

Title Comprehensive characterization of the genes in AP2/ERF family and their involvement in salt–alkali stress response during *Nelumbo nucifera* seed germination

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Abstract

Background: *Nelumbo nucifera* Gaertn. is an important aquatic crop valued for its ornamental, nutritional, and ecological traits. However, soil salinization and alkalization impose major constraints on its growth and productivity. The *AP2/ERF* transcription factor family plays central roles in plant responses to abiotic stresses, but their functions in salt–alkali tolerance of *N. nucifera* remain largely unknown.

Results: We identified 101 *AP2/ERF* transcription factors in the *N. nucifera* genome, exhibiting diverse physicochemical properties and structural variations. Phylogenetic analysis grouped them into five subfamilies, showing both evolutionary conservation and species-specific divergence compared to *Arabidopsis thaliana*. Biochemical assays during seed germination under salt–alkali stress revealed dynamic changes in malondialdehyde, proline, superoxide dismutase, peroxidase, and catalase activities, indicating that *AP2/ERF* genes may enhance stress tolerance by regulating proline metabolism and reactive oxygen species scavenging. Transcriptome analysis of seedlings treated with 150 mM salt–alkali solution for 5 and 10 days identified 7,350 differentially expressed genes, including 29 *AP2/ERFs* with significant expression changes. Among them, 13 genes, such as *AP2-9*, *ERF23*, *ERF15*, *ERF31*, *ERF34*, and *DREB21*, were consistently upregulated under stress, suggesting their key roles in stress adaptation. Furthermore, qRT-PCR validation confirmed the upregulated expression of *AP2-9*, *ERF23*, *ERF34*, and *DREB21* in both 5-day and 10-day salt–alkaline treatments, corroborating the transcriptome results and supporting their involvement in the regulation of salt–alkali stress responses during seed germination.

Conclusions: This study provides the first comprehensive characterization of the *AP2/ERF* gene family in *N. nucifera* and highlights their involvement in salt–alkali stress responses. These findings advance our understanding of the molecular mechanisms underlying stress adaptation in *N. nucifera* and provide a theoretical basis for breeding salt–alkali tolerant varieties, thereby enhancing the ecological and economic value of saline–alkaline wetlands.

Introduction

The APETALA2/ethylene-responsive factor (*AP2/ERF*) superfamily represents one of the largest and most evolutionarily conserved transcription factor families in plants [1, 2], playing pivotal roles in diverse developmental processes and stress adaptation [3–5]. As a plant-specific gene family, *AP2/ERF* members are characterized by the presence of one or two highly conserved AP2 DNA-binding domains, each comprising 60–70 amino acid residues and mediating the regulatory functions by binding to specific cis-elements in target gene promoters [6]. Based on structural variations and functional divergence, this superfamily is classified into five major subfamilies: APETALA2 (*AP2*), dehydration-responsive element-binding (*DREB*), ethylene-responsive element-binding protein (*ERF*), related to ABI3/VP1 (*RAV*), and Soloist [7, 8]. Among these, the *AP2* subfamily members uniquely contain two AP2 domains, whereas *DREB* and *ERF* members possess only a single AP2 domain [9]. The *RAV* subfamily is distinguished by an additional B3 DNA-binding domain, a feature conserved in other plant-specific transcription factors [10]. In contrast, the Soloist subfamily members, despite harboring an AP2 domain, exhibit limited sequence homology with other members, suggesting a distinct evolutionary trajectory [11]. Given their critical regulatory roles in growth, development, and stress responses, *AP2/ERF* transcription factors have been extensively studied across plant species [9, 12], offering valuable insights into their molecular mechanisms and potential biotechnological applications.

Under abiotic stresses such as low temperature, drought, and high salinity, *AP2/ERF* transcription factors play crucial regulatory roles by rapidly activating downstream stress-responsive genes to enhance plant adaptation [13, 14]. For instance, in tomato (*Solanum lycopersicum*), *SlERF15* directly binds to and activates the promoters of cold-responsive genes (*CBF1*, *WRKY6*, and *SAG21-like*), thereby improving chilling tolerance, while its knockout significantly compromises cold resistance [15]. Overexpression of the *Ophiopogon japonicus* *OjERF* gene in tobacco (*Nicotiana tabacum*) upregulates stress-responsive genes, increases chlorophyll and proline accumulation, and enhances the activities of antioxidant enzymes (SOD and CAT), collectively improving drought and salt tolerance [16]. In *Medicago falcata*, transcriptomic analyses revealed that the *ERF* family represents one of the most dynamically regulated transcription factor groups under saline-alkali stress, with 131 *ERF* genes potentially modulating antioxidant systems and ion transport to confer salt tolerance [17]. Functional studies further demonstrate that *GmERF3* overexpression enhances salt and dehydration tolerance in transgenic plants by activating stress-related genes, whereas in potato (*Solanum tuberosum*) [18], *StERF3* acts as a negative regulator—its silencing improves NaCl tolerance, and overexpression increases salt sensitivity [19]. Additionally, cotton (*Gossypium hirsutum*) overexpressing *GhERF13.12* exhibits significantly enhanced salt tolerance [20], and the wild soybean (*Glycine soja*) *GsERF6*, which shares high sequence homology with rice *ERF* proteins, improves saline-alkali tolerance in transgenic rice by boosting ROS scavenging, osmotic adjustment, and stress-responsive gene expression [21]. These findings collectively elucidate the dual characteristics of conservation and functional diversification among *AP2/ERF* transcription factors in plant stress adaptation, particularly in the context of saline-alkali tolerance.

N. nucifera is an important aquatic plant with high ornamental, nutritional, and ecological value, and is widely cultivated worldwide [22]. However, soil salinization and alkalization have become major environmental constraints limiting its growth, productivity, and geographical distribution [23, 24]. In recent years, the adverse effects of salt-alkali stress on both crop and ornamental plant performance have become increasingly prominent due to global climate change and accelerated agricultural land degradation [25, 26]. Although *N. nucifera* generally exhibits moderate salt tolerance, its tolerance capacity varies significantly depending on the cultivar, developmental stage, and environmental conditions [27]. Under low to moderate salinity (e.g., ≤ 50 mM NaCl), certain *N. nucifera* cultivars can adapt to stress conditions through physiological mechanisms, including osmotic adjustment, enhanced antioxidant defense, and maintenance of ion homeostasis [28]. Nevertheless, high salinity levels (>150 mM NaCl) often result in growth inhibition, leaf damage, and reduced flowering [29]. The adaptive capacity of *N. nucifera* to saline-alkaline environments is closely associated with its underlying molecular regulatory networks [29, 30]. Among these, the *AP2/ERF* transcription factor family plays a pivotal role in regulating plant responses to abiotic stresses [30, 31]. Members of this family can activate or repress the expression of downstream stress-responsive genes, thereby participating in key physiological processes including osmotic regulation, reactive oxygen species (ROS) scavenging, and ion homeostasis [32]. Through these mechanisms, *AP2/ERF* transcription factors contribute to enhanced salt-alkali stress tolerance [9, 31]. Despite their critical roles in other plant species, a comprehensive characterization of the *AP2/ERF* gene family in *N. nucifera*, as well as their regulatory mechanisms in salt-alkali stress response, remains largely unexplored.

In this study, we conducted a comprehensive genome-wide analysis of the *AP2/ERF* transcription factor family in *N. nucifera*, identifying 101 members and characterizing their physicochemical properties, gene structures, conserved motifs, and phylogenetic relationships. Comparative analyses revealed notable structural and evolutionary divergence of *AP2/ERF* genes in *N. nucifera* relative to *A. thaliana*, suggesting lineage-specific expansion and potential functional specialization. To explore their roles in stress adaptation, we examined the

physiological and transcriptomic responses of *N. nucifera* seeds under salt-alkaline stress. Significant alterations in germination, growth, and oxidative stress markers were observed, alongside thousands of differentially expressed genes. Notably, 29 *AP2/ERF* family members exhibited stress-responsive expression, with 13 genes, including *AP2-9*, *ERF23*, *ERF15*, and *DREB21*, consistently upregulated. These results highlight the functional diversification of the *AP2/ERF* gene family and underscore their potential as key regulators in salt-alkaline stress tolerance, providing a foundation for future genetic improvement of aquatic crops.

Results

2.1 Identification of 101 *AP2/ERF* genes in *N. nucifera*

To identify *AP2/ERF* family members in *N. nucifera*, we performed a BLASTp analysis using *A. thaliana* At*AP2/ERF* protein sequences as queries, which led to the identification of 101 *AP2/ERF* proteins in *N. nucifera*. After removing redundant sequences corresponding to identical genes and alternative transcripts, the *AP2/ERF* genes were systematically named according to their order in the NCBI genome sequence database (Table 1). Bioinformatic analysis revealed substantial variation in the physicochemical properties of these proteins. The *AP2/ERF* proteins ranged from 116 (*ERF8*) to 679 (*AP2-8*) amino acids in length (Table 1), with corresponding molecular weights (MW) spanning 13.3438 kDa (*ERF8*) to 74.78307 kDa (*AP2-8*) (Table 1). The predicted isoelectric points (pI) exhibited a broad range from 4.24 (*DREB30*) to 10.31 (*ERF37*) (Table 1), with 26 *AP2/ERF* proteins displaying pI values ≥ 7 , indicating enrichment in basic amino acids or low content of acidic residues, resulting in a net positive charge under acidic conditions. Protein stability analysis showed that the instability index varied considerably from 33.03 (*DREB9*) to 85.25 (*DREB11*) (Table 1). Significantly, only three proteins (*DREB9*, *ERF37*, and *AP2-15*) exhibited instability indices below 40, suggesting these proteins possess compact structures with minimal disordered regions. The aliphatic index ranged from 43.42 (*DREB28*) to 85.1 (*DREB9*) (Table 1), with 87 *AP2/ERF* proteins showing values below 70, potentially indicating temperature sensitivity or reliance on alternative stabilization mechanisms, such as disulfide formation. Intriguingly, *DREB9* was the sole protein with an aliphatic index exceeding 80, implying enhanced thermostability. All *AP2/ERF* proteins displayed negative grand average of hydropathicity values (Table 1), consistent with their overall hydrophilic nature and high solubility. Collectively, these comprehensive analyses demonstrate that the 101 *AP2/ERF* proteins in *N. nucifera* exhibit remarkable diversity in their physicochemical characteristics, which may reflect their functional specialization in various biological processes.

2.2 Evolutionary analysis of *AP2/ERF* family genes in *N. nucifera*

To investigate the evolutionary relationships within the *AP2/ERF* gene family, we performed a comprehensive phylogenetic analysis using full-length protein sequences from 147 *A. thaliana* and 101 *N. nucifera* *AP2/ERF* members. The resulting phylogenetic tree clearly delineated these proteins into five well-defined subfamilies (Figure 1). The *ERF* subfamily emerged as the most abundant group, comprising 51 members (50.5% of total), followed by the *DREB* subfamily with 30 members (29.7%). The *AP2*, *RAV*, and *Soloist* subfamilies contained 15 (14.85%), 4 (3.96%), and 1 (0.99%) members (Figure 1), respectively. Comparative analysis revealed a consistent reduction in gene numbers across all subfamilies in *N. nucifera* compared to *Arabidopsis*, suggesting potential lineage-specific gene loss events during evolution.

Furthermore, synteny analysis identified 117 homologous gene pairs between the two species, with 53 *N. nucifera* *AP2/ERF* genes showing orthologous relationships to 73 *Arabidopsis* counterparts (Figure S1). These

orthologous pairs were distributed across 35 chromosomal segments, indicating both conserved and divergent evolutionary patterns in the *AP2/ERF* family between the two species. This systematic characterization not only establishes a framework for understanding *AP2/ERF* gene family evolution in *N. nucifera* but also highlights key differences in gene family expansion between basal and core eudicots.

2.3 Structural characterization and functional diversification of *AP2/ERF* genes in *N. nucifera*

Our comprehensive analysis of gene architecture and conserved domains revealed critical insights into the functional diversification of *AP2/ERF* family members in *N. nucifera*. The exon-intron organization exhibited distinct subfamily-specific patterns (Figure 2A): The *AP2* subfamily displayed the most complex structures, with five members containing 10 exons and others possessing 7-9 exons. In contrast, the *Soloist1* maintained a conserved 7-exon structure. The *RAV* subfamily showed remarkable divergence, with *RAV1-3* being intronless while *RAV4* retained two introns. Within the *ERF* subfamily, 33 genes were single-exon, 14 contained two exons, and *ERF32/40/41/47* uniquely possessed three exons. The *DREB* subfamily predominantly exhibited intron loss, with only *DREB9* and *DREB25* retaining intronic sequences (Figure 2B). UTR analysis uncovered substantial structural variation: while 78 genes maintained typical dual-UTR configurations, 8 genes were identified to be with three UTRs, 11 with single UTRs, and strikingly, *ERF47* and *AP2-12* exhibited unique tetra-UTR architectures (Figure 2B). Protein motif analysis identified 10 conserved motifs (15-50 aa) (Figure S2). Motifs 4 and 5 were nearly ubiquitous (absent only in *DREB30* and *Soloist1*), while motif 6 was widely distributed across *RAV/ERF/DREB* subfamilies, suggesting their roles as core functional elements (Figure 2C). Subfamily-specific motif patterns emerged: the *AP2* subfamily uniquely contained motifs 1-3, with most members possessing duplicate motif 2, while the *RAV* subfamily specifically retained motif 1. The *ERF* subfamily exhibited subgroup specialization, with 10 members containing motif 8 and *ERF37-43* possessing motifs 1 and 9. Domain architecture analysis confirmed structural conservation: all *AP2* proteins except *AP2-6* maintained dual *AP2/ERF* domains, *RAV* members contained characteristic *AP2/ERF-B3* domain combinations, while *ERF* and *DREB* subfamilies possessed single *AP2/ERF* domains - consistent with established paradigms. Remarkably, *DREB* subfamily members displayed motif simplification, with *DREB30* retaining only the minimal motif 6 and motif 10 combination. These findings not only validate conserved structural features but also reveal novel complexities in UTR arrangements, motif combinations, and domain architectures that likely contribute to functional diversification within this crucial transcription factor family. The observed structural variations provide molecular correlates for the evolutionary specialization of *AP2/ERF* proteins in *N. nucifera*.

2.4 Physiological and developmental responses of *N. nucifera* seeds to salt-alkaline stress

The *AP2/ERF* transcription factor family plays a pivotal role in plant responses to abiotic stresses, including salinity and alkalinity. Gene Ontology (GO) enrichment analysis of 147 *AtAP2/ERF* members in *A. thaliana* revealed significant enrichment in both biotic and abiotic stress-responsive pathways, with particularly strong associations with cold and salt stress responses (Figure 3A). We hypothesize that *AP2/ERF* transcription factors in *N. nucifera* may also perform similar biological functions. To elucidate the regulatory role of *AP2/ERF* transcription factors in the salt-alkaline tolerance of *N. nucifera*, we focused on the germination stage of *N. nucifera* seeds, a developmental phase highly sensitive to salt-alkaline stress. Seeds were treated with mixed salt-alkaline solutions ($\text{NaCl}:\text{NaHCO}_3 = 1:1$, molar ratio) at varying concentrations (0, 50, 100, and 150 mM), and germination dynamics along with physiological responses were systematically assessed. After 5 days of treatment, seeds in the 0 and 50 mM groups had germinated and developed visible shoots, whereas shoot elongation was markedly inhibited at 150 mM and completely suppressed at 150 mM. By day 10, the differences

among treatments were more pronounced: shoot lengths reached 100–300 mm in the control group, were moderately suppressed at 50 mM, severely inhibited at 150 mM, and nearly halted at 150 mM (Figures 3B and 3E). Both germination potential and germination rate exhibited a significant negative correlation with increasing salt-alkaline concentration (Figures 3C and 3D). Biochemical analyses revealed that levels of malondialdehyde (MDA), proline (Pro), and superoxide dismutase (SOD) activity increased significantly with salt-alkaline concentrations up to 150 mM, but were markedly reduced at 150 mM (Figure 3F–H). Peroxidase (POD) activity peaked at 50 mM, began to decline at 150 mM, and showed no significant difference from the control at 150 mM. In contrast, catalase (CAT) activity consistently declined with increasing salt-alkaline concentration (Figure 3I–J). These findings suggest that under low to moderate salt-alkaline conditions, members of the *AP2/ERF* family may contribute to stress tolerance by enhancing proline biosynthesis and activating reactive oxygen species (ROS)-scavenging mechanisms, thereby mitigating oxidative damage and osmotic imbalance.

2.5 Identification of key *AP2/ERF* family members responsive to salt-alkaline stress in *N. nucifera*

To further elucidate the molecular regulatory mechanisms by which the *AP2/ERF* transcription factor family mediates salt-alkaline stress responses in *N. nucifera* seeds, transcriptome profiling was performed on four shoot samples collected after 5 and 10 days of treatment under 0 mM and 150 mM salt-alkaline conditions (designated as 0 mM-5d-T5, 150 mM-5d-Y5, 0 mM-10d-T10, and 150 mM-10d-Y10, respectively). Comparative transcriptomic analysis revealed a total of 7,350 differentially expressed genes (DEGs) commonly identified between control and treatment groups (Figures 4A and S3). Specifically, 8,949 DEGs were identified between the 0 mM and 150 mM samples on day 5, including 4,493 upregulated and 4,456 downregulated genes. On day 10, the number of DEGs increased to 11,535, with 5,705 genes upregulated and 5,830 downregulated under salt-alkaline stress (Figures 4A–C). Gene Ontology (GO) enrichment analysis showed that these DEGs were significantly enriched in key biological processes such as metabolism, biological regulation, and responses to stimuli (Figures 4E–F), providing a molecular framework for understanding the regulatory roles of *AP2/ERF* transcription factors under salt-alkaline stress in *N. nucifera*.

Further screening of the 7,350 common DEGs led to the identification of 29 *AP2/ERF* transcription factors with significant expression changes (Figures 4A and 5), comprising 3 *AP2* subfamily members, 18 *ERF* subfamily members, and 8 *DREB* subfamily members (Figure 5). Importantly, seven of these genes were significantly downregulated under salt-alkaline treatment, suggesting their potential roles in basal germination processes rather than stress responses. In contrast, 13 *AP2/ERF* genes exhibited sustained upregulation under 150 mM salt-alkaline conditions, indicating their likely involvement in stress adaptation and regulatory processes (Figure 5). Among them, *AP2-9*, *ERF23*, *ERF15*, *ERF31*, *ERF34*, and *DREB21* showed particularly strong and consistent induction, suggesting these genes may serve as key regulators in the salt-alkaline stress response network of *N. nucifera* seeds. These findings provide critical insights into the transcriptional regulatory networks governed by the *AP2/ERF* family and offer valuable clues for dissecting salt-alkaline tolerance mechanisms in *N. nucifera*.

2.6 Expression validation of key *AP2/ERF* genes during seed germination of *N. nucifera* under salt-alkaline stress

Based on transcriptome analysis, we identified *AP2-9*, *ERF23*, *ERF15*, *ERF31*, *ERF34*, and *DREB21* as potential key members of the *AP2/ERF* transcription factor family involved in the response of *N. nucifera* during seed germination under salt-alkali stress. To validate their expression patterns, *N. nucifera* seeds were treated with 0 mM (control) and 150 mM salt-alkaline solution for 5 and 10 days, followed by quantitative real-time PCR (qRT-

PCR) analysis. The results showed that under 150 mM salt–alkali stress, the expression levels of *AP2-9*, *ERF23*, *ERF15*, *ERF31*, *ERF34*, and *DREB21* were significantly upregulated compared with the control at both 5 and 10 days of treatment (Figures 6A–F). However, when compared with the transcriptome data, only *AP2-9*, *ERF23*, *ERF34*, and *DREB21* exhibited consistent expression trends across both treatments and time points (Figures 6A, C, D, and F). Notably, *ERF15* and *ERF31* did not show significant differential expression in the transcriptome data at day 5 of salt–alkaline treatment, yet were markedly upregulated according to the qRT-PCR results (Figures 6B and E). This discrepancy suggests that their regulatory mechanisms may be more complex, warranting further investigation into their biological functions and regulatory pathways. Overall, these findings indicate that *AP2-9*, *ERF23*, *ERF34*, and *DREB21* exhibit highly consistent expression patterns between transcriptome and qRT-PCR analyses, supporting their pivotal regulatory roles in the response of *N. nucifera* to salt–alkaline stress during seed germination.

Discussion

To elucidate the functional significance of *AP2/ERF* genes in *N. nucifera*, a total of 101 *AP2/ERF* family members were identified. Based on phylogenetic analysis, these genes were classified into five subfamilies, with the majority belonging to the *AP2*, *ERF*, and *DREB* subgroups. This classification is consistent with observations in other plant species, including *A. thaliana* [33], *Passiflora edulis* [34], cucumber [35], barley [36], and tomato [37]. Among these, members of the *ERF* and *DREB* subfamilies accounted for a large proportion, suggesting that they may play widespread and diverse roles in *N. nucifera* growth, development, and stress responses.

The identification of 101 *AP2/ERF* family members in *N. nucifera*, remarkably fewer than the 147 found in *A. thaliana*, highlights a significant contraction in gene family size that is evolutionarily meaningful. This disparity may reflect differences in evolutionary trajectories, especially considering that *N. nucifera* is a basal eudicot while *Arabidopsis* belongs to the core eudicots. Multiple studies have shown that core eudicots like *Arabidopsis* have undergone more recent and extensive whole-genome duplication (WGD) events, which are primary drivers of gene family expansion. Comparative genomic analyses across angiosperms confirmed that species-specific gene retention after WGD, especially in the *AP2/ERF* family, can vary dramatically, often aligning with phylogenetic position and environmental adaptation [38, 39]. For example, in Chinese cabbage (*Brassica rapa*), a close relative of *Arabidopsis* that experienced a whole-genome triplication, 291 *AP2/ERF* genes were identified, a size far exceeding that of *N. nucifera*, likely due to tandem and segmental duplications that are common post-WGD phenomena [40]. Similarly, rice and maize, although monocots, also exhibit large *AP2/ERF* families, with 170 and 214 members respectively, and studies attribute this to their own lineage-specific duplications and adaptive pressures [41, 42]. The extensive expansion of specific subgroups such as the *DREB/CBF* genes in *Eucalyptus grandis* has been associated with adaptation to climate variation, further illustrating how ecological factors shape gene family evolution [43]. Conversely, the relatively smaller *AP2/ERF* family in *N. nucifera* may also stem from its aquatic habitat, which imposes distinct environmental constraints. Aquatic environments often reduce the need for drought-responsive genes that are commonly expanded in terrestrial species. In *Prosopis cineraria*, for instance, a desert species, high *AP2/ERF* gene copy number and enhanced DNA-binding affinity are linked to increased drought tolerance, suggesting selective retention of these genes for stress resilience [44]. Thus, *N. nucifera* may have undergone lineage-specific gene loss due to relaxed selection on drought-related gene subgroups. Evolutionary analyses also suggest that basal eudicots like *N. nucifera* maintain more ancestral configurations of *AP2/ERF* genes, lacking the extensive diversification seen in *Arabidopsis*, which further supports the hypothesis of functional streamlining in response to niche specialization [45, 46]. The extensive variation

observed in the physicochemical properties of AP2/ERF proteins, such as length, isoelectric point (pI), instability index, and hydrophobicity, suggests functionally adaptive diversification that reflects their regulatory roles across different cellular contexts. A wide range of pI values from acidic to basic among AP2/ERF members has been consistently reported across multiple plant species including wheat, foxtail millet, and maize, indicating potential subcellular compartmentalization and the ability to interact with partners bearing varying electrostatic properties [42], thereby expanding their regulatory capacity under diverse cellular conditions [47, 48]. This biochemical diversity may also relate to localization within specific cellular microenvironments, from acidic vacuoles to nuclear or cytosolic compartments. In species such as almond and *Brachypodium*, the diversity in protein size and charge also correlates with tissue-specific expression patterns and responsiveness to abiotic stress, such as freezing and drought, supporting the hypothesis that variable charge profiles may fine-tune DNA or protein-protein interactions in a context-dependent manner [49, 50]. The instability index, where the majority of AP2/ERF proteins fall into the unstable category (index >40), aligns with the functional nature of transcription factors as rapidly inducible and degradable regulatory switches. This pattern has been repeatedly observed in various crops such as *Brassica napus* and chickpea, reinforcing the idea that post-translational turnover is essential for timely stress signaling and development control [51, 52]. However, the existence of a few highly stable AP2/ERFs such as DREB9, with elevated aliphatic indices and reduced disorder, may indicate their role as central regulatory hubs required for sustained activity under prolonged stress. This hypothesis is supported by studies on conserved motifs and protein disorder regions, where thermodynamically stable show fewer disordered regions, suggesting a rigid structure favorable for persistent interaction with target DNA or cofactors under heat or osmotic stress conditions [50, 53]. Furthermore, the aliphatic index, which serves as a proxy for thermostability, has drawn attention in species like foxtail millet and almond, where specific AP2/ERFs such as DREB-like proteins exhibit elevated indices and are consistently upregulated during high-temperature or freezing conditions, hinting at a protective function during environmental extremes [48, 49]. Such features not only inform our understanding of protein evolution in the AP2/ERF family but also provide testable hypotheses for functional studies, especially in identifying transcription factors with long-term or central regulatory roles under persistent abiotic stress. Altogether, the observed physicochemical heterogeneity across AP2/ERFs is not merely structural but is strongly suggestive of their regulatory modularity, enabling plants to rapidly and differentially fine-tune responses to spatial, temporal, and environmental cues.

The association between structural features of AP2/ERF genes and their functional diversification has been a central focus in understanding transcriptional regulation in plants, and comparative studies across species consistently reinforce the observed patterns in *N. nucifera*. The AP2 subfamily is structurally more complex, typically containing multiple introns that facilitate alternative splicing, allowing the production of multiple protein isoforms from a single gene. This structural complexity enhances regulatory plasticity and enables fine-tuning of gene expression in developmental and stress-response contexts, a pattern documented in several crops including almond, chickpea, and *Brachypodium distachyon* [49, 50, 52]. In contrast, ERF and DREB subfamilies, especially those with key roles in abiotic stress signaling, frequently lack introns or contain only one, supporting their rapid transcriptional activation under environmental stress. This structural economy has been reported in maize, foxtail millet, and wheat, and is thought to enhance transcriptional speed and energy efficiency during immediate stress responses [41, 42, 48]. At the level of conserved motifs, the ubiquitous presence of Motifs 4, 5, and 6 across nearly all subfamilies highlights their essential roles in DNA binding and transcriptional activation. These conserved elements likely serve as the core functional units of the AP2/ERF domain. In contrast, the subfamily-specific motifs such as Motifs 1–3 observed predominantly in the AP2 subfamily suggest a role in mediating specific protein–protein interactions or in the regulation of downstream genes with pathway-specific

functions, facilitating functional specialization. Similar motif-based diversification patterns have been described in chickpea, soybean, and grapevine, where subgroup-specific motifs correlate with unique expression profiles and stress responsiveness [52, 53]. Of particular note in *N. nucifera* is the novel observation of tetra-UTR structures, such as those found in *ERF47* and *AP2-12*. This unique feature has not been commonly reported in other species and implies potential involvement in complex post-transcriptional regulatory networks. UTRs are known to harbor microRNA binding sites and RNA-binding protein motifs, influencing mRNA localization, stability, and translational efficiency. Although UTR architecture in *AP2/ERF* genes remains underexplored, recent studies in chickpea and tomato suggest that variations in 3' UTRs can significantly affect stress-responsive gene expression through miRNA-mediated regulation and mRNA turnover rates [52]. Thus, the discovery of tetra-UTRs in *N. nucifera* not only reveals a layer of transcriptional control that may be lineage-specific but also opens up novel avenues for studying untranslated regions as dynamic hubs of gene expression modulation in aquatic plants.

The physiological shift observed in *Nelumbo nucifera* under escalating salt stress, where defense-related indicators such as MDA, Proline, and SOD peak at 150 mM NaCl but decline at 150 mM, corresponds to a threshold in stress resilience capacity, highlighting a critical “inflection point” in the plant’s ability to maintain redox and osmotic homeostasis. This physiological inflection is mechanistically supported by the transcriptomic response observed at 150 mM, where a surge in differentially expressed genes (DEGs) included a suite of *AP2/ERF* transcription factors known for their pivotal roles in abiotic stress regulation. In sugarcane (*Saccharum spontaneum*), a comprehensive genome-wide study identified 218 *AP2/ERF* genes, with several, including *SsERF52* and *SsSoloist4*, exhibiting specific inducibility under salt and ABA treatment, reflecting conserved stress-response mechanisms that are likely mirrored in *N. nucifera* [54]. Similarly, *CaERF2* in pepper was found to enhance salt tolerance by modulating ROS scavenging through upregulation of antioxidant enzymes [55], providing parallel functional evidence supporting the observed SOD elevation in *N. nucifera* at moderate salt levels. Crucially, several of the *AP2/ERF* genes prominently upregulated in *N. nucifera*, such as *DREB21*, *ERF23*, and *ERF15*, have functional orthologs in other species with experimentally validated roles in stress adaptation. For instance, *GmERF105* from soybean was shown to act as a negative regulator of salt stress by suppressing SOD and stress-responsive gene expression, linking transcriptional repression to heightened ROS accumulation [56]. In contrast, *IbRAP2-12* from sweetpotato positively regulates proline biosynthesis and ABA-mediated pathways, enhancing tolerance through osmotic adjustment and redox buffering [57], aligning with the elevated Proline levels in *N. nucifera* under 150 mM NaCl. Especially, similar regulation was observed in *Foxtail millet*, where specific *AP2/ERF* members were found to be induced via ABA-dependent pathways, significantly increasing proline content and ROS-scavenging activities [48]. Further validating the hypothesis of a core regulatory module, the overexpression of genes like *GhERF13.12* in cotton and *FaTINY2* in strawberry enhanced salt tolerance through modulating proline synthesis, SOD/POD activity, and ABA signaling [20, 58]. These responses are consistent with the physiological traits seen in *N. nucifera* and substantiate the functional conservation of *AP2/ERF* regulators across divergent taxa. Conversely, downregulation of genes associated with developmental processes, interpreted as a “growth-defense trade-off”, has also been substantiated, such as in celery, where *AgERF8* overexpression reduced antioxidant enzyme activity and increased sensitivity to NaCl, underlining the energetic cost of growth under stress [59]. The coherence between *N. nucifera* transcriptomic trends and these comparative findings underscores that the *AP2/ERF* family, particularly *DREB* and *ERF* subgroups, orchestrate critical nodes in ABA-responsive and redox-regulating gene networks during early stress responses. These insights reinforce that 150 mM NaCl represents not only a physiologically significant threshold

but also a molecularly active window, where *AP2/ERF*-mediated transcriptional programs are functionally engaged to sustain plant viability under salt stress.

The present study represents the first comprehensive characterization of the *AP2/ERF* gene family in *N. nucifera*, offering novel insights into its structural, evolutionary, and functional dimensions. A distinctive feature uncovered is the presence of tetra-UTR structures in certain *AP2/ERF* genes, which may contribute to complex post-transcriptional regulatory mechanisms. Such structural uniqueness echoes findings in *Arabidopsis* and maize, where cis-regulatory variation in *AP2/ERF* genes, particularly in untranslated regions, was shown to influence transcriptional plasticity and stress responsiveness [38]. Functional predictions, although largely computational, are substantiated by comparative transcriptomic analyses under salinity-alkalinity stress, revealing a suite of differentially expressed genes (DEGs) that are tightly correlated with physiological stress responses. This is consistent with the regulatory behavior of *OsDREB2B* in rice, which controls stress-related genes through gibberellin metabolism and interacts with *OsWRKY21* and *OsAP2-39*, ultimately modifying plant growth and tolerance [60]. The physiological breakpoint observed at 150 mM NaCl, marked by maximal MDA, Pro, and SOD levels, aligns with transcriptional upregulation of key regulators such as *DREB21* and *ERF23*. Similar transcriptional inflection points have been identified in maize and *Arabidopsis*, where transcriptomic shifts under optimal stress conditions precede irreversible cellular damage[42]. The upregulation of *DREB21* in particular points to its probable involvement in ABA-dependent or -independent osmotic stress pathways, a role widely confirmed in model plants [61]. Meanwhile, *ERF15* and *ERF23* may act through ethylene signaling and ROS detoxification, akin to the role of *ERF012* in *Arabidopsis*, which modulates root development and stress adaptation through ethylene biosynthesis and auxin signaling [62]. Furthermore, the growth-defense trade-off mechanism is proposed to underlie the downregulation of genes linked to basic germination processes under high stress, a phenomenon observed in multiple species where energy allocation prioritizes survival over growth [33, 63]. Despite these robust insights, this study remains limited by its reliance on in silico predictions and transcriptional snapshots from the seed germination stage alone. No functional validation has yet been conducted, and the expression profiles of *AP2/ERF* genes across different developmental stages and tissues (e.g., leaves, roots, rhizomes) remain unexplored. Functional assays such as heterologous overexpression in *Arabidopsis* or rice, gene silencing via VIGS, and CRISPR/Cas9-mediated knockouts represent immediate avenues for validating the roles of key candidates like *AP2-9* and *DREB21*. Equally crucial is the elucidation of the downstream gene targets and regulatory networks governed by these transcription factors. Recent studies have highlighted the utility of methods such as ChIP-seq, EMSA, and DAP-seq for delineating direct transcriptional interactions, as employed in *HRE2* regulatory analysis in *Arabidopsis* under salt stress [64]. Moreover, the activation of *AP2/ERF* genes themselves may be mediated by upstream cues such as ABA, ROS, calcium signaling, and ethylene, as proposed in recent network models integrating miRNA interactions and TF regulation [65]. Therefore, mapping the complete regulatory cascade from stress perception to gene expression remains a critical future direction.

Materials and Methods

4.1 Identification and systematic analysis of *AP2/ERF*

To identify and classify *AP2/ERF* family members in *N. nucifera*, we conducted a comparative analysis using *A. thaliana* *AP2/ERF* proteins (AtAP2/ERFs) as reference sequences. Multiple sequence alignment was performed, followed by the construction of a phylogenetic tree in MEGA7 using the neighbor-joining (NJ) method with 1000

bootstrap replicates to ensure statistical reliability. Conserved motif analysis was carried out using the MEME Suite (<https://meme-suite.org/meme/tools/Meme>), and the resulting motifs were graphically represented using TBtools. The domain organization of the proteins was examined through the Conserved Domain Database (CDD) provided by NCBI (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>). In addition, the gene structure of *N. nucifera* AP2/ERFs was illustrated using TBtools.

4.2 Collinearity analysis of *N. nucifera* AP2/ERF genes

Gene duplication events in *N. nucifera* were identified using the Multiple Collinearity Scan toolkit (MCScanX), applying stringent BLASTp criteria with an e-value threshold of less than 1×10^{-5} . To investigate the evolutionary dynamics of gene families in *N. nucifera*, a comparative analysis was conducted using *A. thaliana* as a representative model species. Collinear gene pairs between *N. nucifera* and *A. thaliana* were comprehensively detected and visualized using TBtools.

4.3 Phenotypic analysis of *N. nucifera* under different saline-alkali concentrations

Uniform, fully developed, plump, and disease-free seeds of *N. nucifera* were selected for the germination experiment. Four concentrations of mixed saline-alkaline solutions (NaCl: NaHCO₃ at a molar ratio of 1:1) were prepared: 0, 50, 100, and 150 mmol·L⁻¹. Each treatment included three replicates, with 35 seeds per replicate. A 1000 mL beaker was used as the germination container, and 500 mL of the respective saline-alkaline solution was added for the stress treatment. The beakers were incubated in a growth chamber under controlled conditions: 25 °C with 16 h light and 20 °C with 8 h dark. Germination potential, germination rate, and shoot length were measured on days 0, 5, and 10 to evaluate seed germination and early seedling growth under different concentrations of saline-alkaline stress.

4.4 Determination of MDA, Proline, and Antioxidant Enzyme Activities (SOD, POD, and CAT)

To evaluate the physiological responses of *N. nucifera* under saline-alkali stress, the contents of malondialdehyde (MDA), proline (Pro), and the activities of antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), were measured using commercial assay kits purchased from Solarbio (Beijing, China), following the manufacturer's protocols. Fresh shoots (0.1-0.5 g) were homogenized in appropriate extraction buffers on ice and centrifuged at 8,000-12,000 × g at 4 °C for 10-15 min. The supernatants were collected for subsequent biochemical analyses.

4.5 Transcriptome Sequencing and Differential Expression Analysis

To investigate the transcriptomic responses of *N. nucifera* shoots under salt-alkaline stress, plants were subjected to 0 mM (control) and 150 mM salt-alkaline treatments, and shoot tissues were harvested at 5 and 10 days post-treatment. A total of four treatment groups were established: 0 mM-5d (T5), 150 mM-5d (Y5), 0 mM-10d (T10), and 150 mM-10d (Y10), with each sample labeled accordingly as 0 mM-5d-T5, 150 mM-5d-Y5, 0 mM-10d-T10, and 150 mM-10d-Y10. Total RNA was extracted from shoot tissues using a Plant RNA Isolation Kit (e.g., TIANGEN, China), following the manufacturer's instructions. RNA quality and concentration were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and integrity was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, USA). High-quality RNA samples (RIN ≥ 7.0) were used for library construction and sequencing. RNA-seq library preparation and sequencing were performed by Beijing Genomics Biotechnology Co., Ltd (Beijing, China). Libraries were constructed using the NEBNext® Ultra™ RNA Library Prep Kit for

Illumina® (New England Biolabs, USA), and sequencing was carried out on the Illumina NovaSeq 6000 platform to generate 150 bp paired-end reads. Raw reads were filtered to remove adapters and low-quality sequences using fastp, and clean reads were aligned to the *N. nucifera* reference genome using HISAT2. Read counts were obtained using featureCounts, and gene expression levels were normalized as fragments per kilobase of transcript per million mapped reads (FPKM). Differentially expressed genes (DEGs) were identified using DESeq2 with the criteria of $|\log_2 \text{fold change}| \geq 2$ and adjusted p-value < 0.05. GO and KEGG enrichment analyses of DEGs were performed using the clusterProfiler package in R.

4.5 Quantitative RT-PCR analysis

The *AP2-9*, *ERF23*, *ERF15*, *ERF31*, *ERF34*, and *DREB21* genes were selected for quantitative real-time PCR (qRT-PCR) analysis. Gene-specific primers were designed using Primer 5, and *Actin* was used as the internal reference gene (Table S1). qRT-PCR was conducted with a Bio-Rad CFX Manager system using SYBR Green Master Mix. Each 10 μL reaction mixture contained 5 μL of SYBR Green Mix, 0.5 μL of each primer, 2 μL of cDNA template, and 2.5 μL of nuclease-free water. The amplification program consisted of an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. A melting curve was generated from 60 °C to 90 °C to verify amplification specificity. Each sample was analyzed with three biological replicates and three technical replicates.

Conclusion

This study presents a comprehensive analysis of the *AP2/ERF* transcription factor family in *N. nucifera*, integrating genome-wide identification, structural and evolutionary characterization, and transcriptomic profiling under salt-alkaline stress conditions. A total of 101 *AP2/ERF* genes were identified and classified into five subfamilies, showing considerable diversity in gene structure, motif composition, and physicochemical properties. Phylogenetic and syntenic comparisons with *A. thaliana* revealed both conserved and species-specific evolutionary patterns. Physiological and biochemical assays demonstrated that salt-alkaline stress significantly impairs seed germination and growth, accompanied by dynamic changes in oxidative stress-related indices. Transcriptome analyses identified 29 *AP2/ERF* genes that were differentially expressed under stress, among which 13 genes, including *AP2-9*, *ERF23*, *ERF15*, *ERF31*, *ERF34*, and *DREB21*, showed sustained upregulation, highlighting their potential regulatory roles in salt-alkaline tolerance. Subsequent qRT-PCR validation confirmed the expression trends of *AP2-9*, *ERF23*, *ERF34*, and *DREB21* in both 5-day and 10-day treatments, corroborating the transcriptome results and supporting their critical roles in the regulatory network of salt-alkali stress response during seed germination. These findings offer valuable insights into the molecular mechanisms of stress adaptation in *N. nucifera* and provide a foundation for further functional studies and genetic improvement of salt-alkaline resilience in *N. nucifera* and other aquatic crops.

Abbreviations

AP2, *APETALA2*; *ERF*, ethylene-responsive factor; *DREB*, dehydration-responsive element-binding; *RAV*, related to ABI3/VP1; ROS, oxygen species; MDA, malondialdehyde; Pro, proline; SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; TF, Transcription factors; FPKM, transcript per million mapped reads; DEGs, Differentially expressed genes; GO, Gene Ontology; qRT-PCR, quantitative real-time PCR.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and material: The datasets generated and analysed during the current study are available in the CNCB (China National Center for Bioinformatics) repository: PRJCA049079 (subCRA052378) (<https://ngdc.cnbc.ac.cn/gsub/submit/gsa/subCRA052378>).

Competing interests The authors declare that they have no competing interests.

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Tables

Table 1. Physicochemical properties analysis of AP2/ERF proteins in *N. nucifera*.

LOC ID	Gene ID	Number of Amino Acid	Molecular Weight	Theoretical pI	Instability Index	Aliphatic Index	Grand Average of Hydropathicity
LOC104594736	AP2-1	518	55908.6	5.82	52.48	54.86	-0.631
LOC104599345	AP2-2	511	55576.22	5.9	54.72	51	-0.688
LOC104593481	AP2-3	373	41973.48	5.73	56.57	61.8	-0.791
LOC104605418	AP2-4	441	50380.61	5.95	58.01	58.23	-0.873
LOC104589848	AP2-5	347	38896.92	5.96	46.47	61.38	-0.819
LOC104590512	AP2-6	427	47374.15	8.9	51.11	65.64	-0.796
LOC104591238	AP2-7	677	74523.81	6.42	51.78	60.86	-0.653
LOC104609075	AP2-8	679	74783.07	6.32	50.93	61.94	-0.684
LOC104600583	AP2-9	597	66024.11	6.69	50.67	51.96	-0.879
LOC104609334	AP2-10	597	65778.1	6.61	44.59	54.92	-0.827
LOC104611932	AP2-11	540	59130.38	5.99	54.1	65.48	-0.535
LOC104595508	AP2-12	555	61151.88	6.36	55.29	66.5	-0.521
LOC104597438	AP2-13	552	60509.76	6.58	46.84	59.96	-0.646
LOC104613021	AP2-14	542	59725.9	6.25	53.65	61.61	-0.638
LOC104603172	AP2-15	528	57510.29	6.19	39.82	58.05	-0.583
LOC104586961	S1	232	25958.02	9.6	63.86	52.54	-1.019
LOC104601351	RAV1	365	40720.75	9.07	43.42	71.81	-0.624
LOC104603036	RAV2	365	40798.01	8.91	41.9	75.23	-0.565
LOC104591998	RAV3	366	41939.11	6.29	51.01	65.74	-0.716
LOC104598694	RAV4	397	45259.85	8.02	44.81	63.9	-0.661
LOC104595388	ERF1	178	19545.23	9.12	68.23	69.72	-0.533
LOC104592437	ERF2	189	21016.98	8.87	68.52	73.86	-0.519
LOC104603203	ERF3	150	17052.78	5.74	72.93	61.2	-0.759
LOC104603363	ERF4	185	21162.07	8.46	54.74	66.97	-0.687
LOC104589060	ERF5	187	21116.05	8.81	46.91	72.03	-0.59
LOC104610490	ERF6	304	32693.62	6.77	66.18	63.98	-0.51
LOC104598425	ERF7	309	33296.33	6.78	69.99	67.61	-0.488
LOC104610091	ERF8	116	13343.8	5.76	60.63	68.28	-0.871
LOC104610031	ERF9	135	15363.04	7.87	66.15	66.59	-0.813

LOC104600858	ERF10	131	14742.39	5.48	54.64	61.98	-0.792
LOC104600857	ERF11	143	16178.97	5.86	57.16	62.24	-0.928
LOC104610033	ERF12	154	17026.57	5.94	56.75	49.55	-0.945
LOC104610032	ERF13	155	17238.96	6.84	55.32	53.03	-0.866
LOC104610030	ERF14	253	28696.84	5	66.96	57.43	-0.711
LOC104600859	ERF15	230	26117.91	5.22	70.97	58.52	-0.721
LOC104599517	ERF16	281	31224.74	4.93	51.08	68.47	-0.597
LOC104598426	ERF17	357	39885.54	5.29	57.87	67.79	-0.635
LOC104610488	ERF18	337	37547.08	6.04	52.64	66.26	-0.578
LOC104606510	ERF19	235	25911.64	9.33	64.3	56.94	-0.83
LOC104594121	ERF20	235	26042.77	9.63	60.1	52.85	-0.89
LOC104612420	ERF21	175	19498.27	9.77	45.17	62.51	-0.597
LOC104608169	ERF22	157	17037.27	8.88	62.33	67.26	-0.398
LOC104599450	ERF23	159	17308.63	9.26	63.03	68.18	-0.48
LOC104608167	ERF24	242	25672.25	6.87	54.38	56.16	-0.578
LOC104599451	ERF25	237	25339.87	9	52.61	56.54	-0.63
LOC104593077	ERF26	393	43256.04	5.52	71.67	60.84	-0.598
LOC104591971	ERF27	389	43206.75	5.24	67.66	54.7	-0.731
LOC104586349	ERF28	352	38717.89	4.66	68.19	56.62	-0.495
LOC104592717	ERF29	275	29757.34	4.59	59.66	47.96	-0.521
LOC104591899	ERF30	285	30802.72	4.69	60.3	48.67	-0.461
LOC104592746	ERF31	242	27082.86	6.22	57.7	56.07	-0.842
LOC104592013	ERF32	329	36554.9	8.24	62.21	68.57	-0.729
LOC104599945	ERF33	423	45596.9	5.55	62.83	51.96	-0.612
LOC104589605	ERF34	412	44663.78	6.34	69.83	53.81	-0.605
LOC104606467	ERF35	290	32037.35	5.27	62.8	55.97	-0.753
LOC104601789	ERF36	277	30596.62	5.48	48.75	61.37	-0.689
LOC104607321	ERF37	140	15552.68	10.31	36.33	64.79	-0.801
LOC104606213	ERF38	268	29969.72	7.05	53.73	66.64	-0.688
LOC104598889	ERF39	258	28668.25	8.83	41.84	65.47	-0.719
LOC104609267	ERF40	372	41595.75	4.82	49.25	54.06	-0.85

LOC104600466	ERF41	376	42035.61	4.82	44.12	55.56	-0.741
LOC104606214	ERF42	328	36054.07	5.58	55.03	54.54	-0.761
LOC104598888	ERF43	314	34956.39	5.01	50.93	48.82	-0.875
LOC104600304	ERF44	339	37899.97	5.36	52.58	62.65	-0.627
LOC104593412	ERF45	328	37039.61	5.1	57.08	57.68	-0.838
LOC104602974	ERF46	343	38183.9	4.95	72.53	56.36	-0.846
LOC104601805	ERF47	345	38297.43	5.38	62.31	63.04	-0.745
LOC104597658	ERF48	267	29511.56	6.08	40.74	56.97	-0.738
LOC104600784	ERF49	256	28848.1	7.65	52.56	60.59	-0.705
LOC104588536	ERF50	275	29658.4	9.55	43.87	68.84	-0.349
LOC104603385	ERF51	235	25841.13	9.02	65.97	65.57	-0.459
LOC104605948	DREB1	391	42603.53	6.33	59.63	66.21	-0.542
LOC104600117	DREB2	415	45276.34	6.01	65.26	60.7	-0.565
LOC104588890	DREB3	461	51370.89	6.03	68.7	52.97	-0.863
LOC104586457	DREB4	432	47407.59	6.39	58.44	54.7	-0.724
LOC104600346	DREB5	323	35041.3	8.75	49.51	68.64	-0.493
LOC104609420	DREB6	432	47361.72	5.64	57.15	70.05	-0.472
LOC104586003	DREB7	428	46686.08	5.35	55.52	70.47	-0.453
LOC104599845	DREB8	290	31455.48	8.32	54.92	59.59	-0.676
LOC104604994	DREB9	157	17799.26	4.47	33.03	85.1	-0.401
LOC104609654	DREB10	220	24020.65	5.13	75.42	55.14	-0.609
LOC104608486	DREB11	221	24239.93	5.47	85.25	50.86	-0.619
LOC104586477	DREB12	163	18104.27	5.11	47.53	77.85	-0.543
LOC104609294	DREB13	204	21947.44	5.49	47.2	57.45	-0.488
LOC104609295	DREB14	207	22854.08	4.67	42.53	52.42	-0.627
LOC104600498	DREB15	199	22291.38	4.59	52.93	53.97	-0.721
LOC104606875	DREB16	153	16767.69	9.24	50.37	53.66	-0.779
LOC104601725	DREB17	152	16578.44	9.37	55.41	51.51	-0.795
LOC104593944	DREB18	198	21885.39	5.24	59.03	74.04	-0.509
LOC104589184	DREB19	233	25782	6.39	51.51	77.47	-0.404
LOC104604770	DREB20	190	21140.58	6.33	56.91	57.11	-0.753

LOC104604040	DREB21	187	20786.07	5.3	53.38	59.09	-0.711
LOC104612693	DREB22	187	20161.53	4.87	49.11	70.05	-0.405
LOC104596183	DREB23	236	25237.27	4.68	70.03	64.62	-0.505
LOC104586938	DREB24	211	22425.94	5	45.69	70.9	-0.343
LOC104591308	DREB25	182	19673.14	4.7	49.79	76.15	-0.389
LOC104595123	DREB26	242	26559.67	5.18	57.7	66.53	-0.521
LOC104602399	DREB27	282	31059.44	5.27	50.12	67.2	-0.608
LOC104598459	DREB28	275	29959.54	5.27	63.49	43.42	-0.858
LOC104602444	DREB29	225	25028.77	5.19	50.38	63.02	-0.691
LOC104602443	DREB30	147	15965.66	4.24	52.99	70.61	-0.231

Figures

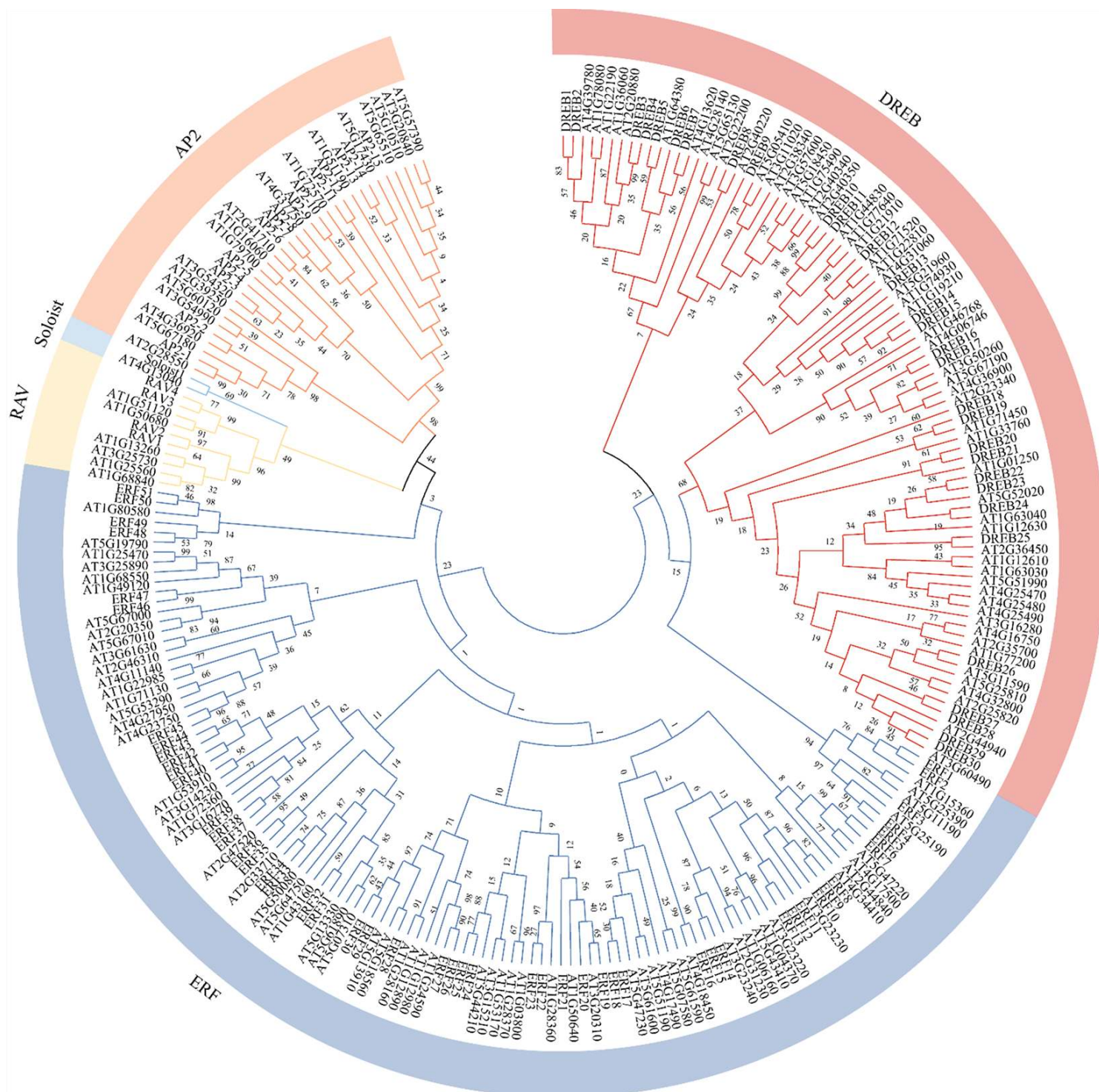


Figure 1

Phylogenetic tree of AP2/ERF gene families in *N. nucifera* and *A. thaliana*.

MEGA7 software was employed to construct the phylogenetic tree, and subgroup classification was visualized using different colors.

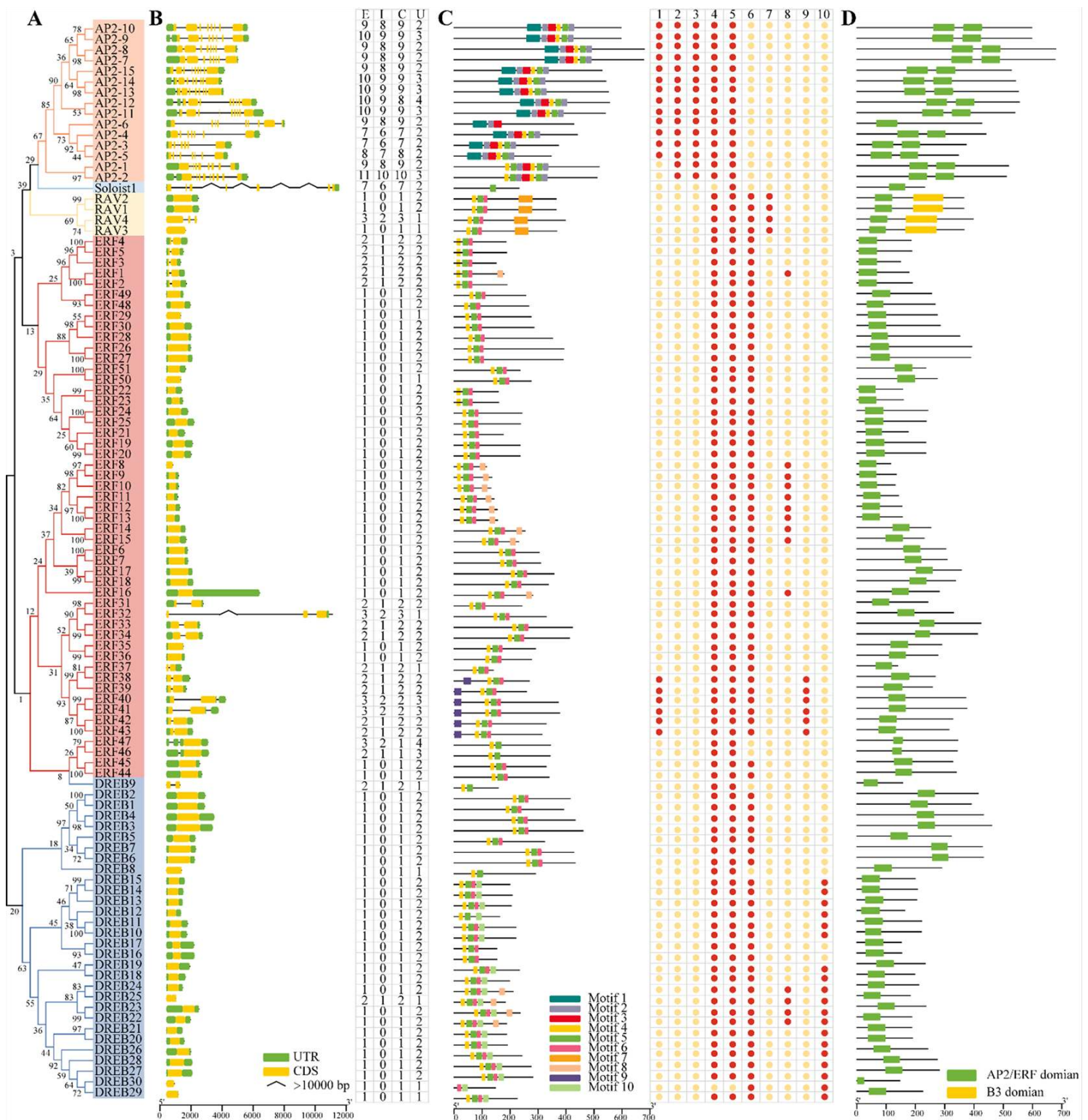


Figure 2

Comparative analysis of phylogenetic relationships, gene structures, conserved motifs, and protein domains of the AP2/ERF gene family in *N. nucifera*.

(A) Phylogenetic tree of the AP2/ERF gene family, constructed based on amino acid sequences using MEGA7 software.

(B) Gene structure analysis of 101 *AP2/ERF* gene. Black lines represent introns; green boxes denote 5' and 3' untranslated regions (UTRs); yellow boxes represent exons. A table showed the number of exons (E), introns (I), coding sequence (C), and UTRs (U) of *AP2/ERF* genes.

(C) Conserved motif analysis. Ten conserved motifs were identified within *AP2/ERF* proteins, with each motif shown in a different color. A table shows whether motifs 1-10 are present in various *AP2/ERF* protein sequences. Orange dots indicate absence, while red dots indicate presence.

(D) Conserved domain analysis. Green boxes indicate AP2 domains, and yellow boxes represent B3 domains.

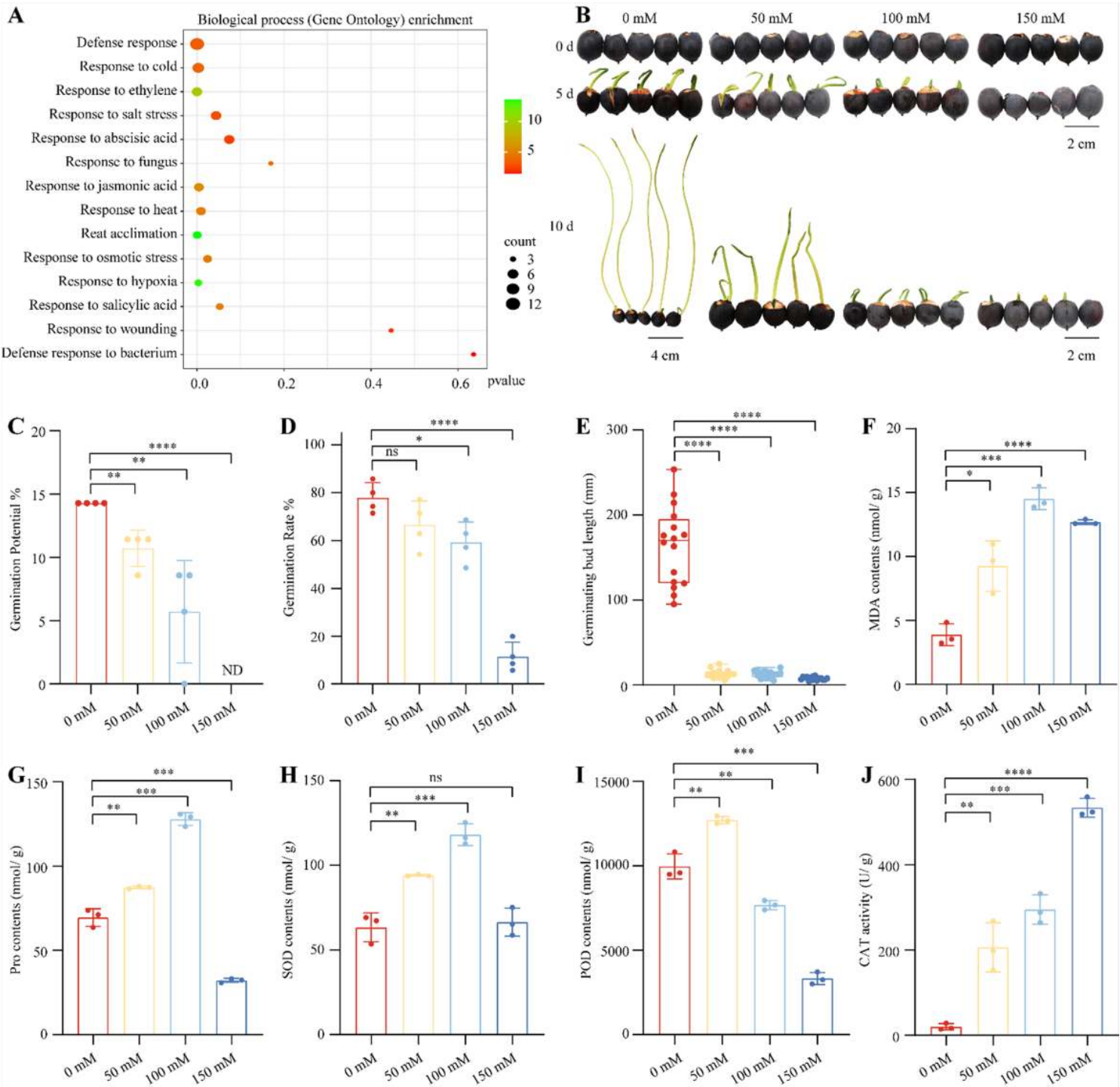


Figure 3

Higher salt-alkaline stress inhibits seedling growth in *N. nucifera*.

(A) GO enrichment analysis of 147 *Arabidopsis* AP2/ERF gene family members, highlighting 23 major enriched pathways.

(B) Germination phenotypes of *N. nucifera* seeds under different salt-alkaline stress levels (0, 50, 100, and 150 mM) at 0, 5, and 10 days after treatment.

(C-D) Germination potential and germination rate of the seeds under various salt-alkaline stress conditions.

(E) Radicle length of germinated seeds under salt-alkaline stress.

(F-I) Malondialdehyde (MDA), Proline (Pro), Superoxide dismutase (SOD), and Peroxidase (POD) contents under different salt-alkaline treatments (0, 50, 100, and 150 mM) at 10 d.

(J) Catalase (CAT) activity in response to salt-alkaline treatments (0, 50, 100, and 150 mM) at 10 d.

Statistical differences were determined using Student's T-test. * significant differences between treatments at $p \leq 0.05$. ** significant differences between treatments at $p \leq 0.01$. *** significant differences between treatments at $p \leq 0.001$. **** significant differences between treatments at $p \leq 0.0001$. ns, not significant.

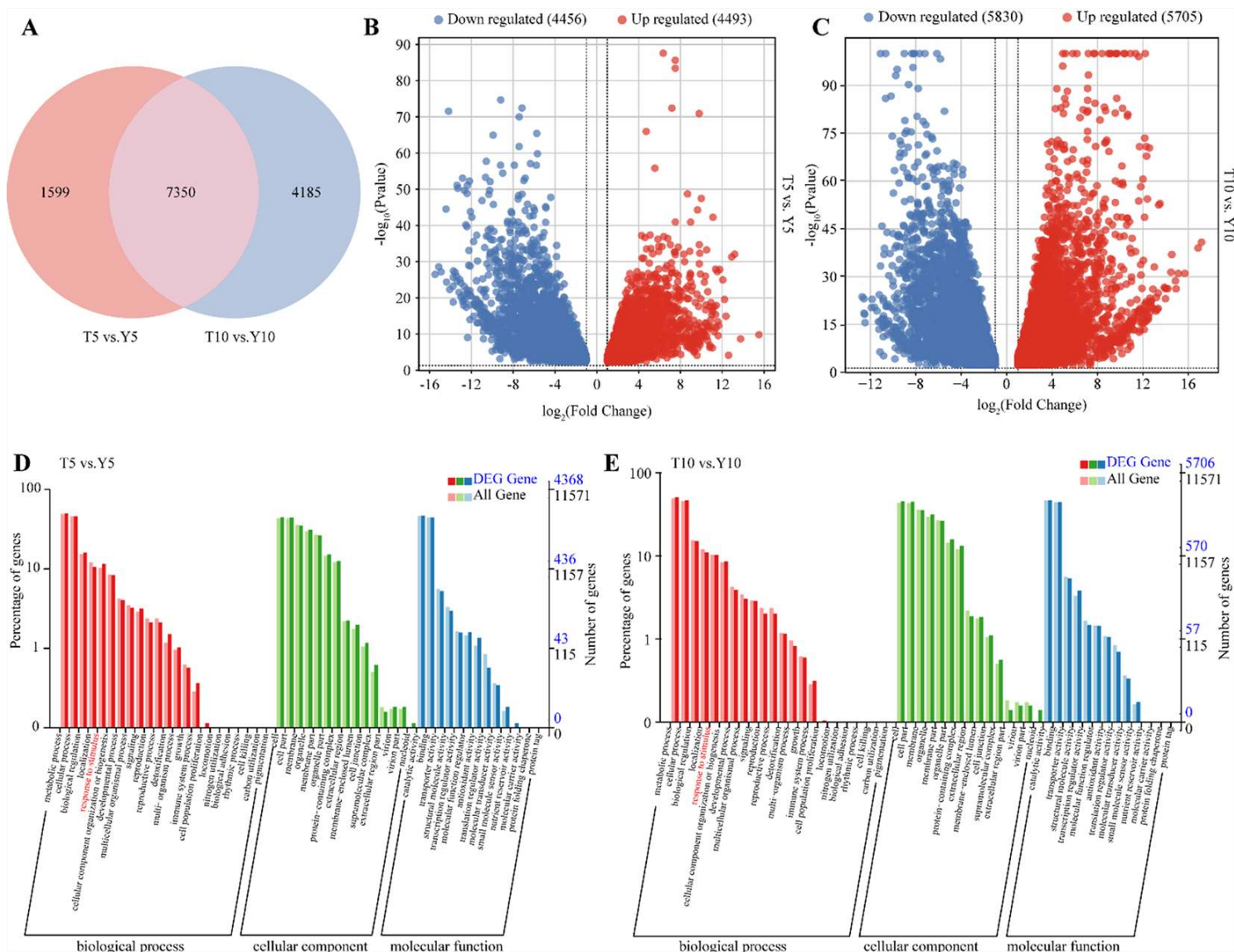


Figure 4

Transcriptomic analysis of *N. nucifera* under salt-alkaline stress.

(A) Venn diagram showing the overlap of differentially expressed genes (DEGs). T5, 0 mM-5 d; Y5, 150 mM-5 d; T10, 0 mM-10 d; Y10, 150 mM-10 d.

(B-C) Volcano plots of DEGs in the T5 vs. Y5 and T10 vs. Y10. Red dots indicate significantly upregulated genes, and blue dots indicate significantly downregulated genes.

(D-E) Gene Ontology (GO) enrichment analysis of DEGs in the T5 vs. Y5 and T10 vs. Y10, showing significant enrichment in key biological processes, cellular component, and molecular function.

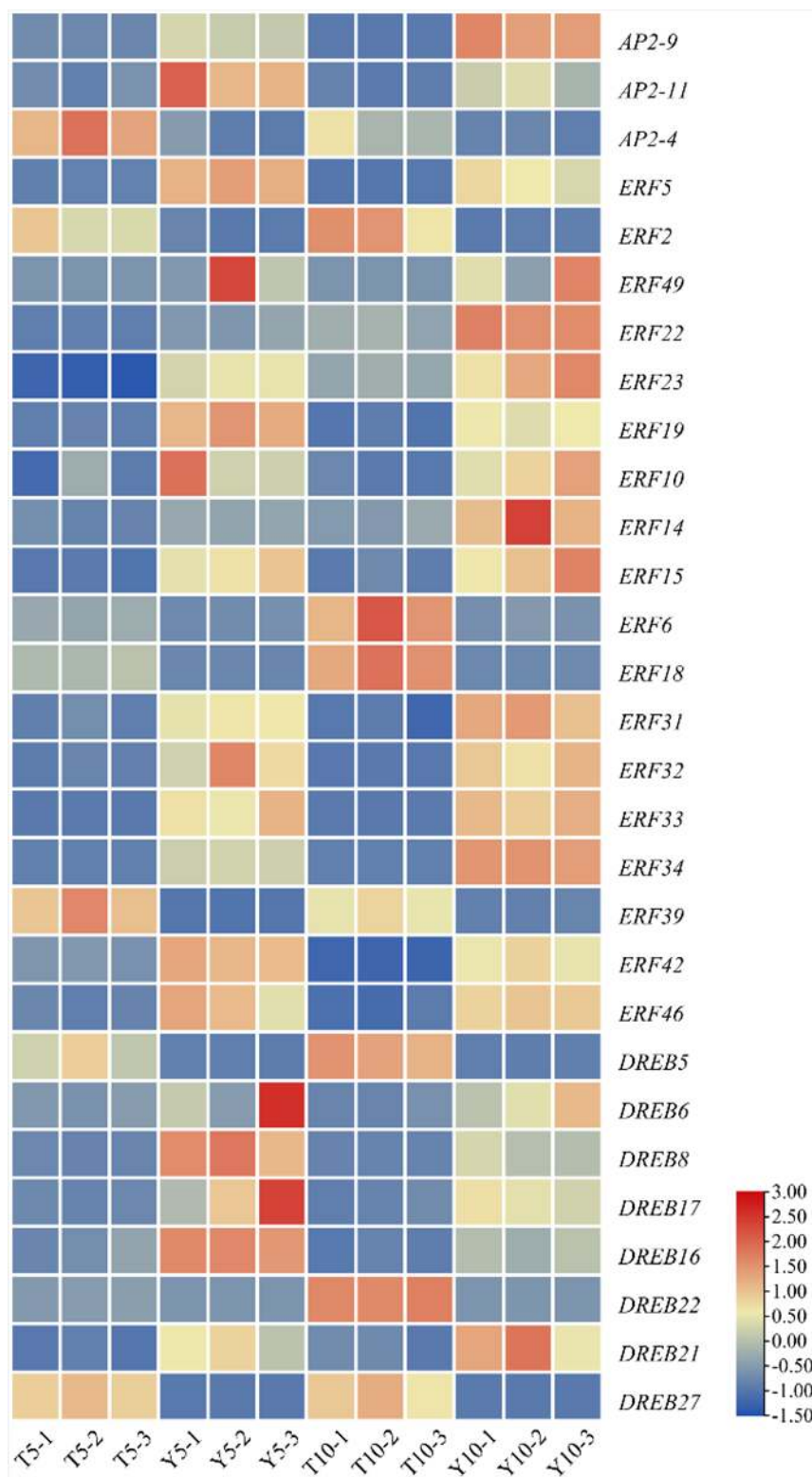


Figure 5

Expression profiles of 29 AP2/ERF transcription factors significantly affected by salt-alkaline stress.

The heatmap displays the expression patterns of 29 AP2/ERF transcription factors in response to salt-alkaline treatment in *N. nucifera*, including 3 AP2 subfamily members, 18 ERF subfamily members, and 8 DREB subfamily members. The color scale represents normalized expression levels (log₂-transformed values).

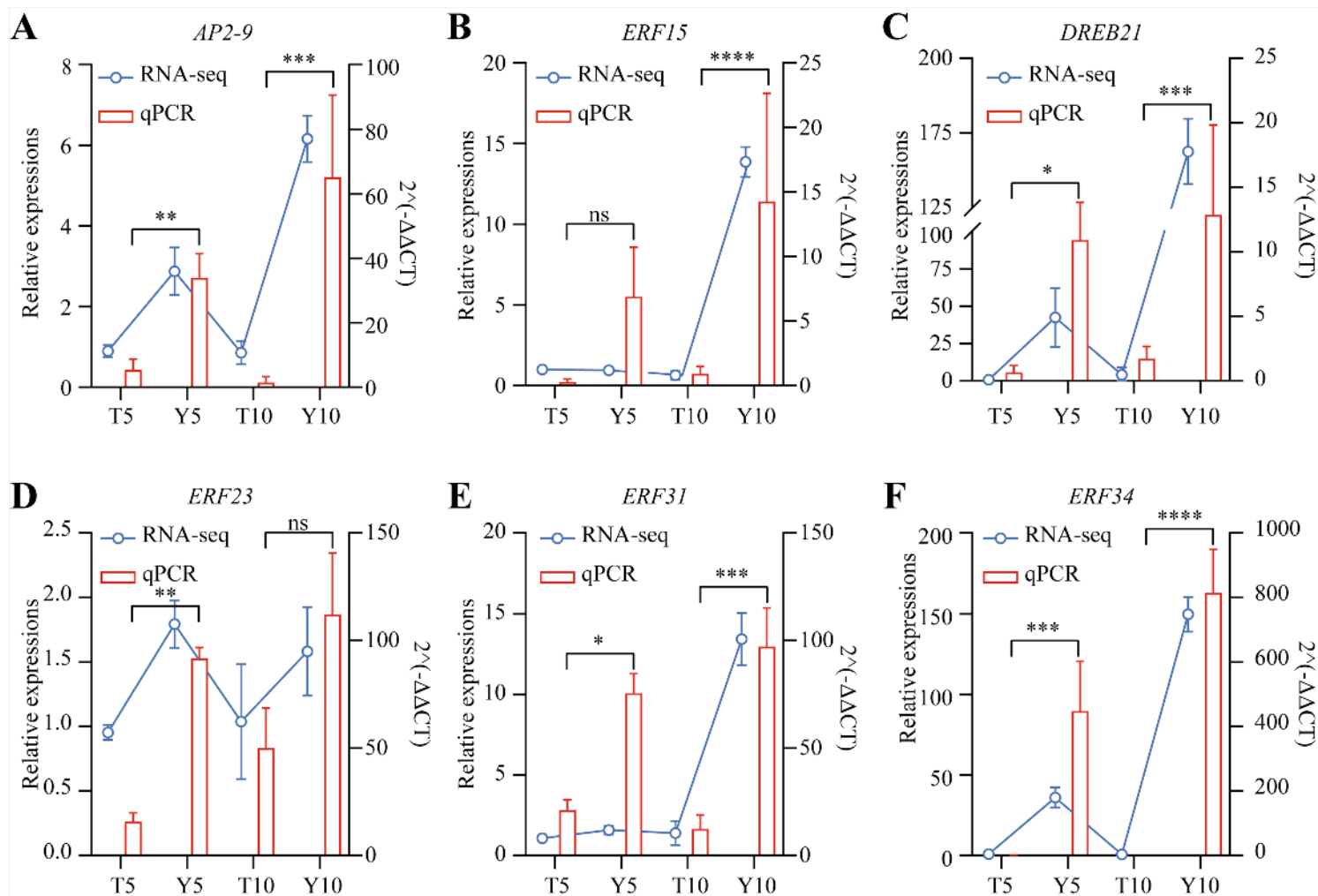


Figure 6

Expression validation of key *AP2/ERF* genes during seed germination of *Nelumbo nucifera* under salt-alkali stress.

Based on transcriptome analysis, *AP2-9*, *ERF23*, *ERF15*, *ERF31*, *ERF34*, and *DREB21* were identified as potential key members of the *AP2/ERF* family. Their expression patterns were validated by qRT-PCR using samples collected from seeds subjected to 0 mM (control) and 150 mM salt-alkali treatments for 5 and 10 days. The results showed that all six genes were significantly upregulated under salt-alkali stress. Among them, *AP2-9*, *ERF23*, *ERF34*, and *DREB21* displayed expression trends consistent with the transcriptome data, while *ERF15* and *ERF31* exhibited markedly higher induction levels in qRT-PCR, suggesting a more complex regulatory mechanism.

Statistical differences were determined using Student's T-test. * significant differences between treatments at $p \leq 0.05$. ** significant differences between treatments at $p \leq 0.01$. *** significant differences between treatments at $p \leq 0.001$. **** significant differences between treatments at $p \leq 0.0001$. ns, not significant.

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