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RESEARCH ARTICLE

Multi-omics analysis reveals *WRKY31* and *MATE* as key regulators of flavonoid-based waterlogging tolerance in Welsh onion (*Allium fistulosum* L.)

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Highlights

- *WRKY31* and *MATE* mediate flavonoid accumulation and confer waterlogging tolerance in Welsh onion.
- The waterlogging-tolerant Welsh onion cultivar BJQC enhances flavonoid accumulation by transcriptionally activating genes in the flavonoid biosynthetic pathway, providing new genetic targets for breeding waterlogging-tolerant crops.

Abstract

Waterlogging poses a major challenge to Welsh onion (*Allium fistulosum* L.) production, exacerbated by climate change-induced extreme weather. Unraveling the molecular mechanisms of waterlogging tolerance is essential for breeding resilient cultivars. Here, we compared two Welsh onion varieties: BJQC (tolerant) and YZDC (sensitive). Waterlogging treatment revealed that YZDC exhibited higher accumulation of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide ions ($O_2^{-}\cdot$), and malondialdehyde (MDA), leading to increased mortality. In contrast, BJQC demonstrated enhanced waterlogging tolerance, which was attributed to its ability to upregulate flavonoid biosynthesis genes, resulting in higher flavonoid accumulation under waterlogging stress. Transcriptomic analysis identified that the activation of flavonoid pathway-related genes in BJQC was central to this response. In addition, genes associated with jasmonic acid and gibberellin signaling were activated. Weighted gene co-expression network analysis (WGCNA) revealed that *WRKY31* and *MATE* likely played critical roles in regulating flavonoid biosynthesis under waterlogging conditions. Genome-wide association study (GWAS) results from natural populations further supported the significance of these genes in waterlogging tolerance. Our comprehensive multi-omics analysis, including phenotypic, physiological, transcriptomic, and genomic approaches, provided new insights into the molecular mechanisms underlying Welsh onion responses to waterlogging. These findings highlight *WRKY31* and *MATE* as key candidates for improving waterlogging tolerance in crop breeding programs.

Keywords: Welsh onion, waterlogging tolerance, GWAS, RNA-Seq

1. Introduction

Welsh onion (*Allium fistulosum* L.), is a globally cultivated vegetable species, renowned for its culinary and nutritional significance (Li *et al.* 2010; Asemani *et al.* 2019; Alam *et al.* 2023). However, waterlogging stress poses a significant challenge to Welsh onion cultivation, often resulting in substantial yield losses and economic setbacks. Under waterlogged conditions, plants initiate a series of tightly coordinated physiological and biochemical responses aimed at survival and adaptation. These responses can be broadly categorized into hormonal regulation, oxidative stress management, and osmotic adjustment.

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Hormonal signaling plays a central role in the early perception and response to hypoxic stress. Phytohormones such as ethylene (ETH), abscisic acid (ABA), gibberellins (GA), brassinosteroids (BR), and jasmonic acid (JA) are rapidly modulated in response to waterlogging, triggering transcriptional reprogramming and morphological changes (Waadt *et al.* 2022; Wang H *et al.* 2022; Sun *et al.* 2024). Additionally, oxidative stress arises from the accumulation of reactive oxygen species (ROS), which can lead to cellular damage. Plants mitigate this damage by activating antioxidant defense systems, involving enzymes like superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) (Renziehausen *et al.* 2024; Zhang Y *et al.* 2024). Lipid peroxidation byproducts, such as malondialdehyde (MDA), serve as markers for oxidative injury (Lu *et al.* 2023; Niu *et al.* 2024). Moreover, osmotic protection mechanisms, including the accumulation of soluble sugars and compatible solutes, help preserve membrane stability and cellular integrity under anaerobic conditions (Goyal *et al.* 2020; Liang *et al.* 2025).

Flavonoids, essential secondary metabolites, play a pivotal role in plant adaptation to waterlogging stress by enhancing antioxidant defenses and modulating metabolic processes (Schenke *et al.* 2019; Dias *et al.* 2021; Cui *et al.* 2024). These compounds are regulated by structural genes (e.g., *PAL*, *C4H*) and transcription factors, contributing to ROS scavenging, signal transduction, and membrane stabilization, all of which are crucial for improving crop resilience to stress (Tanaka *et al.* 2008; Zou *et al.* 2018; Shomali *et al.* 2022). Recent studies have highlighted the importance of flavonoids in stress tolerance across various species. For instance, in *Brassica napus* L., the waterlogging-tolerant variety G230 shows increased flavonoid levels and upregulation of biosynthesis genes such as *FAP3* and *VSR7*, while the sensitive variety G218 exhibits higher oxidative damage (Hong *et al.* 2024). Similarly, in *Paeonia lactiflora* Pall., flavonoid accumulation enhances antioxidant capacity under waterlogging stress (Liu *et al.* 2021). In *Prunus persica*, flavonoid levels are boosted by mycorrhizal fungi, strengthening the plant's oxidative defense (Liang *et al.* 2024). In *Zea mays* L., flavonoids like quercetin and diosmetin are essential for maintaining cellular redox balance under waterlogging and shading stress (Zhao *et al.* 2021).

Acute waterlogging typically results in immediate, short-term responses, including alterations in hormonal signaling, rapid accumulation of ROS, and the activation of aerenchyma formation to alleviate oxygen deficits in plant tissues. In contrast, prolonged waterlogging induces more sustained molecular responses, such as a shift in metabolism towards anaerobic pathways, long-term changes in gene expression related to stress tolerance, and enhanced antioxidant enzyme activities (Harb *et al.* 2010; Peláez-Vico *et al.* 2023). While acute and prolonged waterlogging elicit distinct molecular responses, a comprehensive analysis of these mechanisms in Welsh onion remains lacking (Skirycz *et al.* 2011). Understanding the molecular mechanisms activated at different stages of waterlogging is crucial for unraveling plant performance under such conditions, particularly for crops like Welsh onion, which are sensitive to waterlogging stress (Rymaszewski *et al.* 2017; Chen *et al.* 2021; Nicolas *et al.* 2022).

In this study, we focused on a waterlogging-tolerant variety, 'Beijingqingcong', and a sensitive variety, 'Yuanzangdacong', of Welsh onion. Our findings revealed that 'Beijingqingcong' exhibited greater tolerance to waterlogging, attributed to physiological changes and increased flavonoid accumulation. Through comparative transcriptomic analyses across various tissues under different durations of waterlogging, we identified key gene expression patterns associated with tolerance. Furthermore, by integrating genome-wide association studies (GWAS), we pinpointed candidate genes linked to waterlogging tolerance. The functional roles of these genes were validated *via* heterologous expression in *Arabidopsis*. This study enhances our understanding of the molecular mechanisms underlying waterlogging tolerance in Welsh onion and provides a robust foundation for future breeding programs aimed at developing waterlogging-resistant cultivars.

2. Materials and methods

2.1. Experimental design and sampling

In July 2022, we recorded the post-waterlogging survival rate of 423 *A. fistulosum* germplasms after 7 d of natural waterlogging stress in the field, followed by 7 d of recovery under normal conditions, for subsequent analysis. Based on the survival rate and phenotype, we selected two Welsh onion varieties, 'Beijingqingcong' (BJQC) and 'Yuanzangdacong' (YZDC). In November 2022, the seeds were sown in 50-cell trays and grown under controlled conditions ((23±2)°C, 55–65% humidity, 16-h light photoperiod). After 6 weeks of growth, the seedlings were transplanted to the field in Jimo, Shandong. In July 2023, one group was subjected to waterlogging stress for 7 d under controlled conditions, with the water level approximately 15 cm above the soil surface, followed by 7 d of recovery under normal conditions, and the survival rate was recorded. Another group was subjected to waterlogging treatments for 0 h, 0.5 h, 12 h, and 5 d, with the water depth as described above. After the treatments, three samples were collected from the roots, leaf sheaths, and leaves, rapidly frozen in liquid nitrogen, and stored at –80°C.

2.2. Physiological assays

The growth and treatment conditions were the same as those described in Section 2.1. BJQC and YZDC were sampled at four different time points after waterlogging treatment. Whole plant samples were used for physiological index measurements, except for chlorophyll, which was measured from leaf samples. Commercial assay kits (H_2O_2-1-Y, SA-1-G, POD-2-Y, CAT-2-W, SOD-2-Y, MDA-1-Y, KT-1-Y, GST-1-W, LHT-1-G, CPL-1-G; COMIN, Suzhou, China; http://www.cominbio.com/) were employed to quantify ROS content (H_2O_2, $O_2^{-}\cdot$), and the activities of POD, CAT, SOD, GSTs, and MDA, as well as total flavonoid, chlorophyll, and soluble sugar contents.

2.3. RNA extraction and RNA-Seq data processing

Tissue samples were collected in triplicate, and library

construction and sequencing were performed on the DNBSEQ-T7 platform (Annoroad Gene Technology, Beijing, China) using DNBSEQ™ technology. Quality control was conducted using FastQC software. RNA-seq reads were aligned to the Welsh onion reference genome using HISAT2. Gene expression was normalized to FPKM values using StringTie. Differential expression was calculated using DESeq2 ($|\log_2 FoldChange|>1$, P-value<0.05) (Love *et al.* 2014). Data were visualized with PCA and heatmap analysis using ggplot2.

2.4. Gene clustering and GO enrichment

Gene clustering was performed using the "mfuzz" package (Kumar and Futschik 2007). GO enrichment was conducted using the hierarchical AlliumDB gene functional annotation library and clusterProfiler. Enriched GO terms with a P-value of 0.05 and Q-value≤0.2 were visualized in a bubble chart.

2.5. Weighted gene co-expression network analysis (WGCNA)

Analysis of RNA-seq data and construction of gene co-expression networks were performed using the R package WGCNA v1.72.5 (Yu *et al.* 2012). Gene networks were generated based on the average FPKM of gene expression (average FPKM across replicates treated). For visualization, networks were rendered using Cytoscape v3.10.1.

2.6. Genome-wide association study (GWAS)

A total of 423 *Allium* germplasm accessions were collected across 33 countries: 250 (61%, originating from 12 countries) from Asia, 109 (27%, 15 countries) from Europe; 48 (12%, 4 countries) from the Americas; with 1 each from Sudan and New Zealand; and 14 with unknown geographic origins. (Yang *et al.* 2024). A subset of 337 accessions was selected for further analysis. GWAS was analyzed using the Efficient Mixed Model Association eXpedited (EMMAX) algorithm (Kang *et al.* 2010). The kinship (K) matrix, generated by the emmax-kin program, was used to determine population stratification and hidden relatedness. The P-value threshold for suggestive association loci was set based on the Bonferroni correction estimate, adjusted for the effective number of independent markers (Duggal *et al.* 2008).

3. Results

3.1. Physiological responses of YZDC and BJQC to waterlogging stress

In a waterlogging environment, we evaluated the growth responses of two Welsh onion varieties, YZDC and BJQC, selected from a population of 337 individuals. Phenotypic analysis revealed distinct sensitivities: YZDC exhibited root rot, leaf curling, and wilting, while BJQC showed greater tolerance, primarily as leaf chlorosis (Fig. 1-A). Survival rates further supported this, with YZDC having significantly lower survival (36.31%) compared to BJQC (72.62%) (Fig. 1-B).

Oxidative stress responses were assessed by measuring superoxide anion ($O_2^{-}\cdot$) and hydrogen peroxide (H_2O_2) levels. YZDC exhibited significantly higher $O_2^{-}\cdot$ and H_2O_2 levels as early as 0.5 h post-waterlogging compared to BJQC (Fig. 1-C and F). Antioxidant enzyme activities, including SOD, POD and CAT, were significantly higher in BJQC (Fig. 1-D, E and G). MDA levels, indicating oxidative damage, were higher in YZDC (Fig. 1-H).

Soluble solids decreased with prolonged waterlogging, with YZDC showing a greater decline (Fig. 1-J). Chlorophyll content dynamics diverged between the varieties, but no significant differences were observed after 5 d compared to the control (Appendix A-I–IV).

Prolonged waterlogging resulted in significantly higher glutathione-*S*-transferase (GST) activity and total flavonoid content in BJQC (Fig. 1-I and K). Under short-term waterlogging stress, flavonoid content in YZDC increased slightly but gradually decreased as the stress continued. In contrast, BJQC showed a continuous increase in flavonoid levels throughout the entire stress period, significantly surpassing YZDC after prolonged waterlogging. Similarly, GST activity in YZDC rose initially but declined over time, whereas in BJQC, GST activity steadily increased and remained elevated even after 5 d of waterlogging. The sustained accumulation of flavonoids and consistently high GST activity in BJQC are closely linked to its enhanced stress tolerance, underscoring their roles as key indicators of waterlogging resistance. These findings emphasize the critical functions of flavonoids and GST in managing oxidative stress and improving resilience to prolonged waterlogging.

3.2. Transcriptomic responses to waterlogging in YZDC and BJQC

This study investigated the transcriptional responses of two Welsh onion varieties, BJQC and YZDC, to waterlogging stress across four time points (0, 0.5 h, 12 h, and 5 d) and three tissue types: roots, leaf sheaths, and leaves. Transcriptome sequencing was conducted on 24 biological samples with triplicate replicates, achieving an average sequencing depth of 10.4X. After stringent quality control, 5,447,265,760 high-quality clean reads were obtained and aligned to the *A. fistulosum* reference genome (Liao *et al.* 2022) (Appendix B). Correlation analysis confirmed the consistency among biological replicates (Appendix C-I), ensuring data reliability. Principal component analysis (PCA) and hierarchical clustering revealed that tissue type was the primary factor influencing sample separation (Fig. 2-A; Appendix C-II).

Differential expression analysis was performed on 18 tissue-treatment combinations, with non-waterlogged samples used as controls. A total of 9,178 differentially expressed genes (DEGs) were identified, comprising 4,373 upregulated and 4,805 downregulated genes (Fig. 2-B). Roots exhibited the highest number of DEGs compared to leaf sheaths and leaves. Notably, the number of DEGs in roots increased progressively with waterlogging duration, while those in leaf sheaths and leaves peaked at 12 h before declining. These observations suggest that roots are the primary site for gene expression regulation under waterlogging stress. This tissue-specific transcriptional response reflects adaptive mechanisms employed by Welsh onion to mitigate waterlogging stress.

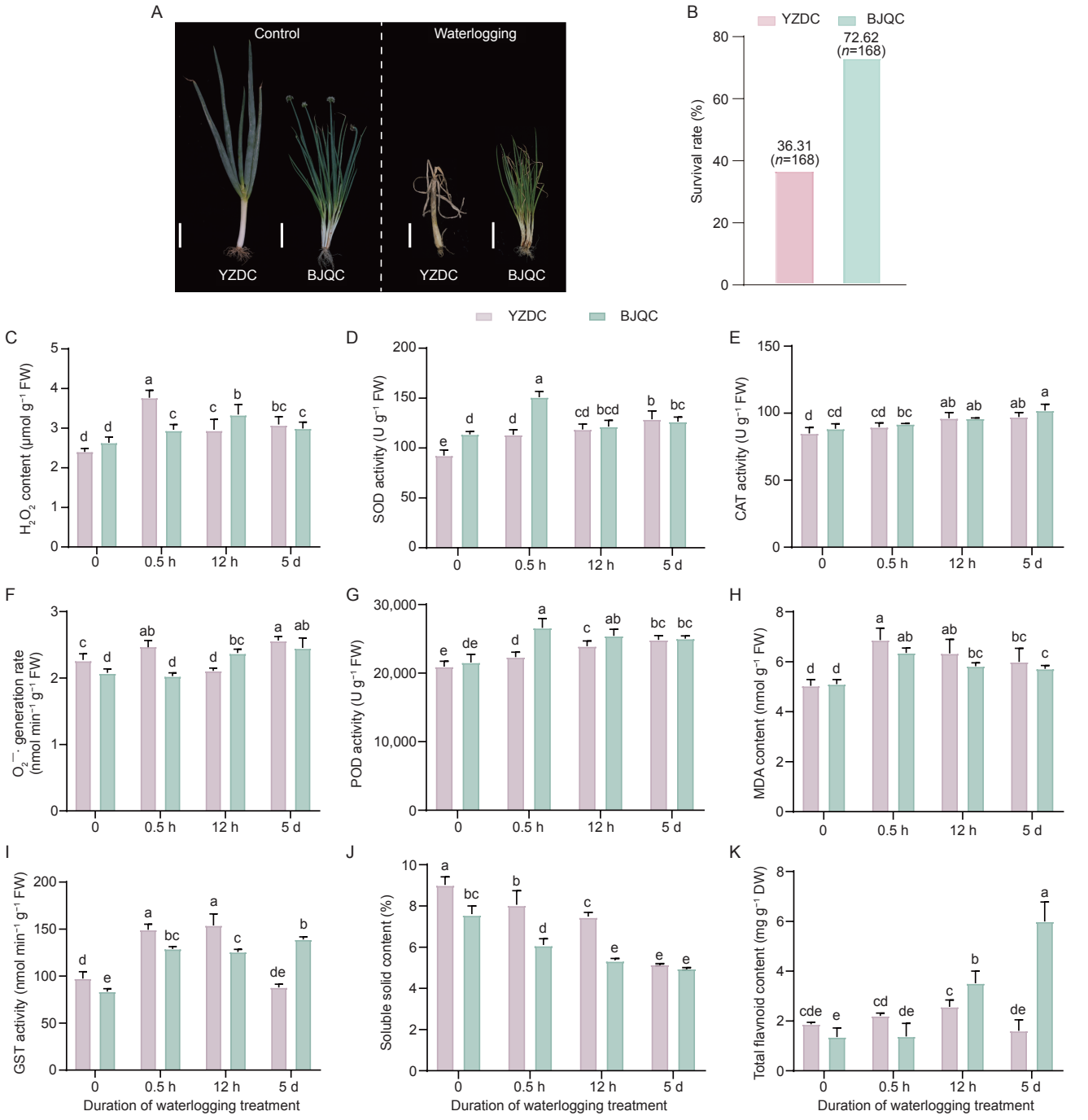


Fig. 1 Phenotypic and physiological differences between 'Beijingqingcong' (BJQC) and 'Yuanzangdacong' (YZDC) under waterlogging stress. A, morphological changes in YZDC and BJQC before and after waterlogging stress (scale bar=10 cm). B, survival rates of YZDC and BJQC following waterlogging, with 168 plants measured per variety. C–K, physiological parameters at 0, 0.5 h, 12 h, and 5 d post-waterlogging, including: hydrogen peroxide (H_2O_2) accumulation (C), superoxide dismutase (SOD) activity (D), catalase (CAT) activity (E), superoxide anion ($O_2^{-}\cdot$) production (F), peroxidase (POD) activity (G), malondialdehyde (MDA) content (H), glutathione *S*-transferase (GST) activity (I), soluble solids content (J), and total flavonoid content (K). Data represent mean values±standard deviation (SD) from three replicates (*n*=3). Means marked with lowercase letters are significantly different based on ANOVA followed by Tukey's HSD test (P<0.05). Leaf samples were collected from plants exposed to waterlogging for the indicated durations, and all measurements were conducted using standard biochemical assays.

Further analysis of transcription factors (TFs) among the DEGs revealed 49 and 124 TFs in YZDC and BJQC, respectively (Fig. 2-C and D). The AP2/ERF family was the most abundant across both cultivars; however, significant differences were observed in the proportions of other TF families, such as bHLH. In BJQC, upregulated genes were enriched in the AP2/ERF, EIL, and Tify families, whereas downregulated genes in YZDC were predominantly associated with WRKY, C2C2-Dof, and MYB families. These findings underscore divergent transcriptional regulatory mechanisms

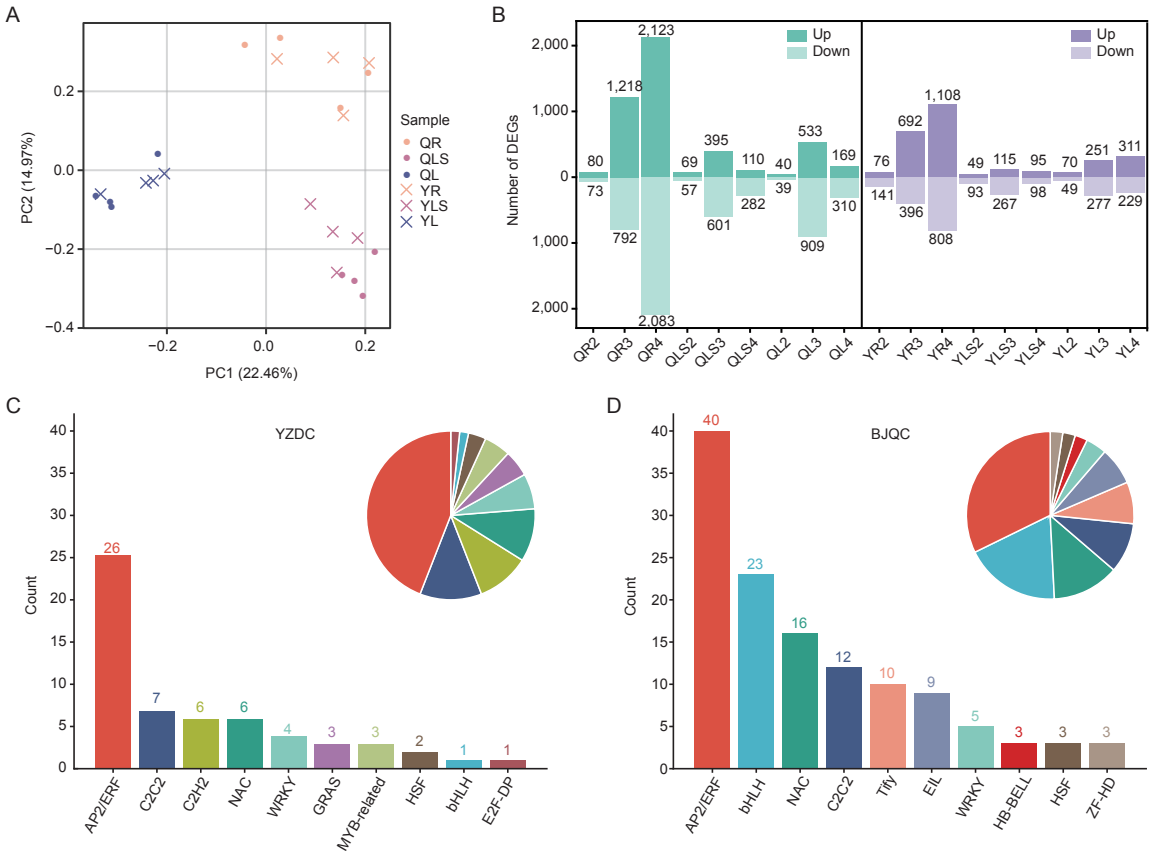


Fig. 2 Transcriptome analysis of 'Beijingqingcong' (BJQC) and 'Yuanzangdacong' (YZDC) under waterlogging stress. A, principal component analysis (PCA) of the RNA-seq data reveals distinct transcriptional responses among samples, with clear separation along the first principal component. B, bar plot displaying differentially expressed genes (DEGs) in treated samples of BJQC (Q) and YZDC (Y) across tissue types (R, root; LS, leaf sheath; L, leaf) and time points (1, 0 h; 2, 0.5 h; 3, 12 h; 4, 5 d). Color intensity reflects $\log_2$-transformed fold change relative to control conditions. C, distribution of transcription factor (TF) families in DEGs of YZDC, showing the relative abundance and diversity of TFs involved in stress responses. D, transcription factor family distribution in DEGs of BJQC, highlighting cultivar-specifc regulatory mechanisms under waterlogging stress.

between the two cultivars under waterlogging stress.

3.3. Differential gene enrichment analysis across varying durations of waterlogging stress and tissues

We performed enrichment analysis of DEGs in various tissues of BJQC and YZDC varieties under varying durations of waterlogging stress. In BJQC, following a 0.5-h treatment, the root tissue exhibited significant enrichment for GO terms GO:0010510 (regulation of acetate biosynthesis from pyruvate) and GO:0016131 (brassinosteroid metabolic process) (Fig. 3-A). Concurrently, leaf sheaths were enriched for GO:0046520 (sphingolipid biosynthetic process) and GO:0051781 (positive regulation of cell division), while leaves displayed enrichment for GO:0009932 (regulation of apical cell growth) (Appendix D-I and IV). After 12 h of waterlogging, root tissue showed pronounced enrichment in GO:0009812 (flavonoid metabolic process), GO:0009718 (anthocyanin-containing compound biosynthetic process), GO:0070483 (hypoxia detection), GO:1901701 (cellular response to oxygen-containing compounds), GO:0009695 (jasmonic acid biosynthetic process), and GO:0080141 (regulation of jasmonic acid biosynthesis) (Fig. 3-B). Leaf sheaths were enriched for GO:0010052 (guard cell differentiation), GO:0071370 (cellular response to gibberellin), and GO:0010371 (regulation of gibberellin biosynthesis), while leaves exhibited enrichment for GO:0010025 (wax biosynthetic process) (Appendix D-II and V). Following prolonged waterlogging for 5 d, roots retained significant enrichment in GO:0009812, GO:0009718, GO:0009717 (isoflavonoid biosynthetic process), and hormone-related processes including GO:0009695 (Fig. 3-C). Leaf sheaths displayed enrichment for GO:0002679 (respiratory burst in defense response) and GO:0009617 (response to bacteria), while leaves were predominantly enriched for GO:0042542 (response to hydrogen peroxide) (Appendix D-III and VI). These findings highlight distinct temporal patterns of gene expression across tissues under waterlogging stress in BJQC.

For the YZDC variety, following 0.5 h of waterlogging, root tissue samples exhibited significant enrichment for GO:0016114 (terpenoid biosynthetic process), GO:0009684 (auxin biosynthetic process), and GO:0071215 (cellular response to abscisic acid) (Fig. 3-D). Leaf sheaths

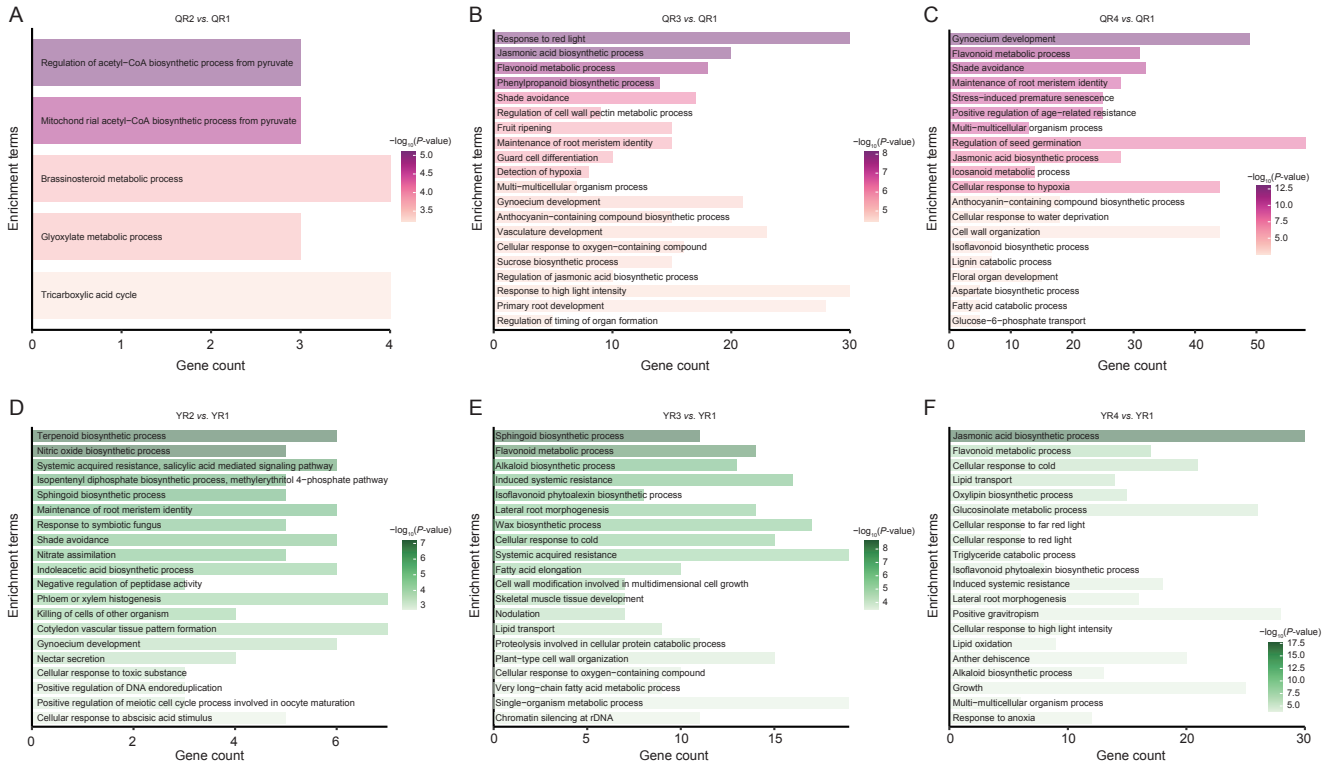


Fig. 3 GO enrichment analysis of differentially expressed genes (DEGs) in roots (R) of 'Beijingqingcong' (BJQC; Q) (A–C) and 'Yuanzangdacong' (YZDC; Y) (D–F) under waterlogging stress at different durations (1, 0 h; 2, 0.5 h; 3, 12 h; 4, 5 d).

and leaf tissues displayed enrichment for GO:0016114 and GO:0009862 (Appendix E-I and IV). After 12 h of waterlogging, root tissue exhibited enrichment for GO:0046520, GO:0009812, and GO:0009701 (isoflavonoid phytoalexin biosynthetic process) (Fig. 3-E), while leaf sheaths showed enrichment for GO:0000305 (response to oxygen radical) (Appendix E-II). Following prolonged waterlogging for 5 d, root tissue retained significant enrichment for GO:0009812, GO:0034440 (lipid oxidation), and hormone-related GO:0009695 (Fig. 3-F). Leaf tissue exhibited enrichment for GO:0009867 (jasmonic acid mediated signaling pathway) (Appendix E-VI). These results demonstrate distinct temporal responses to waterlogging stress in the YZDC variety, highlighting tissue-specific and time-dependent variations in gene expression.

Enrichment analysis revealed that under waterlogging stress, both jasmonic acid (JA) and gibberellin (GA) signaling pathways were significantly activated in BJQC, contributing to its enhanced waterlogging tolerance. Gene expression profiling further highlighted the upregulation of key regulatory genes involved in the synthesis and signaling of JA and GA, with distinct stress-responsive trends observed in downstream regulatory genes (Appendix F). Both cultivars exhibited significant activation of flavonoid metabolism pathways, primarily in roots, underscoring the important role of flavonoids in waterlogging tolerance in Welsh onion. Notably, BJQC displayed more extensive transcriptional changes compared to YZDC, particularly in genes associated with secondary metabolites and hormone biosynthesis. This suggests that BJQC may possess a more comprehensive array of stress-responsive mechanisms, which could be crucial to its superior tolerance under waterlogging conditions.

3.4. WGCNA-based identification of key flavonoid pathway regulators in response to waterlogging stress

Comparative physiological studies revealed significantly higher total flavonoid contents in BJQC roots under waterlogging conditions (Fig. 1-K). These findings were further supported by GO term enrichment analysis, which consistently highlighted the flavonoid biosynthesis pathway as a key process in BJQC roots (Fig. 3-B and C). To elucidate transcriptional regulation of flavonoid biosynthesis, we conducted WGCNA on the transcriptomic data to identify key regulators. In the phenylalanine metabolism pathway, an early step in flavonoid biosynthesis, BJQC exhibited more pronounced upregulation of key genes *AfisC2G00045* (*PAL1*), *AfisC6G01009* (*PAL1*), *AfisC1G02245* (*C4H*), *AfisC4G00795* (*C4H*), *AfisC6G04784* (*C4H*), and *AfisC6G01765* (*4CL2*) in the roots compared to YZDC, with most of these genes showing significant upregulation post-waterlogging (Fig. 4-A and B). We conducted a WGCNA analysis using the FPKM values of four flavonoid biosynthesis genes: *AfisC2G00045* (*PAL1*), *AfisC1G02245* (*C4H*), *AfisC8G05600* (*F3′H*), and the R2R3-type MYB transcription factor *AfisC5G05306* (*MYB9*) as phenotypic data (Fig. 4-C–F). The WGCNA analysis revealed a significant correlation (correlation coefficient>0.7) with the MEbrown module (Fig. 4-G; Appendix G-I–III). Given that the flavonoid pathway is primarily enriched in

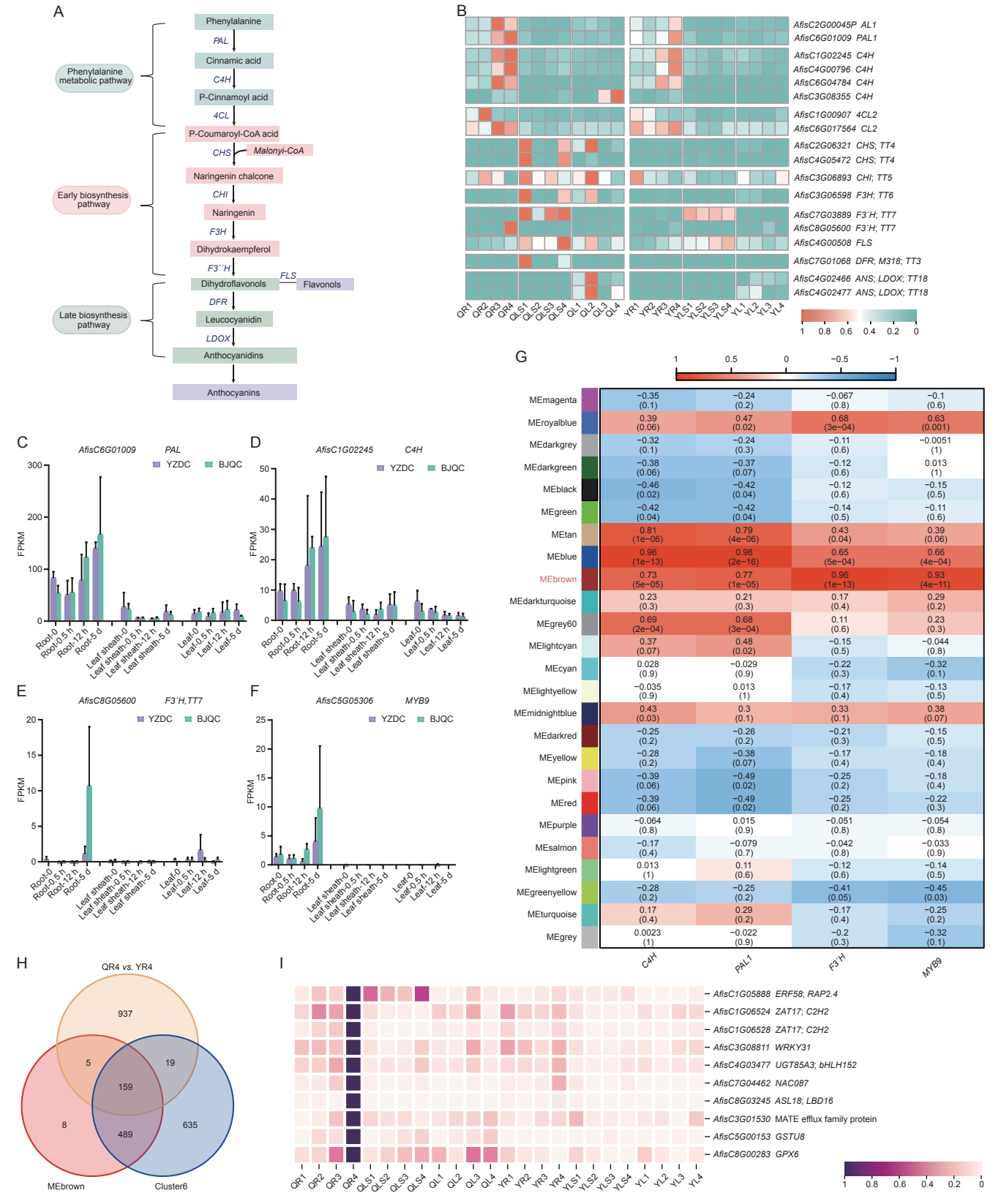


Fig. 4 Flavonoid pathway analysis after waterlogging stress in 'Beijingqingcong' (BJQC) and 'Yuanzangdacong' (YZDC). A, flavonoid synthesis pathway. B, expression profile of differentially expressed genes (DEGs) in the flavonoid synthesis pathway. C, comparative expression analysis of *PAL* in BJQC and YZDC. D, comparative expression analysis of *C4H* in BJQC and YZDC. E, comparative expression analysis of *F3′H* in BJQC and YZDC. F, comparative expression analysis of *MYB9* in BJQC and YZDC. G, correlations of physiological indicators with WGCNA modules. Each row represents a module, labeled accordingly. Columns correspond to physiological indicators. The cell color indicates the correlation coefficient between the module and physiological indicator (the top number in the cell is the correlation coefficient, and the bottom number in parentheses is the *P*-value). H, candidate module Venn diagram. I, expression profiles of transcription factors identified from the overlapping gene list in H.

roots (Fig. 3-B, C and F), and the total flavonoid content was upregulated in BJQC after prolonged waterlogging (Fig. 1-K), we performed a time-series analysis of differentially expressed genes (Appendix G-IV). Cluster6, predominantly upregulated in BJQC roots, was identified. Venn analysis of MEbrown, Cluster6, and differential genes in BJQC *vs.* YZDC roots identified 159 common genes (Fig. 4-H and I; Appendix H). Among these co-expressed genes, 7 transcription factors were annotated, including *ERF58* (*AfisC1G05888*) and *WRKY31* (*AfisC3G08811*), other notable genes included a MATE efflux family protein (*AfisC3G01530*), *GSTU8* (*AfisC5G00153*), and *GPX6* (*AfisC8G00283*). These genes represent promising candidates for enhancing waterlogging tolerance in Welsh onion by increasing flavonoid content in response to prolonged waterlogging stress.

This study provides new insights into the transcriptional regulation of flavonoid metabolism under waterlogging conditions, highlighting key genes and pathways that may play crucial roles in the adaptive responses of welsh onion to environmental stress.

3.5. Genome-wide association studies identify candidate genes conferring waterlogging tolerance

To identify potential causal genes associated with waterlogging tolerance in Welsh onion, we conducted a genome-wide association study by exposing 311 accessions to waterlogging stress over two years (2022 and 2024), monitoring their post-waterlogging survival rates (Fig. 5-A and B). Based on phenotypic data, we selected 112 accessions with stable traits, comprising 21 waterlogging-tolerant and 91 waterlogging-intolerant genotypes (Appendix I). Accession information is archived in the AlliumDB gene functional annotation library. These selected accessions were subjected to association analysis (Fig. 5-C and D), which identified two significant loci associated with waterlogging tolerance: one on chromosome 3 (*P*=3.22e–08) and the other on chromosome 8 (*P*=2.36e–60) (Appendix J). The signal on chromosome 3 includes *AfisC3G08811*, a WRKY transcription factor designated as *WRKY31* (Fig. 5-C and E; Appendix J). The signal on chromosome 8 (*P*=2.36e–60) includes the gene *AfisC8G02885* from the MATE family (Fig. 5-C and F; Appendix J).

Integrating transcriptomic data revealed significant differential expression of *AfisC3G08811* (*WRKY31*) and *AfisC8G02885* (*MATE*) between BJQC and YZDC accessions. Notably, *AfisC3G08811* exhibited the largest expression difference in roots after 5 d of waterlogging (Fig. 5-G). In contrast, *AfisC8G02885* was minimally expressed in YZDC but showed high expression in BJQC across roots, leaf sheaths, and leaves under waterlogging stress (Fig. 5-F). These findings underscore the roles of these genes in waterlogging tolerance.

To validate their functional contributions, we analyzed the conservation of *AfisC3G08811* and *AfisC8G02885* protein sequences across *Arabidopsis*, onion, garlic, and Welsh onion (Appendix K-I and II). Functional validation was conducted by generating *Arabidopsis* T-DNA insertion mutants, *wrky31* and *mate*. These mutants exhibited reduced waterlogging tolerance compared to wild-type plants, as evidenced by phenotypic assessments under waterlogging stress and recovery (Fig. 5-I and J). Additionally, both mutants demonstrated significantly lower total flavonoid content (Fig. 5-K), indicating that *wrky31* and *mate* are critical for flavonoid accumulation and waterlogging tolerance in *Arabidopsis*. These results suggest a conserved regulatory mechanism where *AfisC3G08811* (*WRKY31*) and *AfisC8G02885* (*MATE*) mediate flavonoid accumulation and confer waterlogging tolerance in Welsh onion.

4. Discussion

4.1. Physiological and molecular responses of Welsh onion cultivars under waterlogging stress

Understanding the physiological and molecular responses of Welsh onion to waterlogging stress offers valuable insights into plant adaptation mechanisms to abiotic stress. In this study, we observed distinct differences in the physiological and molecular responses between the waterlogging-tolerant BJQC and the sensitive YZDC cultivars. Notably, BJQC exhibited enhanced tolerance following waterlogging, marked by elevated total flavonoid content and increased glutathione transferase activity (Fig. 6). These findings are consistent with previous studies that have emphasized the importance of flavonoid accumulation in stress adaptation (Pinheiro *et al.* 2021).

Plants have developed various adaptive strategies to cope with waterlogging stress, with root architecture modification being one of the key responses. These include the formation of adventitious roots (ARs), aerenchyma, and radial root thickening (Eysholdt-Derzsó and Sauter 2019). Previous studies in barley and oats have shown a correlation between waterlogging tolerance and tiller number. The differences in root morphology and tillering capacity between BJQC and YZDC observed in our study warrant further investigation to better understand their respective roles in waterlogging tolerance (Asemani *et al.* 2019; Zhang L *et al.* 2024).

4.2. Differential transcriptomic responses to waterlogging in Welsh onion cultivars

The transcriptomic analysis further reveals distinct transcriptional regulatory responses between BJQC and YZDC. Notably, BJQC shows significant upregulation of transcription factor families, including AP2/ERF, TIFY, and EIL, as well as genes involved in flavonoid, jasmonic acid, and gibberellin biosynthesis. These findings are consistent with previous studies on waterlogging-tolerant species such as apple and rapeseed, which have also emphasized the role of flavonoid and hormone signaling pathways in abiotic stress adaptation (Zhang *et al.* 2023; Hong *et al.* 2024). Key regulatory factors in the flavonoid biosynthesis pathway are significantly upregulated in BJQC following waterlogging (An *et al.* 2015; Zhang *et al.* 2022; Zhang L *et al.* 2024). These findings corroborate previous research underscoring the roles of these transcription factors and hormones in plant stress adaptation (Fàbregas and Fernie 2019; Sun *et al.* 2020). In contrast, YZDC exhibited a distinct defense mechanism involving the activation of salicylic acid and terpenoid

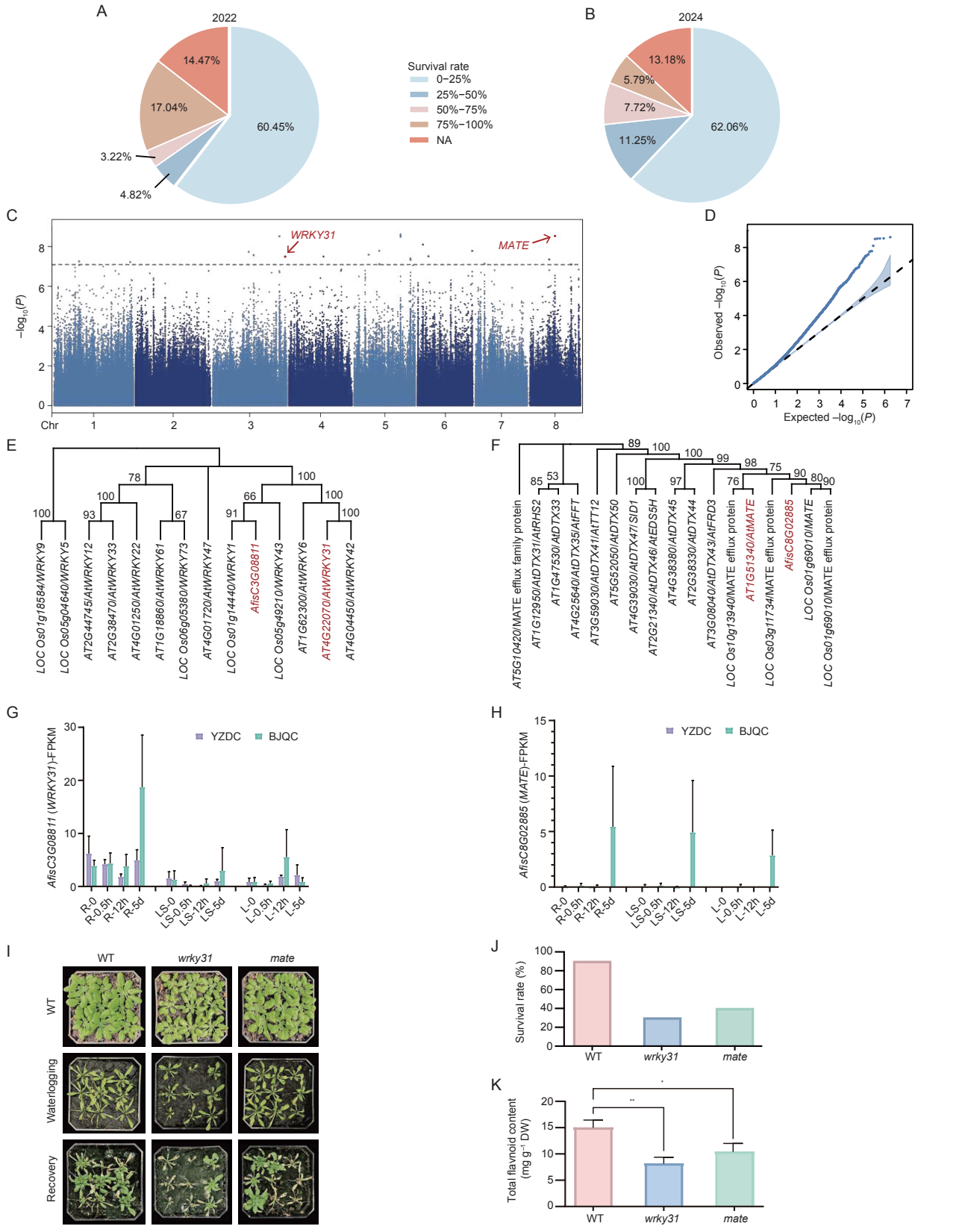


Fig. 5 Genome-wide association analysis (GWAS) of natural populations for screening candidate genes. A and B, survival statistics of 311 natural populations under waterlogging stress in 2022 and 2024, respectively. C, Manhattan plot of GWAS for waterlogging survival rate in Welsh onion. D, quantile-quantile plot of the corresponding GWAS. E and F, phylogenetic analysis of two candidate genes (*AfisC3G08811* and *AfisC8G02885*) with homologs from *Arabidopsis thaliana* and rice. G and H, tissue-specific expression patterns of *WRKY31* (G) and *MATE* (H) in BJQC and YZDC, including root (R), leaf sheath (LS) and leaf (L). I, phenotypes of *A. thaliana* after waterlogging stress and recovery. J, survival rate of WT, *wrky31* and *mate*. K, total flavonoid content of WT, *wrky31* and *mate* (P<0.05).

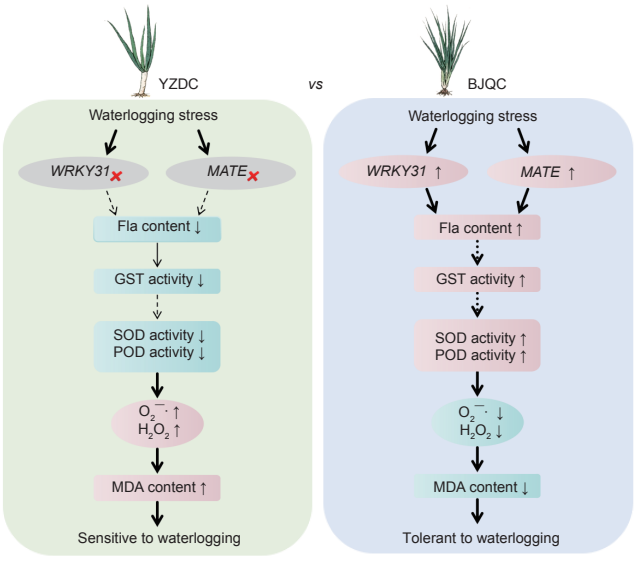


Fig. 6 Response pattern map after waterlogging stress in 'Yuanzangdacong' (YZDC) and 'Beijingqingcong' (BJQC).

biosynthesis pathways, suggesting alternative regulatory strategies under waterlogging conditions.

4.3. Identification of genes linked to waterlogging tolerance in Welsh onion

Genome-wide association studies (GWAS) identified two candidate genes: *AfisC3G08811* (*WRKY31*) and *AfisC8G02885* (*MATE*), as key regulators of waterlogging tolerance. *WRKY31*, a transcription factor involved in flavonoid biosynthesis, is known to interact with *bHLH3* to enhance flavonoid accumulation in response to stress (Li *et al.* 2020; Yin *et al.* 2022). WRKY family members such as *WRKY12*, *WRKY22*, and *WRKY33* have also been implicated in waterlogging tolerance in *Arabidopsis* (Liu *et al.* 2021; Tang *et al.* 2021; Liang *et al.* 2025). To further investigate the regulatory role of *WRKY31* in waterlogging tolerance, a population haplotype analysis was conducted. The analysis revealed two intron variants (r^2=0.98), which could divide the population into three haplotypes. Significant differences in waterlogging tolerance were observed among the different haplotypes (Appendix L-I and II). Additionally, *AfisC8G02885*, a gene from the MATE family, plays a critical role in flavonoid biosynthesis by facilitating the transport of proanthocyanidin precursors into vacuoles (Gani *et al.* 2022). Heterologous expression of these genes in *Arabidopsis* confirmed their involvement in enhancing both waterlogging tolerance and flavonoid accumulation, supporting their potential as breeding targets for improving resistance to waterlogging stress (Marinova *et al.* 2007; Wang S *et al.* 2022).

4.4. Implications for breeding and future research on waterlogging tolerance in Welsh onion

Waterlogging stress is a multifaceted process that involves complex interactions between factors such as hypoxia, ethylene accumulation, ROS, and changes in nutrient and hormone signaling, leading to differential gene expression (Rahman *et al.* 2023). Variability in physiological states or stress perception during sampling can also contribute to differences between biological replicates. To minimize this impact, we employed stringent filtering criteria ($|\log_2 FC \geq 2|$, *P*-value<0.05) and utilized the DESeq2 package, which adjusts for high between-replicate variability, ensuring the robustness of our differential expression results. Our enrichment analysis of DEGs further confirmed that, despite some replicates showing low correlation, the overall conclusions remained consistent. This variability reflects the inherent biological complexity of welsh onion's response to waterlogging stress and does not significantly affect the overall findings.

The results of this study provide valuable insights into the molecular basis of waterlogging tolerance in Welsh onion, identifying key genetic resources that can be leveraged for the development of more resistant cultivars. Given the complexity of plant signaling networks involved in waterlogging responses, future studies should focus on elucidating the specific mechanisms and interactions between the identified genes and other stress-related pathways. Moreover, field validation will be crucial to assess the practical applicability of these findings in breeding programs for waterlogging-resistant varieties.

5. Conclusion

This study elucidates the physiological and molecular mechanisms of waterlogging tolerance in Welsh onion (*A. fistulosum* L.), focusing on the contrasting responses of the tolerant BJQC and the sensitive YZDC varieties. Our results show that BJQC's enhanced tolerance is linked to the upregulation of flavonoid biosynthesis genes, which boost flavonoid content under waterlogging stress. Transcriptomic and genome-wide association studies identify *WRKY31* and *MATE* as key regulators in this process. These genes present valuable targets for breeding waterlogging-resistant Welsh onion varieties. Overall, this study provides critical genetic resources for crop improvement and lays the groundwork for future research on gene interactions and field validation to address climate-related challenges in Welsh onion cultivation.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation

and writing of this manuscript.

Data availability

RNA-Seq data of 24 *Allium* accessions have been submitted to BioProject of PRJNA1228618 in the National Centre for Biotechnology Information (NCBI).

Appendices associated with this paper are available at https://doi.org/10.1016/j.jia.2025.12.068

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