

AREB/ABF-mediated transcriptional regulatory network for salt stress response in *Dendrobium officinale*

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Abstract

Dendrobium officinale, a traditional precious medicinal orchid in China, faces salt stress as a major abiotic constraint limiting its large-scale artificial cultivation, yet the molecular regulatory mechanisms underlying its salt stress response remain unclear. In this study, tissue-cultured seedlings of *Dendrobium officinale* were treated with 250 mM NaCl, and roots and leaves were sampled at 0 h, 4 h, and 12 h post-treatment for transcriptome sequencing. Combined with genome sequences from the NCBI database and homologous *Arabidopsis* sequences from the TAIR database, we performed genome re-annotation, differential expression analysis, WGCNA co-expression network construction, and genome-wide identification of the AREB/ABF gene family, followed by phylogenetic analysis, promoter cis-element analysis, tissue-specific expression analysis, and qRT-PCR validation. The results showed that roots are the core responsive organ to salt stress in *Dendrobium officinale*, with approximately twice as many differentially expressed genes as in leaves. A total of eight AREB/ABF family members were identified genome-wide, among which five Clade A members were confirmed as core regulators of salt stress response. Through multiple lines of evidence, the core regulatory gene *Do07G001659* was identified. This gene exhibits extremely low basal expression under normal conditions but shows a time-dependent significant upregulation in roots under salt stress, thereby regulating the expression of the downstream core target gene *Do17G000728*, which is involved in osmotic regulation and antioxidant processes. This study elucidates the transcriptional regulatory characteristics of salt stress response in *Dendrobium officinale* and defines the ABA-dependent core regulatory pathway mediated by *Do07G001659*, providing key targets and theoretical support for deciphering salt tolerance mechanisms and molecular breeding for stress resistance.

1. Introduction

Dendrobium officinale, a perennial epiphytic orchid, is a valuable traditional Chinese medicine known for its stomach-benefiting, fluid-promoting, and Yin-nourishing effects. Its active components, polysaccharides, alkaloids, and bibenzyl compounds, exhibit immunomodulatory, antitumor, and antioxidant activities [1]. With the depletion of wild resources, artificial cultivation has become the primary source of this medicinal herb [2]. However, improper fertilization and irrigation under protected cultivation have led to increasing soil secondary salinization, making salt stress a major constraint on plant growth, yield, and quality [3, 4]. Therefore, elucidating the molecular mechanisms of salt stress response and identifying key stress-responsive genes in *D. officinale* are essential for developing salt-tolerant germplasm and advancing molecular breeding [5].

Salt stress primarily causes ionic toxicity, osmotic stress, and oxidative damage. Plants have evolved complex signaling and regulatory networks to cope with salt stress, with the abscisic acid (ABA) pathway playing a central role. Upon salt perception, ABA levels rise rapidly; ABA binds to PYR/PYL receptors, inhibiting PP2C phosphatases and activating SnRK2 kinases. Activated SnRK2 then phosphorylates AREB/ABF-type bZIP transcription factors, which bind to ABRE elements in target gene promoters and initiate stress-responsive gene expression [6, 7]. AREB/ABF factors are master regulators

of ABA-dependent osmotic stress responses and have been implicated in drought, high salinity, and low-temperature tolerance in *Arabidopsis*, rice, and maize [8, 9]. However, the AREB/ABF gene family remains largely uncharacterized in orchids [10, 11]. Transcriptome sequencing combined with weighted gene co-expression network analysis (WGCNA) enables genome-wide identification of core gene modules and hub genes associated with target traits. WGCNA has been successfully applied in salt stress studies of *Arabidopsis*, soybean, maize, sesame, tomato, and other species [12–18]. In orchids, transcriptomic research on salt response is still limited, although some progress has been made in *Cymbidium ensifolium* and *Dendrobium officinale* itself [19–22]. In *D. officinale*, the molecular regulatory network under salt stress remains unclear, and core responsive genes have yet to be systematically identified [23, 24].

In this study, tissue-cultured seedlings of *D. officinale* were subjected to 250 mM NaCl stress. Root and leaf samples were collected at 0, 4, and 12 hours after treatment for transcriptome sequencing. Using differential expression analysis and WGCNA-based co-expression network construction, we identified salt stress-regulated genes and inferred putative response pathways. These findings provide a theoretical basis for understanding salt adaptation mechanisms in orchids and offer valuable genetic resources for breeding salt-tolerant *D. officinale*.

2. Materials and methods

2.1. Data sources and experimental treatment

The original plant materials (tissue-cultured plantlets of *D. officinale*) and the experimental treatments were as described by the original authors. Briefly, according to the original study, tissue-cultured plantlets of *D. officinale* with robust growth, consistent morphology, and a height of 8–9 cm were selected for salt stress treatment. The salt stress treatment was conducted by transferring the plantlets from standard culture medium to 1/2 MS medium containing 250 mM NaCl, supplemented with 2% sucrose and 0.6% agar, at pH 5.4. Culture conditions were maintained at temperature (26 ± 1)°C, light intensity $40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and a photoperiod of 12 h light/12 h dark. Root and leaf samples were collected at 0 h, 4 h, and 12 h after salt stress treatment and immediately frozen in liquid nitrogen for subsequent RNA extraction. Additionally, different tissue samples including roots, leaves, stems, and flower buds were collected under normal growth conditions. All raw sequencing data from these samples were retrieved from the NCBI SRA database (accession numbers provided in section 2.2). As this study is a secondary analysis of publicly available data, no formal identification of plant material or deposition of voucher specimens was performed by the authors; such information, if any, should be sought from the original studies that generated the data.

2.2. Genome and transcriptome data

All genome and transcriptome data used in this study were retrieved from publicly available databases. The whole-genome sequence of *D. officinale* was obtained from the NCBI database. Transcriptome data from different tissues were retrieved from the NCBI SRA database, with the following sample accession

numbers: root (SRR5722140), leaf (SRR4431601), green root tip (SRR4431599), white root part (SRR4431598), stem (SRR4431600), flower bud (SRR4431603), sepal (SRR4431597), labellum (SRR4431602), pollinium (SRR5722145), and column (SRR4431596). Salt stress transcriptome data were also obtained from the NCBI SRA database, including root and leaf samples under salt treatment at 0 h, 4 h, and 12 h, with the 0 h samples serving as controls for differential expression analysis (Supplementary Table 1). The amino acid sequences of *Arabidopsis thaliana* AREB/ABF genes were downloaded from the TAIR database (Supplementary Table 2). According to NCBI's official policy, data in the SRA database are freely accessible for re-use without additional permission.

2.3. Genome Annotation of *D. officinale*

Given that the available data only provided the whole-genome sequence of *D. officinale* without a complete genome annotation file, this study performed genome annotation as a supplement. The annotation workflow was as follows: RepeatMasker combined with the Repbase database was used to predict repetitive sequences [25]; Augustus [26] and GlimmerHMM [27] were employed for ab initio gene structure prediction; transcriptome data were utilized for transcript assembly using StringTie [28]; the amino acid sequences of predicted genes were aligned against NR [29], Swiss-Prot [30], Pfam [31], GO [32], and KEGG [33] databases for functional annotation. The final annotation file was generated in standard GFF3 format. Annotation quality assessment revealed a total of 42,433 genes with an average gene length of 3,998 bp, a BUSCO completeness of 98.9% [34], and an NR annotation rate of 87.6% (see Supplementary Tables S4–S6). This annotation provides a reliable foundation for subsequent analyses.

2.4. Transcriptome and differential expression analysis

Raw reads were subjected to quality assessment using Fastp software [35]. Low-quality reads (Q-value < 20), reads containing adapter sequences, and reads with more than 10% ambiguous bases (N) were removed. High-quality reads were aligned to the *D. officinale* reference genome (incorporating the genome annotation generated in this study) using Hisat2 software [36]. Gene expression levels were quantified using featureCounts software [37] to generate a TPM (transcripts per million) expression matrix. Differential expression analysis was performed by comparing the 4 h and 12 h salt-treated samples against the 0 h control samples. Differentially expressed genes (DEGs) were identified using DESeq2 software [38] with the screening criteria of $|\log_2 \text{fold change}| \geq 1$ and $P < 0.05$. Relevant figures were generated using R language [39].

2.5. WGCNA analysis and regulatory network construction

A co-expression network analysis was performed using the R package WGCNA based on the TPM expression matrix of the salt-stress transcriptome [40]. Genes with TPM < 1 in more than 50% of samples were filtered out, and the remaining genes were transformed by $\log_2(\text{TPM} + 1)$. The soft-thresholding power $\beta = 5$ was selected (meeting $R^2 \geq 0.85$ with moderate average connectivity). Modules were identified using the dynamic tree-cut method with a minimum module size of 30 and a merging threshold of 0.25. The correlation between each module and treatment time points (0, 4, and 12 h) was calculated, and modules with significant correlation ($P < 0.01$) were retained. For genes within these

modules, module membership (MM) and gene significance (GS) were calculated; core genes were defined as those with $MM \geq 0.8$ and $GS \geq 0.6$. The amino acid sequences of salt-stress DEGs and WGCNA core genes were submitted to the STRING database (confidence score ≥ 0.7) to obtain protein–protein interaction (PPI) relationships (Szklarczyk et al., 2021). The regulatory network was constructed using Cytoscape software [41], and hub genes (top 10% ranked by degree) were identified using the CytoHubba plugin with the Degree algorithm [42].

2.6. Tissue-specific expression analysis of differentially expressed genes

Raw transcriptome data from different tissues were preprocessed and quantified using Fastp [35], Hisat2 [36], and featureCounts [43] software to obtain gene expression matrices for each tissue. The TPM expression values of salt-responsive differentially expressed genes (DEGs) across different tissues were extracted and normalized using the Z-score method. Expression heatmaps were generated to visualize the expression patterns of salt-responsive DEGs across various tissues (e.g., root, leaf, stem, flower bud). DEGs that were significantly responsive to salt stress and highly expressed in key tissues (e.g., leaf and root) were identified.

2.7. Identification, phylogenetic analysis, and promoter cis-element analysis of gene family members

Using *Arabidopsis thaliana* AREB/ABF amino acid sequences as queries, BLASTp searches ($E \leq 1e-5$, identity $\geq 50\%$) were performed against the protein sequences of the *D. officinale* genome [44]. This was supplemented by HMMER searches using the bZIP domain (PF00170, $E \leq 1e-10$) [45]. Candidate genes were verified using SMART (Letunic et al., 2021), NCBI Conserved Domain Database (CDD) [46], and Pfam [47] databases to confirm the presence of the complete bZIP domain and C1 C4 conserved regions, thereby defining the final family members. All identified *D. officinale* AREB/ABF members together with *Arabidopsis* AREB/ABF homologs were aligned using ClustalX software with default parameters [48]. A phylogenetic tree was constructed using MEGA 11 with the neighbor joining (NJ) method and a Poisson correction model [49]. Branch reliability was assessed by bootstrap analysis with 1000 replicates. The evolutionary relationships and classification of *D. officinale* AREB/ABF genes were analyzed, and members potentially involved in salt stress response were identified according to their phylogenetic branches. The 2000 bp genomic sequences upstream of the start codon (ATG) of *D. officinale* AREB/ABF genes were extracted as promoter regions. Cis acting elements were predicted using the PlantCARE database [50]. Stress related cis elements, including abscisic acid response elements (ABREs), drought inducible MYB binding sites (MBSs), and light response elements (Box II), were statistically analyzed in terms of type, number, and distribution within each promoter.

2.8. Data Statistics and Analysis

All experimental data were obtained from three biological replicates. Data organization was performed using Microsoft Excel, and statistical analyses were conducted using SPSS 26.0 software [51].

Significant differences were determined using Student's t-test [52] or one-way analysis of variance (ANOVA), with $P < 0.05$ considered statistically significant and $P < 0.01$ considered highly significant. All figures were generated using R language to ensure clarity and standardization [39].

3. Results

3.1. Salt-responsive DEGs in *D. officinale* roots and leaves

To systematically characterize the molecular response of *D. officinale* to salt stress, we performed transcriptome sequencing on roots and leaves treated with 250 mM NaCl for 4 h and 12 h, using 0 h as the control. Differentially expressed genes (DEGs) were identified with thresholds of $|\log_2$ fold change (FC)| ≥ 1 and $P < 0.05$ (Fig. 1). Volcano plots illustrated the expression distributions across four comparison groups (roots: R0-vs-R4, R0-vs-R12; leaves: L0-vs-L4, L0-vs-L12; Fig. 1A). Substantial numbers of salt-responsive DEGs were detected in all groups, with markedly higher density in roots than in leaves, indicating that roots, as the primary organ sensing salt stress, exhibit a stronger transcriptional response. As stress duration increased from 4 h to 12 h, the range of DEGs expanded in both tissues, revealing a time-dependent genome-wide transcriptional reprogramming, with response intensity positively correlated with stress duration.

Statistical analysis of DEG counts (Fig. 1B) showed that at 4 h, roots had 8,542 DEGs (4,630 up, 3,912 down), compared to 4,793 DEGs (2,314 up, 2,479 down) in leaves. At 12 h, root DEGs increased to 10,536 (5,475 up, 5,061 down), and leaf DEGs to 5,235 (2,506 up, 2,729 down). The total DEG number in roots was approximately twice that in leaves, confirming roots as the core responsive organ. Venn diagram analysis (Fig. 1C) revealed that many DEGs were shared between the 4 h and 12 h time points in roots, suggesting a persistently responsive core gene cluster. In contrast, leaf DEGs were mostly time-specific, and very few DEGs were common between roots and leaves, indicating highly divergent regulatory strategies between the two tissues.

KEGG pathway enrichment analysis (Fig. 1D, E) showed that root DEGs were significantly enriched in pathways related to stress signal perception and transduction, such as plant hormone signal transduction, MAPK signaling (plant), phenylpropanoid biosynthesis, and starch and sucrose metabolism. Leaf DEGs were enriched in fundamental metabolic pathways, including photosynthesis, carbon metabolism, and amino acid biosynthesis. These results highlight a functional division: roots primarily mediate stress perception and signaling, while leaves focus on maintaining metabolic homeostasis.

3.2. WGCNA co-expression networks and core modules

To identify core gene sets associated with salt stress and elucidate their regulatory relationships, we performed WGCNA on the transcriptomic expression matrix. Sample clustering (Fig. 2A) showed clear separation between roots and leaves, with samples from different time points clustering in time-series

order within each tissue, indicating high biological reproducibility. A soft-thresholding power of $\beta = 5$ was selected (Fig. 2B), yielding a scale-free topology fit index $R^2 \geq 0.85$.

Using this threshold, we identified seven stable co-expression modules via dynamic tree cutting (Fig. 2C). The turquoise module contained the most genes (8,362, 28.3% of total), followed by the blue (7,932, 26.8%) and brown (5,071, 17.2%) modules. Inter-module correlation analysis (Fig. 2D) revealed that the turquoise module was significantly negatively correlated with the blue and green modules, whereas the brown module was positively correlated with the greenyellow module, suggesting functional synergy or antagonism among modules in response to salt stress.

Module-trait association analysis (Fig. 2F, G) showed that the brown module was significantly positively correlated with treatment time ($r = 0.83$, $P < 0.01$), making it the core module most highly associated with salt stress response. The blue module was negatively correlated with treatment time ($r = -0.63$, $P < 0.01$), and the turquoise module was positively correlated with tissue type ($r = 0.68$, $P < 0.01$). KEGG enrichment analysis of the turquoise module revealed significant enrichment in pathways related to ABA signaling and salt stress, including plant hormone signal transduction, MAPK signaling, and phenylpropanoid biosynthesis. This module also contained a significant number of bZIP transcription factors, providing candidate hub regulators. Genes in the brown module were primarily enriched in tissue-specific metabolic pathways such as photosynthesis and carbon fixation, consistent with the module-trait analysis.

3.3. Core co-expression network under salt stress

Using the turquoise module genes and salt-responsive differentially expressed genes, we constructed a core co-expression regulatory network for salt stress response in *D. officinale* (Fig. 3A). In the network, cyan nodes represent five AREB/ABF family bZIP transcription factors (Do05G002378, Do05G002422, Do06G000349, Do07G001659, Do14G001732), and red nodes denote 11 downstream salt-responsive functional genes. These five transcription factors occupy central hub positions, showing strong direct co-expression associations with most downstream genes, confirming their role as core regulators mediating salt stress signal transduction and tolerance by directly regulating numerous stress-responsive genes. Extensive strong co-expression associations were also observed among the 11 downstream genes, suggesting they form a synergistic functional module under unified AREB/ABF regulation.

A heatmap of fold changes (Fig. 3B) revealed typical salt-induced upregulation of all 16 core genes, with significantly higher upregulation in roots than in leaves at both 4 h and 12 h, reflecting roots as the primary responsive organ. Among the transcription factors, Do05G002378, Do06G000349, and Do07G001659 exhibited the highest $\log_2(\text{FC})$ values in the root 12 h vs. 0 h comparison, representing the most central hub genes. Among the downstream functional genes, Do06G001596, Do06G001976, and Do19G000945 showed over 100-fold upregulation in roots after salt treatment, identifying them as core

functional executors and direct targets of AREB/ABF regulation. Furthermore, the upregulation magnitude in roots increased with stress duration, indicating a clear time-dependent response.

3.4. AREB/ABF family in *D. officinale*: identification, phylogeny, and cis-elements

To classify the core AREB/ABF transcription factors and assess their evolutionary conservation and transcriptional regulation, we systematically identified the AREB/ABF gene family in *D. officinale* based on genome annotation, the bZIP conserved domain (PF00170), and homology with *Arabidopsis thaliana*. A total of eight members with complete conserved domains were obtained (Fig. 4). Phylogenetic analysis (Fig. 4A) showed that these eight *D. officinale* AREB/ABF genes, together with nine *Arabidopsis* homologs, clustered into three clades. The five members present in our core regulatory network all fell into Clade A, the stress-responsive clade, grouping with *AtABF1–AtABF4*, indicating high functional conservation in ABA signaling and abiotic stress responses. The remaining three members, not included in the core network, belonged to Clade B, clustering with *Arabidopsis* DPBF2 and EEL genes involved in seed development and embryogenesis, suggesting their primary roles in growth and development rather than salt stress response.

Promoter cis-element analysis (Fig. 4B) supported this functional divergence at the transcriptional level. All eight members contained ABA-responsive elements (ABREs), but the five Clade A genes had significantly more ABREs than those in Clade B. Notably, the core hub genes *Do06G001596* and *Do06G001976* harbored 6 and 13 ABREs, respectively, along with abundant other stress-related cis-elements, including STRE, MYB/MYC binding sites, and ARE, providing a strong cis-regulatory basis for efficient response to ABA and multiple stresses. In contrast, Clade B members had fewer stress-related elements and were enriched in elements associated with seed development and light responsiveness, consistent with their inferred functions from phylogenetic analysis.

3.5. Tissue-specific expression and validation of core regulatory genes

To precisely identify salt-responsive core regulatory genes and exclude constitutively expressed ones, we analyzed the expression patterns of the 16 core genes (5 AREB/ABF TFs and 11 downstream functional genes) across multiple tissues under normal growth conditions, using multi-tissue transcriptome data. The screening criterion was low or basal expression under non-stressed conditions and significant induction upon salt stress (Fig. 5). Based on integration of normal and salt-stress expression profiles, the 16 genes fell into two categories. The first comprised stress-inducible core regulators: four AREB/ABF TFs (*Do05G002378*, *Do06G000349*, *Do07G001659*, *Do05G002422*) and most downstream functional genes.

These exhibited extremely low basal expression (near zero) in vegetative tissues (roots, leaves, stems) under normal conditions, with only trace levels in floral organs. Upon salt stress, they were rapidly and

strongly induced, with upregulation reaching tens to hundreds of fold in roots at 12 h, fully matching the classic signature of stress-responsive regulators. The second category comprised constitutively expressed genes, including *Do14G001732* and a few downstream genes, which maintained consistently high expression across multiple tissues (roots, leaves, flowers) under normal conditions and showed limited upregulation after salt stress, suggesting primary roles in basal growth and development rather than salt response. Through this tissue-specific expression analysis, four of the five AREB/ABF genes in the core network were confirmed as stress-inducible, providing rigorous expression-level evidence for key gene identification.

3.6. Expression validation of core AREB/ABF genes

To identify the core AREB/ABF genes responsible for salt stress response in *D. officinale* and validate the transcriptomic data, we examined the dynamic expression patterns of candidate genes across tissues and time points using qRT-PCR (Fig. 6). The two core AREB/ABF genes (*Do07G001659*, *Do06G000349*) showed highly consistent salt-induced expression: basal levels were extremely low under control conditions (R0, L0); after 4 h of salt stress, both were significantly activated in roots; by 12 h, expression peaked, with upregulation reaching 5 to 100 fold. In leaves, upregulation was more moderate but still significant at 12 h, indicating that these genes cooperatively mediate adaptive responses, with roots as the primary regulatory site. Two downstream target genes (*Do17G000728*, *Do18G000921*) exhibited expression patterns highly synchronized with the core AREB/ABF genes, peaking in roots at 12 h, further supporting their synergistic regulatory relationship. qRT-PCR validation confirmed that expression trends of all core genes were highly consistent with RNA-seq data (correlation coefficients $R^2 > 0.95$), demonstrating the accuracy of the transcriptomic data and reliability of the expression patterns.

3.7 Identification of *Do07G001659* as the master salt-responsive regulator

Through a stepwise screening pipeline integrating differential expression analysis, WGCNA, network analysis, gene family identification, phylogenetic and cis-element dissection, tissue-specific expression validation, and qRT-PCR confirmation, we identified *Do07G001659* as the core regulatory gene governing salt stress response in *D. officinale*. This gene exhibited the highest connectivity in the co-expression network, remained transcriptionally silent under normal conditions, reached a $\log_2$ (fold change) of 8.2 in roots after 12 h of salt stress, and contained six ABRE elements in its promoter, providing a complete basis for ABA responsiveness. Its direct target gene, *Do17G000728*, was upregulated over 100-fold under salt stress and is involved in osmotic regulation and antioxidant defense. We propose the following core regulatory pathway: salt stress activates endogenous ABA signaling, which induces high expression of *Do07G001659*; the encoded protein then binds to ABRE elements in the promoter of *Do17G000728*, driving its transcription and consequently enhancing osmotic adjustment and antioxidant capacity, thereby conferring salt tolerance. This regulatory module provides a precise target for dissecting salt tolerance mechanisms and for molecular breeding in *D. officinale*.

4. Discussion

4.1 Core regulatory role of AREB/ABF transcription factors and functional divergence within the family

This study identified five AREB/ABF transcription factors as hub genes in a co-expression regulatory network, forming high confidence associations with 11 downstream functional genes and constituting a core salt stress response module. This finding aligns with the established role of AREB/ABF in *Arabidopsis thaliana*, where they act as master regulators of ABA dependent gene expression upon activation by SnRK2 phosphorylation [53, 54]. In *D. officinale*, the promoters of these core AREB/ABF genes contain 6–13 ABRE elements and additional stress related cis elements (STRE, MYB/MYC binding sites), providing a cis regulatory basis for efficient ABA responsiveness [55].

Phylogenetic analysis classified the eight *D. officinale* AREB/ABF members into Clade A and Clade B. All five hub genes fell into Clade A, clustering with *Arabidopsis* AtABF1-4, and exhibited typical stress inducible expression (low basal levels under normal conditions, strong upregulation under salt stress) [53]. In contrast, Clade B members were constitutively expressed and associated primarily with growth and development. This functional divergence mirrors that reported for the AREB/ABF family in jute [56], reflecting an evolutionary strategy that balances growth and stress responses via functional specialization. Compared with *Arabidopsis* (9 members) and rice (≥ 10 members), *D. officinale* has a smaller AREB/ABF family (8 members) [57]. Nevertheless, it achieves efficient regulation through a dense downstream target network, exemplifying a “few but fine” strategy for stress tolerance in orchids [58].

4.2 Root-dominated salt stress response and tissue-specific functional partitioning

In this study, the number of DEGs in roots under salt stress was approximately twice that in leaves (10,536 vs. 5,235 at 12 h). Root DEGs were significantly enriched in stress perception and osmotic regulation pathways (e.g., plant hormone signal transduction, MAPK signaling, phenylpropanoid biosynthesis), whereas leaf DEGs were mainly enriched in photosynthesis and carbon metabolism. These results indicate that roots serve as the primary organ for stress perception and signal transduction, while leaves focus on maintaining basal metabolic homeostasis. Similar tissue-specific responses have been reported in chrysanthemum [59] and water spinach [60]; however, a stronger leaf response observed in rose [61] suggests that tissue functional partitioning is closely linked to the ecological adaptability of different plant species. As an epiphytic orchid, *D. officinale* has roots persistently exposed to the external environment, which may have driven the evolution of enhanced stress perception capacity. Venn analysis revealed very few DEGs shared between roots and leaves, indicating highly divergent molecular regulatory strategies between the two tissues. This phenomenon has also been observed in multi-tissue transcriptome studies of grain amaranth [62], suggesting that tissue-specific responsiveness is a widespread molecular basis for plant adaptation to salt stress.

4.3 Effectiveness of the WGCNA strategy and the multi-layered screening system

Using WGCNA, we identified seven co-expression modules. The turquoise module was significantly positively correlated with tissue type ($r = 0.68$), and the brown module with stress duration ($r = 0.83$). The turquoise module was also enriched in pathways related to ABA signaling and salt stress response, confirming that WGCNA effectively captures trait-associated gene modules. Similar approaches have been successfully applied to salt stress studies in soybean [63] and tomato [64]. Here, we further integrated WGCNA with phylogenetic analysis, cis-element dissection, and tissue expression validation to establish a progressive screening pipeline comprising differential expression screening, WGCNA, network analysis, family identification, and expression validation. This integrated strategy aligns with the multi-step screening frameworks employed in salt tolerance studies of other non-model plants, such as peanut [65] and cotton [66]. This pipeline ultimately identified *Do07G001659* as the core regulatory gene, providing a methodological reference for mining stress-responsive genes in non-model plants.

4.4 Regulatory model of salt stress response and innovations

Synthesizing the above results, we propose a core regulatory model for salt stress response in *D. officinale*: salt stress activates endogenous ABA signaling, which induces high expression of Clade A AREB/ABF transcription factors (exemplified by *Do07G001659*). These factors bind to ABRE elements in the promoters of target genes, driving the transcriptional activation of downstream genes involved in osmotic regulation and antioxidant defense (e.g., *Do17G000728*), thereby enhancing cellular tolerance. This model is highly consistent with the classical AREB/ABF-SnRK2-ABRE pathway in *Arabidopsis* [67]. Notably, the number of ABREs in the promoters of AREB/ABF genes correlates positively with the magnitude of stress induced expression: *Do17G000728*, which contains 3 ABREs, was upregulated over 100 fold, whereas members with fewer ABREs showed more moderate upregulation. This suggests that ABRE abundance may serve as a cis regulatory meter determining response sensitivity, a finding supported by the observation in *Arabidopsis* that eight downstream genes containing two or more ABREs were greatly upregulated by AREB1 overexpression [68], while RD29B is known to require two ABREs for full dehydration responsive expression [69]. To our knowledge, this quantitative relationship between ABRE copy number and stress induced expression magnitude is reported for the first time in an orchid species.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors reviewed and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable. This study is a secondary analysis of publicly available genomic and transcriptomic data (NCBI SRA). No plant material was directly collected or handled. No ethical approval was required.

Consent for publication

Not applicable. This manuscript contains no individual person's data.

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Supplementary materials

Accompanies this paper at (<https://doi.org/xxxxx>).

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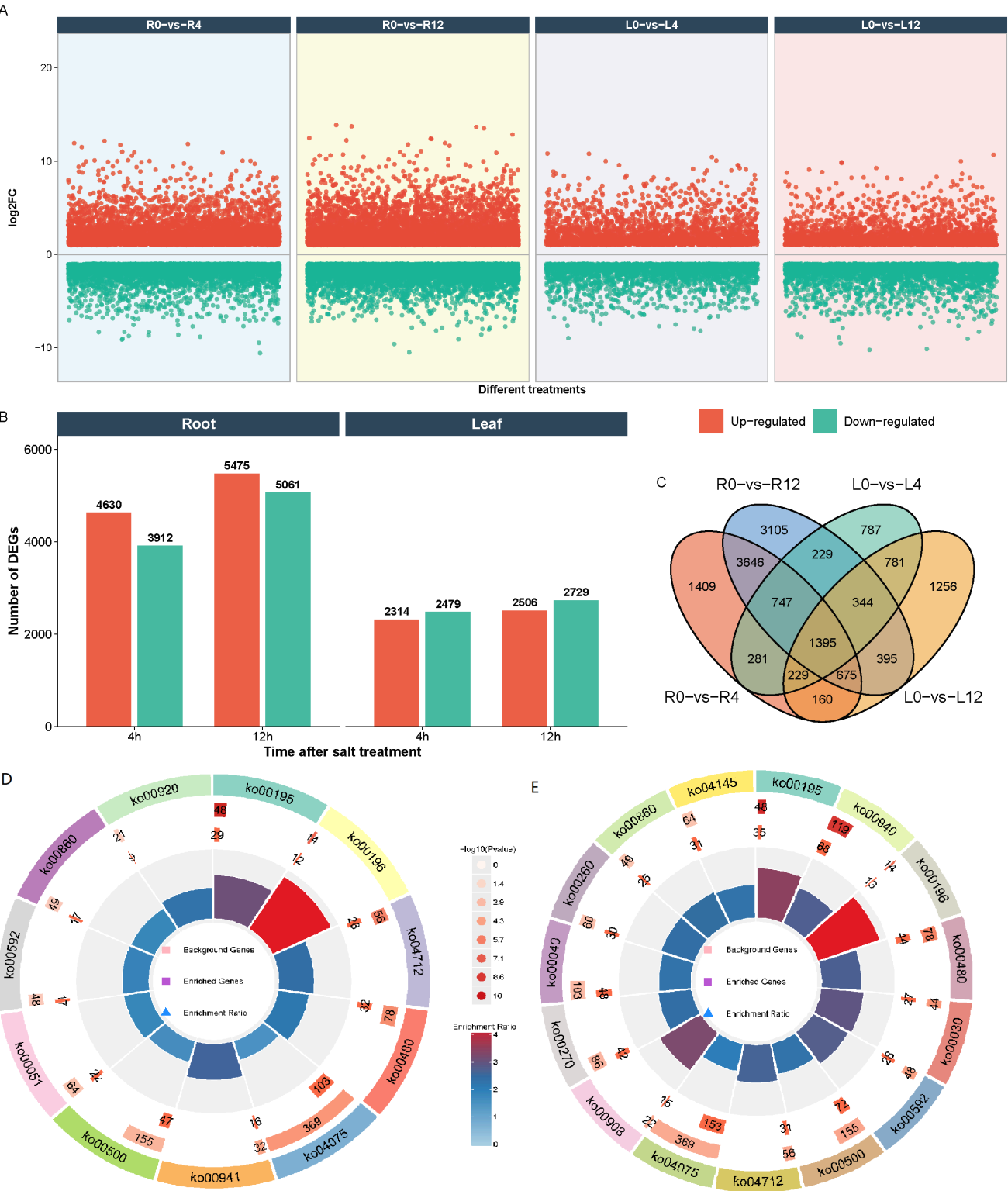
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Figures



Screening and functional enrichment analysis of differentially expressed genes in roots and leaves of *D. officinale* under salt stress. (A) Volcano plots of differentially expressed genes (DEGs) at different salt stress time points compared to the control (0 h). Red, green, and gray dots represent significantly up-regulated genes, significantly down-regulated genes, and genes with no significant differential expression, respectively. (B) Bar chart showing the number of DEGs in each salt stress treatment group. (C) Venn diagram showing the distribution of shared and unique DEGs among different salt stress treatment groups. (D) Functional enrichment analysis of DEGs in root tissues at 12 h of salt stress compared to the control. (E) Functional enrichment analysis of DEGs in leaf tissues at 12 h of salt stress compared to the control.

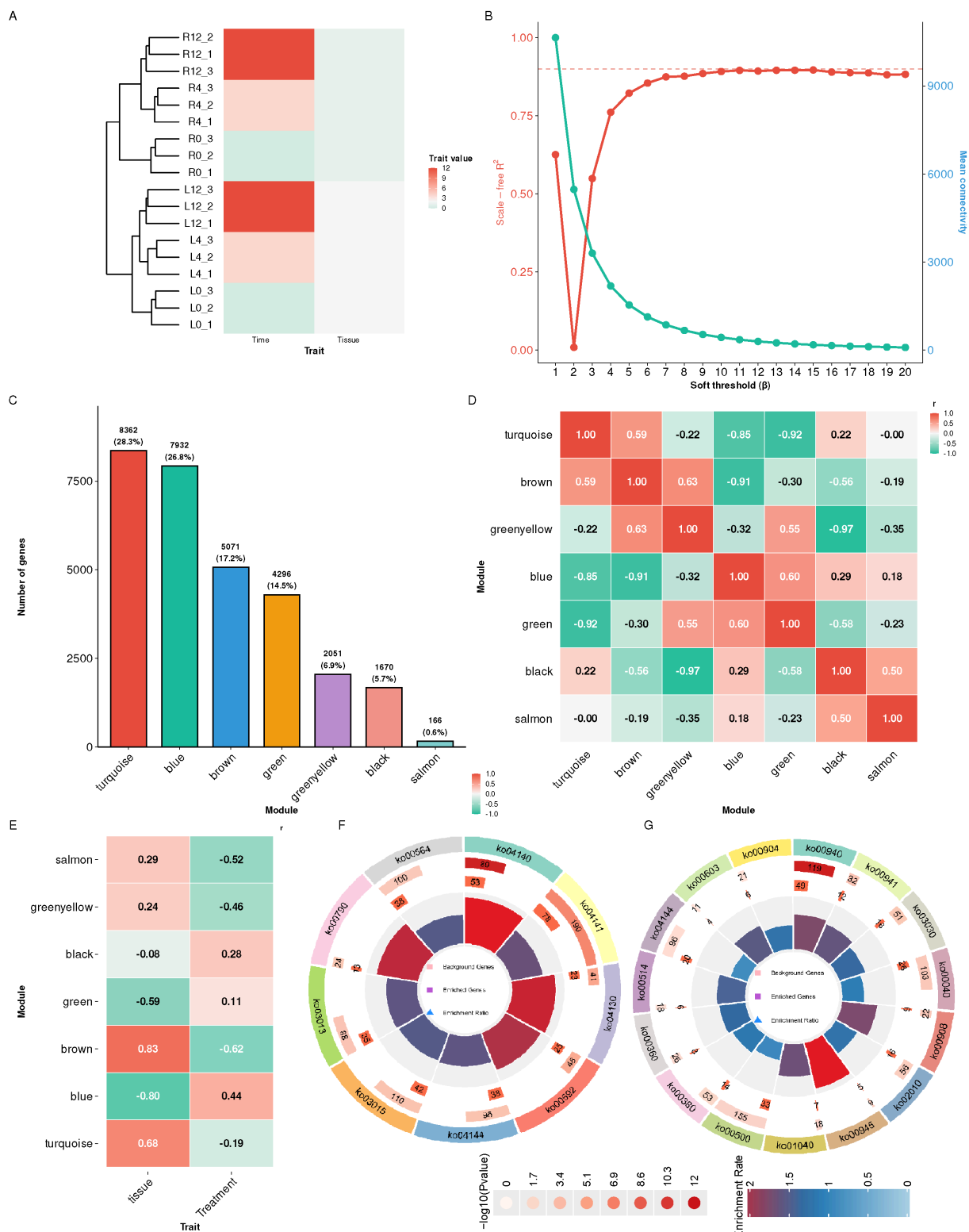


Figure 2

Weighted gene co-expression network analysis (WGCNA) of salt stress response in *D. officinale*. (A) Sample hierarchical clustering tree and trait association heatmap. Left: clustering tree of all sequenced samples; right: heatmap of associations between samples and traits (Tissue, salt stress treatment time), used to validate biological reproducibility of sample expression profiles and trait matching. (B) Soft-thresholding power (β) selection plot for WGCNA. The red curve represents the scale-free topology fit

index (Scale-free R^2), and the green curve represents mean connectivity, used to determine the optimal soft-thresholding power that satisfies scale-free network characteristics. (C) Bar chart showing the number of genes in each co-expression module, with the number of genes contained in each module and their proportion relative to the total number of genes indicated, illustrating the size differences among distinct co-expression regulatory units. (D) Heatmap of correlations among co-expression modules. The color intensity represents the correlation coefficient of gene expression between modules, visually presenting synergistic or antagonistic regulatory relationships among modules. (E) Heatmap of correlations between co-expression modules and traits, showing the correlation of each module with traits (Tissue, salt stress treatment time). The color represents the correlation coefficient (r), used to screen for core modules responsive to salt stress. (F) Circular plot of KEGG pathway enrichment analysis for genes in the turquoise module, showing the enrichment extent and significance of core pathways in this module. (G) Circular plot of KEGG pathway enrichment analysis for genes in the brown module, showing the enrichment characteristics of core pathways in this module.

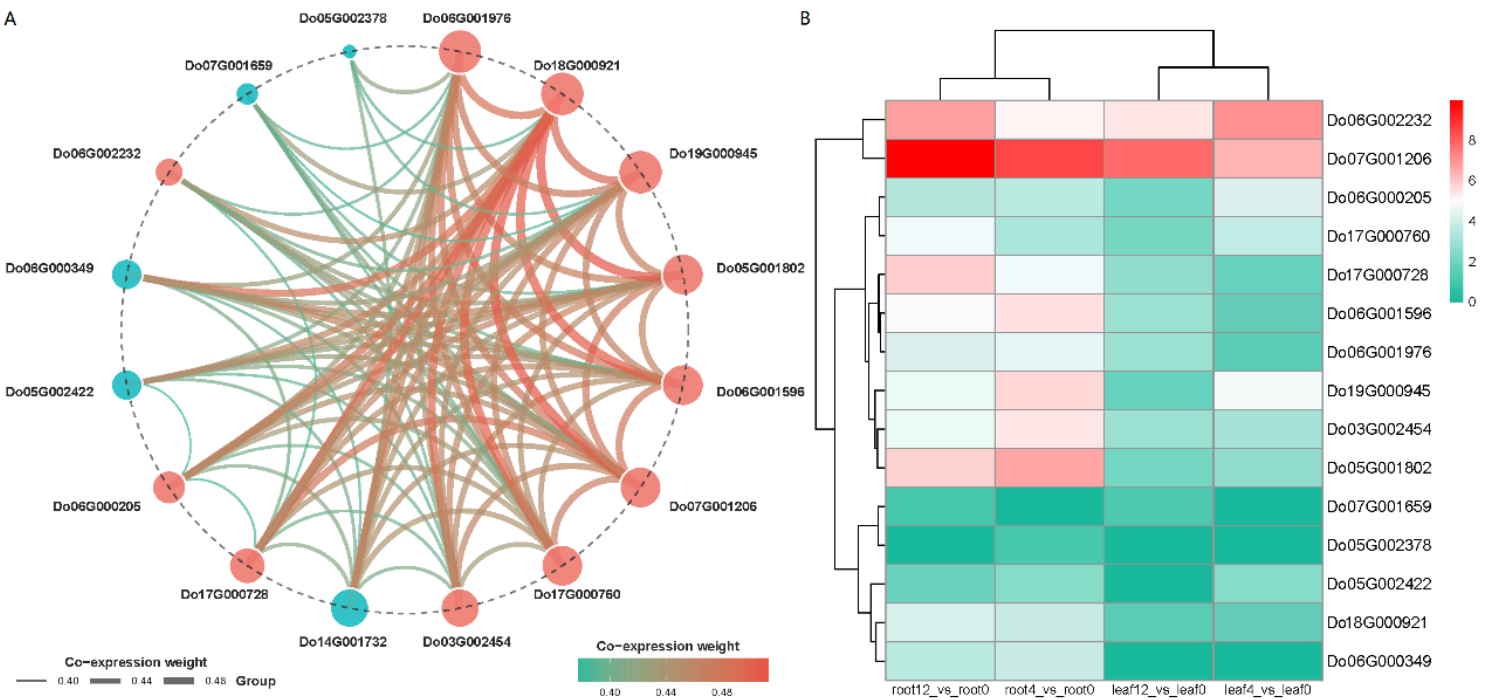


Figure 3

Co-expression regulatory network of differentially expressed genes responsive to salt stress in *D. officinale* and expression analysis of core genes. (A) Core gene co-expression regulatory network. Red nodes represent salt-responsive functional genes, and cyan (green) nodes represent AREB/ABF family transcription factors. The color/thickness of edges reflects the co-expression weight, with higher weights indicating stronger co-expression associations between genes. (B) Heatmap of differential expression of core regulatory genes across different treatment groups. Columns correspond to four comparison groups (root 12 h vs. root 0 h, root 4 h vs. root 0 h, leaf 12 h vs. leaf 0 h, leaf 4 h vs. leaf 0 h). The color scale represents log₂(fold change) values, with red indicating upregulation and green indicating downregulation.

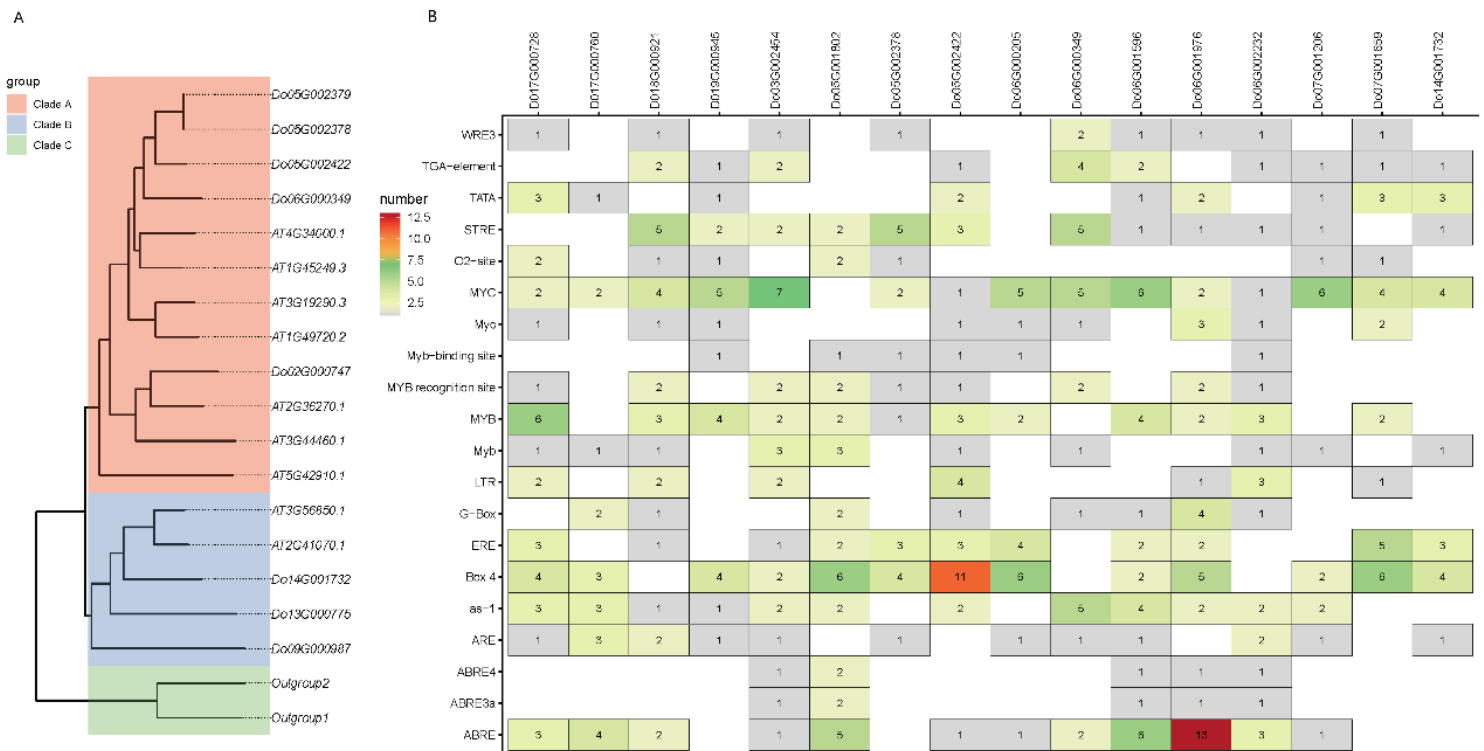


Figure 4

Phylogenetic analysis and cis-acting element analysis of the AREB/ABF gene family in *D. officinale*. (A) Phylogenetic tree of the AREB/ABF gene family from *D. officinale* and *Arabidopsis thaliana*. Different colors represent distinct evolutionary clades. (B) Heatmap of cis-acting element distribution in the promoters of AREB/ABF genes from *D. officinale*. Rows represent types of cis-elements, columns represent family members, and the color intensity indicates the number of elements.

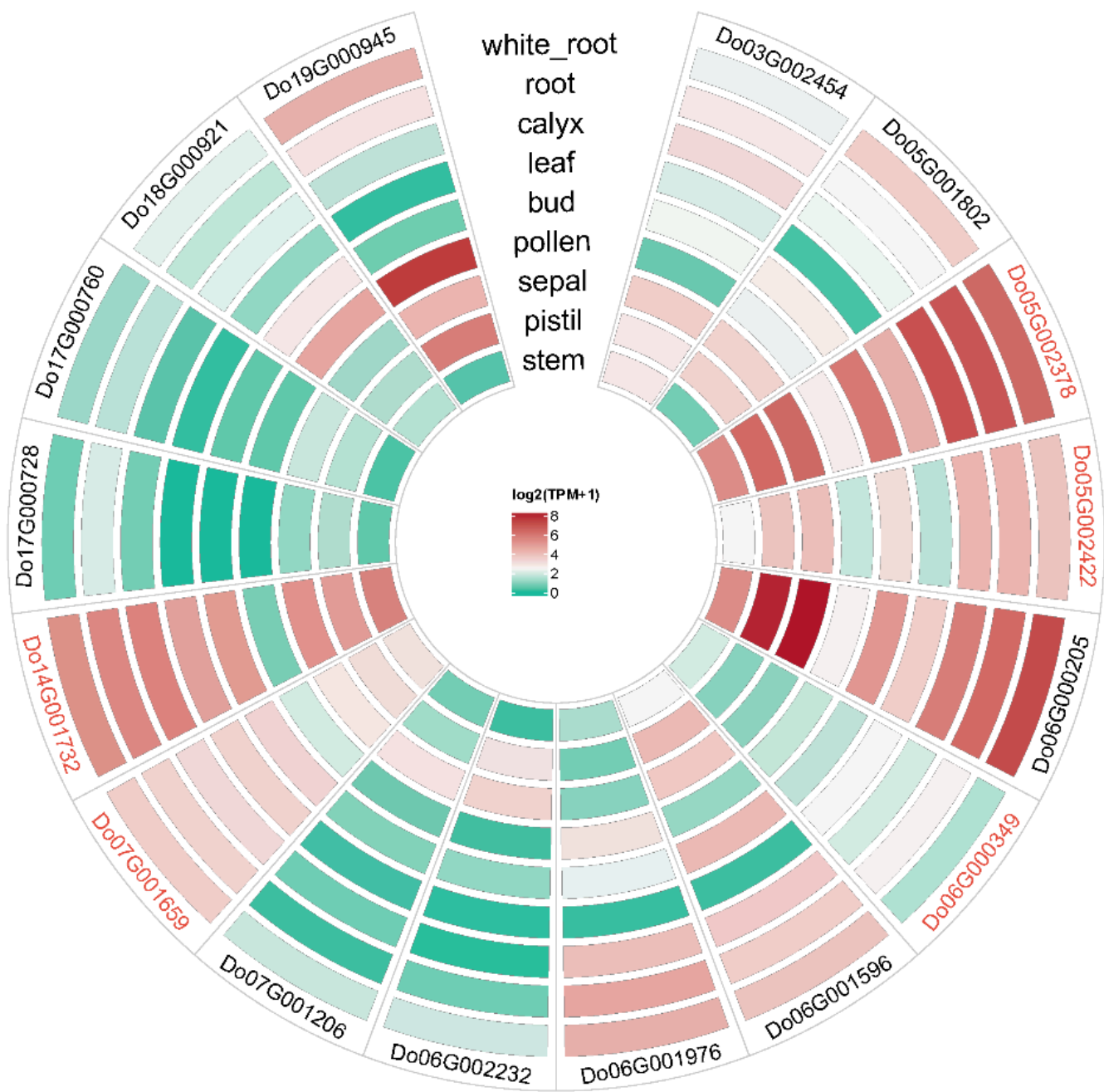


Figure 5

Tissue-specific expression analysis of candidate core genes responsive to salt stress in *D. officinale*.

The figure shows the expression levels of 16 candidate core genes under normal growth conditions across different tissues (white root, pistil, sepal, pollen, bud, leaf, calyx, root, stem). Bars represent the expression level of each gene in the corresponding tissue, used to screen for core regulatory genes characterized by low expression under normal conditions and high expression under stress.



Figure 6

Expression analysis of candidate core salt-responsive genes in *D. officinale* under different treatments.

The figure shows the dynamic expression levels of 16 candidate genes in roots (R0/R4/R12, representing 0 h, 4 h, and 12 h of salt stress, respectively) and leaves (L0/L4/L12). Bars represent the expression level of each gene under the corresponding treatment, and error bars indicate the standard error of biological replicates.

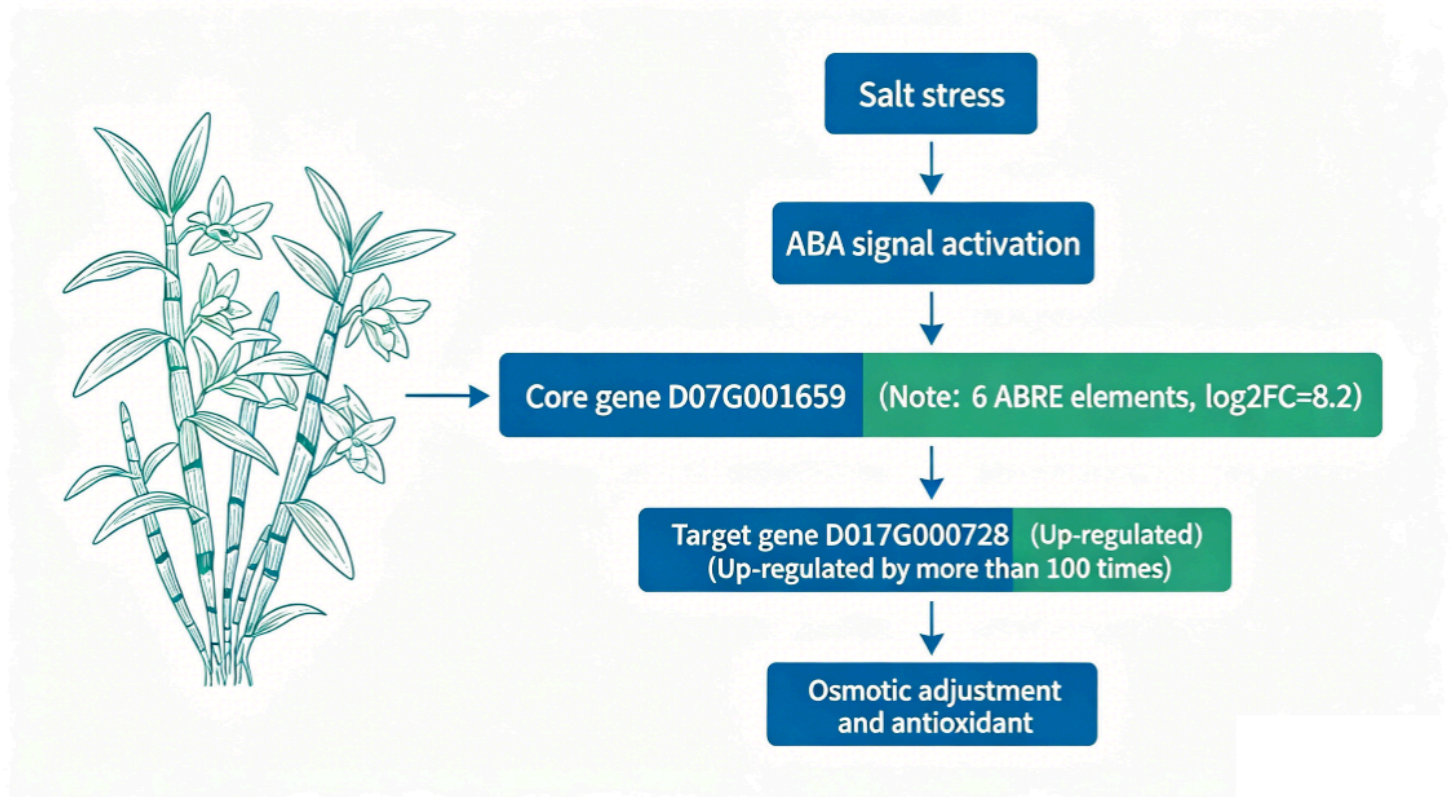


Figure 7

Schematic diagram of the proposed salt stress response regulatory pathway in *D. officinale*.

Supplementary Files

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