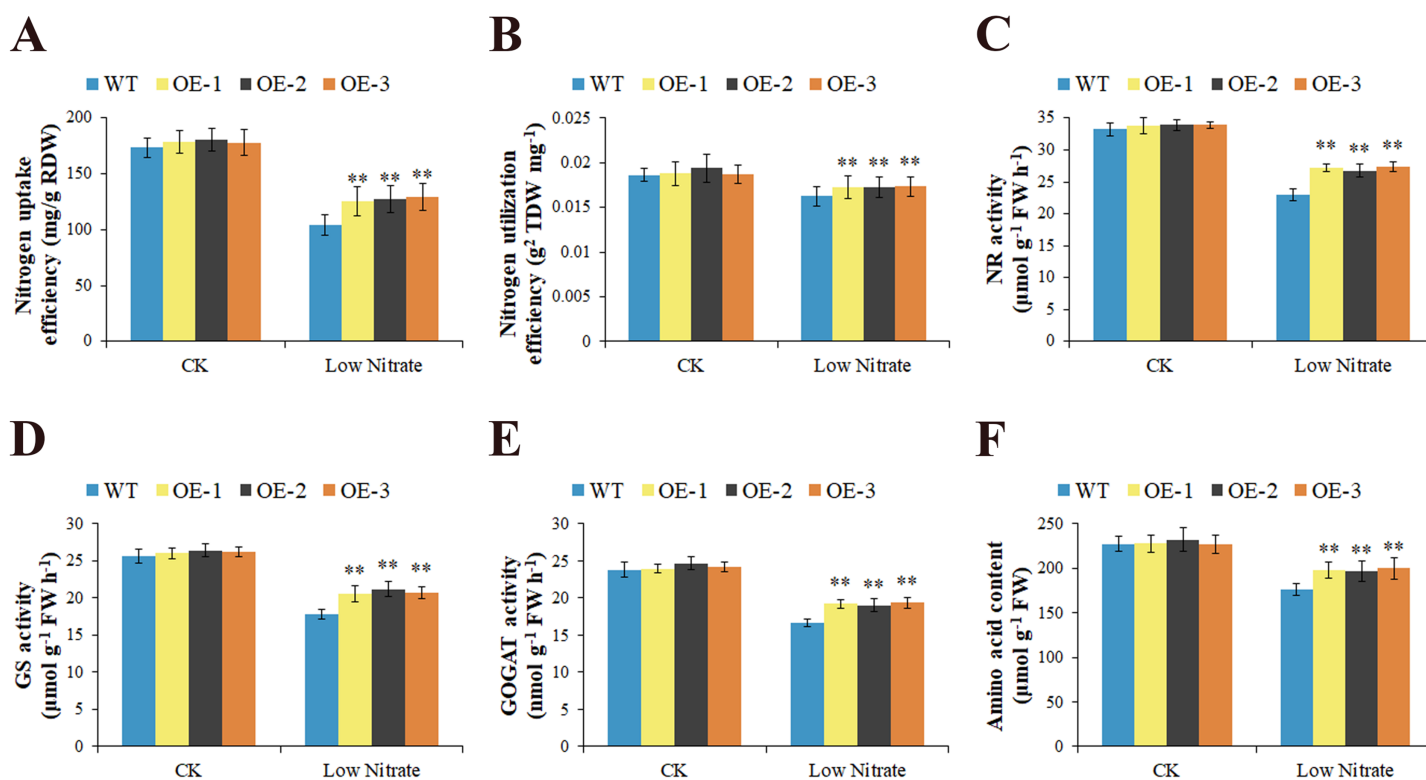


Figure 10 Physiological and biochemical analysis of *GhNRT2.1e*-overexpressed *Arabidopsis*. Nitrogen uptake efficiency (NUPE) (A), nitrogen utilization efficiency (NUPE) (B), Nitrate reductase (NR) activity (C), Glutamine synthetase (GS) activity (D), Glutamate synthetase (GOGAT) activity (E), and amino acid content (F) of WT and overexpression lines grown under 2.5 and 0.25 mM NO₃⁻ conditions. The values are means ± standard deviation (SD) of ten replicates. Student's *t*-test was used to analyze the significance of differences. ***P* < 0.01 indicates significant differences between WT and overexpression lines. "CK" represents a nutrient solution with a nitrate concentration of 2.5 mM; "Low Nitrate" represents a nutrient solution with a nitrate concentration of 0.25 mM; "WT" represents the wild type; "OE" represents overexpression line.

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of overexpressed lines and analyzed the expression level of *GhNRT2.1e* under a normal nitrate environment by qRT-PCR and PCR (Figs. 9B and S1). Results indicated that *GhNRT2.1e* had been successfully transferred into *Arabidopsis* and could be stably expressed. Three T3 generation overexpressed lines were screened for further functional studies of *GhNRT2.1e*. Plants were supplied with 0.25 mM and 2.5 mM of nitrate to create a nitrogen deficiency and normal supply. After 6 weeks of growth, plants were evaluated for phenotypic, physiological, and biochemical traits. There were no phenotypic differences between overexpressed lines and WT under 2.5 mM nitrate conditions. However, the overexpressed lines exhibited better growth than the WT when watered with 0.25 mM NO₃⁻ (Fig. 9A). We further measured dry weight to reflect differences between plants and found that the overexpressed lines had higher biomass than WT (Fig. 9C). To reflect physiological differences between plants, we measured nitrogen and nitrate content (Figs. 9D and 9E). There were no differences between plants under normal conditions, but overexpressed lines had higher levels of nitrogen and nitrate at low nitrate concentrations. Additionally, we found that nitrogen accumulation in overexpressed lines was increased under low nitrate conditions (Fig. 10A). We also calculated the NUPE and

NUtE of plants and found that the NUpE and NUtE were higher in the overexpressed lines (Figs. 10B and 10C). We speculated that the overexpressed lines may promote nitrate uptake, allowing plants to accumulate and utilize more nitrogen and nitrate. To further analyze nitrate uptake and utilization in plants, we measured nitrogen-metabolizing enzyme activity (Figs. 10D–10F). Nitrogen-metabolizing enzyme activity was higher in the overexpressed lines at low nitrate concentrations, but there were no apparent differences at normal concentrations. We also observed higher amino acid contents in overexpressed lines (Fig. 10G). In conclusion, *Arabidopsis* undergoes low-affinity transport under normal conditions, and *GhNRT2.1e* plays a minor role, so there is no apparent difference between overexpressed *Arabidopsis* and WT. In contrast, under low nitrate conditions, *GhNRT2.1e* plays an essential role as a high-affinity transporter protein to absorb more nitrate into nitrogen metabolism, which produces more nitrogen assimilation products for plant growth with the action of nitrogen metabolizing enzymes.

Evaluation of morphology, physiological indexes, and biochemical characters of *GhNRT2.1e*-silenced plants

To further elucidate the function of *GhNRT2.1e*, we selected the TRV vector for the VIGS assay. To verify the effectiveness of the vector, we injected TRV2:PDS into cotton leaves. Cotton leaves showed an albino phenotype after 14 days TRV2:PDS injection, indicating that the vector used was effective (Fig. 11A). After injection, plants were given nutrient solutions with a nitrate concentration of 2.5 and 0.25 mM to create normal and low nitrate environments, respectively. To evaluate gene silencing efficiency, we determined the expression levels of *GhNRT2.1e* in WT, TRV2:00, and TRV2:*GhNRT2.1e* plants by qRT-PCR after 2 weeks of plant growth (Fig. 11C). The expression levels of the *GhNRT2.1e* gene were not significantly different in WT and TRV2:00, indicating that the injection of an empty vector did not affect the plants. However, the expression level of the *GhNRT2.1e* gene was significantly down-regulated in TRV2:*GhNRT2.1e* plants compared with WT and TRV2:00, which indicated that gene silencing was successful. After 4 weeks of growth, there were no apparent differences between plants under 2.5 mM nitrate conditions. However, silenced plants showed dwarf and slow growth under low nitrate conditions compared to WT and TRV2:00 (Fig. 11B). To further analyze the morphological differences between VIGS and control plants, we evaluated physiological traits including dry weight, nitrogen content, and nitrate content (Figs. 11D–11F). In the low nitrate environment, VIGS plants had lower dry weight, nitrogen, and nitrate content than WT and TRV2:00. We further analyzed NUpE and NUtE and found that the uptake and utilization efficiencies of VIGS plants were low as compared to WT and TRV2:00 (Figs. 12A and 12B). Additionally, we also found lower total nitrogen accumulation in VIGS plants (Fig. 12C). The differences exhibited by the plants in normal and low nitrate environments could be due to the high nitrate concentration in the normal environment, where plants mainly undergo low-affinity transport. However, *GhNRT2.1e* is a high-affinity transporter protein that may not function or have less effect in environments with high nitrate concentrations. In low nitrate environments, plants mainly undergo high-affinity transport, and *GhNRT2.1e* plays an essential role as a critical gene. Therefore,

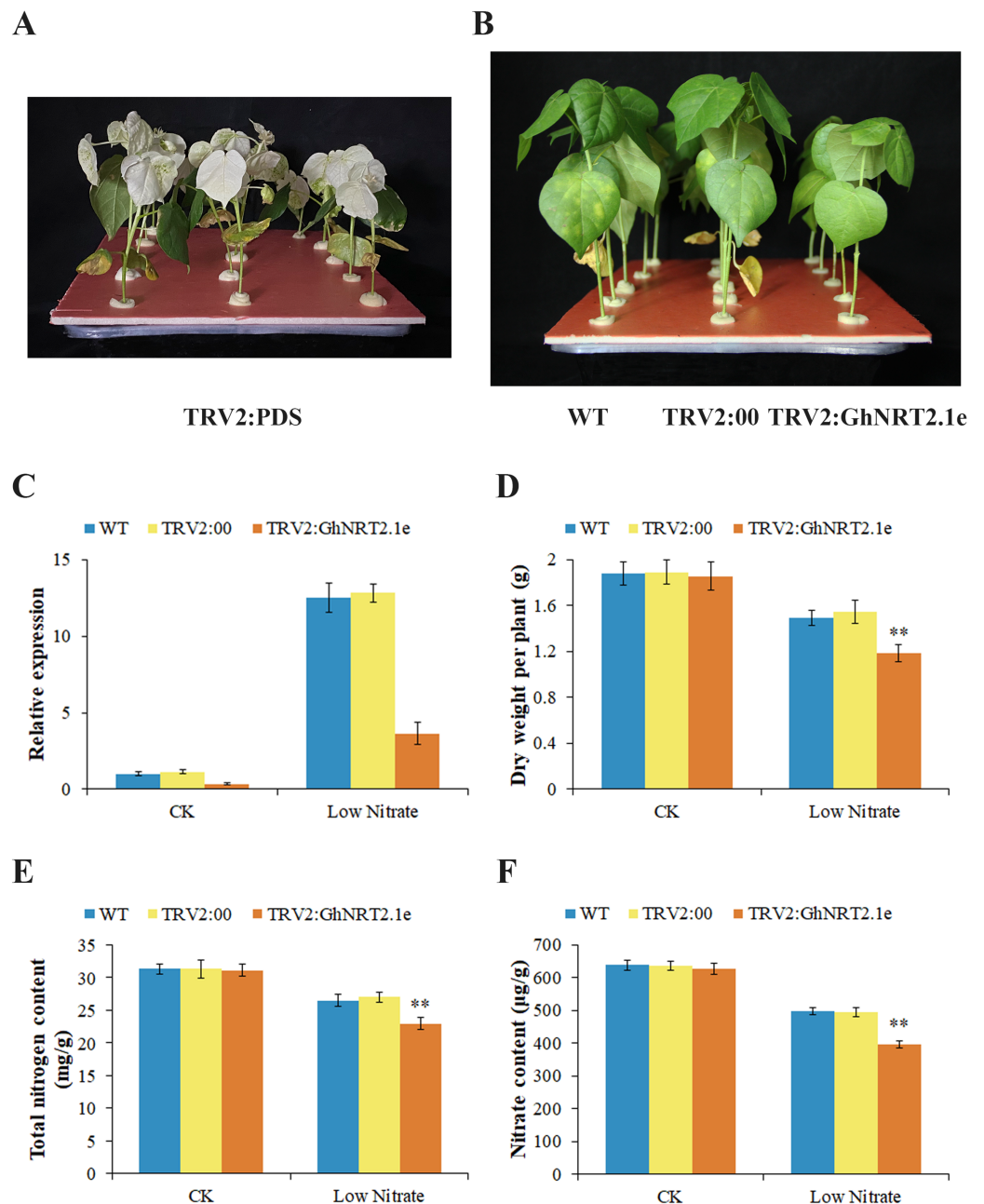


Figure 11 Phenotypic observation and identification of silenced plants. (A) Albino phenotype appearance on the leaves of the TRV2:PDS infused plants. (B) The phenotype of WT, TRV2:00, and TRV2:GhNRT2.1e plants grown at 0.25 mM nitrate concentration. (C) The expression levels of *GhNRT2.1e* in WT, TRV2:00, and TRV2:GhNRT2.1e plants grown at 0.25 mM and 2.5 mM nitrate concentrations were analyzed by qRT-PCR. The *GhActin* gene was used as the internal control. Comparison of dry weight (D), total nitrogen content (E), and nitrate content (F) of WT, TRV2:00, and TRV2:GhNRT2.1e plants. The values are means \pm standard deviation (SD) of ten replicates. Student's *t*-test was used to analyze the significance of differences. $**P < 0.01$ indicates significant differences between *GhNRT2.1e*-silenced plants and control plants. "WT" represents the wild type; "TRV2:00" represents the plants carrying control the TRV2 empty vector; "TRV2:GhNRT2.1e" represents the *GhNRT2.1e*-silenced plants; "CK" represents a nutrient solution with a nitrate concentration of 2.5 mM; "Low Nitrate" represents a nutrient solution with a nitrate concentration of 0.25 mM.

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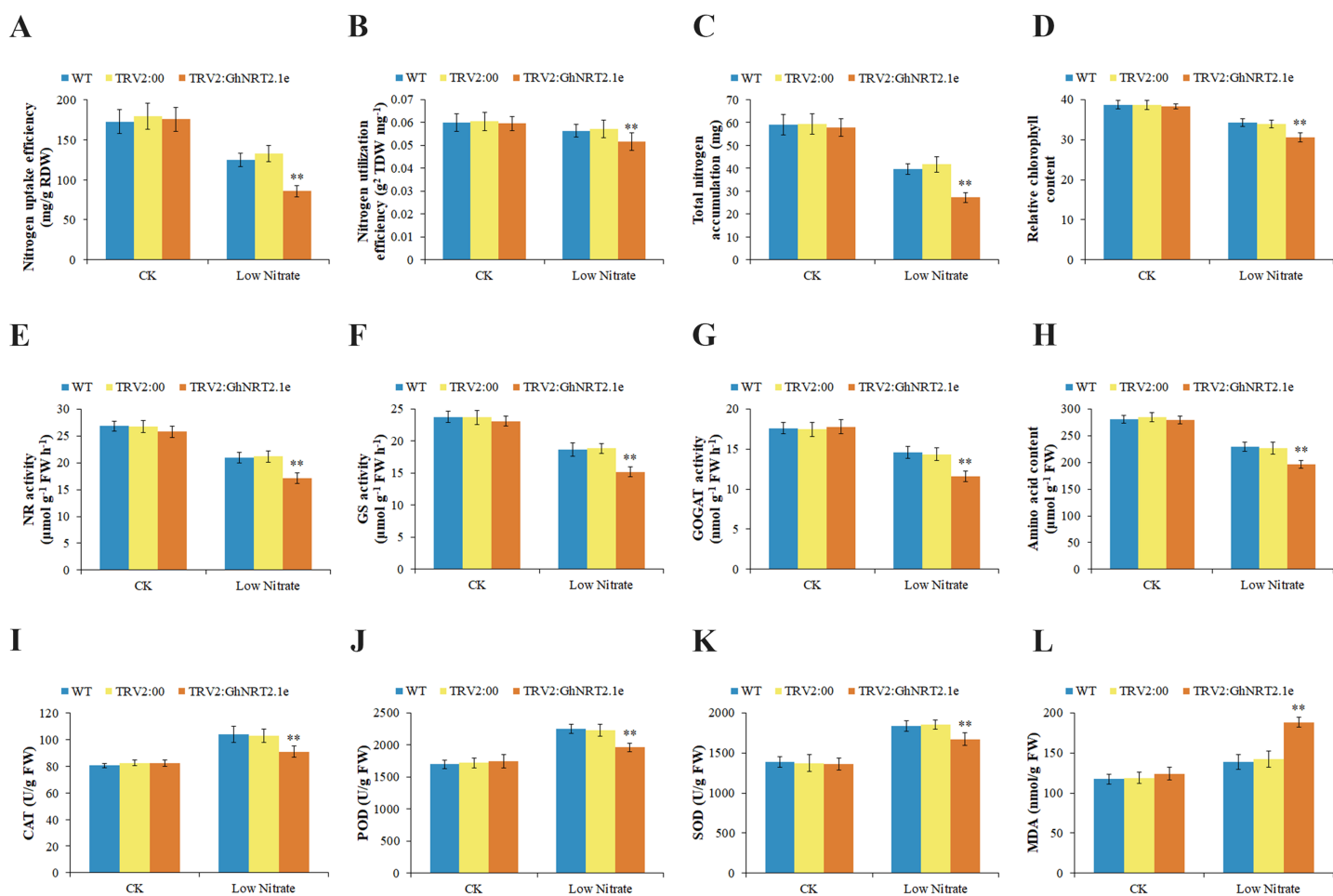


Figure 12 Physiological and biochemical evaluation of *GhNRT2.1e*-VIGS cotton plants. Quantitative determination of nitrogen uptake efficiency (NUpE) (A), nitrogen utilization efficiency (NuTE) (B), total nitrogen accumulation (C), chlorophyll content (D), Nitrate reductase (NR) activity (E), Glutamine synthetase (GS) activity (F), Glutamate synthetase (GOGAT) activity (G), amino acid content (H), catalase (CAT) activity (I), peroxidase (POD) activity (J), superoxide dismutase (SOD) activity (K) and malondialdehyde (MDA) concentration (L) in *GhNRT2.1e*-silenced and control plants grown under 2.5 mM NO₃⁻ and 0.25 mM NO₃⁻ conditions. The values are means ± standard deviation (SD) of ten replicates. Student's *t*-test was used to analyze the significance of differences. ***P* < 0.01 indicates significant differences between *GhNRT2.1e*-silenced plants and control plants. "WT" represents the wild type; "TRV2:00" represents the plants carrying control the TRV2 empty vector; "TRV2:GhNRT2.1e" represents the *GhNRT2.1e*-silenced plants; "CK" represents a nutrient solution with a nitrate concentration of 2.5 mM; "Low Nitrate" represents a nutrient solution with a nitrate concentration of 0.25 mM. Full-size [DOI: 10.7717/peerj.15152/fig-12](https://doi.org/10.7717/peerj.15152/fig-12)

nitrate uptake by *GhNRT2.1e* silenced plants is inhibited, which affects the accumulation of nitrogen and nitrate and ultimately reduces plant biomass. We also found that chlorophyll content decreased in VIGS plants under low nitrate conditions (Fig. 12D). Nitrogen is a vital chlorophyll component, essential for photosynthesis and plant growth. Therefore, reducing chlorophyll content also reflects the inhibition of nitrogen uptake and utilization by VIGS plants. We measured nitrogen metabolizing enzyme activity to reflect whether nitrate uptake by silenced plants was affected (Figs. 12E–12G). The activity of nitrogen metabolizing enzymes was reduced in VIGS plants compared with controls. We also observed that the amino acid content was reduced in VIGS plants (Fig. 12H). These results suggested that silencing of *GhNRT2.1e* affects nitrate uptake by plants, thereby reducing

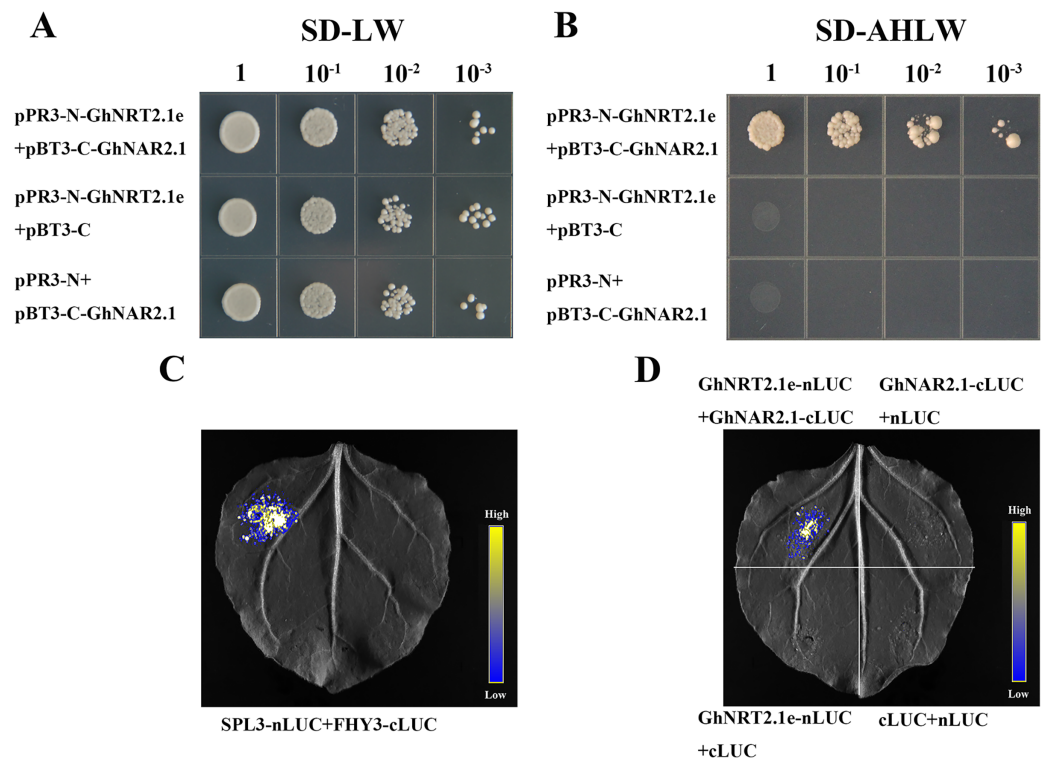


Figure 13 The interaction between GhNRT2.1e and GhNAR2.1 was verified by yeast two-hybrid assay and luciferase complementation imaging assays. (A) Yeast cells were grown on SD-LW (SD-Leu-Trp) medium. (B) Yeast cells were grown on SD-AHLW (SD-Ade-His-Leu-Trp) medium. (C) Imaging of luciferase complementation between SPL3-nLUC and FHV3-cLUC as a positive control. (D) Imaging of luciferase complementation between GhNRT2.1e and GhNAR2.1.

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nitrogen-metabolizing enzyme activity and amino acid content. We assessed the concentrations of oxidants and antioxidant enzymes in plants including CAT, POD, SOD, and MDA (Figs. 12I–12L). Under low nitrate conditions, the concentrations of CAT, POD, and SOD in VIGS plants decreased, and the concentration of MDA increased compared with control plants. Under stress conditions, elevated levels of ROS lead to membrane damage and an increase in MDA levels. High concentrations of MDA in silenced plants indicated increased peroxidation of plant cells and oxidative damage to the plants, as further showed by reduced antioxidant enzyme activity. These results showed that plants suffer from nutrient stress in low nitrate environments, and GhNRT2.1e perform high-affinity transport to maintain a partial nitrate supply. However, silencing of *GhNRT2.1e* resulted inhibition of nitrate uptake and reduced tolerance to low-nitrate environments, leading to oxidative damage in plants.

GhNRT2.1e interacts with GhNAR2.1

We performed a yeast two-hybrid assay to verify interaction between GhNRT2.1e and GhNAR2.1 in cotton as we identified the homologous gene GhNAR2.1 of AtNAR2.1 in *G. hirsutum*. On SD-LW medium, yeast carrying pPR3-N-GhNRT2.1e/pBT3-C-GhNAR2.1, pPR3-N-GhNRT2.1e/pBT3-C, and pPR3-N/pBT3-C-GhNAR2.1 could grow normally

(Fig. 13A). On the SD-AHLW medium, only yeast carrying pPR3-N-GhNRT2.1e/pBT3-C-GhNAR2.1 could grow normally (Fig. 13B). Among them, pPR3-N and pBT3-C were empty vectors, and the constructs pBT3-C-GhNAR2.1 and pPR3-N-GhNRT2.1e were connected with empty vectors as negative controls. The results indicated that GhNRT2.1e interacts with GhNAR2.1, which was further verified by LCI assay. SPL3-nLUC and FHY3-cLUC served as positive controls (Fig. 13C). In tobacco leaves, only GhNRT2.1e-nLUC/GhNAR2.1-cLUC could fluoresce (Fig. 13D). The results showed that GhNRT2.1e and GhNAR2.1 could also interact *in vivo*.

DISCUSSION

Identification and characterization of the *NRT2* genes

Nitrogen fertilizer can promote cotton growth but over-application of nitrogen fertilizer causes waste of resources and environmental pollution. Therefore, it is necessary to apply nitrogen fertilizer rationally and improve the NUE to save resources and protect the environment (Xu, Fan & Miller, 2012). We can explore key genes regulating nitrogen uptake and utilization through molecular methods and breeding new cotton cultivars to improve NUE. *NRT2* proteins have been found in plants, which can uptake and transport nitrate at low nitrate concentrations and are particularly important for increasing NUE (Chen *et al.*, 2016).

In this research, the numbers *NRT2* genes were twice in tetraploid cotton as in diploid cotton species, verifying that *G. hirsutum* was developed by crossing two diploid cotton species (Huang *et al.*, 2020). Gene duplication promotes functional differentiation, improves adaptation to the environment during evolution, and expands the gene family (Cannon *et al.*, 2004). In the replication analysis of *NRT2* genes, segmental duplication, tandem repeats, and WGD were observed to expand the *NRT2* gene family. Besides, the *NRT2* genes may have diverged during the duplication process to generate new functions. Almost all *NRT2* genes have undergone purifying selection during evolution. Similar results were observed for changes in the number of *NRT2* proteins in *Brassica* families (Tong *et al.*, 2020). Purification selection is essential to reduce deleterious mutations in the *NRT2* genes and increase their stability. We performed subcellular localization predictions and found that most of the cotton *NRT2* proteins were present in the plasma membrane, and only *NRT2.7* was on the vacuolar membrane. These results are similar to the localization of *Arabidopsis* *NRT2* proteins (Lezhneva *et al.*, 2014). Furthermore, our prediction of the transmembrane helix of *NRT2* proteins confirmed their localization to membranes. The plasma membrane controls the inflow and outflow of substances during the exchange of ions, metabolites, and nutrients (Barberon *et al.*, 2014). Therefore, *NRT2* proteins on membranes are essential for cellular uptake and transport of nitrate. Predicting cis-acting elements helps us speculate on gene function (Feng *et al.*, 2021). Some hormone-related elements were found in the promoter region of *GhNRT2* genes. Among those, ABA, auxin, and cytokinin are closely related to regulating nitrogen signaling and nitrogen uptake (Kiba *et al.*, 2011). Nitrate controls ABA accumulation in root tips, while ABA regulates lateral root formation by nitrate (Ondzighi-Assoume, Chakraborty & Harris, 2016). *Arabidopsis* can regulate auxin levels in their roots according to their

nitrogen status (Kiba et al., 2011). In watermelon, SA regulates nitrate uptake and assimilation (Vega, O'Brien & Gutiérrez, 2019). The expression of *GhNRT2* genes in roots and their response to low nitrate may be related to ABA, auxin, and SA response elements and phytohormone regulation of nitrogen signaling.

Expression analysis of the *GhNRT2* genes

Tissue differential expression analysis facilitates the investigation of gene functions (Yang et al., 2019). We identified differential expression of *GhNRT2* genes by RNA-seq and qRT-PCR (Figs. 6 and 7). The *GhNRT2* genes were expressed in roots, which might be related to nitrate uptake by the roots. Both *GhNRT2.5a* and *GhNRT2.5b* had the high expression in several tissues and may be implicated in root uptake and inter-tissue transport. *GhNRT2.7a* and *GhNRT2.7b* were expressed mainly in ovules and leaves and may participate in nitrate storage and accumulation. There was a similar expression in *Arabidopsis*, where *AtNRT2.7* was expressed in leaves and seeds, while other genes were expressed in roots (Orsel, Krapp & Daniel-Vedele, 2002). Similar situations were identified in cassava and rapeseed (Du et al., 2022; You et al., 2022). The similarity in the expression of homologous genes in different species suggests that *NRT2* genes are relatively conserved during evolution and have an essential role in plants. The tissue specificity of *GhNRT2* genes suggested that they function at different sites to jointly promote nitrate uptake, transport, storage, and utilization.

The expression levels of *GhNRT2* genes were investigated after nitrate supplementation. The *GhNRT2.1(a-e)* genes were strongly induced by nitrate, whereas *GhNRT2.4b*, *GhNRT2.5a* and *GhNRT2.5b* were significantly repressed (Fig. 8). The cHATS is active without nitrate supply and can initiate nitrate-induced genes (Kiba et al., 2012). In lack of nitrate supply, iHATS is barely expressed, whereas expression is high hours of nitrate induction (Kiba et al., 2012; Orsel, Krapp & Daniel-Vedele, 2002). In *A. thaliana*, iHATS activity is mainly dependent on *AtNRT2.1* (Li et al., 2007). In contrast, *AtNRT2.4* and *AtNRT2.5* belong to cHATS and are expressed under very low nitrate environment or nitrogen starvation conditions (Kotur & Glass, 2015). Therefore, we speculated that the *GhNRT2.1(a-e)* proteins might belong to iHATS and the *GhNRT2.4b*, *GhNRT2.5a* and *GhNRT2.5b* proteins belong to cHATS. Although they perform different functions, their interactions are necessary for the growth of plants under a restricted nitrogen supply. In addition, the expression of *GhNRT2.1(a-e)* genes first increased and then decreased, which was consistent with *CsNRT2* (Li et al., 2018). Thus, we speculated that plant nitrogen requirements may regulate the expression of *NRT2* genes and that many plants have similar conditions. In cassava, *MeNRT2.2* was strongly induced in nitrate deficiency (You et al., 2022). In barley, *HvNRT2* genes also have a similar expression (Guo et al., 2020).

The potential role of *GhNRT2.1e* in increasing NUE

GhNRT2.1e was the most up-regulated gene at low nitrate concentration and may be a key gene regulating nitrate uptake and transport. We validated the function of *GhNRT2.1e* using overexpression in *A. thaliana* and silencing in *G. hirsutum*. As transgenic cotton

takes longer, but *Arabidopsis* is a convenient model plant to study gene function, we investigated the role of *GhNRT2.1e* by heterologous expression in *Arabidopsis* (Xiao et al., 2022). The *NRT2* genes were overexpressed and can promote nitrate uptake and transport, and increase plant biomass. The cassava *MeNRT2.2* gene was overexpressed and increased the fresh weight of transgenic *Arabidopsis* (You et al., 2022). The study of the *NRT2* genes in chrysanthemum demonstrated that nitrate uptake was increased when *CmNRT2.4* was used in transgenic *Arabidopsis* and that overexpression of *CmNRT2.1* also contributed to the uptake of nitrate (Gu et al., 2016). The *HvNRT2.1* gene was overexpressed and increased nitrate and nitrogen content (Guo et al., 2020). The *CsNRT2.4* gene was overexpressed, increasing biomass and promoting lateral root development (Zhang et al., 2021). The results of this study demonstrated that in the presence of low nitrate supply levels, transgenic *Arabidopsis* promotes nitrate uptake, increasing nitrate and nitrogen accumulation and ultimately contributing to biomass accumulation (Figs. 9 and 10). Therefore, *GhNRT2.1e* promotes nitrate uptake and transport, resulting in the better growth potential of transgenic *Arabidopsis* under limited nitrate conditions. The current research results on *Arabidopsis* overexpression lines will provide some reference for future research on transgenic cotton.

VIGS is often used to assess the function of genes, which can inhibit gene expression (Gao et al., 2011). This study revealed no significant difference between *GhNRT2.1e*-silenced plants and control at normal nitrate concentrations. At low nitrate concentrations, *GhNRT2.1e*-silenced plants had lower dry weight, nitrogen and nitrate accumulation, chlorophyll content, and nitrogen uptake and use efficiency than control plants (Figs. 11 and 12). Similar results were obtained by knocking out or silencing the *NRT2* gene in other plants. For example, HATS activity was significantly reduced in *AtNRT2.1*-deficient mutants (Filleur et al., 2001). In cucumber, knockdown of *CsNRT2.1* also inhibited nitrate uptake (Li et al., 2018). We speculate that silencing of *GhNRT2.1e* affects nitrate uptake by VIGS plants, thereby inhibiting nitrate and nitrogen utilization and accumulation. The reduced nitrogen supply to stems and leaves affects plant photosynthesis and biomass accumulation. Therefore, the *GhNRT2.1e* gene increased nitrate uptake and transport and promoted cotton growth at low nitrate concentrations.

Previous studies of *Arabidopsis* and rice nitrate transporter Km data demonstrated that nitrogen transporters regulate the activities of nitrogen-metabolizing enzymes (Glass et al., 2002). Nitrogen absorbed by transporters is converted into amino acids by metabolic enzymes (Iqbal et al., 2020b). Therefore, the activity of the nitrate transporter can be judged by measuring metabolic enzyme activity and amino acid content. We found that nitrogen metabolizing enzyme activity and amino acid content were higher in transgenic *Arabidopsis* (Fig. 10). However, nitrogen metabolizing enzyme activity and amino acid content were found to be lower in *GhNRT2.1e*-silenced plants (Fig. 12). It might be due to the silencing of the *GhNRT2.1e* gene that inhibits nitrate absorption by VIGS plants and reduces the NO_3^- that enters the assimilation process, thereby affecting the assimilation process and reducing metabolic enzyme activity and amino acid content. However, transgenic *Arabidopsis* can absorb more NO_3^- and promote the metabolic process, so metabolic enzyme activity and amino acid content are increased. Previous studies on other

nitrogen-related genes had similar results. The *CPSF30* promoted nitrate signaling, while NR activity and amino acid content were reduced in mutants (Hou et al., 2021). *BnaA2.Gln1;4* was expressed in root and shoot with a high affinity for nitrogen and *BnaA2.Gln1;4* overexpression increased GS and GOGAT activity (Zhou et al., 2022). *FIP1* can regulate nitrate uptake and transport, and nitrogen-metabolizing enzyme activity and nitrate content were reduced in *fip1* mutants (Wang et al., 2018b).

When stressed, plants generate excessive reactive oxygen species (ROS), and cells are damaged by oxidation, which may result in plant death (Caverzan, Casassola & Brammer, 2016). MDA is a product of lipid breakdown, and the degree of peroxidation in plant cells can be measured by detecting MDA content (Wang et al., 2018a). Antioxidant enzymes function in maintaining the homeostasis of plant organelles (Hasanuzzaman et al., 2020). In this research, the oxidant and antioxidant enzyme activities were similar between *GhNRT2.1e*-silenced plants and controls at normal nitrate concentrations. However, at low nitrate concentrations, VIGS plants had higher MDA concentrations and lower activities of SOD, POD, and CAT than control plants (Fig. 12). Similar results were found in other studies that *GhNPL5* enhanced plant tolerance under nitrogen-limiting conditions but also showed lower antioxidant enzyme activity and higher oxidant content in VIGS plants (Magwanga et al., 2019). It may be because the production and elimination of ROS are in a dynamic equilibrium in plants at normal nitrate concentrations so that they do not cause damage to plant cells. Plants are subjected to abiotic stress at low nitrate concentrations, but *GhNRT2* genes can carry out high-affinity nitrate transport, maintain partial nitrogen supply, and reduce plant oxidative damage. The results indicated that *GhNRT2.1e* could improve cotton tolerance under low nitrogen conditions.

Interactions between proteins provide essential information for characterizing cells' biological activities and metabolic processes (Mehari et al., 2021). NAR2 is a chaperone protein and has been identified in many plants. All AtNRT2 proteins can interact with AtNAR2.1 except AtNRT2.7 (Kotur et al., 2012). AtNRT2.5 interacts with AtNAR2.1 to create a 150 kDa complex in plasma membrane to facilitate nitrate transport (Kotur & Glass, 2015). In rice, OsNRT2.1, OsNRT2.2, and OsNRT2.3a were demonstrated to interact with OsNAR2.1 by yeast two-hybrid experiments (Yan et al., 2011). In chrysanthemum, the interaction of CmNRT2.1 and CmNAR2 *in vivo* was confirmed by yeast two-hybrid and BiFC assays (Gu et al., 2016). In this research, we demonstrated that *GhNRT2.1e* interacts with *GhNAR2.1* using yeast two-hybrid and LCI assays (Fig. 13). We speculated that *GhNRT2.1e* and *GhNAR2.1* can also form a complex in the plasma membrane, and the interaction promotes nitrate uptake and transport.

CONCLUSIONS

In this work, genome-wide identification and functional characterization of *GhNRT2* genes in *G. hirsutum* was performed. Most NRT2 proteins were predicted at the plasma membrane. The results of phylogeny, gene structure, and conserved motifs indicated that *GhNRT2* proteins were conserved during evolution. Most *GhNRT2* genes were found expressed in roots by RNA-seq and qRT-PCR. When 0.25 mM nitrate was resupplied, the *GhNRT2* genes showed different expression patterns. *GhNRT2.1e* was the most highly

up-regulated and essential gene involved in nitrate uptake at low concentrations. We performed functional validation of *GhNRT2.1e* using overexpression in *A. thaliana* and silencing in *G. hirsutum*. *Arabidopsis* plants overexpressing *GhNRT2.1e* exhibited increased biomass, nitrogen and nitrate accumulation, nitrogen uptake and utilization efficiency, nitrogen-metabolizing enzyme activity, and amino acid content at low nitrate concentrations. VIGS plants exhibited reduced biomass, chlorophyll content, nitrogen and nitrate accumulation, nitrogen uptake and use efficiency, nitrogen-metabolizing enzyme activity, amino acid content, and tolerance under low nitrate conditions. These results demonstrated that *GhNRT2.1e* promoted nitrate uptake, regulation of nitrogen metabolism, and biomass accumulation. We demonstrated the interaction of GhNRT2.1e with GhNAR2.1 by yeast two-hybrid and LCI assays and speculated that they work together to promote nitrate transport. This study illustrates the function of *GhNRT2* genes and provides new ideas for improving NUE, increasing yield, and developing new varieties with high nitrogen utilization.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by the National Natural Science Foundation of China (Grant No. 31621005 and Grant No. 31901581), the Central Public-interest Scientific Institution Basal Research Fund (Grant No. 1610162022054 and Grant No. 1610162022016) and the “Agricultural Science and Technology Innovation Program of Chinese Academy of Agricultural Sciences”. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:

National Natural Science Foundation of China: 31621005 and 31901581.

Central Public-interest Scientific Institution Basal Research Fund: 1610162022054 and 1610162022016.

Agricultural Science and Technology Innovation Program of Chinese Academy of Agricultural Sciences.

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Xinmiao Zhang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Jiajia Feng analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Ruolin Zhao performed the experiments, authored or reviewed drafts of the article, and approved the final draft.

- Hailiang Cheng analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Javaria Ashraf analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
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- Youping Zhang analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Guoli Song conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Dongyun Zuo conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw data is available in the [Supplemental Files](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.15152#supplemental-information>.

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