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Genome-wide analysis of plant-specific calmodulin-binding IQD proteins in tobacco identifies key regulators of drought stress responses

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Abstract

Background Plants rely on complex signaling networks to perceive and respond to environmental stresses. Calcium ions (Ca^{2+}), acting as central second messengers, have their signals decoded by sensors such as calmodulin (CaM) and relayed to downstream target proteins. IQ67-Domain (IQD) proteins are a class of plant-specific CaM-binding proteins that function as molecular scaffolds integrating calcium signals with diverse cellular processes, playing crucial roles in growth, development, and stress responses. However, the IQD gene family has not yet been systematically investigated in tobacco (*Nicotiana tabacum*), an important economic crop and model plant species.

Results In this study, a total of 77 *NtIQD* genes were identified in the tobacco genome through genome-wide analysis. Phylogenetic analysis classified them into four subgroups, with Group IV showing species-specific expansion within Solanaceae. Analyses of gene structure and conserved motifs revealed that while maintaining the core IQ domain, the family exhibits diversity in auxiliary motifs and domains. Cis-acting element analysis of promoters indicated that all *NtIQD* genes are enriched with elements responsive to light, phytohormones (e.g., jasmonic acid, abscisic acid), and abiotic stresses. Expression profiling demonstrated that *NtIQD* genes exhibit significant tissue specificity and developmental-stage specificity, and display differentiated response patterns to various stresses such as drought, cold, and pathogen infection, suggesting functional specialization. Protein–protein interaction network predictions implicated their potential role as signaling hubs. Further experimental validation showed that *NtIQD1*, *NtIQD13*, and *NtIQD42* localize to distinct cellular compartments. Virus-induced gene silencing (VIGS) of these three genes significantly increased drought sensitivity in tobacco plants, confirming their positive regulatory roles in drought resistance.

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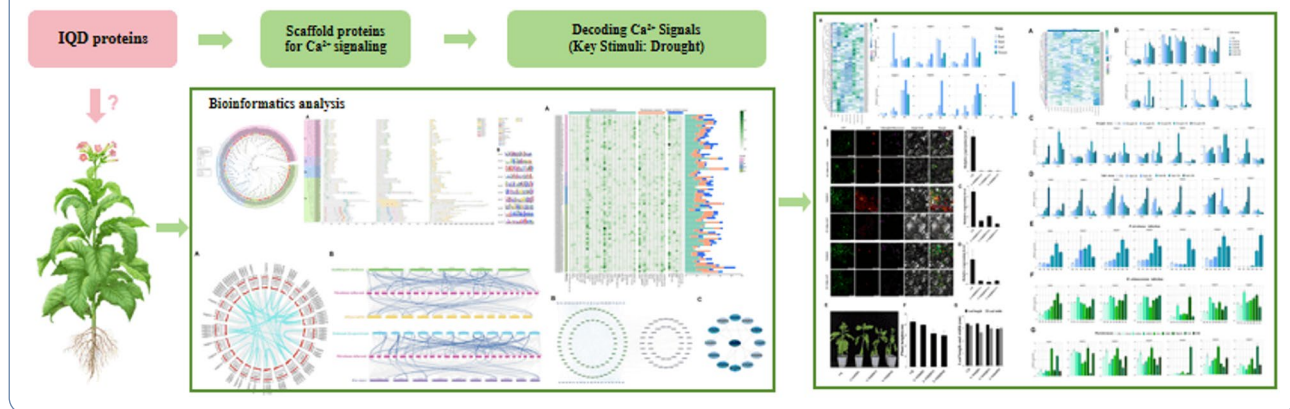
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Conclusion This study presents the first comprehensive genome-wide identification and analysis of the IQD gene family in tobacco. It reveals the diversity of this family in evolution, structure, and expression, and validates the key functions of *NtIQD1*, *NtIQD13*, and *NtIQD42* in drought stress response. These findings provide new insights into the decoding and transduction mechanisms of calcium signaling in Solanaceae plants and offer valuable candidate gene resources for crop genetic improvement aimed at enhancing stress resistance.

Keywords IQD gene family, Calcium signaling ($\text{Ca}^{2+}/\text{CaM}$), Abiotic/biotic stress, Tobacco

Graphical abstract



Background

Plants inhabit complex natural environments and are continuously exposed to diverse abiotic stresses [1]. Understanding the molecular mechanisms by which plants perceive and transduce stress signals and activate adaptive responses remains a central objective of crop genetic improvement and sustainable agriculture [2]. In this context, Ca^{2+} function as a universal second messenger that generate spatiotemporally defined signals, forming a core regulatory network for plant responses to environmental cues [3]. CaM and CaM-related proteins act as primary Ca^{2+} sensors that decode these calcium signatures. Through interactions with downstream target proteins, these signals are translated into specific cellular responses, thereby coordinating plant growth, development, and stress adaptation [4, 5].

IQD proteins represent an important class of plant-specific calmodulin-binding proteins [6]. Their defining feature is a highly conserved IQ67 domain of approximately 67 amino acids [7]. This domain contains multiple tandem calmodulin-binding motifs, including the IQ motif that mediates Ca^{2+} -independent binding and the 1–5–10 and 1–8–14 motifs that confer Ca^{2+} -dependent interactions [8]. This modular motif architecture enables IQD proteins to interact dynamically with CaM and calmodulin-like proteins (CMLs), allowing them to function as molecular scaffolds that couple calcium signaling to diverse downstream cellular processes [9]. Genome-wide analyses further indicate that the IQD gene family has undergone significant expansion across land plants

[10], supporting its broad involvement in plant physiology and environmental adaptation.

Research increasingly shows that IQD proteins function beyond simple calcium signal transduction. Instead, they regulate diverse biological processes, consistent with their role as hub components within complex signaling networks [11]. For example, in tomato, SUN, a member of the IQD gene family, is a key determinant of fruit elongation [12]. In *Arabidopsis*, *AtIQD1* binds to the microtubule cytoskeleton and participates in kinesin-dependent transport [13], while also regulating glucosinolate biosynthesis in response to biotic stress [14]. Accumulating evidence further indicates that IQD family genes play widespread roles in plant responses to abiotic stress. In poplar, wheat [15], and maize [16], multiple IQD genes were significantly induced by drought, salinity, low temperature, and related stress conditions. Notably, *BrIQD31* from Chinese cabbage functions in drought stress responses, and its heterologous expression alters physiological traits of tobacco under drought conditions [17]. Together, these findings suggest that IQD proteins act downstream of calcium signaling pathways, where they integrate environmental stress cues to modulate adaptive growth and development [18].

Tobacco is both a globally important economic crop and a well-established model system for plant molecular and functional genomics research [19]. Its allotetraploid genome, high-quality genome annotation [20], and efficient genetic transformation system [21] make tobacco particularly suitable for heterologous expression and functional validation of stress-responsive genes [22, 23].

With recent advances in genomics, genome-wide identification and characterization of gene families in tobacco has become a powerful approach for dissecting complex traits and identifying candidate genes for crop improvement [24, 25]. However, despite extensive functional studies in diploid model species such as *Arabidopsis*, the genomic organization and functional diversification of the IQD gene family in tobacco remain poorly characterized. Consequently, their specific roles in tobacco growth, development, and stress adaptation are still largely unknown.

Therefore, a comprehensive bioinformatic identification and expression analysis of the IQD gene family in tobacco is both scientifically and practically important. This study aims to systematically identify IQD family members in the tobacco genome, characterize their gene structures, evolutionary relationships, conserved motifs, and promoter cis-acting elements, and examine their expression patterns under environmental stress conditions. Thus, this study will advance understanding of the functional diversity of IQD proteins as calcium signal targets in Solanaceae species and provide candidate genes and theoretical support for improving stress resistance through genetic breeding.

Methods

Identification and physicochemical properties of the gene family

To systematically identify members of the tobacco IQD gene family, the study first downloaded the reference sequences of all 33 *Arabidopsis* IQD proteins from the Ensembl Plants database [26]. Using these sequences as queries, a bidirectional BLAST-P similarity search was performed using TBtools-II software [27] against the tobacco genome protein sequences sourced from the Tobacco Omics Database (*Nicotiana tabacum* SR1) (<http://lifenglab.hzau.edu.cn/Nicomics/index.php>). Sequences with an E-value of less than $1e^{-5}$ were preliminarily selected as candidates. Simultaneously, the hidden Markov model file PF00612 for the IQD domain was obtained from the Pfam database (<http://pfam.xfam.org/>), and the [28] HMMER 3.0 software [29] was used to scan the tobacco whole-genome protein sequences. After merging the screening results from the above two methods, the candidate proteins were further validated for conserved domains using the SMART (<http://smart.embl-heidelberg.de/>), NCBI [30] CDD [31], and Pfam databases. Redundant and domain-incomplete sequences were finally removed to obtain the tobacco IQD gene family members.

The ExPASy ProtParam online tool (<https://web.expasy.org/protparam/>) was employed [32] to analyze the physicochemical properties of the identified IQD proteins, including the number of amino acids, molecular weight,

theoretical isoelectric point, instability index, aliphatic index, and grand average of hydropathicity. Furthermore, subcellular localization prediction for all members was conducted using the CELLO v.2.5 online [33] tool (<http://cello.life.nctu.edu.tw>).

Gene chromosomal localization

Based on the tobacco whole-genome data [20] and genome annotation files, the specific positional information of each *NtIQD* gene on scaffolds or chromosomes was extracted. Using MapChart 2.32 software, the gene locus information anchored to chromosomes was visualized and plotted on a map of 24 tobacco chromosomes.

Phylogenetic analysis

To elucidate the evolutionary relationships of tobacco IQD genes, reported IQD protein sequences from tomato, *Arabidopsis*, rice [34], and maize [35] were collected. ClustalX 2.1 was used to perform multiple alignments of the full-length IQD protein sequences from tobacco and the aforementioned species [36]. Based on the alignment results, an unrooted phylogenetic tree was constructed using the neighbor-joining method in MEGA 11 software, with the Bootstrap method set to 1000 replications to assess branch confidence [37]. The final phylogenetic tree was annotated and beautified using the iTOL online platform [38].

Gene structure, functional domain, and protein motif analysis

The GSDS 2.0 online tool (<http://gsds.cbi.pku.edu.cn/>) was used [39] to align the CDS of each *NtIQD* gene with its corresponding genomic sequence to generate exon-intron structure diagrams. The conserved functional domains present in each protein were identified and visualized using the NCBI-CDD database online tool (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Additionally, the MEME online program (<http://meme-suite.org/tools/meme>) was utilized [40] to identify conserved protein motifs within the family, with the maximum number of motifs to discover set to 10. The results were visualized using TBtools-II.

Gene collinearity analysis

To analyze the expansion mechanism of the *NtIQD* gene family, the MCScanX module in TBtools-II [41] was first used to analyze the tobacco genome for internal collinearity to identify gene clusters generated by tandem duplication events. Subsequently, a whole-genome collinearity comparison between tobacco and tomato, *Arabidopsis*, rice, and maize was performed using BLASTP alignment and MCScanX analysis to identify conserved inter-species IQD homologous gene pairs. To further clarify the selection pressure, TBtools-II was used to

calculate the ratio of non-synonymous substitution rate to synonymous substitution rate (Ka/Ks) for each col-linear gene pair. Based on the calculation results, a box plot of the Ka/Ks value distribution was generated using R language.

Cis-acting element analysis

To decipher the transcriptional regulatory basis of the *NtIQD* genes, this study predicted the cis-acting elements in their promoter regions. Using TBtools-II software, the promoter sequences (2,000 bp upstream of the transcriptional start site) for each gene were extracted. These sequences were submitted to the PlantCARE online database (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) for batch scanning to identify all contained cis-regulatory elements [42]. Following verification and deduplication of the prediction results, element types that were prevalent within the family and had well-defined functions were screened. Finally, key selected elements and their distribution were visualized using plotting packages in the R language.

Protein–protein interaction network prediction

To preliminarily explore the potential functional network of *NtIQD* proteins, this study predicted protein–protein interactions and upstream regulatory relationships. First, the identified *NtIQD* protein sequences were submitted to the STRING database (<https://cn.string-db.org/>), with the organism restricted to tobacco and a minimum interaction confidence score threshold set to 0.7, to obtain core interaction prediction information [43]. This network integrated multiple lines of evidence including known experimental data, text mining, and homology mapping. Second, to investigate transcriptional and post-transcriptional regulation, the PlantTFDB database (<https://plantregmap.gao-lab.org/>) was used to predict transcription factors (TFs) potentially targeting *NtIQD* gene promoters. Simultaneously, the psRNATarget database (<http://plantgrn.noble.org/psRNATarget/home>) was employed to predict miRNAs (Micro RNAs) potentially binding to *NtIQD* gene mRNAs [44, 45]. All predicted interaction and regulatory relationships were imported into the Cytoscape software for the visual construction of an integrated network. Its built-in NetworkAnalyzer plugin was used to calculate and analyze the topological properties of the network [46].

Transcriptome expression profile analysis based on RNA-seq data

To reveal the expression patterns of the *NtIQD* gene family, this study analyzed RNA-seq data from public databases. Transcriptome datasets covering various tobacco tissues, as well as cold, drought, and pathogen (*Phytophthora nicotianae*) stresses, were downloaded from the

NCBI SRA (Sequence Read Archive) database (sample details in Supplementary Table S1). The data processing pipeline first used Fastp software for quality control and filtering of raw sequencing reads [47]. Subsequently, high-quality reads were aligned to the tobacco reference genome using Hisat2. Next, FeatureCounts was used to count reads mapped to each gene [48]. The FPKM value for each *NtIQD* gene in each sample was calculated, followed by log2 transformation. Finally, a heatmap of gene expression patterns was generated using the R language.

Plant growth conditions and stress treatments

The tobacco material used in this study was the cultivar K326. Plants were cultivated in an artificial climate chamber under the following conditions: daytime temperature 26–28 °C, photoperiod 16 h light/8 h dark, and relative humidity approximately 60%. To analyze tissue-specific gene expression, samples of roots, stems, leaves, and floral organs were collected from untreated control plants at the seedling, vigorous growth, and mature stages.

To study gene responses under abiotic stress, five-leaf-stage seedlings were subjected to the following treatments: Drought stress: Watering was withheld from potted seedlings for 11 days, after which roots and leaves were collected [49]. Cold stress: Plants were placed in a 4 °C illuminated incubator, and samples were taken at 0, 6, 24, and 48 h after treatment. To analyze hormone-induced expression patterns, seedlings were treated with solutions of Indole-3-acetic acid (IAA), 6-Benzylaminopurine (6-BA), abscisic acid (ABA), gibberellin (GA), strigolactone (GR24), methyl jasmonate (MeJA), salicylic acid (SA), and brassinosteroid (BR) for 6 h, a time point selected on the basis of preliminary time-course experiments that showed optimal gene expression responses, after which root and leaf samples were collected.

In the biotic stress analysis, two pathogens were used. For the oomycete pathogen *Phytophthora nicotianae*, mycelial plugs from cultures grown on oat medium for 14 days were co-cultured with plant roots. Samples were taken at 0, 6, 12, 24, 60, and 72 h post-inoculation [50]. For the bacterial pathogen *Ralstonia solanacearum*, plants were inoculated with a bacterial suspension at OD₆₀₀=0.1; treatment with an equal amount of nutrient solution served as the control. Root and stem tissues were collected at the same time points [51]. All samples had three biological replicates. They were immediately flash-frozen in liquid nitrogen after collection and stored at -80 °C until RNA extraction.

Quantitative analysis of gene expression

The relative expression levels of target genes under different treatment conditions were measured by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Total RNA extraction was completed using the Plant

SuperPure Plantpoly RNA Kit (Takara Biomedical Technology, Beijing). Subsequently, purified RNA was used as a template to synthesize first-strand cDNA using a reverse transcription kit. This cDNA was then used as a template for qRT-PCR amplification. The qRT-PCR reaction was performed in a 15 μ L volume containing cDNA template, gene-specific primers, and SYBR Green master mix. Primers were designed using Primer Premier 6.0 software, with *GAPDH* used as an internal reference gene. The reaction program was set as follows: initial denaturation at 94 °C for 10 min; followed by 45 cycles of amplification, each cycle consisting of denaturation at 94 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 20 s. All reactions were performed with three technical replicates. The relative gene expression level was calculated and analyzed using the $2^{-\Delta\Delta CT}$ method. All primer sequences used in this section of the experiment are detailed in Table S2.

Subcellular localization of genes

To investigate the potential functional sites of the *NtIQD1*, *NtIQD13*, and *NtIQD42* proteins within the cell, this study conducted subcellular localization analysis on these three genes. First, fusion expression vectors were constructed. For the three genes *NtIQD1*, *NtIQD13*, and *NtIQD42*, specific primers (sequence information provided in Table S2) were designed and synthesized based on their respective sequences, with the stop codon excluded during primer design. Using cDNA from the tobacco cultivar K326 as a template, the coding sequence fragments of the three target genes were separately amplified by PCR. Concurrently, the empty backbone vector PC1300S-GFP was selected and linearized via double digestion with the restriction enzymes KpnI and BamHI. Subsequently, using homologous recombination cloning technology, the purified PCR products for each gene were directionally ligated with the linearized PC1300S-GFP vector, respectively, to construct the fusion expression vectors PC1300S::*NtIQD1*-GFP, PC1300S::*NtIQD13*-GFP, and PC1300S::*NtIQD42*-GFP. The recombinant plasmids were transformed into *E. coli* competent cells and selected on kanamycin-resistant plates. Positive clones were verified by colony PCR and sequencing to ensure correct gene insertion and proper reading frame.

Finally, *Agrobacterium*-mediated transient expression in tobacco leaves and fluorescence observation were performed. The fusion expression vectors constructed above, along with the empty vector PC1300S::GFP as a control, were separately transformed into *Agrobacterium* and then mixed at an equal ratio with *Agrobacterium* harboring the endoplasmic reticulum marker AtWAK2-RFP to prepare the infiltration solution. Young, healthy leaves of *Nicotiana benthamiana* were selected, and the infiltration

solution was injected into the abaxial side of the leaves using a needleless syringe. The plants were then cultured at 25 °C for 48 h [52]. A Leica STELLARIS 8 confocal microscope was used to detect the green fluorescence of GFP::*NtIQD1*, GFP::*NtIQD13*, and GFP::*NtIQD42*, the red fluorescence of the RFP marker, and the autofluorescence of chloroplasts. Bright-field images were captured using differential interference contrast microscopy to confirm cell morphology. Composite images were generated, showing the overlay of bright-field, green fluorescence, red fluorescence, and chloroplast autofluorescence (pseudo-colored purple). Fluorescence signal colocalization was captured and analyzed to determine the subcellular distribution patterns of the *NtIQD1*, *NtIQD13*, and *NtIQD42* proteins.

VIGS and drought stress assay

The VIGS experiment was assisted by Wuhan Towin Biotechnology Co., Ltd. First, the VIGS vector was constructed and *Agrobacterium* cultures were prepared. For the three target genes *NtIQD1*, *NtIQD13*, and *NtIQD42*, the SGN online VIGS design tool was used to select optimal specific target fragments of approximately 300 bp from their respective genomic confirmed sequences, aiming for high specificity and optimal silencing efficiency. Each target fragment was obtained via PCR amplification and separately cloned into the viral silencing vector pTRV2, which had been digested with NcoI and MluI, successfully constructing the recombinant plasmids pTRV2-*NtIQD1*, pTRV2-*NtIQD13*, and pTRV2-*NtIQD42*. Concurrently, pTRV2-PDS (which induces an albino phenotype) was set as a positive control, and the empty pTRV2 vector was set as a negative control. All recombinant plasmids were transformed into *Agrobacterium tumefaciens* GV3101 competent cells via electroporation for subsequent use.

Subsequently, plants were inoculated and the silencing efficiency was verified. *Agrobacterium* cultures containing the aforementioned recombinant pTRV2 vectors and the helper vector pTRV1 were mixed at equal concentrations and used to infect young leaves of four-leaf-stage *Nicotiana benthamiana* plants via injection [53]. After inoculation, plants were cultivated under normal conditions. Once the positive control (pTRV2-PDS) plants displayed the typical albino phenotype, indicating the successful establishment and function of the VIGS system, systemically infected leaves from the experimental groups (*NtIQD1*, *NtIQD13*, *NtIQD42* silenced) and the negative control group plants were collected separately. Total RNA was extracted and reverse-transcribed into cDNA. qRT-PCR was used to detect the relative expression levels of each target gene in its corresponding silenced plants to verify silencing efficiency (primers used are listed in Table S2).

Finally, drought treatment was applied and phenotypic observations were made. Plants with confirmed significant silencing efficiency via qRT-PCR and negative control plants were selected for a two-week progressive drought stress treatment. During the treatment period, overall wilting degree and leaf drooping status, among other morphological changes, were observed at regular intervals. After the treatment concluded, samples were collected for the subsequent measurement of indices (such as plant height, leaf length and width) to comprehensively evaluate the impact of gene silencing on the tobacco plants' drought resistance capability.

Results

Identification of IQD genes and phylogenetic tree classification

A total of 77 IQD genes were identified in the tobacco genome using HMM and BLASTP searches with 33 *Arabidopsis* IQD sequences as queries. These genes were named *NtIQD1* to *NtIQD77* according to their chromosomal positions (Table S3). The physicochemical properties of the 77 tobacco IQD proteins were systematically characterized, revealing substantial intra-family diversity (Table S4). Protein length varied widely from 130 to 1695 aa, with molecular weights (MW) ranging from 14.9 to 191.98 kDa. Theoretical isoelectric points (pI) spanned from acidic (5.02) to highly alkaline (12.11). Instability index analysis showed pronounced variation (31.85–72.6), with only approximately 4% of the proteins predicted to be stable (index < 40), while most were potentially unstable. All members were hydrophilic, with Grand Average of Hydropathy (GRAVY) values between -1.018 and -0.303, and exhibited aliphatic indices of 55.47 to 92.75, indicating that some proteins may have relatively high thermostability. Subcellular localization predictions suggested that most *NtIQD* proteins localize to the nucleus (approximately 54 members, 70%), followed by chloroplasts (14 members), cytoplasm (4 members), and mitochondria (3 members). *NtIQD14* was uniquely predicted to localize to the plasma membrane. A clear association was observed between protein length and subcellular localization: long-chain proteins (>1000 aa) like *NtIQD5* (1695 aa) were mainly chloroplast and nuclear-localized, whereas shorter proteins (<500 aa) tended to localize to chloroplasts or mitochondria.

To investigate the phylogenetic evolution of tobacco IQD genes, a phylogenetic tree was constructed to examine homology and evolutionary relationships among IQD family members. As shown in Fig. 1, IQD genes from tobacco and four additional species, tomato, *Arabidopsis*, rice, and maize, were classified into four subgroups (I, II, III, and IV), comprising 26, 7, 11, and 33 tobacco IQD genes, respectively. Tobacco genes were unevenly distributed across subgroups, with Subgroups I

and IV containing the largest numbers. Notably, Group IV formed a Solanaceae-enriched clade dominated by tobacco and tomato members, suggesting a lineage-specific expansion that may underlie specialized physiological functions in Solanaceae species. In contrast, monocot IQD genes from rice and maize were mainly clustered in Groups I and III. This distinct phylogenetic pattern indicates divergent evolutionary trajectories of IQD genes in dicots and monocots, with selective expansion of specific subfamilies to support lineage-specific biological roles.

Structural analysis of the IQD gene family

To further characterize structural variation within the *NtIQD* family, conserved motifs and gene architectures were analyzed across all members. As shown in the figure (Fig. 2A), the distribution of conserved motifs across the *NtIQD* family was heterogeneous, with each protein containing 3 to 6 motifs. Members belonging to the same phylogenetic subgroup, particularly Groups I and IV, exhibited highly similar motif compositions and arrangements, indicating strong structural conservation. For instance, Motif 3 and Motif 7 were consistently retained at specific positions within particular clades, suggesting their potential importance in core protein functions. In contrast, genes located at the terminal branches of the phylogenetic tree often contained unique or additional motifs, implying functional diversification or potential neofunctionalization during evolution.

Domain analysis of tobacco IQD proteins revealed substantial diversity in domain composition among family members. The DUF4005 and IQ domains were widely conserved and represented the predominant structural features of the family. Most IQD proteins, especially those in Subgroups I, II, III, and part of IV, contained both domains, which were typically adjacent or closely positioned, forming a conserved core functional module characteristic of IQD proteins. The evolutionary conservation of this module suggests a fundamental role in essential IQD functions (e.g., cytoskeleton regulation, signal transduction). Besides this core architecture, additional domains, including CH, Arm, Myosin_tail_1, and Motor_domain, were detected. The presence of these subgroup-specific domains likely contributes to functional versatility within the family, enabling the integration of calcium signaling with diverse cellular processes, including cytoskeletal dynamics and hormone-responsive pathways.

Analysis of gene structure revealed substantial variation in exon–intron organization among *NtIQD* genes. Exon numbers ranged from 2 to 41, with corresponding intron numbers (1–40). Genomic sequence lengths also varied widely, from approximately 500 bp (*NtIQD9*) to approximately 34.5 kb (*NtIQD25*). Notably, gene structure showed a strong association with phylogenetic

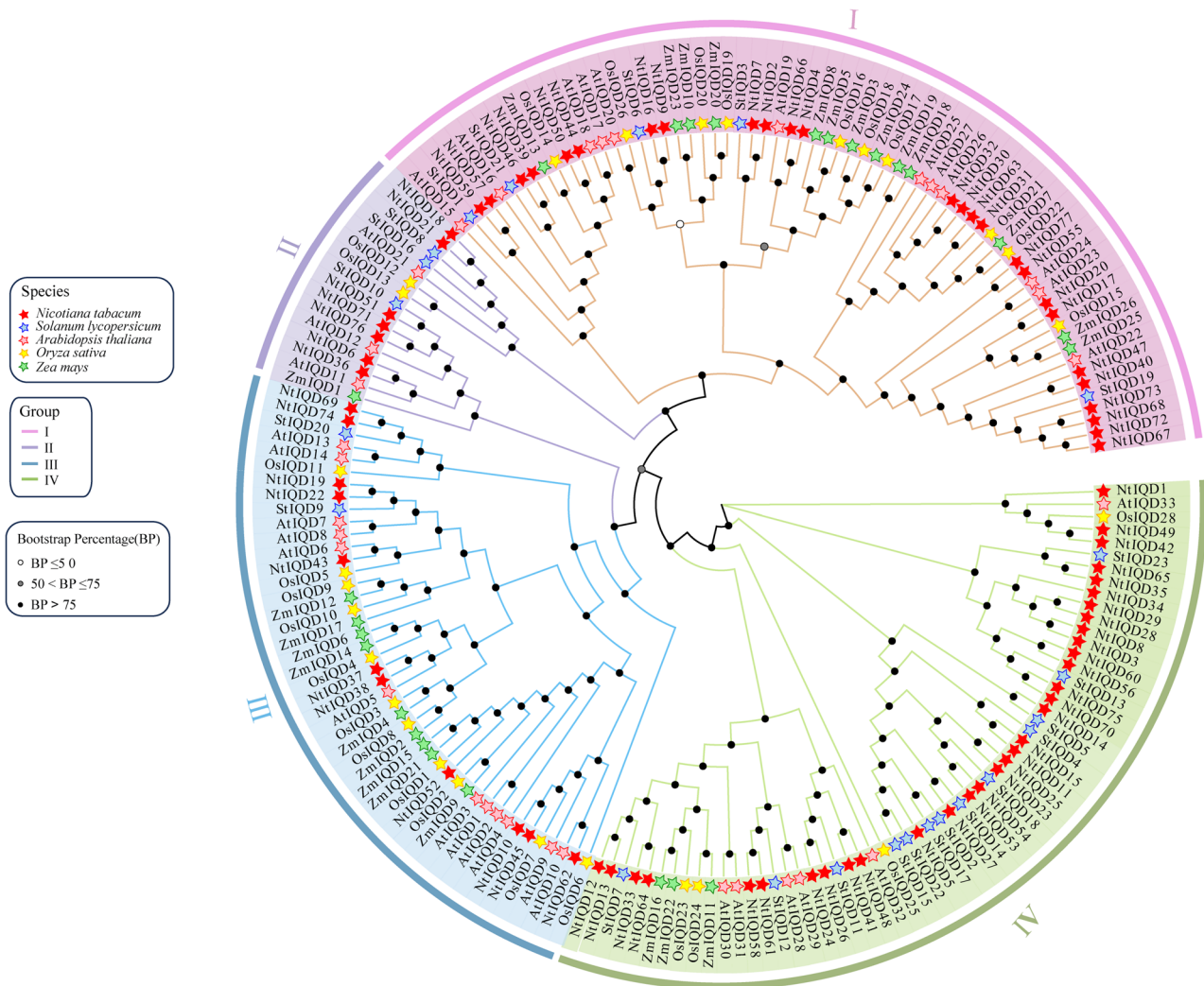


Fig. 1 Phylogenetic analysis of IQD family members from tobacco, tomato, *Arabidopsis*, rice, and maize

classification. Members within the same subgroup generally shared similar exon–intron patterns and gene lengths, supporting the robustness of the phylogenetic classification. For example, certain Group IV members exhibited longer genomic sequences with numerous exons, whereas many Group I members were comparatively compact. Additionally, most *NtIQD* genes contained both 5' and 3' untranslated regions (UTRs). The pronounced structural divergence among subgroups suggests that the *NtIQD* family has experienced extensive evolutionary remodeling, including intron gain and loss events.

Through MEME analysis, a total of 10 conserved motifs were identified in the *NtIQD* family, designated Motif 1 to Motif 10, with their sequence information shown in the figure (Fig. 2B). In summary, the combined analysis of phylogeny, motifs, domains, and gene structures indicates that while the *NtIQD* family retains a conserved core architecture, it has undergone significant structural

diversification to accommodate distinct biological functions.

Chromosomal localization and duplication analysis

The 77 tobacco IQD gene family members are distributed across all 24 chromosomes (Chr01–Chr24), but their chromosomal distribution is uneven. Chr15 harbors the highest number of genes, with seven members including *NtIQD39–45*, whereas Chr10, Chr13, Chr14, and Chr18 each contain only a single gene (Supplementary Fig. S1). No obvious relationship was observed between chromosomal location and gene function or structural features based on current data. For example, genes predicted to localize to chloroplasts, such as *NtIQD5* and *NtIQD17*, are not confined to specific chromosomes, and nuclear-localized genes, including *NtIQD1* and *NtIQD3*, as well as genes of varying lengths, are broadly dispersed throughout the genome. These patterns indicate that the structural and functional attributes of the *NtIQD* gene family



Fig. 2 Gene structure analysis of *NtIQD* genes. **A** From left to right, the phylogenetic tree of 77 *NtIQD* genes, conserved motifs, conserved domain structure, and gene structure are shown **B** Sequences of the top 10 conserved motifs

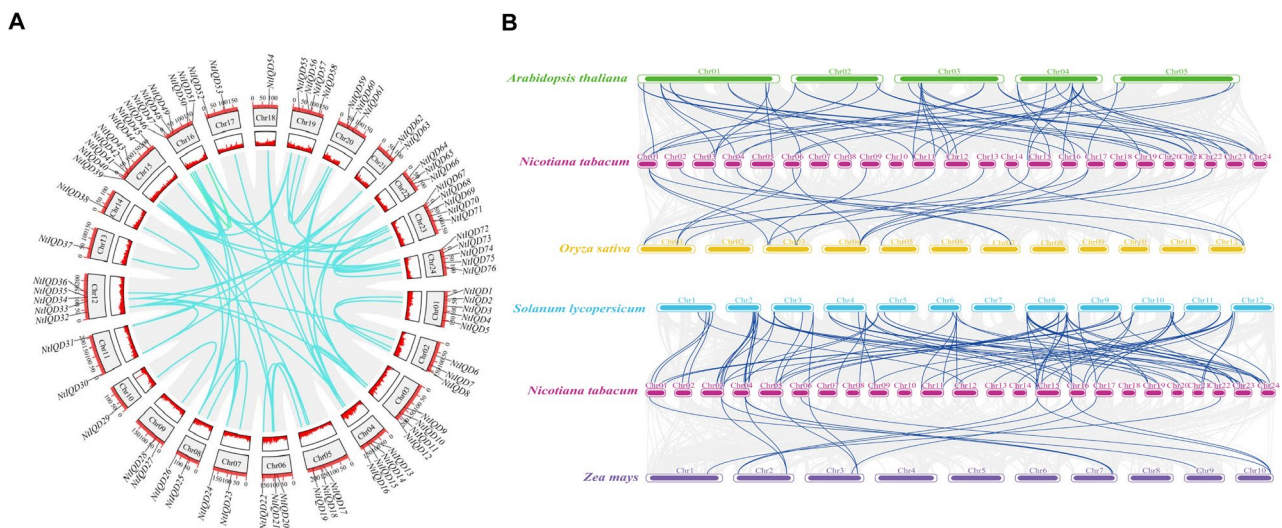


Fig. 3 Distribution and duplication of *NtIQDs*. **A** Intra-species collinearity analysis of *NtIQD* genes. **B** Collinearity analysis of IQD family genes between common tobacco, *Arabidopsis*, rice, tomato, and maize

are randomly distributed, with no evident chromosomal clustering or positional regulatory bias.

We further examined duplication events within the *NtIQD* gene family (Fig. 3A; Table S5). Tandem duplication was detected, exemplified by *NtIQD46* and *NtIQD50* on Chr16. In addition, 55 *NtIQD* gene pairs located on different chromosomes were identified, indicating widespread segmental duplications across the tobacco genome. Selection pressure analysis (Ka/Ks ratio) was performed to reconstruct the phylogeny and elucidate relationships between protein-coding sequences. All 56 duplicated pairs exhibited Ka/Ks values < 1, ranging from

0.034 (*NtIQD30/NtIQD32*) to 0.815 (*NtIQD58/NtIQD61*), indicating strong purifying selection (Supplementary Fig. S2). These results suggest limited functional divergence and a high degree of evolutionary conservation within the *NtIQD* family.

To clarify the evolutionary origin of *NtIQD* genes, synteny analysis was conducted with four representative species, including two dicots (*Arabidopsis* and tomato) and two monocots (rice and maize) (Fig. 3B). Tomato showed the highest number of collinear gene pairs with tobacco (82), followed by *Arabidopsis* (51), rice (20), and maize (15), consistent with the close evolutionary relationship

between tomato and tobacco within Solanaceae. Interestingly, no collinear *NtIQD* genes were detected on tobacco Chr 10 and 18 in any of the examined species, implying possible tobacco specificity or substantial sequence divergence. Overall, the stronger syntenic relationships with dicots than monocots are consistent with established plant phylogeny.

Cis-elements and protein regulatory networks

To investigate the transcriptional regulation of *NtIQD* genes, cis-acting elements within their 2,000 bp upstream promoter regions were analyzed. A total of 44 element types were identified and classified into three functional categories: plant growth and development (27 types), phytohormone responsiveness (11 types), and stress responsiveness (6 types) (Fig. 4A; Table S6). Among plant growth and development-related elements, 64 genes contained the light-responsive element (Box 4), followed by 61 genes harboring the conserved light-responsive G-box. Box 4 was the most abundant element and accounted for the highest proportion. For phytohormone-responsive elements, 58 genes contained the abscisic acid-responsive element ABRE, while 50 genes harbored the jasmonic acid-responsive CGTCA-motif and TGACG-motif. Additionally, 36 genes contained auxin-related elements, including the TGA-element, AuxRR-core, and TGA-box. Regarding abiotic and biotic stress-related elements, 62 genes were associated with anaerobic induction elements (ARE and GC-motif), 30 genes contained low-temperature responsiveness elements (LTR), and 28 genes harbored MYB binding sites (MBS) involved in drought inducibility.

Notably, *NtIQD25* exhibited the highest number of cis-elements associated with plant growth and development, whereas *NtIQD64* showed the greatest enrichment of phytohormone-responsive elements. In contrast, *NtIQD44* contained the highest number of stress-related cis-elements. Collectively, these findings indicate that the *NtIQD* gene family may participate in complex phytohormone-mediated regulatory networks, with functional diversification reflected by distinct cis-element compositions (Fig. 4A).

To further characterize the regulatory architecture of *NtIQD* genes, TFs and miRNA interaction networks were constructed (Fig. 4B). Regulatory relationships between TFs and *NtIQD* genes, as well as between miRNAs and *NtIQD* genes, were predicted using publicly available databases. A total of 823 potential regulatory interactions involving 38 TF families were identified, highlighting their extensive involvement in *NtIQD* regulation (Fig. 4B; Table S7). Among these, ERF, LBD, C2H2, MYB, GATA, bZIP, Nin-like, and bHLH transcription factors were the most prevalent.

Based on psRNATarget predictions, 46 miRNA families comprising 131 tobacco miRNA members were identified, forming 182 putative regulatory interactions. In total, 131 distinct miRNAs were predicted to regulate 62 *NtIQD* genes. Several miRNAs targeted multiple *NtIQD* genes; for example, nta-miR6024 targeted 13 *NtIQD* genes, while nta-miR482b-3p targeted six. Conversely, individual *NtIQD* genes were often regulated by multiple miRNA families. Notably, *NtIQD8* was targeted by eight families, including nta-miR159, nta-miR172, nta-miR395, nta-miR6144, nta-miR6145, nta-miR6151, nta-miR6158, and nta-miR6162 (Fig. 4B and Table S7). Together, these results reveal an extensive post-transcriptional regulatory network, indicating that miRNAs play an important role in fine-tuning *NtIQD* expression during development and stress responses.

To investigate potential functional associations among *NtIQD* proteins, a PPI network was constructed based on orthologous interactions in *Arabidopsis* (Fig. 4C and Table S7). This analysis identified interaction relationships among 13 *NtIQD* family members. *NtIQD10* showed the highest connectivity, interacting with five proteins *NtIQD4*, *NtIQD63*, *NtIQD66*, *NtIQD26*, and *NtIQD58*. *NtIQD1*, *NtIQD4*, *NtIQD66*, *NtIQD26*, and *NtIQD62* each interacted with three other members.

Analysis of *NtIQD* gene expression in different tissues at various growth stages

To characterize the *NtIQD* gene family expression during tobacco growth and development, tissue-specific expression of seven genes, *NtIQD1*, *NtIQD13*, *NtIQD28*, *NtIQD29*, *NtIQD42*, *NtIQD45*, and *NtIQD49*, was quantitatively analyzed at the seedling, vigorous growth, and mature stages (Fig. 5 and Table S8). Overall, most genes showed maximal expression at the mature stage, following a consistent trend of mature stage > vigorous growth stage > seedling stage. In contrast, *NtIQD1* displayed a distinct pattern, with peak expression during the vigorous growth stage.

The expression heatmap and qRT-PCR data were highly consistent, jointly validating the major expression features of these genes. Both approaches showed that *NtIQD13*, *NtIQD28*, *NtIQD29*, *NtIQD42*, *NtIQD45*, and *NtIQD49* were strongly expressed during the mature stage, with the particularly high expression of *NtIQD13* at this stage showing close agreement between datasets. Additionally, the dominant expression of *NtIQD1* during vigorous growth, the consistently high leaf expression of *NtIQD42* across all stages, and the higher root than leaf expression of *NtIQD29* during the seedling and vigorous growth stages were all confirmed by heatmap and qRT-PCR.

Further analysis demonstrated that these genes display pronounced stage- and tissue-specific expression

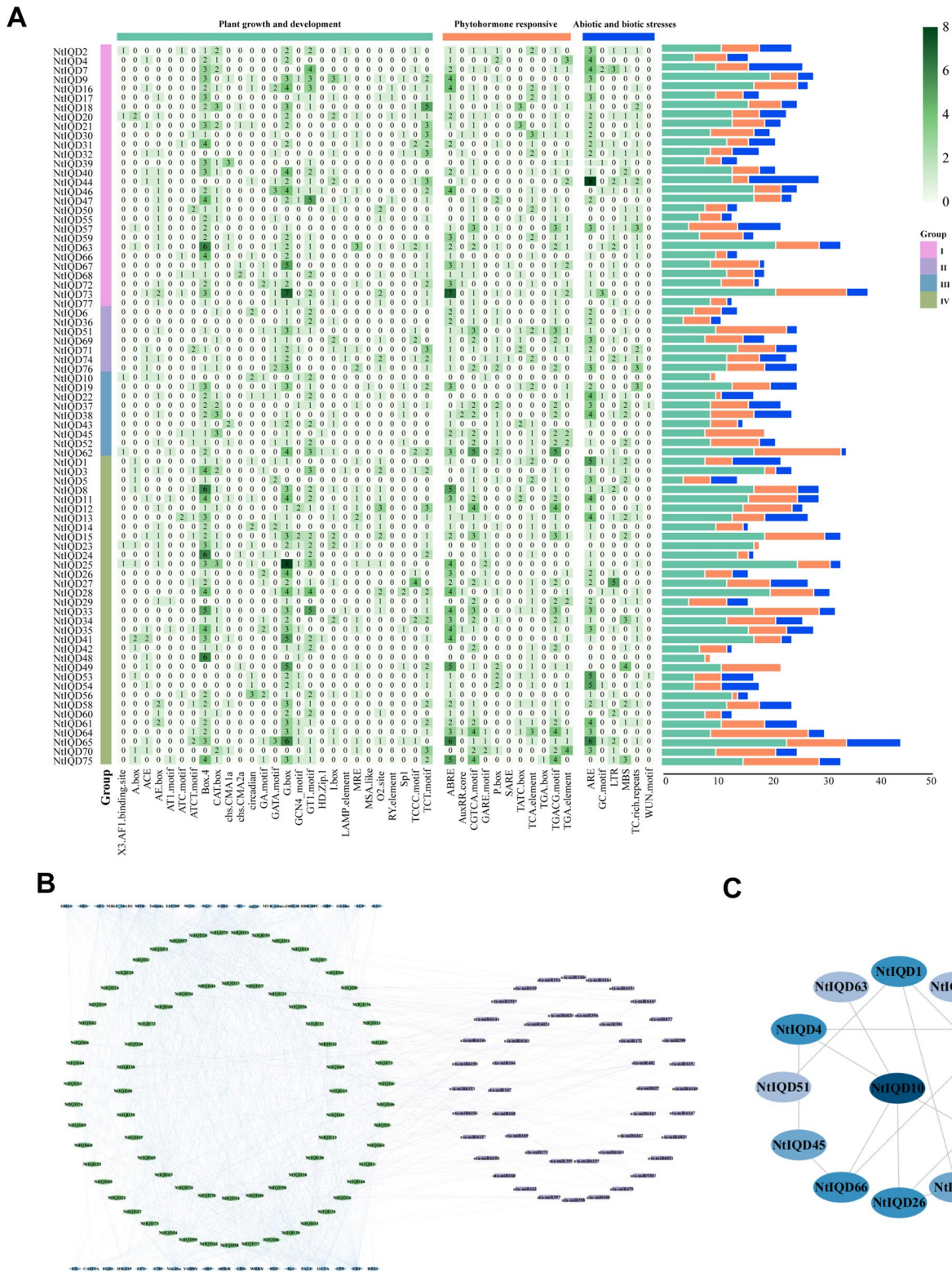


Fig. 4 Cis-acting elements and protein regulatory networks. **A** Predicted cis-elements of *NtIQD* genes. **B** Network of TFs and miRNAs regulating *NtIQD* in tobacco. **C** Protein–protein interaction network of *NtIQD* proteins in tobacco

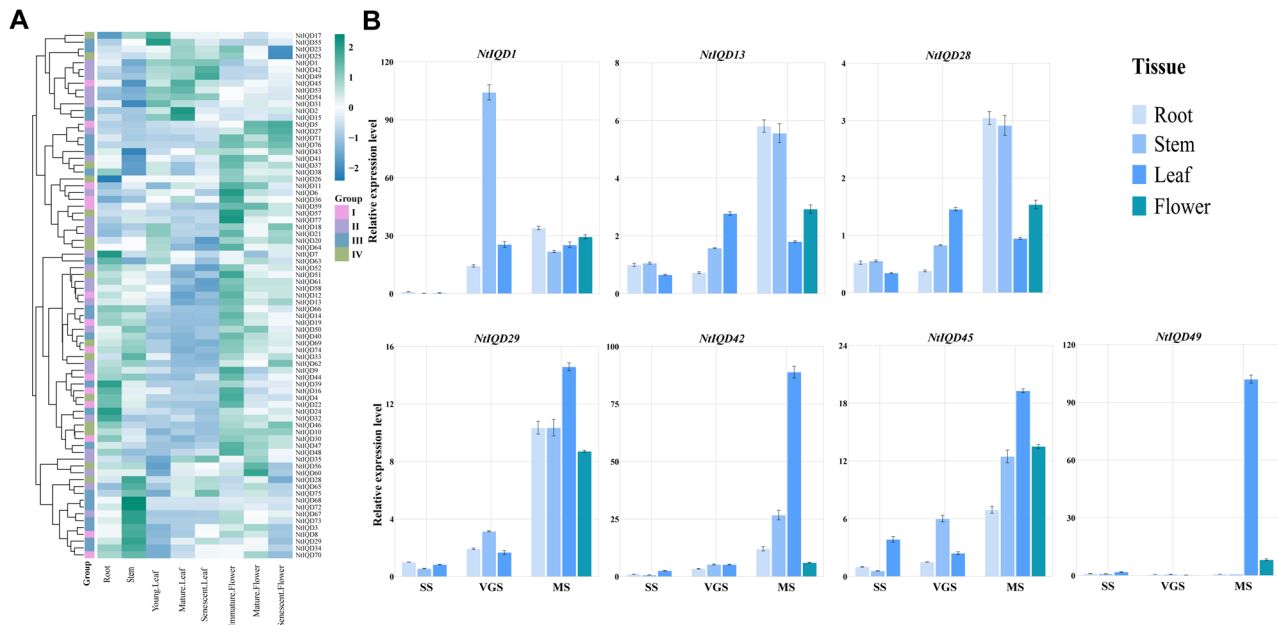


Fig. 5 Tissue-specific expression profiles of *NtIQD* genes. **A** Heatmap of expression in different tissues. Primary sequence data were downloaded from the SRA database. **B** qRT-PCR analysis results of *NtIQD* genes in different tissues at three developmental stages (seedling stage (SS), vigorous growth stage (VS), mature stage (MS))

patterns. At the seedling stage, expression was relatively dispersed across tissues: *NtIQD1* and *NtIQD29* were predominantly expressed in roots, *NtIQD13* and *NtIQD28* showed higher expression in stems, and *NtIQD42* and *NtIQD45* were most active in leaves. During the vigorous growth stage, dominant expression patterns shifted, with *NtIQD1*, *NtIQD29*, and *NtIQD45* showing higher expression in stems, whereas *NtIQD13* and *NtIQD28* were preferentially expressed in leaves. At the mature stage, *NtIQD1*, *NtIQD13*, and *NtIQD28* exhibited higher expression in roots, while the other four genes showed stronger expression in leaves. Additionally, several genes showed a gradual increase in expression from roots to stems to leaves. This gradient pattern was consistently observed for *NtIQD42* across all developmental stages, and for *NtIQD45* and *NtIQD49* specifically during the mature stage. Collectively, these distinct spatiotemporal expression patterns indicate that *NtIQD* family members may participate broadly and precisely in regulating tobacco development and tissue differentiation.

Expression analysis of *NtIQD* genes in response to abiotic and biotic stress

To further characterize the regulatory behavior of *NtIQD* genes under abiotic and biotic stresses, this study conducted analysis based on transcriptome data (Fig. 6; Table S9). Transcriptome profiling under five stress treatments revealed strong stress-type specificity and clear temporal dynamics in *NtIQD* gene expression. Under abiotic stress conditions, cold treatment consistently induced

upregulation of *NtIQD28* and *NtIQD29*, with peak expression at 8 h, whereas *NtIQD42* and *NtIQD49* exhibited distinct response patterns (Fig. 6A). Heat stress elicited pronounced time-dependent effects, with *NtIQD42* maintaining high expression during prolonged exposure (20–60 days), while *NtIQD45* was strongly repressed. Drought stress triggered more complex transcriptional responses. *NtIQD28*, *NtIQD42*, and *NtIQD49* displayed a characteristic V-shaped expression pattern, with an initial sharp decline followed by partial recovery at 8 h. In contrast, *NtIQD1* and *NtIQD45* were rapidly induced during the early stages of drought stress. In response to biotic stress, Cucumber Mosaic Virus infection had limited regulatory effects on most genes at the early stage (1 day) but selectively altered expression at later stages (8–15 days). By comparison, *Phytophthora nicotianae* infection produced broader transcriptional responses at 72 h, significantly inducing several genes, including *NtIQD13* and *NtIQD45*.

Further qRT-PCR validation focused on genes showing significant expression changes. Under 4 °C cold stress, responses differed markedly among tissues (Fig. 6B). In roots, *NtIQD1*, *NtIQD42*, *NtIQD45*, and *NtIQD49* were significantly downregulated relative to the control, whereas *NtIQD28* and *NtIQD29* were significantly upregulated. In contrast, leaves showed upregulation of *NtIQD1*, *NtIQD28*, and *NtIQD42*, with *NtIQD28* displaying a consistent induction pattern in both roots and leaves. Overall, most genes exhibited higher expression levels in leaves than in roots; for example, *NtIQD1*,

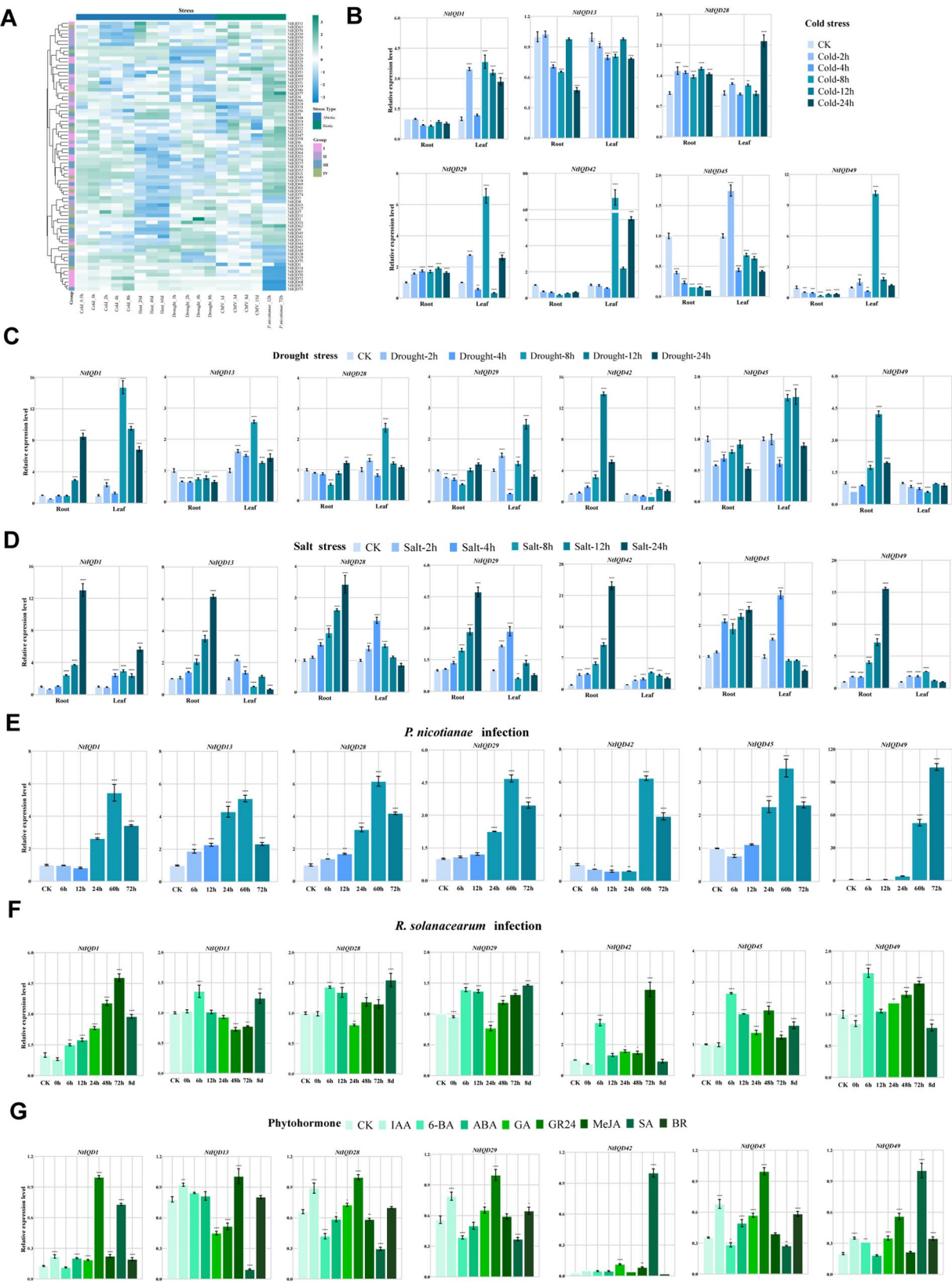


Fig. 6 (See legend on next page.)

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Fig. 6 Expression profiles of *NtIQD* genes under biotic and abiotic stress treatments. **A** Heatmap of *NtIQD* gene expression under various stresses (cold, heat, drought, CMV infection, *P. nicotianae* infection). Primary sequence data were downloaded from the SRA database. The heatmap color gradient represents gene expression levels. **B–G** qRT-PCR analysis results of *NtIQD* genes for cold stress **B**, drought stress **C**, salt stress **D**, *P. nicotianae* infection **E**, *R. solanacearum* infection **F**, and plant hormone treatments **G**. The figure shows the relative gene expression under various stress treatments. Statistical significance was determined by two-way ANOVA with Sidak's test **B–D** and one-way ANOVA followed by Dunnett's test **E–G**. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

NtIQD29, *NtIQD42*, *NtIQD45*, and *NtIQD49* followed this trend at most time points. Notably, *NtIQD42* expression in leaves increased sharply to 66.8 after 8 h of cold treatment, while *NtIQD49* and *NtIQD29* also reached relatively high levels. These qRT-PCR results were highly consistent with the heatmap analysis, providing clear mutual validation, particularly for the expression pattern of *NtIQD29*.

Under drought stress, *NtIQD* genes exhibited distinct spatiotemporal expression dynamics (i.e., varying across different times and tissues). *NtIQD1* was significantly upregulated in both roots and leaves, whereas other members, including *NtIQD13*, *NtIQD42*, and *NtIQD45*, showed pronounced tissue-specific responses (Fig. 6C). *NtIQD13* was significantly downregulated in leaves but upregulated in roots, indicating differentiated regulatory roles across organs. With increasing drought duration, most genes displayed a general pattern of initial downregulation followed by recovery. This dynamic trend was also evident in the heatmap, with changes in *NtIQD28*, *NtIQD42*, and *NtIQD49* being particularly prominent. Regarding expression peaks, *NtIQD42* and *NtIQD49* reached higher maximum levels in roots than in leaves, whereas *NtIQD1*, *NtIQD13*, *NtIQD28*, *NtIQD29*, and *NtIQD45* peaked more strongly in leaves. Among these responses, the upregulation of *NtIQD1* (leaf, 8 h, 14.7) and *NtIQD42* (root, 12 h, 13.83) was especially pronounced.

In the *Phytophthora nicotianae* infection experiment, all seven genes exhibited a consistent upregulation trend after 72 h of stress treatment (Fig. 6E). Most genes (*NtIQD1*, *NtIQD13*, *NtIQD28*, *NtIQD29*, *NtIQD42*, and *NtIQD45*) peaked at 60 hpi. In contrast, *NtIQD49* showed a pronounced and specific induction, increasing to 103.8-fold at 72 hpi. This exceptional response suggests that *NtIQD49* may function as a key regulator in defense against *Phytophthora* infection.

From 72 h to 8 days post-infection with *Ralstonia solanacearum*, all seven examined *NtIQD* genes showed varying degrees of upregulation (Fig. 6F). *NtIQD1* and *NtIQD42* exhibited intermediate expression peaks at 72 hpi, with 4.78- and 5.54-fold increases, respectively. In contrast, *NtIQD28* and *NtIQD29* displayed delayed induction, reaching maximum expression at 8 days post-infection. This temporal differentiation indicates a coordinated defense response involving early activation followed by sustained gene induction.

Phytohormone response analysis revealed that the promoter regions of *NtIQD* genes contain multiple hormone-responsive elements, and their expression patterns varied depending on the hormone treatment (Fig. 6G). Under IAA treatment, all seven genes were upregulated. GA treatment induced upregulation of all genes except *NtIQD13*, while BR treatment upregulated all genes except *NtIQD42*. Notably, under SA induction, *NtIQD42* exhibited an exceptionally strong upregulation that far exceeded its response to other hormones. Simultaneously, *NtIQD1* and *NtIQD49* were significantly upregulated, whereas *NtIQD13* was significantly downregulated. Additionally, *NtIQD1*, *NtIQD29*, and *NtIQD45* showed significant upregulation under GR24 treatment. Together, these complex expression patterns indicate that *NtIQD* family members likely function as integrative hubs coordinating multiple hormone signaling pathways, particularly the SA-mediated defense pathway, to balance plant immunity and growth.

Subcellular localization and the role of *NtIQD* in drought resistance

To further clarify the cellular functions of key candidate genes, subcellular localization and VIGS analyses were conducted for *NtIQD1*, *NtIQD13*, and *NtIQD42*, which showed significant responses under drought stress. Subcellular localization analysis (Fig. 7A) showed that *NtIQD1* localized to both the nucleus and the cytoplasm/endoplasmic reticulum, indicating potential roles in nuclear transcriptional regulation and cytoplasmic signal transduction. *NtIQD13* was mainly distributed in the cytoplasm/endoplasmic reticulum and the cell membrane, suggesting involvement in membrane-associated signaling or material transport. In contrast, *NtIQD42* was exclusively localized to the cytoplasm/endoplasmic reticulum. Despite sharing conserved domains, these distinct localization patterns indicate that *NtIQD* family members have diversified to operate in different cellular compartments, thereby supporting functional specialization.

To validate candidate genes that may function as positive regulators of drought stress, VIGS was used to suppress *NtIQD1*, *NtIQD13*, and *NtIQD42* expression in tobacco. A phytoene desaturase (PDS) vector served as the positive control. Three weeks post-inoculation, plants displayed a clear albino phenotype, confirming the effective operation of the VIGS system. Following verification of silencing efficiency, drought stress was imposed on both gene-silenced plants and empty vector

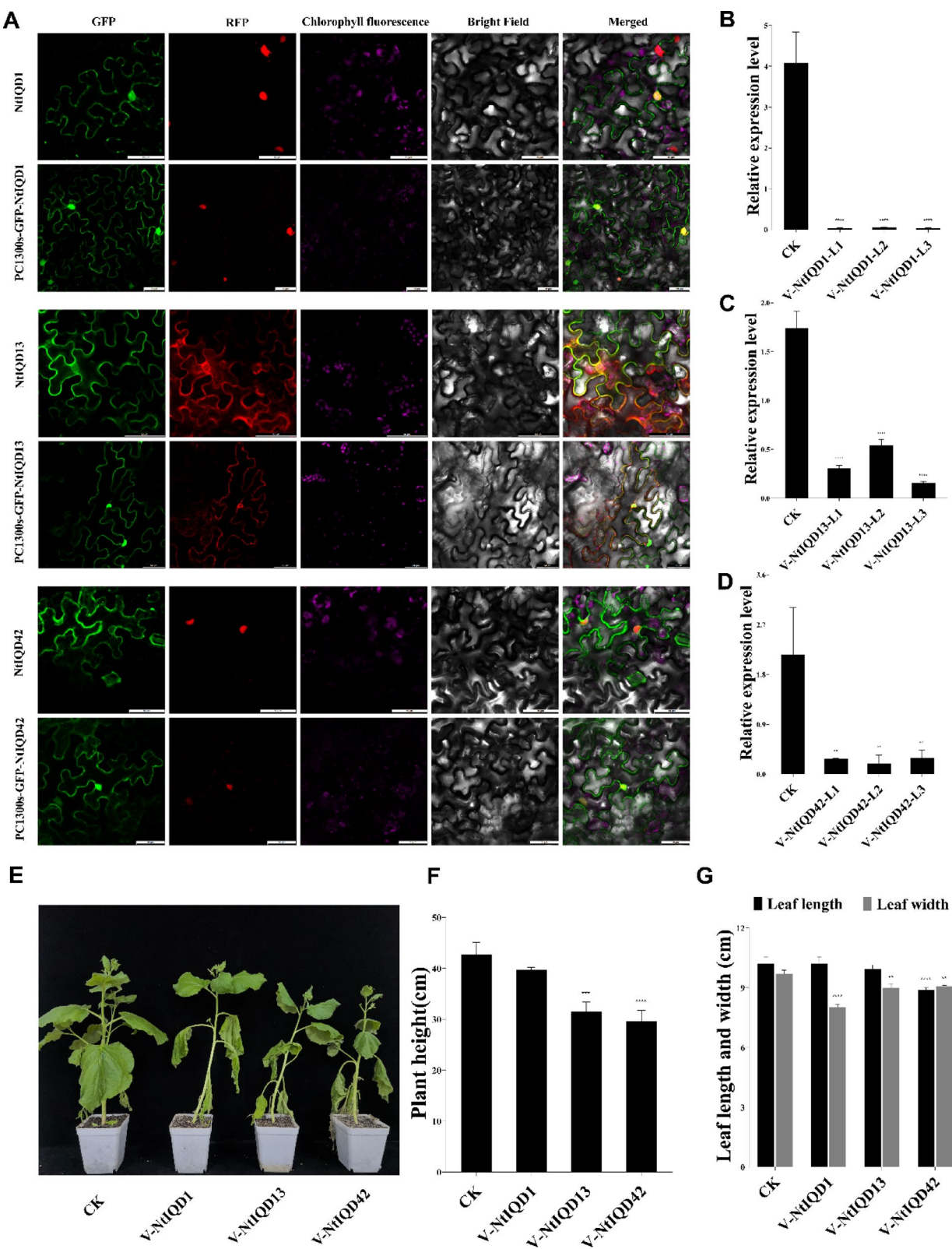


Fig. 7 (See legend on next page.)

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Fig. 7 Subcellular localization and drought phenotype of *NtIQD* proteins in silenced plants. **A** Subcellular localization of *NtIQD1*, *NtIQD13*, and *NtIQD42* in *Nicotiana benthamiana* leaf epidermal cells. **B** Expression levels of *NtIQD1* in control and silenced plants. **C** Expression levels of *NtIQD13* in control and silenced plants. **D** Expression levels of *NtIQD42* in control and silenced plants. **E** Phenotypes of control plants CK and silenced plants affected by drought stress. **F** Height statistics of control plants CK and silenced plants. **G** Leaf length and width statistics of control plants CK and silenced plants. Statistical significance was determined by one-way ANOVA followed by Dunnett's test **B–F** and two-way ANOVA followed by Tukey's test **G**. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

controls. qRT-PCR analysis showed that the expression of all three target genes was significantly reduced in their respective VIGS lines compared with controls. Among them, *NtIQD1* exhibited the strongest downregulation (Fig. 7B–D). This result is consistent with its pronounced drought-induced upregulation reported previously, further supporting its proposed role as a key positive regulator of drought tolerance.

All gene-silenced plants displayed growth inhibition and enhanced drought sensitivity, although the magnitude of morphological changes varied among genes. Plant height was significantly reduced in *NtIQD13*- and *NtIQD42*-silenced plants, whereas *NtIQD1*-silenced plants showed a milder but consistent reduction. Under drought conditions, all silenced plants exhibited leaf wilting and yellowing. Quantitative measurements further indicated that leaf width was significantly decreased in all VIGS lines, with the most pronounced reduction observed in *NtIQD1*-silenced plants, followed by *NtIQD13*- and *NtIQD42*-silenced plants (Fig. 7E–G). Reduced leaf width is a typical consequence of diminished cell turgor caused by water loss and directly reflects the severity of wilting.

In summary, subcellular localization analysis revealed that *NtIQD1*, *NtIQD13*, and *NtIQD42* are distributed in distinct cellular compartments. VIGS assays demonstrated that, despite differences in silencing efficiency and effects on specific growth traits (e.g., plant height), suppression of each gene consistently resulted in severe leaf wilting and yellowing under drought stress. These findings provide strong evidence that *NtIQD1*, *NtIQD13*, and *NtIQD42* act as positive regulators of drought stress responses in tobacco, likely through complementary or functionally specialized mechanisms.

Discussion

Plants respond to environmental stresses through complex regulatory networks initiated by Ca^{2+} signalling [54]. As a major class of CaM-binding proteins, the IQD family plays a central role in coupling calcium signals with downstream cellular responses [7]. In this study, we conducted the first genome-wide identification and characterization of the IQD gene family in tobacco and identified 77 *NtIQD* members. We further analyzed their phylogenetic relationships, structural features, expression patterns, and potential roles in drought resistance.

The following discussion integrates these findings with previous reports.

Expansion and evolution of the IQD gene family

Gene family expansion is a key driver of plant adaptive evolution and functional diversification [55]. The tobacco IQD family contains 77 members, substantially exceeding the numbers reported in *Arabidopsis* (33) and rice (29) [10]. Similar large-scale expansions have been documented for other gene families in tobacco and are closely associated with multiple rounds of whole-genome duplication and polyploidization, consistent with its origin as an ancient allotetraploid [56]. Gene redundancy generated by whole-genome duplication provides opportunities for sub-functionalization or neo-functionalization [57]. Supporting this view, collinearity analysis revealed a large number of conserved gene pairs between tobacco and tomato, another Solanaceae species, far exceeding those detected in comparisons with *Arabidopsis* or monocot species. This pattern agrees with previous analyses of Solanaceae gene families [25] and supports lineage-specific expansion of the IQD family following Solanaceae divergence. Notably, phylogenetic Group IV showed the highest enrichment of tobacco and tomato IQD members. The Ka/Ks ratios of homologous gene pairs within this group were consistently < 1 , indicating strong purifying selection during expansion [58]. The long-term retention of multiple copies under purifying selection suggests functional specialization or dosage-sensitive roles in growth, development, or stress adaptation in Solanaceae plants [59]. For instance, the tomato IQD gene SUN is a key regulator of fruit elongation, and its functional emergence is linked to a specific duplication event. Together with our results, these observations suggest that expansion of *NtIQD* genes, particularly within Group IV, may support fine-tuned regulation of complex Solanaceae-specific traits, including fruit development, specialized secondary metabolism, and stress resistance.

Diversity in gene structure and regulatory elements

Variation in gene structure and cis-regulatory elements underlies functional differentiation [60]. This study revealed substantial diversity among *NtIQD* genes in coding sequence length, exon–intron organization, conserved motifs, and protein domains. Notably, the shared architecture of a conserved core functional module (IQ

domain), coupled with variable auxiliary modules mirrors patterns reported in *Arabidopsis* and rice IQD families and represents a common mechanism for gene family diversification [61]. For example, *Arabidopsis AtIQD5* differs from other members in subcellular localization and function because it contains a unique coiled-coil domain. Similarly, the CH and Arm domains identified here have been reported in IQD proteins from other plant species and are associated with specific protein–protein interactions or cellular localization. Promoter cis-acting element analysis further highlights the regulatory complexity of *NtIQD* genes. All members are enriched in elements related to responses to abiotic stresses (e.g., drought, low temperature) and hormonal signals (e.g., ABA, MeJA, SA). These features are consistent with previous reports describing the involvement of IQD genes in diverse stress responses across species [1]. Moreover, distinct combinations of promoter elements are likely to confer gene-specific responsiveness to particular stresses or hormones [62]. This interpretation is supported by the differential expression of *NtIQD* genes under hormone treatments (e.g., the strong induction of *NtIQD42* by SA) [63]. Collectively, these findings suggest that *NtIQD* genes function as integrative nodes linking calcium signaling with multiple hormonal pathways to coordinate plant adaptive growth.

Functional specialization suggested by subcellular localization and expression patterns

The subcellular localization of a protein is closely linked to its functional roles. Both computational prediction and experimental validation in this study indicate that *NtIQD* members are distributed across multiple compartments, including the nucleus, cytoplasm, endoplasmic reticulum, and cell membrane. Similar localization diversity has been systematically reported for the *Arabidopsis* IQD family and is strongly associated with functional differentiation across biological processes. For example, nuclear-localized *AtIQD1* is implicated in transcriptional regulation [14], whereas IQD proteins associated with the plasma membrane or microtubules primarily participate in signal transduction and cytoskeletal organization [64]. Consistent with this framework, the three *NtIQD* proteins (*NtIQD1*, *NtIQD13*, *NtIQD42*) exhibited distinct localizations: nucleus/cytoplasm, membrane/cytoplasm, and endoplasmic reticulum/cytoplasm, respectively. These patterns suggest functional stratification across hierarchical cellular processes, ranging from signal perception (membrane), signal transduction and integration (cytoplasm/ER), to transcriptional regulation (nucleus). Expression profiling further supports functional specialization among *NtIQD* genes. Clear tissue specificity and developmental stage specificity were observed, with particularly high expression during the mature stage. This

pattern is notable because reproductive processes such as pollen tube elongation depend strongly on calcium gradients and cytoskeletal dynamics [65], suggesting an important role for *NtIQD* proteins in tobacco reproductive development. *NtIQD* genes also displayed subgroup-specific expression responses to abiotic stress [66]. For example, under cold stress, some Group II members were upregulated, whereas many Group III members showed downregulation or no significant change. In contrast, drought stress induced upregulation in some Group III members. Similar subgroup-dependent expression patterns have been reported for IQD families in maize and pepper, indicating conserved functional modularization. Distinct IQD subgroups may therefore be selectively recruited to respond to specific stress conditions by regulating different downstream pathways (e.g., osmotic adjustment, reactive oxygen species homeostasis, or stomatal movement).

Functional validation of *NtIQD1/13/42* in drought stress response

Based on expression profiling, *NtIQD1*, *NtIQD13*, and *NtIQD42* were selected for loss-of-function analysis. VIGS assays demonstrated that silencing each of these genes caused a significant wilting phenotype in tobacco under drought stress, confirming their positive roles in drought resistance [67]. This finding is consistent with functional analyses of IQD genes in other plant species [68]. For example, overexpression of Chinese cabbage *BrIQD31* improved drought tolerance in transgenic tobacco, while wheat *TaIQD3* enhanced drought tolerance in *Arabidopsis*. Notably, the three *NtIQD* genes exhibited functional heterogeneity. Although silencing any of them increased drought sensitivity, their effects on growth parameters such as plant height and leaf width differed. When considered alongside their distinct subcellular localizations, these differences suggest that the genes contribute to drought resistance through partially divergent molecular mechanisms. Specifically, the nuclear localization of *NtIQD1* implies a role in regulating drought-responsive gene transcription. In contrast, the membrane-associated localization of *NtIQD13* suggests involvement in early stress perception or transmembrane signal transduction, whereas the ER/cytoplasmic localization of *NtIQD42* points to functions related to ER-microtubule interactions or protein homeostasis under stress conditions. Similar combinations of functional divergence and localization diversity have been reported for other gene families [69]. Collectively, these observations reflect a common plant strategy in which multiple members of a gene family operate across cellular compartments to form a multilayered, partially redundant, yet functionally specialized stress response network [70].

Summary and outlook

In summary, this study presents the first comprehensive genome-wide characterization of the *NtIQD* gene family in tobacco. The results show that this family has undergone marked lineage-specific expansion and diversification, giving rise to members with distinct protein structures, subcellular localizations, and spatiotemporal expression patterns. Importantly, functional validation using VIGS identified *NtIQD1*, *NtIQD13*, and *NtIQD42* as positive regulators of drought tolerance. These findings support a model in which *NtIQD* proteins function as versatile hubs downstream of calcium signaling, integrating environmental signals to coordinate plant growth and stress adaptation. This study provides a foundation for elucidating the molecular mechanisms of IQD proteins in crop species. Future research could be advanced in several directions [71]. First, CRISPR-Cas9-mediated generation of stable mutants or overexpression lines for key *NtIQD* genes would enable functional validation under more rigorous genetic backgrounds and facilitate detailed phenotypic analyses. Second, interaction partners of *NtIQD* proteins, including CaM/CML proteins, transcription factors, and cytoskeletal components, could be identified using yeast two-hybrid screening, co-immunoprecipitation, or proximity labeling approaches to clarify their roles within protein–protein interaction networks [72]. Third, combining chromatin immunoprecipitation sequencing (ChIP-seq) using antibodies against IQD proteins with RNA-seq analysis would enable identification of direct downstream targets [73, 74]. Finally, the roles of *NtIQD* genes in additional biological processes (e.g., biotic stress resistance, secondary metabolism, or organ development) merit investigation, along with assessments of their potential value for molecular breeding [75, 76]. As functional insights into the IQD family continue to expand, their application in improving crop stress tolerance is expected to become increasingly feasible [77, 78].

Conclusions

A total of 77 IQD genes were identified in tobacco, constituting a plant-specific gene family involved in calcium signal transduction. Comprehensive analyses revealed their diversity in phylogenetic evolution, protein structure, and cis-regulatory elements, and demonstrated their tissue-specific and stress-responsive expression patterns. We validated the positive regulatory roles of *NtIQD1*, *NtIQD13*, and *NtIQD42* in drought stress response through subcellular localization and VIGS assays. However, the detailed molecular mechanisms by which these IQD proteins decode calcium signals and orchestrate downstream stress-responsive networks require further elucidation.

Abbreviations

Ca ²⁺	Calcium ions
CaM	Calmodulin
IQD	IQ67-Domain
VIGS	Virus-induced gene silencing
CMLs	Calmodulin-like proteins
Ka/Ks	The ratio of non-synonymous substitution rate to synonymous substitution rate
TFs	Transcription factors
miRNAs	Micro RNAs
SRA	Sequence read archive
IAA	Indole-3-acetic acid
6-BA	6-Benzylaminopurine
ABA	Absciscic acid
GA	Gibberellin
GR24	Strigolactone
MeJA	Methyl jasmonate
SA	Salicylic acid
BR	Brassinosteroid
qRT-PCR	Quantitative real-time polymerase chain reaction
MW	Molecular weight
pI	Theoretical isoelectric point
GRAVY	Grand average of hydropathy
UTRs	Untranslated regions
LTR	Low-temperature responsiveness elements
MBS	MYB binding sites
PDS	Phytoene desaturase

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40538-026-00947-7>.

Supplementary Material 1

Supplementary Material 2

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Not applicable.

Author contributions

P.L., T.T., and Y.J. conceived and designed the experiments. T.T., Y.J., W.C., and R.H. collected and processed the transcriptome data. Y.J., J.Z., J.C., and J.T. performed the bioinformatics data analysis. J.T., Y.J., and Z.F. conducted the qRT-PCR experiments. Y.J., T.T., W.C., and P.L. contributed to writing the manuscript. X.M. and J.Z. provided technical and material support. All authors have reviewed and approved the final version.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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