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Phenotypic Changes in Chamaecyparis hodginsii Seedlings Induced by New Environments and Mediated by Gene Expression

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Abstract

In the context of climate change, assessing the adaptive potential of species and populations is crucial for developing effective conservation strategies. Changes in plant gene expression play a significant role in the adaptation process to climate change. This study aims to explore the adaptive responses of the near-threatened conifer species *Chamaecyparis hodginsii* to climate change and analyze the molecular-level reactions of these long-lived trees to climatic shifts. It seeks to understand their phenotypic responses to climate change, identify key environmental factors driving adaptive gene expression, and provide information for transplantation conservation strategies based on genetic adaptability. By conducting mixed-tissue RNA sequencing on seeds from multiple provenances and employing redundancy analysis (RDA), weighted gene co-expression network analysis (WGCNA), and partial least squares path modeling (PLS-PM), the study assesses the impact of climatic variables on gene expression and phenotype. It identifies key gene groups associated with environmental responses and elucidates the complex relationships between environmental factors, functional gene groups, and phenotypic traits. The findings reveal that *C. hodginsii* adapts to environmental stresses by regulating specific gene activities related to morphological trait adjustments. Moreover, environmental factors such as the impact on tree architecture emphasize the importance of Precipitation Seasonality, Isothermality, and Precipitation of Driest Quarter for adapting to climate stresses. This research not only unveils the complex adaptive responses of *C. hodginsii* to climate change but also provides critical insights for the management and conservation of long-lived tree species facing climate change threats.

1 Introduction

Climate change is one of the major threats to biodiversity, with studies showing it has led to localized extinctions of animal and plant species [1]. Assessing the current adaptive state of species and populations is essential for effective conservation strategies [2,3]. For long-lived species, such as forest trees, rates of mutation and migration may be insufficient to match the pace of environmental change, thus limiting adaptive potential [4–6]. Consequently, shifts in gene expression are critical for plant adaptation to changing environments and can, to some extent, reflect the adaptive status of species. Rapid environmental shifts can induce changes in gene expression programs, thereby regulating plant metabolism to adapt to new conditions and increasing phenotypic variation [7,8]. Changes in gene expression not only directly reflect the species' adaption to current environments but are also essential for assessing the viability of natural species under climate change and for developing effective ecological and forest management strategies. During the process of adapting to antibiotic stress, plants undergo extensive changes in gene expression [9,10], providing a basis for natural selection on short evolutionary timescales [11,12].

Differences in gene expression patterns can explain the varying abilities of different populations to adapt to environmental changes [11,13]. Traditional transplant or garden experiments are the gold standard for assessing plant adaptability [14]. Translocating organisms from their native habitats to new environments

also serves as an effective method to conserve genetic diversity [15–17], especially for threatened and rare species [18]. Notably, seedlings, being in the early stages of growth, may be more sensitive to environmental changes [19,20]. RNA-seq has been successfully applied to adaptive studies in conifer species [21,22]. Additionally, Weighted Gene Co-expression Network Analysis (WGCNA) can identify gene sets closely related to climate adaptation, revealing how plants regulate gene expression to cope with rapidly changing climates, thereby deepening the current understanding of species adaptation mechanisms.

C. hodginsii is classified as Near Threatened (NT) on the International Union for Conservation of Nature (IUCN) Red List [23] and is listed as a Category II protected plant in China (https://www.gov.cn/gongbao/content/2000/content_60072.htm). This Tertiary relict species has survived since the glacial periods [24,25]. In some regions where it currently exists, it remains a dominant species; however, historical human activity and climate change have led to population decline and habitat fragmentation, which are the primary threats to its persistence [26]. Additionally, fossil records indicate that this genus was once widely distributed across the Northern Hemisphere, including locations such as Saskatchewan, Canada [27], Jilin in northeastern China [28], and Zhejiang in eastern Chin [29]. However, climate shifts have led to its disappearance in these areas, and *C. hodginsii* is now limited to southern China, northern Vietnam, and northern Laos [24]. It is highly likely that *C. hodginsii* will face further population declines and heightened survival challenges under the current context of climate change. The recently released *C. hodginsii* genome [25] offers an excellent opportunity to evaluate its environmental plasticity at the genetic level.

Trees act as effective carbon sinks and are crucial in fighting climate change [30,31]. Their long generation times and frequent extreme climate events make them vulnerable to climate change [6,32]. This study focuses on *C.s hodginsii*, a near-threatened species. We aim to understand its adaptability to climate change using mixed-tissue RNA sequencing. Our goals include revealing tree phenotypes' response to climate change at the gene expression level, identifying key environmental factors affecting adaptability, and analyzing gene expression regulation. This work seeks to provide insights and strategies for protecting long-lived tree species against climate change.

2 Materials and Methods

2.1 Plant Materials

All plant samples in this study were sourced from the Bailai State-owned Forest Farm in Hutou Town, Anxi County, Quanzhou City, Fujian Province, China (118°3' E, 25°49' N, 590-600 m above sea level). The plant experiments and field studies, including the collection of plant materials, complied with relevant institutional, national, and international guidelines and regulations. The germination and sprouting experiments of cypress seeds were conducted in the greenhouse of Fujian Agriculture and Forestry University, with seeds disinfected before sowing. At the end of 2015, seed germination began under

controlled environmental conditions, maintaining a temperature of 22°C to 25°C and a relative humidity of 70%-80%. Successfully germinated seeds were then transferred to seedling trays filled with standard nutrient soil to promote seedling growth. Seedlings received at least 8 hours of natural light, supplemented by grow lights to ensure adequate lighting, and were watered and fertilized according to standard care procedures. In April 2016, when the seedlings reached about 15cm in height, they were transplanted to outdoor plots with similar soil texture, drainage conditions, and light exposure. The experiment employed a completely randomized block design (CRD) with 8 plots, each containing representative seedlings from 14 different provenances (Table S1; Figure S1). Five seedlings from each provenance were randomly assigned to each plot to ensure even distribution across the experimental area, with a planting distance of 2m to minimize competition. Appropriate measures were taken to alleviate transplant shock, and the growth and adaptability of the seedlings were regularly monitored post-transplantation. In April 2020, four years after transplantation, key growth parameters of the surviving seedlings were measured, including Height, Diameter at Breast Height (DBH), Crown Width (CW), and the Average Angle of Dips, the latter obtained by calculating the average angle of the three lowest secondary branches of each plant.

2.2 Transcriptome Sequencing

In April 2020, we randomly selected seven individuals from each seed provenance and collected their stem-differentiating xylem, leaves, branches, and roots. These tissues were mixed by category and disinfected uniformly with alcohol for 30 seconds to one minute, then rinsed in deionized water for 2 minutes to remove any alcohol residue and dead microbes. One gram of mixed tissue sample from each type was immediately weighed, flash-frozen in liquid nitrogen, and stored at -80°C for later use. Total RNA was extracted using the RNAprep Pure Plant Kit (Tiangen, Beijing, China) and the quality of RNA was assessed by 2% agarose gel electrophoresis. The concentration of RNA was determined by the NanoPhotometer® spectrophotometer (IMPLEN, California, USA) and the Qubit® 2.0 Fluorometer in conjunction with the Qubit® RNA Assay Kit (Life Technologies, California, USA). RNA integrity was evaluated using the RNA Nano 6000 Assay Kit on the Agilent® 2100 Bioanalyzer (Agilent Technologies, California, USA), with an RNA integrity number (RIN) greater than 7 considered satisfactory. Subsequently, libraries were constructed using the NEB-Next® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) and sequenced on the Illumina® NovaSeq 6000 platform to produce 150 bp paired-end reads.

2.3 RNA-seq Reads Mapping and Quantification

We performed initial processing of the raw RNA-Seq reads using fastp v0.23.2 [33] with parameters set to ensure quality (-qualified_quality_phred 15 --unqualified_percent_limit 50 --n_base_limit 15). Subsequently, we obtained the C. hodginsii reference genome and structural annotations [25]. The resulting clean reads were aligned to the genome using the Two-pass alignment mode of STAR v2.7.8a [34] with parameters of --alignIntronMin 20 --alignIntronMax 50000 --sjdbOverhang 149 --

outFilterMismatchNmax 2 --outSJfilterReads Unique --outSAMmultNmax 1 --outSAMmapqUnique 60 to enhance the accuracy and reliability of the alignment. After alignment, gene expression levels for each gene were quantified using featureCounts v2.0.1 [35] with default parameters.

2.4 Statistical Analysis

First, we obtained information on 19 environmental factors from various seed provenance locations (Table S1), sourced from the WorldClim database [36]. Spearman correlation analysis was employed to assess the interrelationships among these environmental factors. High correlations were observed among many environmental factors, likely due to the close proximity of certain provenance locations. Such high correlations can lead to multicollinearity among predictors in redundancy analysis (RDA), reducing model accuracy. Therefore, following the method of Chen et al. [17], we selectively retained the most significant variables from highly correlated groups. We conducted gradient forest model analysis using the R package gradientForest v0.1-37 [37], setting the tree count to 500, to evaluate the impact of each environmental factor on gene expression levels and determine their importance. Based on the results of the gradient forest analysis and the correlations between environmental factors, we selected factors that were both significant and had low intercorrelations to reduce potential collinearity issues. These selected environmental factors were then used as explanatory variables in subsequent Redundancy Analysis (RDA) to delve deeper into their relationship with gene expression data. RDA, known for its low false-positive rate [38], was conducted using the R package vegan v2.6-4 [39] to further reveal how environmental factors influence gene expression patterns. Significant environment-associated expressed genes were defined by their loadings on the tails of the distribution along one or more RDA axes.

To gain a deeper understanding of the modular characteristics of gene expression, this study employed WGCNA. We utilized the Python package PyWGCNA v2.0.1 [40] with default parameter settings. This analysis clusters genes into modules based on similar expression patterns, aiding in exploring the correlations among these modules, environmental factors, and morphological traits of each seed provenance. It helps identify groups of genes that may be influenced by specific environmental factors. Within each module significantly related to environmental and phenotypic factors, we further filtered to select genes with a KME value greater than 0.6 as key genes. These key genes are considered to play central roles within the modules and may be sensitive to environmental changes. Functional annotation and enrichment analysis identified genes with significant functions and strong correlations to environmental factors. Finally, we selected these genes for constructing a Partial Least Squares Path Model (PLS-PM) using the R package plspm v0.5.1 [41], setting the maximum iteration count to 300. We began by hypothesizing potential connections among three variable groups based on the prior assumption that environmental changes, mediated by gene expression, influence phenotypic traits. Using PLS-PM, we then assessed the strength, direction, and reliability of relationships between each factor within the environmental and gene expression datasets. This was followed by analyzing

connections between gene expression and phenotypic changes, ultimately linking the three variable groups through the combined results. The PLS-PM model reveals the direct and indirect effects among these variables, offering profound insights into how gene expression is regulated by environmental factors at the seed provenance location, impacting the phenotype.

3 Results

3.1 Assessing and Addressing Collinearity in Environmental Factors Affecting Gene Expression

In this study, we initially conducted a correlation analysis for all considered environmental factors to identify potential collinearity issues (Figure 1A). The results revealed strong positive correlations among some temperature indices such as Annual Mean Temperature, Mean Temperature of Driest Quarter, and Mean Temperature of Warmest Quarter. Additionally, precipitation indices like Annual Precipitation, Precipitation of Coldest Quarter, and Precipitation of Wettest Quarter also showed very strong positive correlations. These highly correlated factors could affect the accuracy and interpretability of the RDA. Therefore, we assessed the impact of each environmental factor on gene expression using the gradient forest model. The results indicated significant differences in the importance of various environmental variables for model prediction. Precipitation of Driest Quarter and Precipitation of Driest Month had the highest importance scores, highlighting the critical impact of drought conditions on the prediction model. Mean Temperature of Driest Quarter and Precipitation of Wettest Month also showed high importance, emphasizing the effects of temperature and extreme wet conditions.

Furthermore, we grouped highly correlated environmental factors (correlation coefficient $|r| > 0.65$) together. During processing, we considered the importance scores of collinear factors within each group and selected the factor with the highest importance score as the representative for each group (Figure 1B). Based on the analysis, we ultimately chose five relatively independent and important environmental factors to avoid collinearity issues while retaining the most explanatory factors. These factors include Precipitation of Driest Quarter, Mean Temperature of Driest Quarter, Temperature Annual Range, Isothermality, and Precipitation Seasonality. This selection process ensured our model accounted for the importance of environmental factors while avoiding potential problems caused by collinearity.

3.2 Linking Environmental Variability to Gene Expression Patterns

The RDA results revealed a strong connection between gene expression and environmental factors, with the first axis explaining 38.6% of the environmental variation, while the second and third axes accounted for an additional 21.7% and 16.5% of variation, respectively, highlighting the profound impact of environmental factors on gene expression (Figure 2 A&B; Table S2). Notably, the significant orientation of Precipitation Seasonality on the first axis indicated it as a key environmental factor driving major

variations in gene expression. The similar direction of arrows for Isothermality and Precipitation of Driest Quarter suggested some similarity in their effects on gene expression. In contrast, the unique direction of Mean Temperature of Driest Quarter pointed to a different pattern of influence compared to other factors. Furthermore, the directions of Temperature Annual Range and Precipitation of Driest Quarter nearly overlapped on the second and third axes (Figure 2B), suggesting similarities in their effects on gene expression patterns in these specific dimensions. However, this does not imply a lack of differences between these two factors; rather, such differences may not be evident on these two axes.

Further analysis revealed that 2941 genes had significant loadings on at least one RDA axis, indicating high sensitivity to specific environmental factors and significant expression variation. Through 999 permutation tests, our model was statistically significant ($p < 0.05$), confirming the substantial impact of Precipitation of Driest Quarter and Mean Temperature of Driest Quarter on gene expression (Table S3). Overall, environmental factors accounted for 25.06% of the total variance, with the remaining variance likely due to other unconsidered variables. These findings highlight the complexity of plant gene regulatory networks and suggest that plants may respond to diverse environmental conditions through transcriptional regulatory mechanisms.

3.3 Identifying Key Gene Modules Linked to Environmental and Phenotypic Variations

Through WGCNA, we categorized the genes into 14 modules, each containing a cluster of genes with similar expression patterns (Figure S2). The analysis indicated significant correlations between specific modules and environmental factors or phenotypic traits, suggesting that certain genes may respond to environmental stresses or phenotypic changes. For instance, the Rosybrown module showed a positive correlation with Precipitation of Driest Quarter ($r = 0.65$, $P < 0.05$), while the Brown module was negatively correlated with DBH ($r = -0.63$, $P < 0.05$) (Figure 3). To identify genes most sensitive to environmental changes, we selected those with module membership (KME) values greater than 0.6 as key genes, highly correlated with the representative gene expression patterns of their respective modules. Integrating these findings with RDA results, we further pinpointed key genes within the Dimgrey, Firebrick, Rosybrown, and Snow modules. These modules demonstrated strong correlations with environmental factors or phenotypic traits, reinforcing the credibility of the RDA findings. Notably, the Dimgrey module contributed the majority of key genes, with fewer key genes from the other three modules (Figure S3), suggesting that while multiple modules are associated with environmental or phenotypic characteristics, the Dimgrey module may play a leading role in responding to environmental changes. Functional enrichment analysis of these key genes revealed their involvement in the synthesis of cyclic and aromatic compounds and various enzymatic activities (Figure 4). These functions are crucial for cellular metabolic activities, the transmission of genetic information, and genome integrity, highlighting the pivotal role of these genes in plant adaptive responses and phenotypic shaping.

3.4 Modeling the Influence of Environmental Factors on Phenotypic Traits via Key Gene Pathways

To further understand how environmental factors influence plant phenotypic traits through key genes, we employed PLS-PM. This analysis revealed a network of relationships among environmental variables, functional gene groups within the key genes, and phenotypic traits ($p<0.05$). The environmental variables included only Precipitation Seasonality, Isothermality, and Precipitation of Driest Quarter. These factors significantly impacted two groups of genes: one involved in the biosynthesis of cyclic and aromatic compounds, and the other related to peptidase and nucleotidyltransferase activities (Figure 5; Table S4). These relationships were further linked to two phenotypic traits of trees: CW and Average Angle of Dips. Precipitation Seasonality and Isothermality directly influenced the genes associated with cyclic and aromatic compound biosynthesis, subsequently affecting CW. In contrast, the mean temperatures of the three environmental variables influenced the gene groups related to peptidase and nucleotidyltransferase activities, which then affected the Average Angle of Dips. Additionally, our model explained 76.15% of the variance in CW and 68.24% of the variation in Average Angle of Dips (Table S5), indicating that we could substantially explain the variations in phenotypic traits. This model helps visualize the complexity of gene responses to environmental cues and their potential impact on phenotypes, emphasizing the significance of specific environmental factors in the adaptive responses of the species.

4 Discussion

The capacity of plants to adapt to environmental stress, particularly under rapidly changing climate conditions, is a central topic in ecological and evolutionary biology research. Previous studies have shown that plants can achieve local adaptation through genetic variation even over short geographical and temporal scales [12,42], suggesting that plants can cope with diverse environmental challenges by selecting specific life-history traits [13]. However, long-lived and highly specialized species may face issues of adaptive lag, especially pronounced against the backdrop of rapid climate change [4,5,17]. In response to this challenge, using the translocation of *C. hodginsii* as an example, explored the potential adaptability of conifer trees under environmental change pressures. Climate change has significantly increased the intensity of droughts in humid regions, posing major challenges to plant growth and development [43,44]. Temperature and moisture are key factors affecting plant growth and development, with changes in environmental temperature directly impacting plant physiological processes [45,46].

The strong El Niño event of 2015–2016, part of the El Niño Southern Oscillation (ENSO) phenomenon [47], coincided with the transplantation period of our *C. hodginsii* seedlings. This severe drought may lead to a significant increase in tree mortality rates [32], presenting a unique challenge to plant growth and development. Under such harsh conditions, the early growth stages of plants are likely to be profoundly affected. Notably, transcriptomic analyses were conducted on these four-year-old transplanted *C. hodginsii*. The results showed a range of transcriptional changes closely related to the climate of their

seed provenance locations (Fig. 2 and Fig. 3), especially genes involved in the biosynthesis of cyclic and aromatic compounds (Fig. 4). This suggests that *C. hodginsii* might cope with early environmental stress by regulating the synthesis of aromatic compounds. These compounds not only play roles in signaling between plants, enhancing stress tolerance [48,49], but may also prime surrounding plant tissues for stronger responses to subsequent stresses [50]. This mechanism is particularly important for coniferous trees known for their aromatic wood and contributes to their adaptation to rapidly changing environments.

Gymnosperms often have large genome sizes, largely attributed to historical transposon activity causing genomic redundancy [31]. In this study, we found a positive correlation between the climate of seed provenance locations and genes involved in peptidase and nucleotidyltransferase activities, enzymes associated with transposon activity [51,52]. This suggests that there might be differences in transposon activity levels among *C. hodginsii* provenances. Additionally, the activity changes in these genes seem to be related to a decrease in the Average Angle of Dips (Figs. 4 and 5). Transposon activity plays a unique role in species diversification and gene function variation, even in species with narrower gene pools [53]. Specifically, Genic transposable element (TE) insertions can lead to the production of multiple transcriptional isoforms, thereby increasing the diversity of gene expression [54,55]. As a tertiary relict gymnosperm [56], transposon activity in the evolutionary history of *C. hodginsii* has provided significant momentum [57,58]. In the context of dramatic global climate changes, active transposons may offer ongoing dynamism for the environmental adaptability of *C. hodginsii*, facilitating phenotypic variation.

Precipitation and sunlight significantly influence tree architecture, where smaller branch angles and shorter branch lengths help reduce the risk of photodamage and cavitation in the water transport system [59–61]. Our findings indicate a negative correlation between changes in Precipitation Seasonality and both the CW and Average Angle of Dips of *C. hodginsii* (Fig. 5). This suggests that *C. hodginsii* from regions with more seasonal precipitation may be more sensitive to extreme drought conditions.

Additionally, transposon activity may mediate the regulation of gene expression associated with plant hormone signaling and branch development, thereby indirectly influencing branching patterns [62]. For instance, a class of aromatic plant hormones, such as strigolactones, can regulate plant architecture [63,64]. Therefore, we speculate that transposon activity may regulate the branching pattern of *C. hodginsii* by influencing the expression of genes in hormone signaling pathways that regulate plant architecture, thereby helping it better adapt to its growing environment.

While our study provides insights into the potential interactions between environmental factors, transposon activity, and plant hormones, the way these elements collectively influence the adaptive development of relict plants like *C. hodginsii* requires further investigation. In particular, understanding how *C. hodginsii* utilizes these mechanisms to adapt to changing environments remains an open question. Future research should delve into the specific mechanisms of these interactions through genetic and physiological experiments to deepen our understanding of plant adaptive evolution.

Moreover, considering that transcriptional regulatory networks and epigenetic mechanisms may be associated with transposon activity [65], exploring their roles in rapid environmental adaptation will be crucial. This necessitates not only broader genomic data but also specific studies on the role of epigenetic modifications in adaptive development.

Declarations

Author Contribution

P.-K. Z.: Conceptualization, Data Curation, Formal Analysis, Writing - Original Draft. Y.-P. X.: Data Curation, Investigation, Writing- Reviewing and Editing. R.-J. D.: Data Curation, Investigation. K. L.: Data Curation, Investigation. T.-Y. H.: Funding Acquisition. L.-G. C.: Funding Acquisition. Y.-S. Z.: Resources. L.-Y. C.: Supervision, Writing- Reviewing and Editing.

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Data Availability

The sequencing data and phenotypic measurements in the current study are available in the NGDC (National Genomics Data Center) repository, PRJCA023136. Further information may be requested from the corresponding author.

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Figures

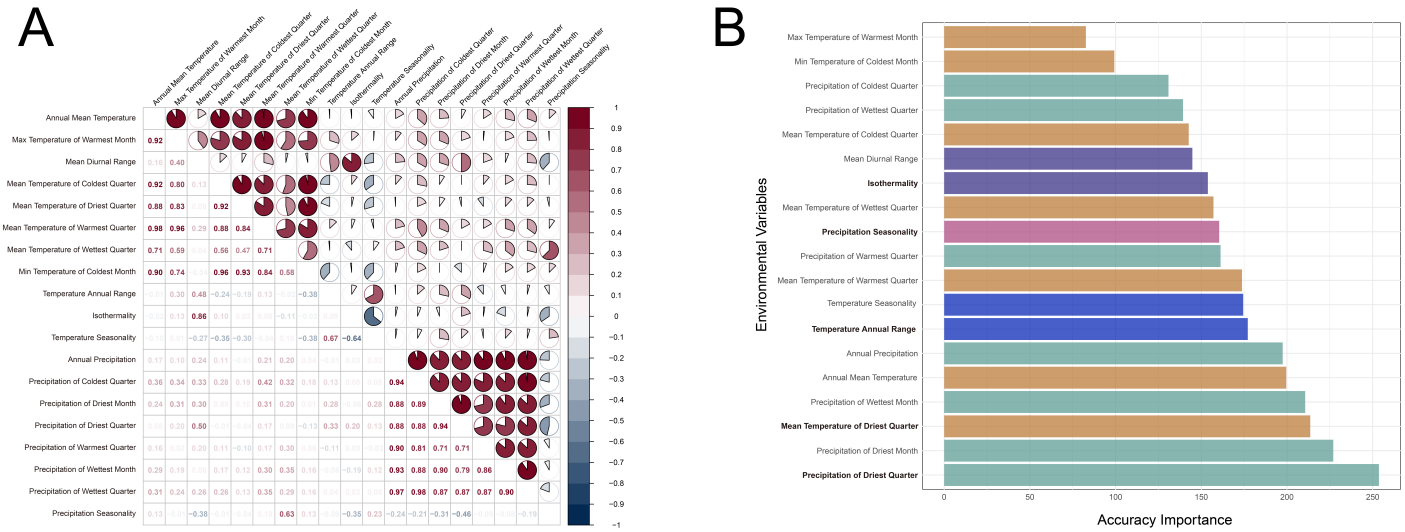


Figure 1

Correlation matrix (A) and importance barplot (B) of the 19 environmental factors. The depth of color and the fill level of the shapes are both used to represent the strength of the correlation, with dark colors and solid fills indicating strong correlations, and white and hollow shapes indicating no correlation. Environmental factor importance was calculated using the random forest algorithm. Within the highly correlated groups (correlation coefficient |r| > 0.65), the most important factors are highlighted in bold and the bars for the same factor group are colored the same.

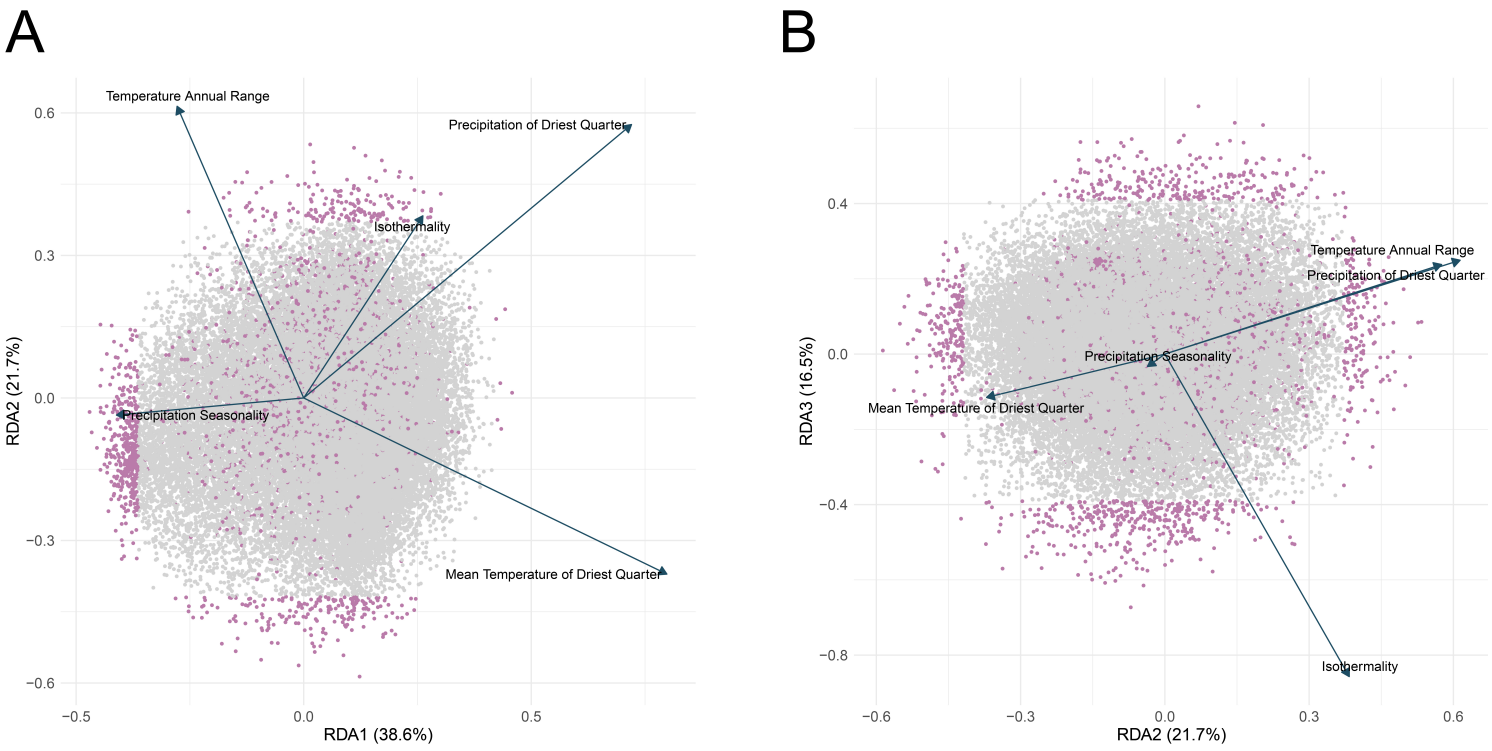


Figure 2

Ordination plots of redundancy analysis (RDA) of gene expression under different environmental conditions, showing RDA1 vs. RDA2 (A) and RDA2 vs. RDA3 (B). In both plots, individual genes are depicted as small points, with those more affected by environmental factors highlighted in purple. The vectors represent the direction and magnitude of the environmental factors' influence on gene expression, with the length of the vector corresponding to the strength of the influence.

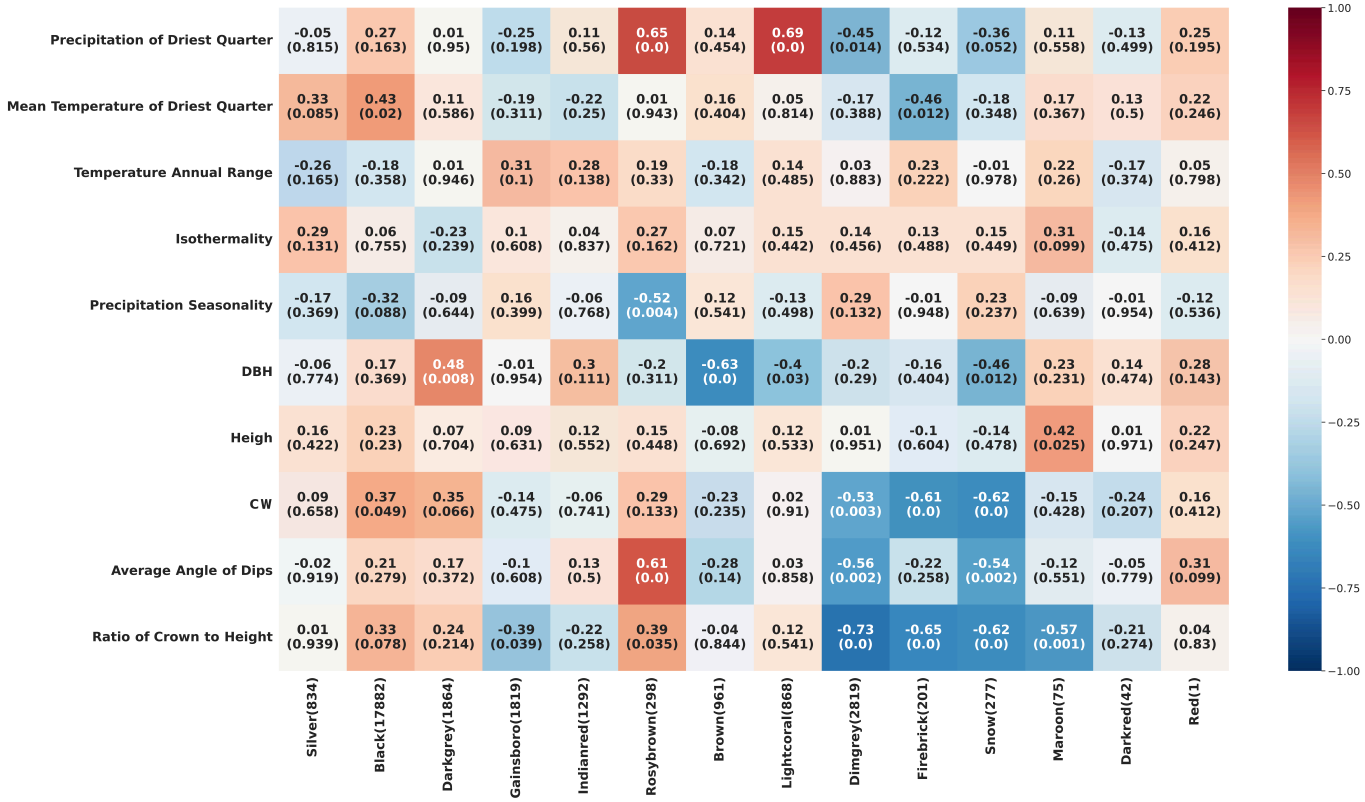


Figure 3

Heatmap showing the results of weighted gene co-expression network analysis (WGCNA) associating gene modules with environmental and phenotypic variables. Each cell in the heatmap represents the correlation coefficient and its significance in parentheses between a gene module and an environmental or phenotypic trait. The color scale on the right indicates the strength of the correlation.

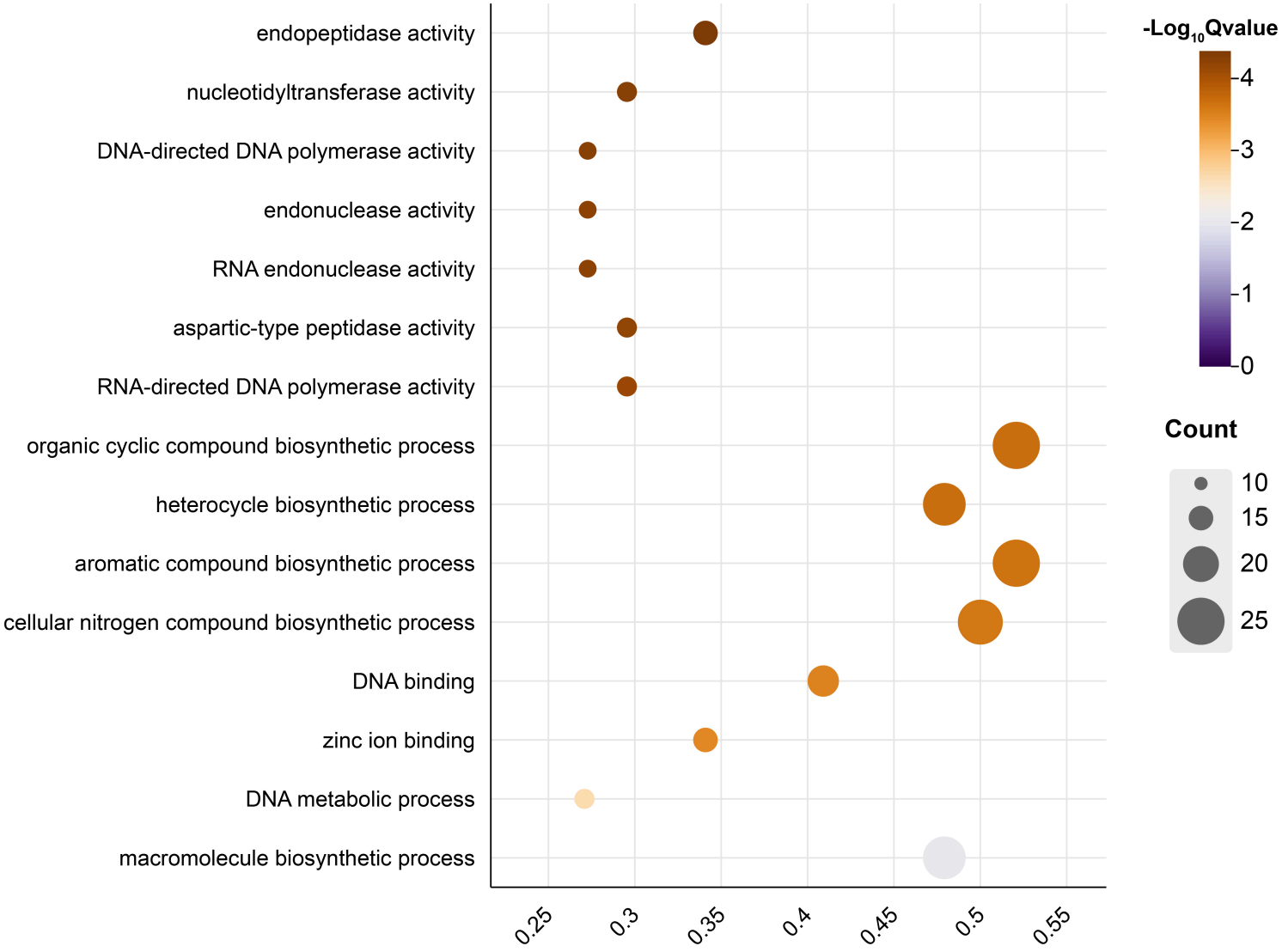


Figure 4

Gene ontology (GO) functional enrichment analysis of key genes identified with KME > 0.6 by WGCNA and RDA. The bubble plot represents various biological processes and molecular functions, with the size of each bubble reflecting the number of genes associated with that term and the color representing the -$\log_{10}$Qvalue of the enrichment significance.

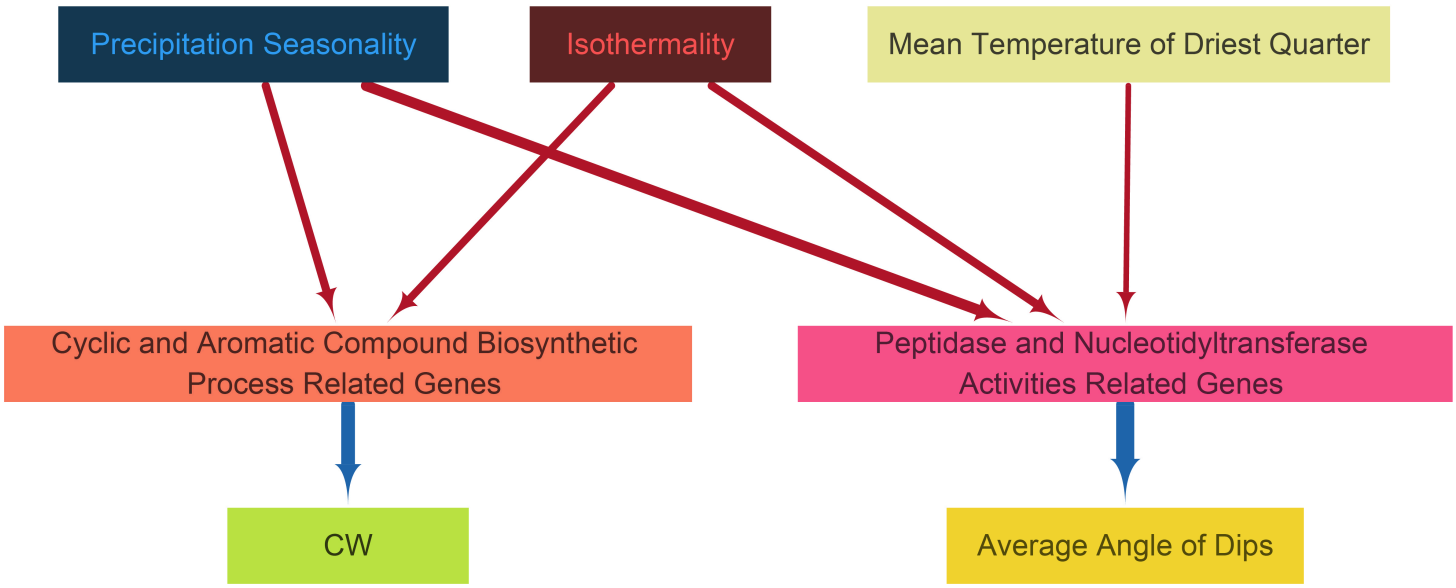


Figure 5

Partial least squares path modeling (PLS-PM) plot showing the relationships between specific functional gene categories and environmental and phenotypic data. These relationships are represented by the color and thickness of the arrows, with red indicating positive effects and blue indicating negative effects, and the thicker the arrow, the stronger the relationship.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xlsx
- TableS2S5FigureS1S3.docx