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Genome-wide identification and analysis of ERF transcription factors related to abiotic stress responses in *Nelumbo nucifera*

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Abstract

Background Ethylene-responsive factor (ERF) transcription factors belong to the APETALA2/ERF (AP2/ERF) superfamily, and play crucial roles in plant development process and stress responses. However, the function of ERF proteins (especially for their role in response to abiotic stresses) remains scarce in *Nelumbo nucifera*, which is an important aquatic plant with high ornamental, economic, and ecological values.

Results A total of 107 *ERF* genes were identified from the *N. nucifera* genome, and phylogenetic analysis classified these genes into 11 groups. The *NnERF* genes in the same group exhibited similar gene structure and conserved motifs, and they were unevenly distributed across the 8 chromosomes, with three pairs of tandem duplications and 21 pairs of segmental duplications. Synteny analysis revealed 44 and 39 of *NnERF* genes were orthologous to those in *Arabidopsis thaliana* and *Oryza sativa*, respectively. Tissue-specific expression patterns analysis of *NnERF* showed that 26 *NnERF* genes were expressed in all tested tissues, in which five genes exhibited high expression levels. Furthermore, 16 *NnERF* genes were selected for exploring their responses to different abiotic stresses, including cold, salt, drought, and Cd stresses. qRT-PCR analysis revealed that all these 16 investigated genes were regulated by at least one stress treatment, and 12 genes responded to all the stress treatments with different expression patterns or levels, suggesting their potential roles in diverse abiotic stress tolerance of *N. nucifera*. Additionally, two representative stress-related NnERFs (Nn3g19628 and Nn1g06033) were confirmed to be nuclear-localized proteins and displayed transcriptional activation.

Conclusions In this study, we conducted a genome-wide identification and analysis of *NnERF* gene family related to abiotic stress responses in *N. nucifera*, which provides valuable information for further functional validation of these genes in stress responses, and forms a foundation for stress tolerance breeding in *N. nucifera* and other aquatic ornamental plants.

Keywords Sacred lotus, *ERF* gene family, Gene duplication, Expression patterns, Subcellular localization

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Background

During the growth and development, plants are inevitably exposed to various adverse conditions, such as extreme temperature, drought, excessive salt and heavy metals [1–4]. To combat with these environmental stresses, plants have evolved complex mechanisms at morphological, physiological and molecular levels, including modulating a series of genes in response to stresses [2, 4]. Transcription factors (TFs) act as a critical switch for regulation of the stress-responsive genes expression by binding to their *cis*-regulatory elements (CREs) [5, 6], and therefore they are of great importance in abiotic stress regulatory networks and improving plant resistance to multiple abiotic stresses [7].

The ethylene response factor (ERF) TFs belong to the APETALA2 (AP2)/ERF superfamily, which is one of the largest TF families in plants involved in plant development and stress responses [6–8]. The AP2/ERF superfamily is characterized by at least one highly conserved AP2 domain with 60–70 amino acids, and it can be categorized into four families, named AP2, ERF, related to ABI3/VP1 (RAV) and Soloist [6, 7]. Among them, the AP2 family members usually feature double AP2 domains, the ERF family members process a single AP2 domain, the RAV family members are composed of both an AP2 domain and an additional B3 DNA-binding domain, and the remaining small group containing only one AP2 domain but a different structure is termed as Soloist [6, 9]. Compared with other AP2/ERF families, ERF family is much larger, and it can be subdivided into ERF subfamily and dehydration-responsive element binding protein (DREB) subfamily based on the conserved amino acids in the AP2 domains. Furthermore, the DREB and ERF subfamilies are further classified into 12 groups, including I-X, VI -L and Xb-L [6, 9, 10].

To date, the ERF family has been comprehensively identified in many plant species, including *Arabidopsis thaliana*, *Oryza sativa*, *Solanum tuberosum*, *Vitis vinifera*, *Zea mays*, *Ananas comosus*, and *Sesamum indicum* [8, 9, 11–14]. Previous studies have demonstrated that ERF family proteins are implicated in various plant biological processes by regulating target gene expression via binding to CREs such as the GCC-box or DRE/C-repeat (DRE/CRT) element in the promoter, particularly the regulation of abiotic stress responses [6]. For example, three DREB subfamily members C-repeat binding factor 1 (CBF1), CBF2, and CBF3 in *A. thaliana* were demonstrated to be required for optimal seed germination, and led to basal freezing tolerance through regulation of cold-responsive gene expression by binding to the DRE/CRT motif [1]. Expression of *Glycine max GmERF3*, which belongs to group IV, was induced by high salinity, drought, abscisic acid, salicylic acid, jasmonic acid, and ethylene, and its overexpression in transgenic *Nicotiana tabacum* gave tolerance to high salinity and dehydration stresses [15]. It was reported that group IX member AtERF98 modulated ascorbic acid (AsA) biosynthesis by directly regulating the expression of AsA synthesis genes, and therefore contributing to enhanced salt tolerance in *A. thaliana* [16]. Recently, ectopic expression of *V. amurensis VaERF092* gene in transgenic *A. thaliana* transcriptionally induced cold stress-related TF gene *VaWRKY33* through binding to its promoter GCC-box, and increased cold tolerance [5]. Therefore, numerous studies have revealed that *ERF* family genes are extensively involved in responses to environmental stresses in terrestrial plants, however, their roles are rarely systematically reported in aquatic plants.

Nelumbo nucifera Gaertn. is a famous aquatic plant with an extensive native distribution throughout Asia and northern Australia. It has been cultivated for about 7000 years in Asia, for its attractive flowers and its edible seeds and rhizomes [17, 18]. With high ornamental, economic, and ecological values, *N. nucifera* is popular and widely used in wetland landscape and water remediation around the world nowadays [17, 19, 20]. Additionally, as a basal eudicot species, *N. nucifera* processes important values in evolutionary and taxonomic studies [17, 21, 22]. Thus, this plant species has gained increasing interests from the scientific community, and its global market is expanded [17, 23, 24]. Unfortunately, *N. nucifera* is facing more and more adverse conditions due to the changeable climate and water pollution, which seriously influenced the growth and quality of *N. nucifera* and limited the rapid development of *N. nucifera* industry [20, 25, 26]. Therefore, the cultivation and breeding of stress-adaptable *N. nucifera* varieties has become a major concern. In the past decades, conventional breeding methods have contributed considerably to development of *N. nucifera* varieties resistant to abiotic stresses, which are time consuming and labor intensive [24]. Alternatively, current genetic-engineering technique has great potential for molecular improvement of plant stress resistance with high efficiency and short periods [26–28], but that requires understanding regulatory mechanisms of stress resistance, especially for the specific role of TF gene families. To the best of our knowledge, the molecular function and mechanism of *ERF* gene family in *N. nucifera* responding to environmental stresses remains largely unknown. With the completion of its whole genome sequencing, molecular biology researches on *N. nucifera* have entered a new stage [17, 18, 21, 22, 29], and genome-wide identification and characterization of *ERF* family genes in *N. nucifera* can facilitate the understanding of stress tolerance mechanisms and accurate identification of *NnERF* genes corresponding to stress resistance.

In the current study, we performed genome-wide identification of ERF family in *N. nucifera*, and then comprehensively analyzed the *NnERF* gene family, covering their phylogenetic relationship, gene structure, conserved motifs, putative CREs, chromosome localization, gene duplication, and synteny analysis. Meanwhile, *NnERF* gene expression in different tissues of *N. nucifera* were analyzed combined with the transcriptome data, and expression patterns of 16 selected *NnERF* genes under different abiotic stresses were detected using quantitative real-time PCR (qRT-PCR) analysis. Furthermore, subcellular localizations and self-activation activities of some representative stress-related NnERFs were determined. Our findings will explore the function and regulatory mechanisms of *NnERF* genes in plant development and abiotic stress responses, and offer important *NnERF* gene resources for genetic improvement of *N. nucifera* resistance to abiotic stresses.

Materials and methods

Identification of *ERF* genes in *N. nucifera*

The *N. nucifera* genome protein sequences (version 2.0) were downloaded from Nelumbo Genome Database (http://nelumbo.biocloud.net) [18]. The hidden Markov model (HMM) profile of the AP2 domain (PF00847) was downloaded from the Pfam database (http://pfam.xfam.org/) [30], and the AP2/ERF family TFs were retrieved from *N. nucifera* genomic data by HMMER3 (v. 3.3) software (http://hmmer.janelia.org/) [31] with an E-value threshold of 0.1. Next, a Basic Local Alignment Search Tool protein (BLASTp) alignment against all *N. nucifera* protein sequences was subjected to perform an extensive search for candidate *AP2/ERF* genes, using AP2/ERF protein sequences from *A. thaliana* downloaded from Tair (https://www.arabidopsis.org/index.jsp/) as queries [9]. After removing all redundant sequences, the candidate-specific proteins were further validated using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) [32] and the NCBI Conserved Domain Search Service (CD Search).

The basic information of NnERF proteins, including amino acid length (aa), molecular weight (Mw) and theoretical isoelectric point (pI), was acquired from ExPasy (https://web.expasy.org/protparam/) [33]. Subcellular localization of NnERF was predicted using the Cell-PLoc2 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) [34].

Sequence alignments, phylogenetic relationship, gene structure and conserved motifs analysis of *ERF* genes in *N. nucifera*

Multiple sequence alignments of full-length amino acid sequences of ERFs in *N. nucifera* and *A. thaliana* were performed using ClustalX (vision 1.83) with the default parameters, and based on the results of the sequence alignment, a phylogenetic tree was constructed using the Maximum Likelihood (ML) method with bootstrap tests of 1,000 replications in MEGA 7.0 software. The alignment of amino acid sequences of the AP2 domains was also performed by ClustalX (vision 1.83), and visualized with Easy Sequencing in ESPript 3.0 (https://espript.ibcp.fr/ESPript/ESPript/) [35].

The exon–intron organization of *NnERF*s was obtained using TBtools software [36]. Conserved motifs in NnERFs were predicted by the Multiple Expectation Maximization for Motif Elicitation (MEME) suite 5.1.1 (http://meme-suite.org/index.html) [37], with the following parameters: optimum width 10 ~ 200 amino acids of a motif, and the maximum number of motifs being 25 [38]. The combined visualization of the phylogenic tree, the gene structure and the conserved motif of NnERFs were conducted with TBtools [36].

Chromosomal localization, gene duplication and genome synteny analysis of *ERF* genes in *N. nucifera*

The chromosomal locations of *NnERF* genes were investigated against the *N. nucifera* genome by TBtools software. Collinearity analysis within *N. nucifera* was performed using the TBtools and MCScanX with default parameters [36]. Non-synonymous (Ka) and synonymous (Ks) substitution rates of each duplicated *ERF* gene pair were calculated using the Simple Ka/Ks Calculator (NG) in the TBtools software, and the divergence time (T) was calculated by $T = Ks/(2 \times 6.1 \times 10^{-9}) \times 10^{-6}$ Mya [39]. Additionally, the syntenic relationship between *ERF* genes from *N. nucifera* and other plans (*A. thaliana* and *O. sativa*) was determined by TBtools [36].

Cis-regulatory element analysis of ERF genes in N. nucifera

To survey CREs in the promoter regions of *NnERF*s, a 2000 bp upstream sequence of the start codon ATG of each gene was analyzed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [40].

In silico expression patterns analysis of *ERF* genes in different tissues of *N. nucifera*

To analyze the expression patterns of *NnERF* genes in different tissues and developmental stages of *N. nucifera*, the published transcriptome data of *N. nucifera* downloaded from Nelumbo Genome Database (http://nelumbo.biocloud.net) [18] were used. Normalized gene expression values expressed as Fragments Per Kilobase of transcript per Million (FPKM) were transformed using $\log_2$ FPKM, and then the heatmap was drawn with TBtools [36]. The FPKM values of *NnERF* genes below 1 were considered as not expressed.

N. nucifera plant materials and diverse abiotic stresses exposure

N. nucifera 'Weishanhuhonglian' seeds were germinated in distilled water with the top part of the blunt end pierced for a week, and then the healthy and uniformly developed seedlings were transferred to 5% Hoagland solution and grown hydroponically for next two weeks at an average day/night temperature of 30 °C/23 °C under natural conditions.

Uniform three-week-old seedlings (two-leaf stage) were subjected with low temperature (4 ℃), 300 mmol/L NaCl, 20% polyethylene glycol 6000 (PEG 6000), and 30 μmol/L $CdCl_2$, respectively. The experiment was performed with three biological replicates. Leaf samples were collected at different time intervals (0, 6, 12, 24 and 48 h) after diverse stress treatments, immediately frozen in liquid nitrogen, and then transferred to an ultra-low temperature freezer at -80 °C.

qRT-PCR analysis of *ERF* genes in *N. nucifera* under different abiotic stress treatments

Total RNA was isolated from leaf samples (0.2 g fresh weight) using a plant RNA extraction kit (Vazyme, NanJing, China). cDNA synthesis was performed with the PrimeScript™ RT reagent Kit with gDNA Eraser (Vazyme, NanJing, China). A 20 μL final reaction was prepared in a 96-well plate containing 10 μL of ChamQ SYBR qPCR Master Mix (2X), 0.4 μL of ROX Reference Dye 2 (50X), 1 μL cDNA, 7.8 μL ddH_2O, and 0.4 μL of each primer. The *NnActin* gene was used as an internal reference [41]. All primers used in this study are shown in Table S1. The qRT-PCR was carried out with the QuantStudioTM Real-Time PCR system (QuantStudio5, Applied Biosystems, Hammonton, NJ, USA) as follows: 95℃ for 30 s for pre-incubation, 40 cycles at 95℃ for 10 s and 60℃ for 30 s, and the collection program of melting curve was default in the system. Each gene was tested in biological triplicates with three technical repeats. The expression analysis was calculated using the $2^{-\Delta\Delta Ct}$ method [42]. Statistical analysis was performed with Student's *t*-test at significance level of $P < 0.05$.

Subcellular localization assay

The open reading frame (ORF) sequences of Nn3g19628 and Nn1g06033 genes without stop codons were ligated to the pFAST-R05-GFP vector (CaMV 35S promoter) and pORE-R4-GFP vector (CaMV 35S promoter), respectively. Then, these constructed fusion vectors and negative control GFP were separately transformed into *Agrobacterium tumefaciens* Strain GV3101. Subsequently, the transformed bacteria were injected into transgenic *N. benthamiana* leaves stably transformed with a nuclear marker [43] for transient expression analysis. After incubation for 48 h, the injected leaves were observed on a laser confocal microscope (LSM800, Zeiss, Jena, Germany).

Transcriptional activation activity assay

The ORFs of Nn3g19628 and Nn1g06033 were cloned and ligated to the pGBKT7 vector using double restriction sites, and the fusion expression vectors of pGBKT7-Nn3g19628, and pGBKT7-Nn1g06033 were constructed. Subsequently, the fusion expression vectors, the co-transformation vector pGBKT7-53/PGADT7-T (positive control), the co-transformation vector pGBKT7-Lam/pGADT7-T and the pGBKT7 vector (negative controls) were separately transferred into yeast strain Y2HGold. The transformed yeast cells with the co-transformation vectors were cultured on SD/-Trp/-Leu medium containing X-α-gal to ensure test results reliable. Finally, the transformants with pGBKT7-Nn3g19628 and pGBKT7-Nn1g06033 were cultured on SD/-Trp deficient plates at 30℃ for 3–5 days, and then the positive colonies were transferred to SD/-Trp medium containing X-α-gal to evaluate their transcriptional activation.

Results

Genome-wide identification and phylogenetic analysis of *ERF* genes in *N. nucifera*

Putative *AP2/ERF* genes were identified from the *N. nucifera* genome using two strategies HMM search and BLASTp search. After removing the redundant and alternate forms of the same gene, a total of 133 genes containing at least one complete or partial AP2 domain were obtained in *N. nucifera*. According to the number of conserved AP2 domains and their sequence similarities, these 133 NnAP2/ERF proteins can be divided into four families: AP2 (18 members, 17 with two repeated AP2 domains and 1 with a single AP2 domain), ERF (107 members, with a single AP2 domain), RAV (7 members, with a single AP2 domain and a B3 domain), and Soloist (1 member, with a single AP2 domain which showed high homology with At4g13040 designated as a soloist [9]). Among these, the ERF family is most dominant in *N. nucifera*. The basic information for these NnERF sequences was further analyzed, and results showed that the 107 NnERF proteins varied from 75 (Nn3g19405.2) to 461 (Nn1g06634.1) aa in length, with Mw ranging from 8.43 (Nn3g19405.2) to 51.62 (Nn2g11006.2) kDa, and the pI from 4.59 (Nn8g38575.1 and Nn3g16240.1) to 10.95 (Nn1g05310.6) (Table S2). Subcellular localization prediction showed that the majority of NnERF proteins (87 of 107, 81.3%) were localized in the nucleus (Table S2).

To clearly explore evolutionary relationships and facilitate the classification of NnERFs, a phylogenetic tree was constructed based on the alignments of the full-length

amino acid sequences of 107 NnERF proteins, and 122 AtERF proteins from *A. thaliana* as reference. The phylogenetic tree showed that all these ERF proteins were categorized into twelve groups and major groups were clustered by interspecies members (except for Xb-L) (Fig. 1), indicating that the ERFs are homologous between *N. nucifera* and *A. thaliana*. According to an innovative classification criteria in *A. thaliana* [9], the NnERF family with two subfamilies (DREB and ERF subfamilies) in *N. nucifera* was further classified into 11 groups: I to X and VI-L, but lacked a Xb-L group that was present in *A. thaliana* (Fig. 1). Groups I to IV composed the DREB subfamily in *N. nucifera*, containing 7, 8, 21, 12 members, respectively; and the rest seven groups (V-X, and VI-L) constituted the ERF subfamily, with 7, 6, 7, 16, 15, 6, and 2 members, respectively (Fig. 1). Among them, group III was the largest group, which was followed by groups VIII and IX. To gain a more comprehensive insight on the DREB and ERF subfamily members in NnERF family, multiple sequence alignment analyses of the conserved amino acid sequences AP2 domain were carried out. As shown in Fig. 2, the majority of NnERF family members possessed two conserved elements YRG and RAYD within the AP2 domain regions. Almost all DREB subfamily proteins contained Val at position 14, and more than 60% had Glu at position 19; In comparison, Ala and Asp residue at position 14 and 19, respectively, were highly conserved in most ERF subfamily proteins in *N. nucifera* (Fig. 2).

Gene structure and motif composition analysis of *ERF* genes in *N. nucifera*

To characterize the structural diversity of ERF family in *N. nucifera*, we analyzed the gene structure and conserved motif of the 107 NnERFs. For gene structure, 79 *NnERF* genes were found to possess a single exon with no introns, accounting for 73.83% of the total number of *NnERF* genes, while the remaining *NnERF* genes

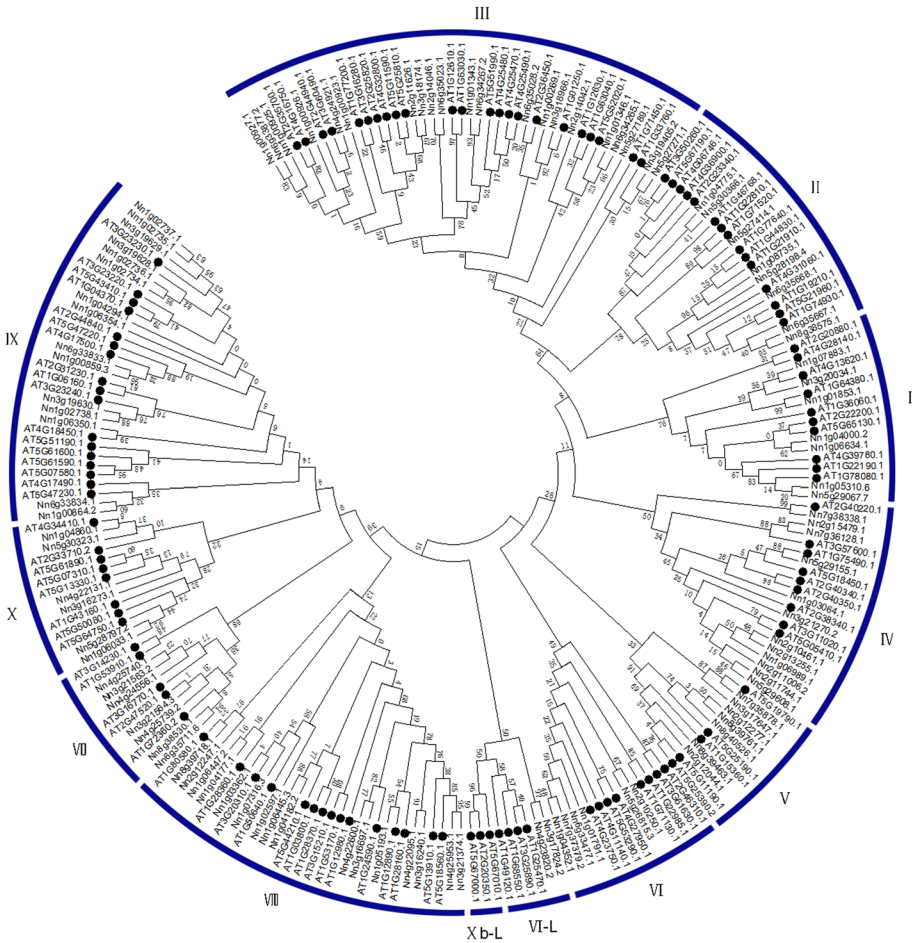

Fig. 1 Phylogenetic analysis among the ERF proteins from *N. nucifera* and *A. thaliana*. The phylogenetic tree was constructed using the Maximum Likelihood (ML) method with bootstrap tests of 1,000 replications by MEGA 7.0. The tree was divided into twelve groups, which contained I-X, VI -L and Xb-L. The ERF proteins from *A. thaliana* are indicated by black circles

Fig. 2 Multiple sequence alignment of the AP2 domain of NnERF proteins

contained introns ranging from 1 to 3 (Fig. 3b). In general, the number of exon and intron differed among the eleven groups of *NnERF* family, and members from the same group had similar gene structures. For example, all genes of groups II and VI-L and most genes of groups I, III, VIII, and IX were intronless, while the majority of genes in groups V, VII and X contained 1–3 introns (Fig. 3).

The motifs of 107 NnERF family members were analyzed by the MEME software. As shown in Fig. 3c, a total of 25 conserved motifs were investigated. Among these, motifs 1, 2, 3 and 5 were related to the AP2 domain, while the remaining 21 motifs were outside of the AP2 domain. Motifs 1–3 located in the AP2 domain regions were found in almost all NnERF family members except several proteins with incomplete AP2 domains. It is noteworthy

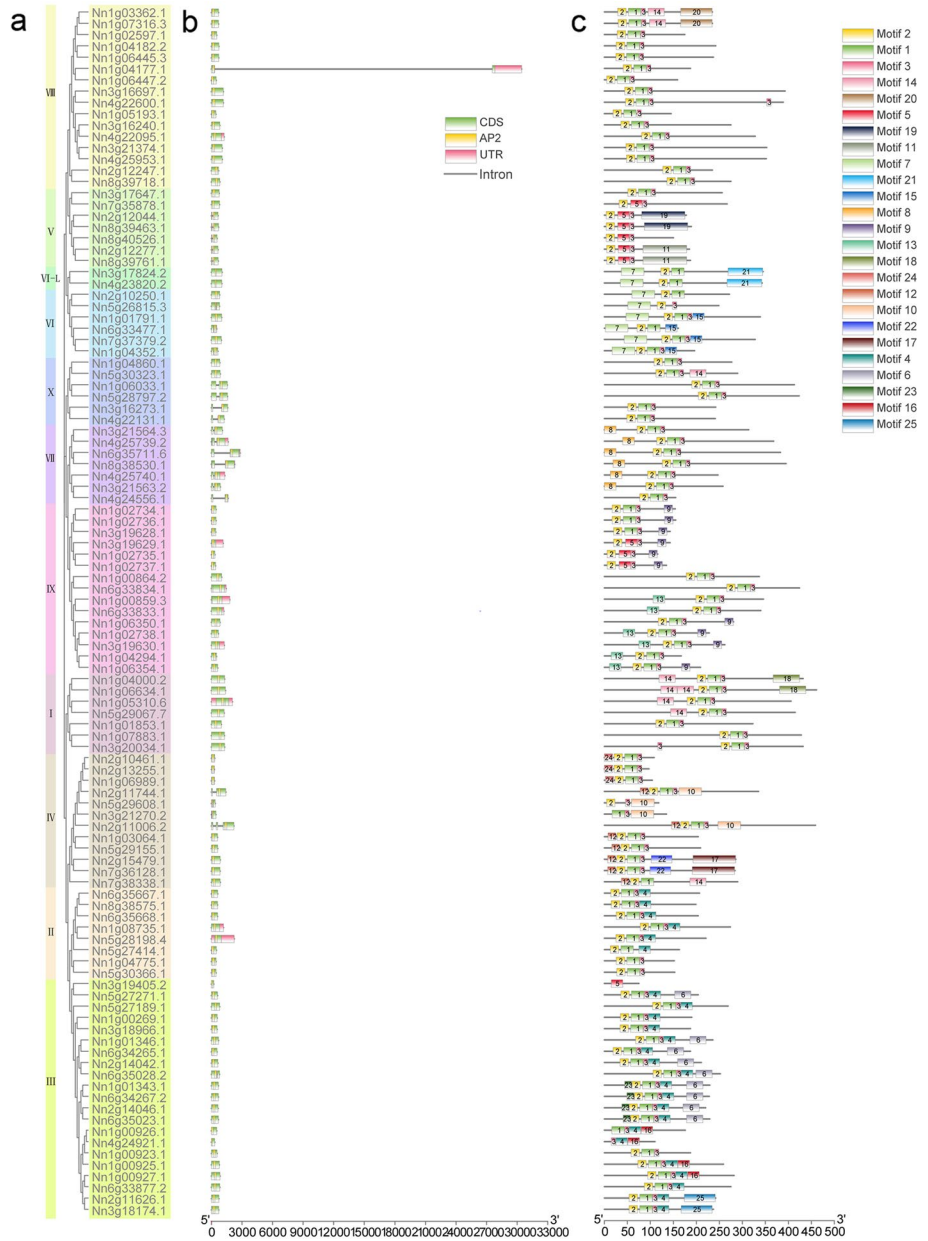

Fig. 3 Phylogenetic relationship, gene structure, and conserved motif analyses of *NnERF* genes in *N. nucifera*. **a** The unrooted tree was constructed with MEGA 7.0 using the full-length amino acid sequences of NnERF proteins. **b** Gene structure of *NnERF* genes. The length of each *NnERF* gene can be estimated using the scale at the bottom. **c** Putative conserved motifs in NnERF proteins were analyzed by MEME. The boxes with different colors represent different putative conserved motifs. The scale bar at the bottom represents the length of proteins in terms of amino acids

that the motifs outside of the AP2 domains were divergent among different groups, and NnERF members in the same group generally shared similar motif composition. For example, only the NnERF proteins of groups II and III commonly processed motif 4, proteins of groups VI and VI-L all contained motif 7, while motifs 6, 8, 9, 12, 13, 15, and 20 were specifically found in groups III, VII, IX, IV, IX, VI, and VIII, respectively (Fig. 3).

Chromosomal distribution, gene duplication and synteny analysis of *ERF* genes in *N. nucifera*

The chromosomal location of *NnERF* genes was determined from the genomic data of *N. nucifera*, and results revealed that 107 *NnERF* genes were unevenly distributed on all 8 chromosomes in *N. nucifera*, with each chromosome containing mixture of DREB and ERF subfamilies genes. Chr 1 was the largest chromosome with the highest number of 38 *NnERFs*, accounting for 35.51% of the total *NnERF* genes, whereas Chr 7 processed the lowest number (only 4 genes) (Fig. 4, and Table S2).

Given that duplication events usually contribute to the expansion of gene families and genome evolution in plants, we analyzed tandem and segmental duplication events of *NnERF* genes in the *N. nucifera* genome. Five *NnERF* genes formed three tandem duplication pairs that were located on two chromosomes, Chr 1 and Chr 6 (Fig. 4). Among these, three genes related to group III of DREB subfamily (Nn1g00925.1, Nn1g00926.1, and Nn1g00927.1) were two tandem duplication gene pairs on the same chromosome (Chr1) and adjacent to each other; Besides, another tandem duplication gene pair was assigned to group II of DREB subfamily (Fig. 4, and Table S3). In parallel to tandem duplications, 21 pairs of 42 *NnERF* segmental duplication genes were identified on the 8 chromosomes in *N. nucifera*, including 14 DREB subfamily genes (14/42, 33.33%) and 28 ERF subfamily genes (28/42, 66.67%) (Fig. 4, and Table S3).

The substitution rate (non-synonymous/synonymous, Ka/Ks) is an effective criterion to evaluate the selective pressure during gene duplication. The Ka/Ks ratio for

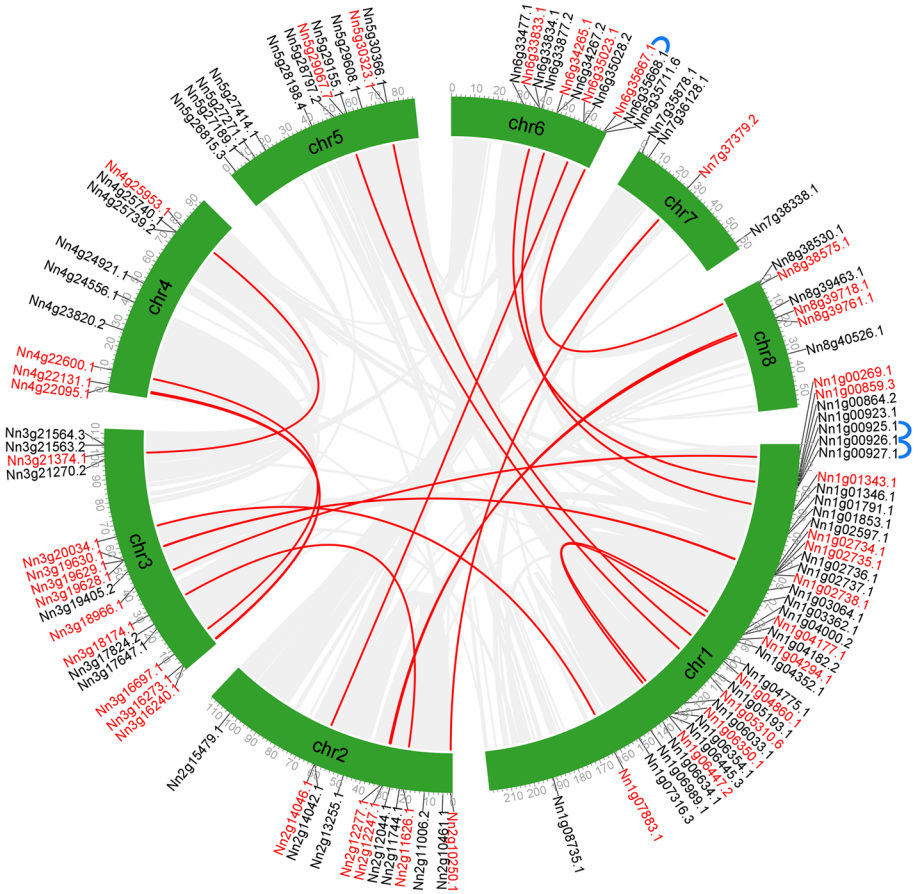

Fig. 4 Chromosome location and duplication of *NnERF* genes on the chromosomes of *N. nucifera*. 107 *NnERF* genes were mapped on the eight chromosomes of *N. nucifera*. Grey lines represent all collinear blocks in the *N. nucifera* genome. The red lines and gene ID indicate segmentally duplicated *NnERF* gene pairs, and the tandem duplicated gene pairs are indicated with blue bracket. The chromosome numbers are indicated in the middle of each chromosome, and the gray scale bar marked on the chromosome represents the length of the chromosome (Mb)

NnERF tandem duplication gene pairs varied from 0.14 to 0.42, and that for segmental duplication gene pairs ranged from 0.10 to 0.48 (Table S3). In addition, the divergence time for these *NnERF* duplication gene pairs were estimated based on Ks values, and it ranged from 17.46 to 289.74 million years ago (Mya), with a peak taking place at ~ 65 Mya (Fig. S1 and Table S3).

In order to further study the evolutionary origin of ERF family in *N. nucifera*, a syntenic map of *N. nucifera* and two representative plant species *A. thaliana* and *O. sativa* was carried out. Based on the syntenic results, 44 and 39 of *NnERF* genes in *N. nucifera* were synonymous with genes in *A. thaliana* and *O. sativa*, respectively, resulting in 96 and 72 orthologous gene pairs (Fig. 5, and Table S4). These orthologous gene pairs were distributed across all chromosomes of *N. nucifera*. Some *NnERF* genes were associated with at least four pairs of orthologous genes (especially between *N. nucifera* and *A. thaliana*), such as Nn1g00859.3, Nn6g33833.1, and Nn3g19628.1; Besides, 26 *NnERF* genes had orthologous gene pairs both in *A. thaliana* and *O. sativa*, such as Nn1g05310.6, Nn5g29067.7, and Nn3g19628.1 (Fig. 5, and Table S4).

Cis-regulatory element analysis in the promoters of ERF genes in N. nucifera

The CREs are related to the regulation of gene expression via interacting with the *trans*-acting element. To understand the response mechanisms of *NnERF* family, 2000 bp upstream sequences of *NnERF* genes were applied to predict CREs by PlantCARE online website. As a result, four main kinds of CREs involved in plant growth and development responsiveness, light responsiveness, hormones responsiveness, and stresses responsiveness were identified in the *NnERF* promoter regions. Among them, TATA-box, CAAT-box, G-Box, ABA-responsive element (ABRE), and Box 4 were the most common CREs, in which TATA-box and CAAT-box processed the highest frequency (4331 times and 3944 times, respectively) and were present in all *NnERF* genes (Table S5). It is noteworthy that a number of CREs related to hormone and stress responsiveness were found in the promoters of *NnERF* genes, such as ABRE, anaerobic response element (ARE), CGTCA-motif, TGACG-motif, W box, MBS, ethylene-responsive element (ERE), low temperature response element (LTR), and dehydration responsive element (DRE) core, with ABRE, CGTCA-motif, TGACG-motif, and ARE present in > 80% of the *NnERF* family (Table S5). These observations indicated the potential roles of most *NnERFs* in adaption to environmental stresses in *N. nucifera*. In addition, some duplication gene pairs in the same group shared almost identical CREs. For example, Nn1g05310.6 and Nn5g29067.7 assigned to group I had similar proportion of CREs, while group IX members Nn1g02734.1 and Nn3g19628.1 harbored the same hormone and stress-responsive CREs (Table S5).

Expression pattern analysis of *ERF* genes in different tissues of *N. nucifera*

To investigate the potential function of 107 *NnERF* genes during growth and development process of *N. nucifera*, their expression patterns in various plant tissues including root, rhizome, leaf, petiole, petal, receptacle (immature and mature), stamen (immature and mature), carpel (pollinated and unpollinated), and seed (seed coat and cotyledon) were explored based on publicly available gene expression data [18]. Totally, 84 of 107 (76.50%) *NnERF* genes were expressed (FPKM > 1 or $\log_2$ FPKM > 0) in at least one of the 13 tissues and development stages tested, covering 37 members in DREB subfamily and 47 members in ERF subfamily (Fig. 6). These *NnERF* genes showed diverse expression patterns in different tissues. For example, 26 *NnERF* genes were expressed in all tested tissues and stages, in which five genes including members of groups VII (Nn3g21564,

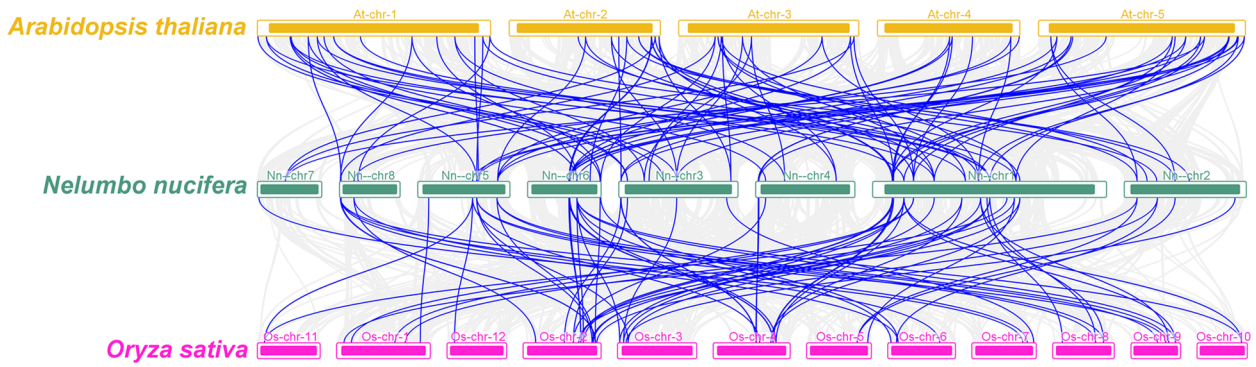

Fig. 5 Syntenic analysis of *ERF* genes between *A. thaliana*, *O. sativa*, and *N. nucifera*. Gray lines in the background represent collinear blocks within *N. nucifera*, *O. sativa*, and *A. thaliana* genomes, while blue lines highlight the syntenic *ERF* gene pairs

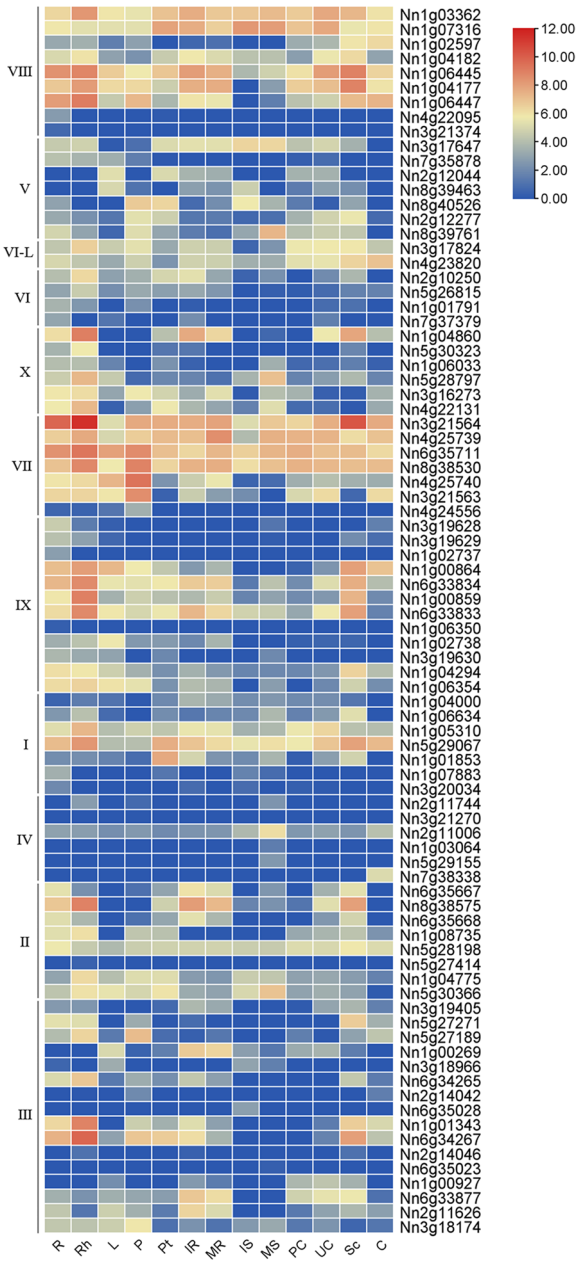

Fig. 6 Heat map for gene expression of 84 *NnERF* genes in various tissues of *N. nucifera*. 13 tissues and development stages including root (R), rhizome (Rh), leaf (L), petiole (P), petal (Pt), immature receptacle (IR), mature receptacle (MR), immature stamen (IS), mature stamen (MS), pollinated carpel (PC), unpollinated carpel (UC), seed coat (Sc) and cotyledon (C) were investigated. The color scale represents log2 of the FPKM values

Nn6g35711 and Nn8g38530) and VIII (Nn1g03362 and Nn1g07316) exhibited relatively high expression levels (FPKM > 32 or $\log_2$ FPKM > 5), indicating these genes may have important roles in plant growth and development. While the transcriptional abundances of three genes including Nn3g21374 (group VIII), Nn1g02737 (group IX), and Nn1g06350 (group IX) were specifically detected in root tissue. Moreover, 32, 36, 19, 23, 18, 29 and 17 *NnERF* genes had high expression levels (FPKM > 32 or $\log_2$ FPKM > 5) in root, rhizome, leaf, petiole, petal, seed coat and cotyledon, respectively; and 21, eight, and 14 genes showed high expression levels (FPKM > 32 or $\log_2$ FPKM > 5) in all selected development stages of receptacle, stamen and carpel, respectively (Fig. 6). These results suggest that these genes might be involved in regulating the growth of special tissues. Interestingly, some *NnERF* family members of the same group shared similar expression patterns, especially duplication gene pairs. For instance, Nn1g05310 and Nn5g29067 belonging to group I harbored similar expression in all tested tissues and stages, with the maximum expression levels in rhizome and relatively lower levels in leaf, petiole and stamen in the immature and mature stages (Fig. 6), suggesting their functional redundancy.

Expression pattern analysis of *ERF* genes in *N. nucifera* under different abiotic stress treatments

In order to further explore the responses of *NnERF* genes under abiotic stresses, 16 *NnERF* genes were selected based on their high homology with *A. thaliana* stress-related *ERF* genes. These *AtERF* genes were AT1G78080 (*RAP2.4*), AT4G36900 (*RAP2.10*/*DEAR4*), AT4G25480 (*CBF1*/*DREB1A*), AT5G51990.1 (*CBF4*/*DREB1D*), AT5G05410.1(*DREB2A*), AT3G16770.1 (*EBP*/*RAP2.3*/*ERF72*), AT1G53910.1 (*RAP2.12*), AT1G50640 (*ERF3*), AT3G23230.1(*TDR1*), AT4G17490.1 (*ERF6*), and AT5G13330.1 (*RAP2.6L*), which have been demonstrated to be involved in plant response to diverse stresses [1, 6, 7, 9, 10, 16, 27, 44–47]. The expression patterns of the 16 *NnERF* genes were examined under different abiotic stress treatments including cold, salt, drought, and Cd by qRT-PCR analysis. Overall, all these 16 investigated genes responded to at least one treatment; and 12 genes responded to all the treatments with different expression patterns or levels under different stresses, such as Nn1g01343, Nn3g19628, Nn1g02734, and Nn1g06033 (Figs. 7, 8, 9 and 10). These results suggested that *NnERF* genes may have multiple functions in *N. nucifera* response to abiotic stresses.

In the case of cold stress treatment, the majority of these 16 *NnERF* genes (13 genes) showed differential up-regulated expression patterns in comparison with those in CK (Fig. 7). Out of the 13 genes, six genes including Nn6g34265 (group III), Nn1g01343 (group III), Nn4g25739 (group VII), Nn3g19628 (group IX), Nn1g02734 (group IX), Nn6g33834 (group IX) were significantly induced at all time points after cold treatment, with most of their transcript levels increased by more

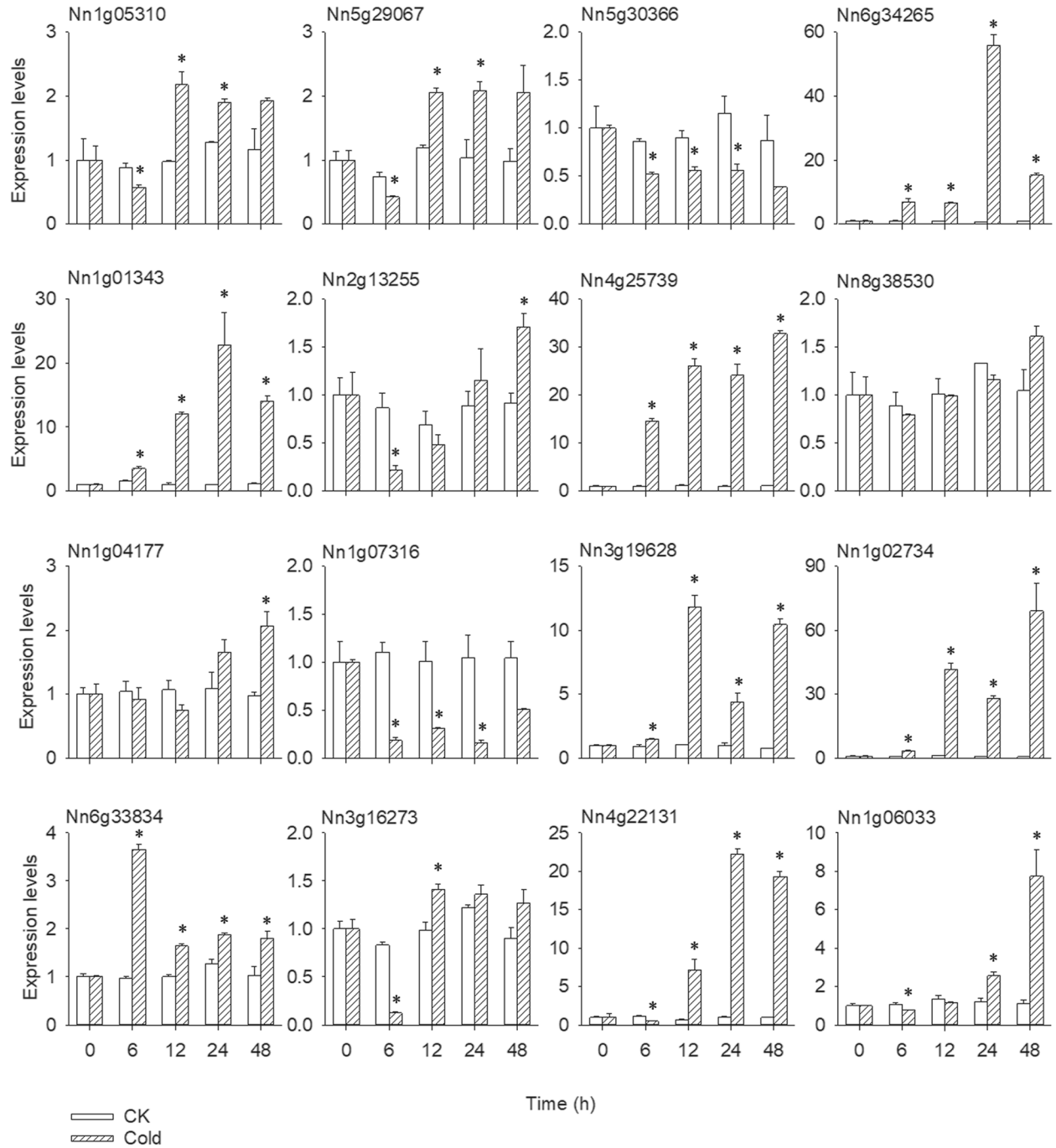

Fig. 7 Expression patterns of the 16 selected *NnERF* genes in leaves of *N. nucifera* under cold stress treatment. qRT-PCR data was normalized using *NnActin* and represent the mean ± SE of biological triplicates with three technical repeats. Asterisks (*) represent a significant difference in transcript abundance between the treatment and the control at the same time ($p < 0.05$)

than tenfold; six gene transcripts including Nn1g06033 (group X) were initially downregulated at 6 h and then significantly upregulated by cold stress; and one gene named Nn1g04177 (group VIII) exhibited similar expression levels in the early stage of cold stress response, but strongly induced at 48 h compared with CK. Among the remaining genes, two genes (Nn5g30366 and Nn1g07316) showed an obvious downward trend while 1 gene did not significantly change after cold stress. It was worth noting that some duplication gene pairs in the same group

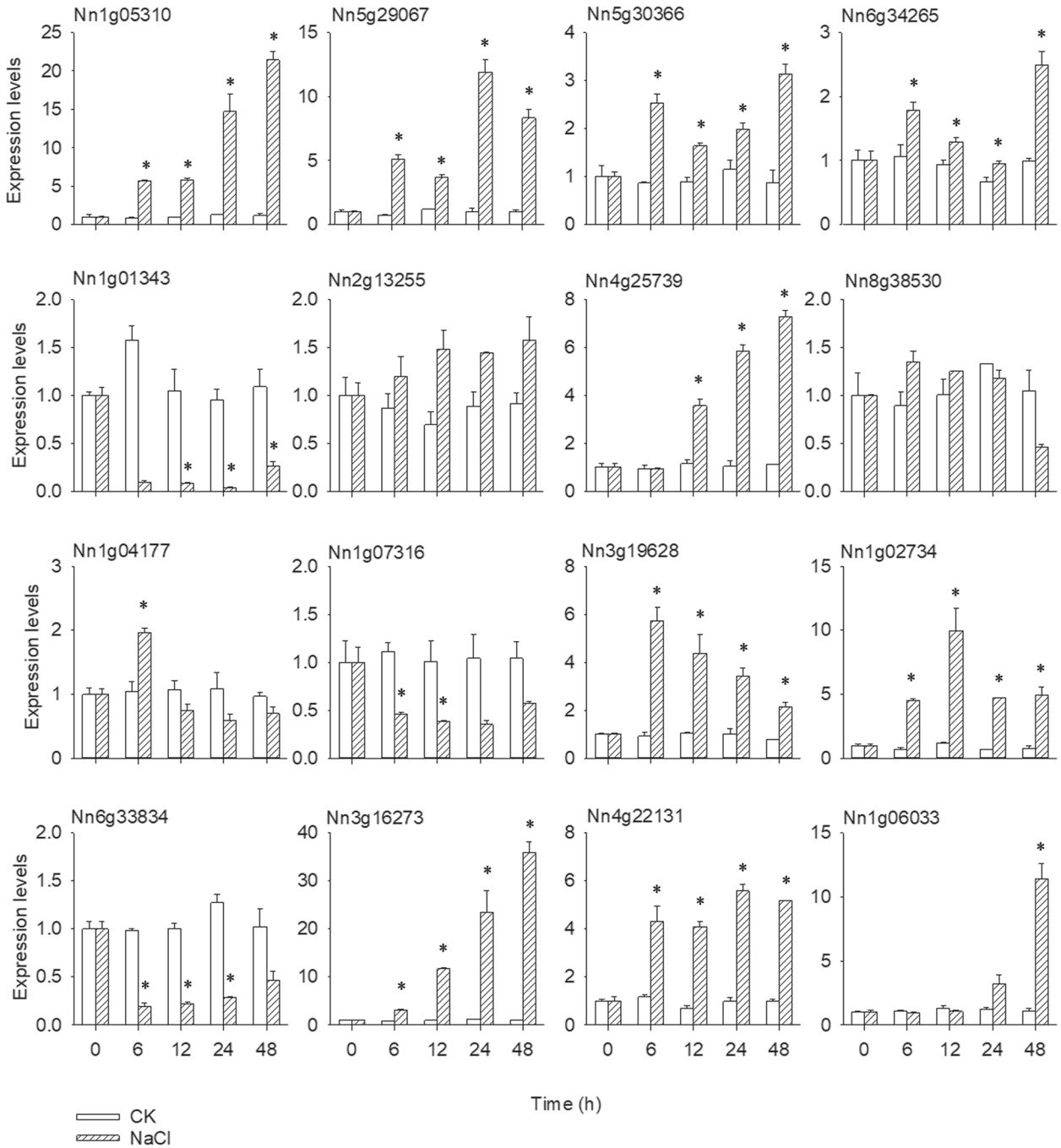

Fig. 8 Expression patterns of the 16 selected *NnERF* genes in leaves of *N. nucifera* under NaCl stress treatment. qRT-PCR data was normalized using *NnActin* and represent the mean±SE of biological triplicates with three technical repeats. Asterisks (*) represent a significant difference in transcript abundance between the treatment and the control at the same time ($p < 0.05$)

had similar expression patterns under cold stress, such as Nn1g05310-Nn5g29067 in group I, Nn6g34265-Nn1g01343 in group III, and Nn3g19628-Nn1g02734 in group IX (Fig. 7).

Compared with CK, 11 *NnERF* genes exhibited upregulated expression trends under salt stress treatment (Fig. 8). Among these genes, eight genes including duplication gene pairs of Nn1g05310 and Nn5g29067 in group I, Nn3g19628 and Nn1g02734 in group IX, and Nn3g16273 and Nn4g22131 in group X were significantly induced at all time points. Additionally, three genes (Nn1g01343, Nn1g07316 and Nn6g33834) were

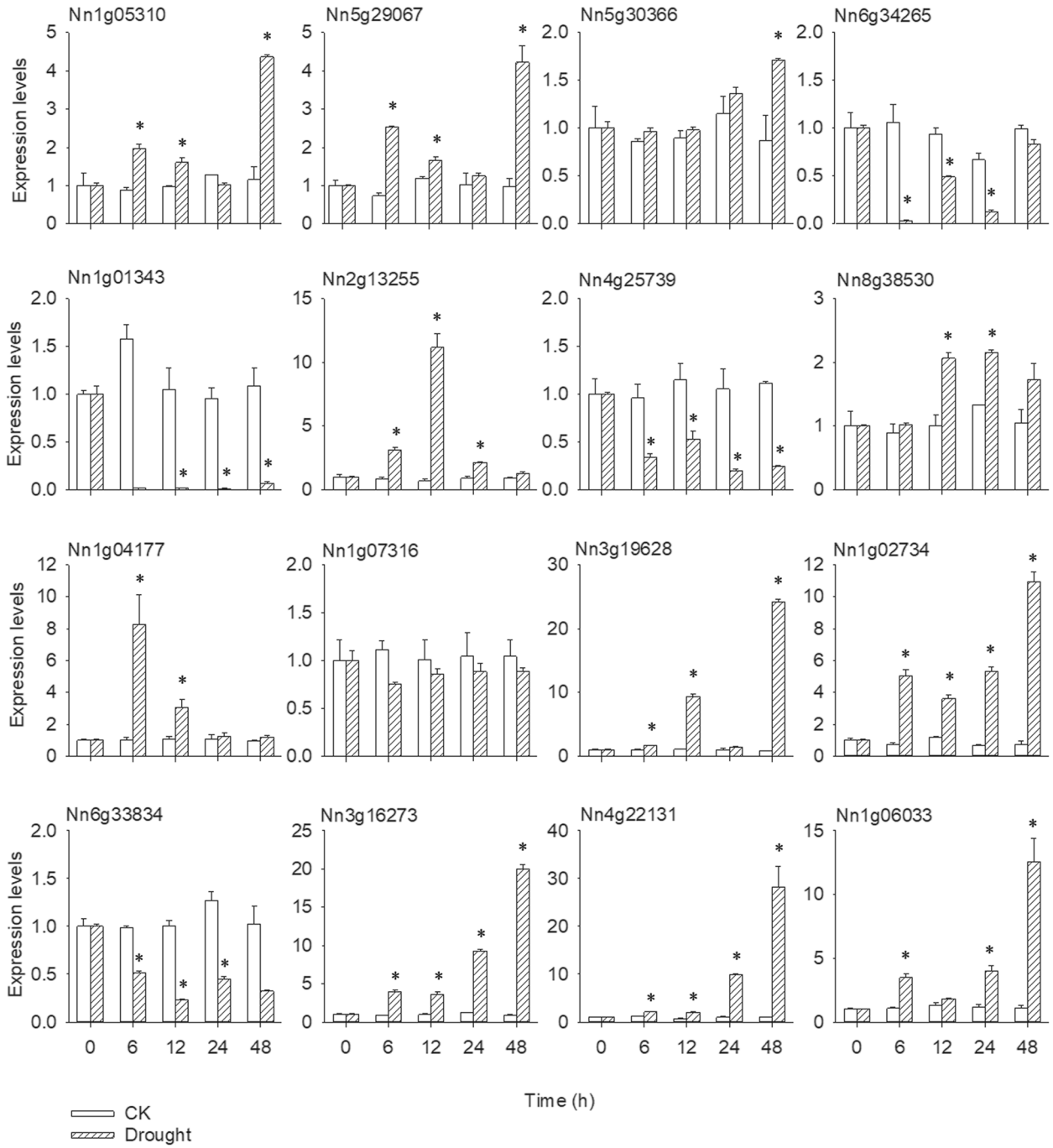

Fig. 9 Expression patterns of the 16 selected *NnERF* genes in leaves of *N. nucifera* under drought stress treatment. qRT-PCR data was normalized using *NnActin* and represent the mean±SE of biological triplicates with three technical repeats. Asterisks (*) represent a significant difference in transcript abundance between the treatment and the control at the same time ($p < 0.05$)

significantly suppressed by salt stress, whilst other 2 genes exhibited similar expression levels under salt treatment with those in the control (Fig. 8).

Drought stress treatment upregulated the expression of 11 *NnERF* genes. Notably, six genes including group IV member Nn2g13255, group IX members Nn3g19628 and Nn1g02734, and group X members Nn3g16273, Nn4g22131, and Nn1g06033 were significantly induced and reached their maximum values with tenfold greater than their untreated levels, respectively. Moreover,

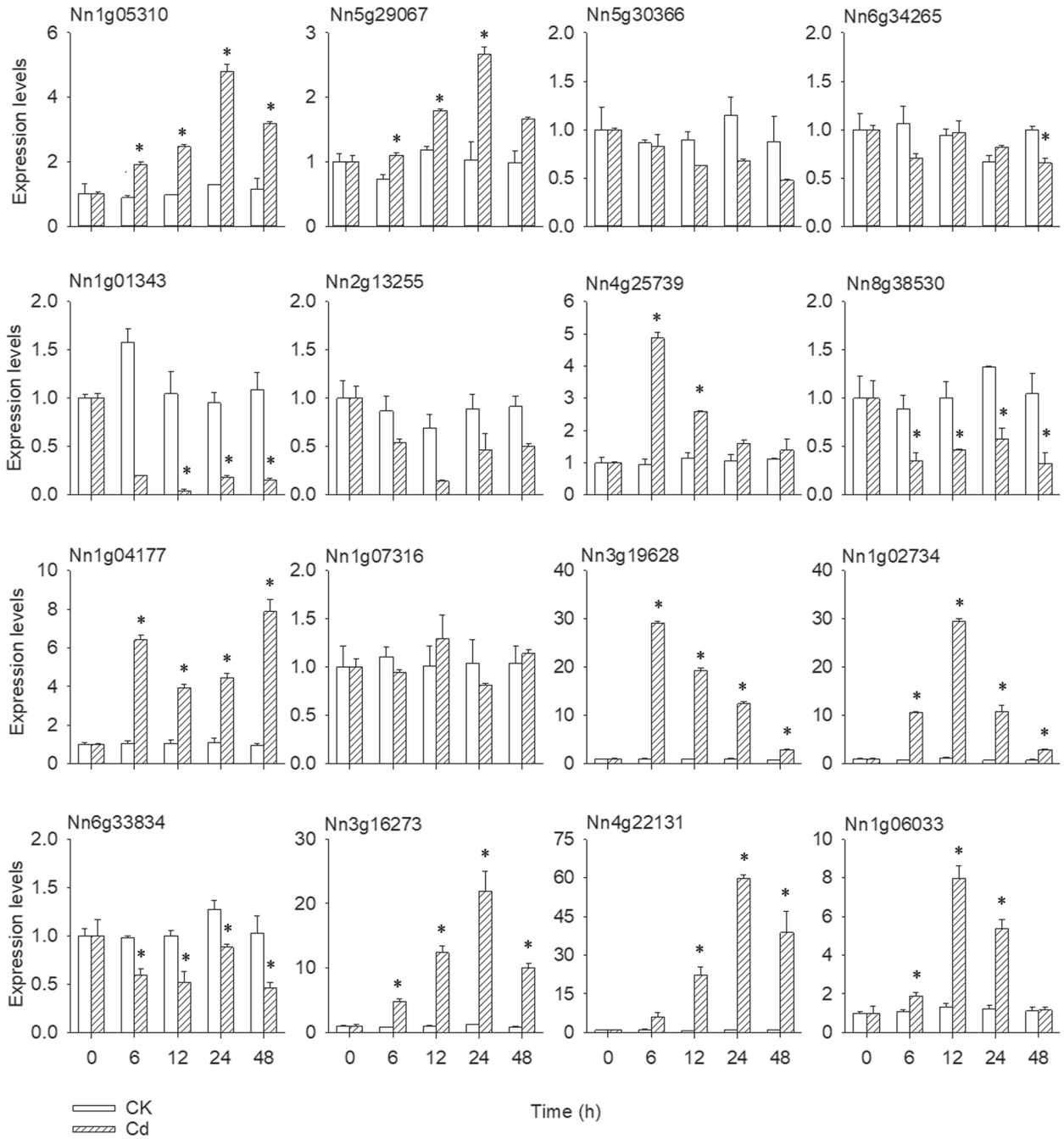

Fig. 10 Expression patterns of the 16 selected *NnERF* genes in leaves of *N. nucifera* under Cd stress treatment. qRT-PCR data was normalized using *NnActin* and represent the mean ± SE of biological triplicates with three technical repeats. Asterisks (*) represent a significant difference in transcript abundance between the treatment and the control at the same time ($p < 0.05$)

four genes (Nn6g34265, Nn1g01343, Nn4g25739, and Nn6g33834) were declined by drought stress; However, one remaining gene showed no response to drought stress (Fig. 9).

Under Cd stress treatment, all 16 investigated *NnERF* genes showed different expression patterns compared with those in the control (Fig. 10). Among the nine genes with upregulated expression patterns, five genes (Nn3g19628 and Nn1g02734 in group IX, and Nn3g16273, Nn4g22131 and Nn1g06033 in group X) showed much stronger response to Cd stress, with their transcripts significant up-regulation and peaking at more

than eightfold greater levels after Cd treatment. Moreover, four genes (Nn6g34265, Nn1g01343, Nn8g38530, Nn6g33834) were down-regulated by Cd stress with two gene transcripts significantly lower than those in CK at all time points, and the remaining three genes were not significantly influenced under Cd stress (Fig. 10).

Subcellular localization and transcriptional activity analysis

For subcellular localization assay of NnERF proteins, we chose two representative stress-related *ERF* genes, Nn3g19628 and Nn1g06033. The two genes were independently cloned and fused with GFP under the control of the CaMV 35S promoter, and then these fusion proteins were separately transformed into *N. benthamiana* leaves. As shown in Fig. 11a, the green florescence signal of the control group (35S::GFP) filled the whole cell, whereas Nn3g19628-GFP and Nn1g06033-GFP fusion proteins only stimulated green fluorescence in the nucleus. These findings confirmed that the Nn3g19628 and Nn1g06033 proteins were localized in the nucleus, which were in agreement with the bioinformatics results (Table S2).

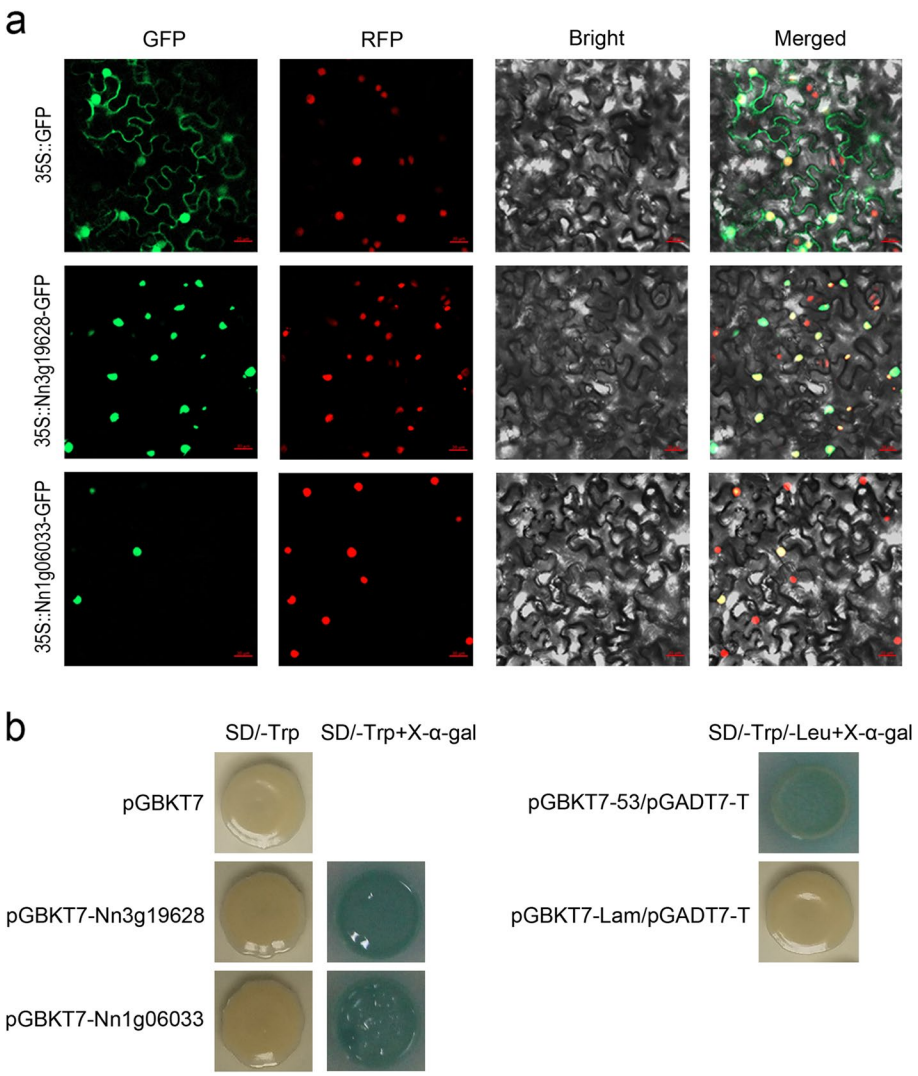

Fig. 11 Subcellular localization and transcriptional activity assays of Nn3g19628 and Nn1g06033. **a** Subcellular localization of Nn3g19628 and Nn1g06033 in *N. benthamiana*. GFP, green fluorescence protein; RFP, red fluorescence protein (used as a marker of nucleus localization signals). Scale Bars = 20 μm. **b** Transcriptional activation activity of Nn3g19628 and Nn1g06033.The ORF fragments of Nn3g19628 and Nn1g06033 were separately ligated with pGBKT7 to construct fusion vectors then expressed in yeast strain Y2HGold. The transformed yeast cells harboring Nn3g19628 and Nn1g06033 fusion vectors were cultured on the SD/-Trp and SD/-Trp + X-α-gal solid medium, respectively

To determine the transcriptional activity of Nn3g19628 and Nn1g06033, the ORF fragments of the two proteins were fused into the pGBKT7 vector, and subsequently, the fusion expression vectors, positive and negative controls were separately transferred into yeast strain Y2HGold. As shown in Fig. 11b, the yeast cells transformed with pGBKT7-Nn3g19628, pGBKT7-Nn1g06033, and pGBKT7 plasmids could normally grow on SD/-Trp medium, whilst the transformants containing pGBKT7-Nn3g19628 and pGBKT7-Nn1g06033 turned blue on SD/-Trp + X-α-gal solid medium. The results showed that Nn3g19628 and Nn1g06033 proteins had self-activation activity.

Discussion

The ERF family is one of the most important TF families in plants, and it belongs to AP2/ ERF superfamily which also contains AP2 and RAV families [6, 7, 9]. It has been demonstrated that ERF family members participate in regulating plant growth and development, and coping with unfavorable environmental conditions [1, 5, 6, 15, 16]. Identification and analysis of gene families provide a basis for systematic study of biological function of the family members. To date, comprehensive investigations of *ERF* family genes have been accomplished in many crops and terrestrial plants [8, 9, 12–14], but relevant researches of this family in aquatic ornamental plant *N. nucifera* is limited. Only a recent study has provided some information about *AP2/ERF* superfamily genes in *N. nucifera* by Cao et al. [48]. However, the study of Cao et al. emphasized on the differences among three distinct families (AP2, RAV, and ERF families) and the role of AP2 family during *N. nucifera* rhizome formation, yet lack of their underlying function under abiotic stress conditions [48]. Additionally, the characterization of these genes in that study mainly came from the previous 1.1 version of the *N. nucifera* genome [17, 23]. Given the complexity of *N. nucifera* genome and the constant updating of Nelumbo Genome Database [18, 29], a genome-wide identification and characterization of ERF family in *N. nucifera* under abiotic stresses still need to be systematically elucidated with a new version of the *N. nucifera* genome. In this study, we identified 133 *AP2/ERF* genes after searching against *N. nucifera* genome protein sequences (version 2.0), including 107 *ERF* family members. The total number of ERF family members in *N. nucifera* is comparable with those in *A. thaliana* (122), *O. sativa* (139), *S. tuberosum* (155), *V. vinifera* (113), *S. indicum* (114), and *Z. mays* (166) [8, 9, 11, 14, 38], though the *N. nucifera* genome size (929 Mb) is larger than those of *A. thaliana* (125 Mb), *O. sativa* (466 Mb), *S. tuberosum* (844 Mb), *V. vinifera* (490 Mb), and *S. indicum* (274 M), but smaller than that of *Z. mays* (2.3 Gb). This finding indicates that the evolution of *ERF* gene family is highly conserved among different species, and their number has no absolute correlation with the plant genome size.

Phylogenetic analyses revealed that 107 members of *N. nucifera* ERF family consisted of ERF and DREB subfamilies were further categorized into 11 groups, namely I to X and VI-L, according to the classification of *A. thaliana* ERF family [9], and each group was clustered by ERF family members from the two species *N. nucifera* and *A. thaliana* (Fig. 1), suggesting that these ERF proteins may have similar functions and a close evolutionary relationship. Among them, the number of NnERF proteins in groups III (21), VIII (16), and IX (15) was the largest in *N. nucifera*. Members of groups III and IX from *A. thaliana* ERF family have been demonstrated to play important roles in environmental stress responses [1, 16, 28, 49], and group VIII members were involved in regulation of hormone signals [9, 50]. As a result, the increased number of genes in the three groups may be the evolutional consequence of *N. nucifera* adaption to complex environmental changes. In addition, the number of ERF in each group was various among different species. For example, the number of group VII was small in many terrestrial plants, including *A. thaliana* (5), *Aethionema arabicum* (4), *Trifolium pratense* (3), *Fragaria vesca* (3), *Cucumis sativus* (5), and *Mimulus guttatus* (5), but it was obviously expanded in aquatic plants *Zostera marina* (17) and *O. sativa* (12), and slightly expanded in *N. nucifera* (7) and *Spirodela polyrihiza* (7) as reported by Wang et al. [51]. Considering that members of group VII often refer to the tolerance of submergence and hypoxia availability, such as *O. sativa* Sub1A [52] and *A. thaliana* RAP2.12 [27], we speculated that *N. nucifera* with small expansion of VII members might be beneficial to cope with its hypoxic aquatic environments.

By analyzing the exon–intron structure of the 107 *NnERF* genes, we found that *NnERF* in *N. nucifera* possessed 0–3 introns, and most of the genes (73.83%) were intronless (Fig. 3). Similar results have also been reported in ERF family of many other plants [9, 13, 38]. Given that genes with fewer introns may have easier function in response to environmental challenge [53], it appears that intronless or intron-poor *NnERF* genes may react rapidly when external environment changed. Conserved motif and multiple alignment analyses could provide more information about functional differences among the ERF family members. In this work, a total of 25 conserved motifs were investigated in 107 NnERF proteins. Among them, motifs 1, 2 and 3 located in the AP2 domain regions were generally present in almost all the NnERF proteins, suggesting that AP2 domain is conserved in NnERF family proteins. Multiple sequence alignment of the AP2 domain further showed highly conserved "YRG" and

"RAYD" elements in members of NnERF family (Fig. 2), and both the elements had been reported to be important for AP2 domain function and involved in DNA binding [6, 54]. The conserved amino acid residues in 14th and 19th positions in AP2 domains are demonstrated to be vital for recognition of DNA-binding sequences, and functional divergence of ERF and DREB subfamilies [6, 10]. Here, Val-14 and Glu-19 residues in AP2 domains from the NnDREB subfamily proteins were highly conserved, especially Val-14, which is more crucial than Glu-19 for detection of DREB subfamily [10]; whereas Ala-14 and Asp-19 residues were highly conserved at the corresponding position in NnERF subfamily proteins, similar to the status in many other plants [10, 13, 15, 38, 55]. Furthermore, according to conserved motif analyses, motifs outside the AP2 domain regions showed a group-specific distribution (Fig. 3), suggesting that ERF proteins may be functionally identical within the same group, but exist functional divergence among groups. For example, the motif 4 was only present in NnERF members of groups II and III, and it contained LPR (P/A) and D (I/V) QAA residues, which were reported to be essential signatures for CBL-interacting serine/threonine protein kinase-12 and ERF037, respectively [56]. The motif 8 was uniquely detected in most members of group VII, with the consensus sequence of MCGGAI (I/L) in the N-terminal regions. Previous studies reported that the MCGGAI (I/L) residue was regulated by the N-end rule pathway of protein degradation and important for plant hypoxia tolerance [57], implying similar functions for group VII members in *N. nucifera*. The motifs 9 and 13 were group IX specific, and contained EDLL motif and DMLV motif, respectively. Both of them function as activation domains, and have the ability to activate the transcription process [9, 58]. In contrast, the motif 20 consisted of an EAR motif, (L/F)DLN(L/F)xP, was specifically existed in members of group VIII, and the EAR motif has been demonstrated to act as a transcriptional repression domain and usually participated in repression mechanism in plants [6]. For example, the EAR motif of AtDEAR4, CmERF12 and PyERF4.1/PyERF4.2 were found to repress transcriptional activity during plant development [44, 59, 60].

Gene duplication events are one of the main evolutionary mechanisms for gene family amplification, and a crucial way to cope with environmental changes during plant development [61]. In this study, a total of three pairs of tandem duplication genes and 21 pairs of segmental duplication genes were observed in *N. nucifera* genome (Fig. 4). The high segmental duplications may be a major driving force for expansion of *NnERF* gene family, which was consistent with the results in other species, such as *Z. mays* [12], *Cucurbita moschata* [62], and *Triticum aestivum* [55]. Generally, the Ka/Ks ratio can represent different selection categories for duplication genes. Ka/Ks > 1 represents accelerated evolution with positive selection, Ka/Ks = 1 means neutral selection, whereas Ka/Ks < 1 stands for negative selection [39]. The Ka/Ks radio of duplicated *NnERF* gene pairs was less than 1, suggesting that these *NnERF* genes had experienced intense negative selection during the evolution of *N. nucifera*. The divergence time for *NnERF* duplication gene pairs ranged from 17.46 to 289.74 Mya, with a peak occurring approximately 65 Mya in this study. Interestingly, *N. nucifera* reportedly underwent recent whole-genome duplication (WGD) event at about 65 Mya [17]. This result illustrated that the expansion of the *NnERF* gene family was largely influenced by the recent WGD event occurring in the *N. nucifera* genome.

A syntenic analysis was carried out to reveal the evolutionary connections between *NnERF* genes and other two representative plants including *A. thaliana* and *O. sativa*. Here, 41% (44) and 36% (39) of *NnERF* genes in *N. nucifera* were synonymous with *ERF* genes in *A. thaliana* and *O. sativa*, respectively (Fig. 5). Often orthologous genes among different species share similar functions during plant development and environmental responses [63]. Some *NnERF* genes has multiple orthologous gene pairs, suggesting these genes might play prominent roles in *NnERF* family evolution. Some *NnERF* genes could be found orthologous gene pairs both in *A. thaliana* and *O. sativa*. This situation has also been reported by Huang et al. in *A. comosus* [13], suggesting that these *ERF* genes might already exist before speciation.

To understand possible functions of NnERF family members during *N. nucifera* growth and development processes, the expression patterns of 107 *NnERF* genes were analyzed in different tissues according to transcription data from the Nelumbo Genome Database [18]. As shown in Fig. 6, 84 *NnERF* genes were expressed in at least one investigated tissue of *N. nucifera*, indicating their wide involvement in developmental processes regulation. Among these *NnERF* genes, five genes were relatively highly expressed in all tested tissues, including Nn3g21564, Nn6g35711 and Nn8g38530 in group VII, and Nn1g03362 and Nn1g07316 in group VIII. Some members of groups VII and VIII in plants are reported to play essential roles in plant growth and development [27, 50, 59]. For instance, oxygen-regulated *RAP2.12* (AT1G53910.1) is demonstrated to be indispensable for plant develpment progression and metabolic performance under both normoxic and hypoxic conditions [27]. Therefore, it is likely that its orthologous genes (Nn6g35711 and Nn8g38530) in *N. nucifera* possess similar function. We found most *NnERF* genes displayed special spatial and temporal expression patterns among

different tissues in this study. For example, Nn3g21374 in group VIII, and Nn1g02737 and Nn1g06350 in group IX were specifically expressed in root tissue, implying their functional specificity for root development. A recent study by Illgen et al. has demonstrated that group IX members ERF102 to ERF105 strongly affected root elongation, lateral root number and density [45]. Meanwhile, there were 19 genes with relatively high transcription levels in leaves, such as Nn6g35711, Nn4g25740, and Nn5g30366, and the orthologous gene of Nn5g30366 in *A. thaliana*, AT4G36900 (*DEAR4*), has been confirmed to be involved in leaf senescence via regulating reactive oxygen species (ROS) production [44].

To further investigate potential roles of *NnERF* genes in response to abiotic stresses, qRT-PCR experiments were performed to analyze the expression patterns of 16 selected *NnERF* genes under cold, salt, drought, and Cd treatments. We observed that 16 *NnERF* genes exhibited differential expression patterns under these abiotic stresses, and most of them were responsive to multiple stresses (Figs. 7, 8, 9 and 10). The co-expression of *NnERF* genes under various stress conditions might be due to the extensive presence of multiple hormone and stress-responsive CREs in their promoter regions, including ABRE, ARE, CGTCA-motif, TGACG-motif, W box (Table S5). These CREs identified in this study have also been reported in the promoter of TF genes responsible for activating the expression of abiotic stress-responsive genes in other plants, resulting in enhanced environmental adaptation [14, 64, 65]. Furthermore, subcellular localization and transcriptional activity analysis showed that two representative stress-related NnERF proteins (Nn3g19628 and Nn1g06033) were located in the nucleus and were self-activating (Fig. 11), which further supports their functions as TFs [3, 66, 67].

Low temperature is one of major environment abiotic stress factors that adversely affects plant growth and development [1, 5]. The well-known classical mechanism underlying plant response to cold stress is CBF/DREB signaling cascade, in which CBF1, CBF2, and CBF3 in group III of ERF family act as central regulatory elements [7, 49]. All the three *CBF* genes were rapidly induced when *A. thaliana* was exposed to cold treatment, and their loss-of-function analysis demonstrated that these *CBF* genes contribute to freezing tolerance and cold acclimation [1]. In this study, qRT-PCR verification revealed that 13 *NnERF* genes distributed across seven groups were significantly upregulated after cold stress treatment, including Nn6g34265 and Nn1g01343 in group III, Nn4g25739 in group VII, Nn3g19628 and Nn1g02734 in group IX, and Nn1g06033 in group X (Fig. 7), indicating that these *NnERF* genes were correlated with cold stress response. Recent studies have demonstrated that apart from group III members of ERF family, overexpression of group IX genes such as *AtERF105* or *VaERF092* positively regulated transgenic plants cold stress tolerance, partially through enhancing activities of antioxidant enzyme system under cold stress [5, 49].

Multiple studies have shown that salt stress induced expression of *ERF* gene family members in many species, such as *CmERF3* in *G. max* [15], *AtERF98* in *A. thaliana* [16], and *ZmERF135* in *Z. mays* [38]. Here, we found 11 *NnERF* genes in *N. nucifera* were significantly upregulated under salt treatment, including duplication gene pairs of Nn1g05310 and Nn5g29067, Nn3g19628 and Nn1g02734, and Nn3g16273 and Nn4g22131 (Fig. 8). Meanwhile, following drought stress treatment, 11 *NnERF* genes in *N. nucifera* were highly up-regulated, with six genes including Nn2g13255, Nn3g19628, Nn1g02734, Nn3g16273, Nn4g22131, and Nn1g06033 expression levels strongly increased by up to more than tenfold compared with CK (Fig. 9), similar to other species findings including the increase in expression of *ERF* family genes *AtDREB2A* in *A. thaliana* [10], *MdERF38* in *Malus*×*domestic* [2] and *SiERF002* in *S. indicum* [14] when exposure to drought stress. The above results suggested that these *ERF* genes might played essential roles in plants response to salt stress or drought stress. Accumulating evidence has indicated that genes expressed during drought and salt stress responses in plants partially overlapped. For example, transgenic plants that overexpressed *OsMYB2* or *TaWRKY24* showed enhanced drought and salt tolerance [64, 65]. In this study, we observed that the expression change trends of 11 individual *NnERF* genes under drought stress were consistent with those under salt stress (Figs. 8 and 9). Among them, Nn3g16273 and Nn4g22131 (a duplication gene pair from group X) were significantly induced by both drought and salt treatments at all time points, implying their functional redundancy. Interestingly, their orthologous gene AT5G13330 (*RAP2.6L*, group X) in *A. thaliana* was previously revealed to exhibit similar upregulated expression patterns, and its overexpression conferred transgenic *A. thaliana* greater tolerance to drought and salt stresses [46]. Thus, it is likely that these drought- and salt-regulated *NnERF* genes, especially group X members Nn3g16273, Nn4g22131 and Nn1g06033, were the suitable candidate genes for *N. nucifera* resistance to both drought and salt stresses.

A few reports have been found for *ERF* genes regulation by heavy metals in some plant species. For instance, *AtERF96* was rapidly upregulated under selenite stress in *A. thaliana* [28], and *TdSHN1* expression was strongly induced by Cd, copper, and zinc in *T. turgidum* [68]. However, the specific role of *ERF* in response to heavy metals, especially Cd, remains poorly characterized and

has become a hot research topic recently [3, 66, 69]. In this study, nine *NnERF* genes in *N. nucifera* were induced after exposure to Cd stress, in which five genes were most strongly upregulated, including group IX members Nn3g19628 and Nn1g02734, and group X members Nn3g16273, Nn4g22131 and Nn1g06033 (Fig. 10). Lin et al. reported that PvERF15 displayed high similarity with *A. thaliana* At2g31230 in group IX, and knockdown of *PvERF15* by RNA interference resulted in a decreased Cd tolerance in *P. vulgaris* [66]. Therefore, we speculate the orthologous genes of At2g31230 in *N. nucifera*, Nn3g19628 and Nn1g02734, have possible role against Cd stress, which needs further studies.

Conclusions

In this study, a total of 133 putative *AP2/ERF* superfamily genes were identified in the *N. nucifera* genome. Among them, 107 genes belonged to the NnERF family comprised DERB and ERF subfamilies, which were further divided into eleven groups based on phylogenetic analysis. A comprehensive analysis for gene structure, motif composition, chromosome location, evolutionary characteristics, CREs, and expression patterns in different tissues of *N. nucifera* was performed on these 107 *NnERF* genes. In addition, expression levels of 16 selected *NnERF* genes were further assessed under cold, salt, drought, and Cd stresses by qRT-PCR analysis, and results indicated their potential regulatory roles in response to multiple abiotic stresses. Meanwhile, the subcellular localization and transcriptional activation activities of two representative stress-related NnERF Nn3g19628 and Nn1g06033 were demonstrated. These findings will be beneficial to screening appropriate candidate *NnERF* genes for further functional characterization, and provide valuable foundation for exploring molecular mechanisms underlying abiotic stress responses in *N. nucifera*, hence contributing to the breeding of *N. nucifera* and other aquatic ornamental plants with enhanced stress tolerance.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions
Y. X. prepared materials, analyzed data and wrote the manuscript. J. J., L. Z., H. L. and P. Z. performed experiments. Q. J. revised the manuscript. Y. W. designed the research, analyzed data and wrote the manuscript. All authors reviewed the manuscript.

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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 no competing interests.

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