Folia Hort. 35(1) (2023): 149–162

DOI: 10.2478/fhort-2023-0011

FOLIA HORTICULTURAE

Published by the Polish Society for Horticultural Science since 1989

ORIGINAL ARTICLE | Open access | https://sciendo.com/journal/FHORT

MAP kinase and plant–pathogen interactions govern male *Zizania latifolia* responses to *Ustilago esculenta* during the early stages of infection

Pengfei Guo[1], Huimin Zhou[1], Longfei Bai[1], Yayu Lin[1], Yalong Zhang[1], Bichen Wang[1], Xiaomei He[2,*], Defang Gan[1,*]

[1] Department of Vegetable Science, School of Horticulture, Anhui Agricultural University, Hefei, Anhui 230036, China
[2] Department of Biotechnology, College of Biological and Pharmaceutical Engineering, West Anhui University, Lu'an, Anhui 237012, China

ABSTRACT

This study explored the effect of *Ustilago esculenta* infection on differentially expressed genes (DEGs) in male Manchurian wild rice (*Zizania latifolia*). We injected male Jiaobai plants at the five-leaf stage with *U. esculenta* suspension, and leaves at different timepoints after inoculation were collected for transcriptome sequencing. Transcriptome sequencing identified 1,226 DEGs mainly enriched in resistance-related processes. Weighted gene coexpression network analysis (WGCNA) showed that 'plant–pathogen interaction' and 'MAPK signalling pathway-plant' were the two most important upregulated pathways, and 16 candidate genes related to response to *U. esculenta* infection were screened. The results provide a basis for determining the molecular mechanisms of the response of male *Z. latifolia* to *U. esculenta* infection.

Keywords: differentially expressed genes, stem expansion, transcriptome analysis, *Ustilago esculenta*, *Zizania latifolia*

INTRODUCTION

Zizania latifolia is a perennial aquatic herb, the swollen stems of which are a delicious vegetable (called Jiaobai in China) when infected by *Ustilago esculenta*. *Z. latifolia* is very popular in North America and East Asia due to its nutritional and medicinal value (Yu et al., 2020; Wang et al., 2020a). *U. esculenta* is an endophytic smut fungus that invades *Z. latifolia* and induces host stems to form swollen culm galls. Three phenotypes of *Z. latifolia* have been identified in the field: male Jiaobai (without *U. esculenta* infection, with no galls but normal flowering), grey Jiaobai (inedible galls filled with dark teliospores, with an unacceptable taste) and normal Jiaobai (edible swollen galls in stem bases filled with hyphae). These three phenotypes are closely related to the growth status of *U. esculenta* in *Z.latifolia* (Wang et al., 2020b). Male Jiaobai is free from *U. esculenta* infection, grey Jiaobai is formed following infection with the T strain and normal Jiaobai contains the M-T (mycelial) strain of *U. esculenta* (Ye et al., 2017). Grey Jiaobai and male Jiaobai have no economic value in cultivation, and are removed manually (Zhang et al., 2021). Research shows that population diversity or physiological race differentiation of *U. esculenta* may exist among *Z. latifolia* plants (Tu et al., 2019). The molecular mechanism underpinning the male *Z. latifolia* responses to *U. esculenta* infection remains unknown, although the mechanism of swelling and stem enlargement in infected *Z. latifolia* has been a focus of attention (Li et al., 2021, 2022; Zhang et al., 2021).

Proteomic studies have explored the stem expansion process of *Z. latifolia*. The number and expression

*Corresponding author.
e-mail: 2023437521@qq.com (Xiaomei He); dfgan1@163.com (Defang Gan).

level of unique proteins in stems of *Z. latifolia* at different developmental stages vary significantly, suggesting that formation of the fleshy stem bases of *Z. latifolia* result from interactions between the plant and the endophytic fungus (Liu et al., 2010). Wang et al. (2017) reported transcriptome assembly and expression profiles based on RNA sequencing (RNA-Seq) data, identified genes responsible for culm gall formation in *Z. latifolia* induced by *U. esculenta*, and detected 8,089 genes (4,389 upregulated, 3,700 downregulated) and 5,251 genes (3,121 upregulated, 2,130 downregulated) differentially expressed in JB and HJ, respectively. The authors also identified 376 host and 187 fungal candidate genes displaying stage-specific expression patterns that might be responsible for gall formation at the initial and later phases, respectively (Wang et al., 2017). Jose et al. (2019) detected differentially expressed proteins in response to local smut formation during the interaction between *Z. latifolia* and *U. esculenta*, and found that the fungus participates in at least seven metabolic pathways and five major biological processes in order to subdue host defences and colonise *Z. latifolia* tissues. A further study found that *U. esculenta* produced proteases and energy acquisition proteins that enhanced its defences and survival in the host (Jose et al., 2019).

In our previous studies on the *Z. latifolia–U. esculenta* interaction, we established an *in vitro* regeneration system for *Z. latifolia*, and carried out inoculation experiments with *U. esculenta* (Yang, 2018). We also studied the physiological responses of male *Z. latifolia* to *U. esculenta* infection, and found that expression of most enzymes was significantly different at 3 hr or 12 hr post-infection, suggesting that responses of *Z. latifolia* to *U. esculenta* infection mainly occurred in the early stages of the colonisation process (Zhou, 2021). However, the mechanism underpinning the interaction between *U. esculenta* and *Z. latifolia* that leads to enlargement of swollen stem galls remains unclear. In order to explore the molecular mechanisms of the *Z. latifolia* responses to *U. esculenta* infection, male (uninfected) *Z. latifolia* plants at the five-leaf stage were used as experimental material. Transcriptome sequencing and identification of differentially expressed genes (DEGs) were performed on leaves at different timepoints after inoculation, and candidate genes of male *Z. latifolia* potentially involved in the responses to *U. esculenta* infection were screened. This work provides an important baseline for subsequent studies into the mechanism of culm gall formation in *Z. latifolia*.

MATERIALS AND METHODS

Transcriptome sequencing and analysis of DEGs

Male *Z. latifolia* plants at the five-leaf stage were injected with the resulting *U. esculenta* cell suspension (TR), while male Jiaobai plants injected with potato dextrose liquid medium served as controls (CK). Inoculation and sampling were performed as described previously in the studies of Zhou (2021) and Xu et al. (2023). Male *Z. latifolia* leaves at 3 hr, 12 hr and 24 hr (TR-3 hr, TR-12 hr and TR-24 hr) after inoculation and controls (CK) were frozen in liquid nitrogen. Total RNA was extracted using an RNAprep Pure Plant Kit (Tiangen Biotech [Beijing] Co. Ltd., Beijing, China), and mRNA was purified by magnetic beads with Oligo dT. Transcriptome sequencing was conducted by Beijing Biomarker Technologies Co. Ltd. (Beijing, China). To ensure the accuracy of the analysis, strict quality control was exercised in relation to the original data. Reads containing joints, reads with a proportion of N >10%, reads with a mass value of Q ≤10, and alkali bases accounting for >50% of the whole read were removed. The remaining reads were aligned against the *Z. latifolia* genome database (http://ibi.zju.edu.cn/ricerelativesgd/download.php). The HSAT2 system was used for comparison, and StringTie software (https://ccb.jhu.edu/software/stringtie/index.shtml) was used to assemble and quantify the data. Three biological replicates were included, and r (Spearman's correlation coefficient) was used to evaluate the correlation of biological replicates. The closer r^2 is to 1, the higher the correlation between biological replicates, and the more reliable the results. DEGs among sample groups (CK-3 hr vs. TR-3 hr, CK-12 hr vs. TR-12 hr and CK-24 hr vs. TR-24 hr) were analysed using DESeq2 software (Love et al., 2014). Among the comparisons, CK-3 hr vs. TR-3 hr represents the comparison between the treatment group (TR-3 hr) and the control group (CK-3 hr) at 3 hr after inoculation. DEGs were screened using the criteria of a fold change in expression level of ≥2 and $p \leq 0.05$ for up- and downregulated genes.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed to clarify the biological functions of DEGs. The Goseq R package (Young et al., 2010) was used for GO enrichment analysis of DEGs, and KOBAS software (Bu et al., 2021) was used to assess pathways enriched in DEGs. The plant TF database (PlantTFDB, http://planttfdb.gao-lab.org/index.php) was used to identify TFs among DEGs, and the number of TFs and their families were obtained.

Quantitative PCR verification of DEGs

Twenty DEGs were selected and real-time fluorescence quantitative PCR (qPCR) was used to confirm the reliability of the transcriptome data. Total RNA from male *Z. latifolia* leaves of the same batch was extracted with ReverTra Ace qPCR RT Master Mix (Code No. FSQ-201: Toyobo [Shanghai] Biotechnology Co., Ltd., Shanghai, China) and reverse-transcribed into cDNA. Specific primers were designed with

Primer Premier 5.0 (Kakhki et al., 2019). Sequences of primers are shown in Table S1 in Supplementary Materials. A CFX96 Real-time System (BIO-RAD, CA, USA) and TB Green Premix Ex Taq II fluorescent reagent (Takara, Dalian City, China) were used for detection following the manufacturer's instructions, and *ZlActin2* served as an internal reference gene (Wang et al., 2017). The $2^{-\Delta\Delta Cq}$ method (Zhou et al., 2020), where ΔCq = Cq (gene of interest) – Cq (*Actin2*) and ΔΔCq = ΔCq (treatment, inoculated with *U. esculenta*) − ΔCq (CK, inoculated with potato dextrose liquid medium), was used to estimate the relative expression levels of DEGs.

Screening of candidate genes in male Z. latifolia in response to U. esculenta infection

WGCNA was used to cluster differential genes with common expression and high correlations into the same module, then select target modules; KEGG enrichment analysis was then performed on the target module to identify important metabolic pathways, the complete metabolic pathway was drawn and relevant DEGs were screened. DEGs were detected by qPCR using the primers shown in Table 1. Combined with the transcriptome results, candidate genes of male *Z. latifolia* involved in response to infection by *U. esculenta* were determined.

Data processing

Microsoft Excel 2016 software (Microsoft Corp., Redmond, WA, USA) was used for basic processing of test data, IBM SPSS statistics 25 software (Jackson and Ukwe, 2022) was used for analysis of variance ($p \leq 0.05$ indicated statistical significance), and SigmaPlot 14.0 (Khata et al., 2019) and Origin 2019b 32bit (Rusnac et al., 2021) were used for mapping.

RESULTS

Analysis of transcriptome data and screening of DEGs

Analysis of DEGs and the associated cluster heatmap

DEGs in treatment and control groups were statistically analysed using DESeq2 software with screening criteria of fold change ≥2 and $p \leq 0.05$. The results showed that there were 934 DEGs at 3 hr after inoculation, including 530 upregulated and 404 downregulated genes; there were 34 DEGs at 12 hr after inoculation, including 10 upregulated and 24 downregulated genes; and there were 258 DEGs at 24 hr after inoculation, including 127 upregulated and 131 downregulated genes (Figure S1 in Supplementary Materials).

A total of 1,226 DEGs were identified in the three comparison groups (CK-3 hr vs. TR-3 hr, CK-12 hr vs. TR-12 hr and CK-24 hr vs. TR-24 hr), and hierarchical cluster analysis was carried out according to the fragments per kilobase of exon per million mapped fragments (FPKM) values of DEGs. The results showed that the three biological repeats of treatment and control groups were clustered together at each timepoint, indicating tight correlations and reliable results that could be used for subsequent analysis (Figure 1).

GO and KEGG enrichment analysis of DEGs

The top 20 GO nodes with the most significant enrichment *p*-values were selected to annotate the GO functions of DEGs. The results showed that in the CK-3 hr vs. TR-3 hr comparison, the major enriched genes were linked to membrane component, response to stimulation, ATP binding, stress response, protein

Table 1. Real-time fluorescence qPCR primers for candidate genes

Gene name	Forward primer (5′ – 3′)	Reverse primer (5′ – 3′)
Zlat_10042236	AGTGGTGACTCTGGAATTGG	G-CCCATGAGTGTGTTGTGATCT
Zlat_10016445	CATGGAAGGAAGGCAGATAC	CAAAGCCACCCTCACCTATT
Zlat_10028687	GAGTCAAGACAGGGAGTAAAGG	CCTTCCACACAATAGCCATAAAG
Zlat_10030446	GCAGAGAATCACAGTTGAGGAG	GCTTCATCTGGTCCTCGTTTAG
Zlat_10002195	TAT-CCACCTCACGCCCAGT	TA-TCCTTGACGACGCCTCC
Zlat_10008237	TCCTACTCAGACTTCTCGTTCC	CTGCTGCTGCTGACATCTATAC
Zlat_10020312	CACGACAACGAGAACTCC	GATCTCAATCTCCGACCT
Zlat_10028790	GGATTCCAAGAGATGGAGGAAAG	TCGATGTCGCTCATGGTTTG
Zlat_10045823	GC-TGACCACCAAATCTTCGACTAC	GCTCATGGAGTTCTCGTTGT
Zlat_10008367	C-TATGTGCACGGCAGATGTT	C-GCTTGTAATGACGCTCCTATC
Zlat_10029338	AACGTGTTGTGGCGCTTA	TTGCAGCCCGTTCAAACT
Zlat_10007178	GACAAGACGGTGGTATGGTATG	GGGATCTCGAAGAGAAAGAACC
Zlat_10004780	G-ACAAGGCGGGCTCTTATTT	GGTACTAGGAGTTGCTGTGAAG
Zlat_10023424	CAAGTAGGTCAGGGTGGATTTG	CTTGTTTGGTGCCAGGAGT
Zlat_10024926	GCAGAGAATCACAGTTGAGGAG	GCTTCATCTGGTCCTCGTTTAG
Zlat_10004358	TGGGTATCAATGGCGGAAG	CCTTCTTCTTCACCGGACAC
Zlat_10003815	ACGGAAGGCAACGTTTGA	GGTCGAAAGCTGGGTAGTATG
ZlActin2	C-TAACCGGCCACGTGTATTT	AGAGCAGAGGCATTCCAAGT

qPCR, quantitative PCR.

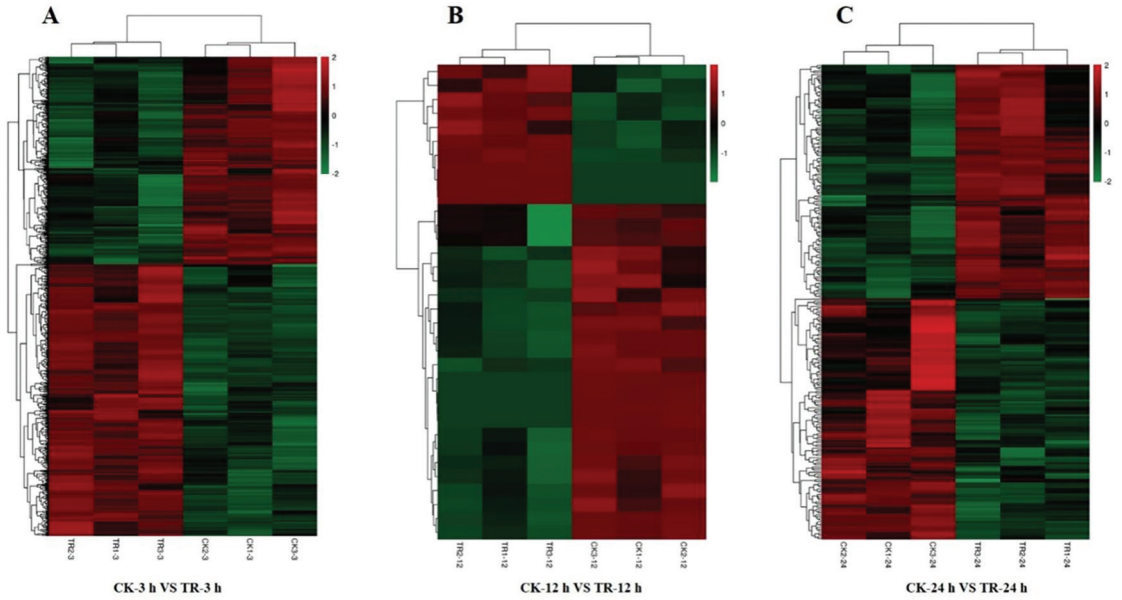

Figure 1. Clustering heat map of DEGs. (A) CK-3 hr vs. TR-3 hr; (B) CK-12 hr vs. TR-12 hr; and (C) CK-24 hr vs. TR-24 hr. Red indicates upregulation whereas green indicates downregulation, and the darker the colour, the greater the up- or downregulation. DEGs, differentially expressed genes.

kinase activity and response to abiotic stress categories, indicating that these genes might participate in the early defence response of male *Z. latifolia* to *U. esculenta* (Figure S2A in Supplementary Materials). In the CK-12 hr vs. TR-12 comparison, the major enriched genes were associated with transmembrane transporter activity, transmembrane transport, heme binding, DNA template and extracellular domain categories, which suggested that *U. esculenta* infection might affect the structure and the function(s) of the cell wall/external surface of male *Z. latifolia* (Figure S2B in Supplementary Materials). In the CK-24 hr vs. TR-24 hr comparison, the most enriched genes were related to the cell periphery, vacuole, cell wall, thermal reaction, cadmium ion, external packaging structure and host cell nucleus categories, which suggested that gene enrichment influenced plant defences and possibly other functions involved in the regulation of *Z. latifolia* response to *U. esculenta* infection (Figure S2C in Supplementary Materials).

Pathway analysis of compared DEGs revealed that the number of pathways enriched in the CK-3 hr vs. TR-3 hr comparison was the most (100), followed by CK-24 hr vs. TR-24 hr (65) and CK-12 hr vs. TR-12 hr (13). At 3 hr after inoculation, the most enriched DEGs were related to the plant–pathogen interaction pathway, indicating that these genes play a key role in the interaction between male *Z. latifolia* and *U. esculenta*. DEGs were also enriched in MAPK signal pathway-plant, diterpene biosynthesis, and flavone and flavanol biosynthesis pathways, all of which are related to plant defence responses (Figure S2D in Supplementary Materials). At 12 hr after inoculation with *U. esculenta* (CK-12 hr vs. TR-12 hr), there were few DEGs, and the most enriched category was sesquiterpenoid and triterpenoid biosynthesis. Additionally, there were a small number of DEGs enriched in glucosinolate biosynthesis, plant–pathogen interaction, flavonoid biosynthesis and cyano amino acid metabolism, and other pathways, which may also participate in the defence response of male *Z. latifolia* (Figure S2E in Supplementary Materials). At 24 hr after inoculation (CK-24 hr vs. TR-24 hr), DEGs were enriched in vitamin B6 metabolism, terpenoid main chain biosynthesis, glycine, serine and threonine metabolism, alanine, aspartic acid and glutamate metabolism, tropine, piperidine and pyridine alkaloid biosynthesis, and valine, leucine and isoleucine biosynthesis, all of which are related to primary plant metabolism, indicating that *Z. latifolia* reorganises primary metabolism following *U. esculenta* infection. Additionally, there was enrichment of some DEGs related to photosynthesis, plant circadian rhythm, carotenoid biosynthesis and other pathways that may also affect the primary metabolic pathways of male *Z. latifolia*. Some DEGs were enriched in fructose and mannose metabolism, galactose metabolism and other pathways, suggesting that *U. esculenta* infection also affects the sugar metabolism of its host plant (Figure S2F in Supplementary Materials).

Analysis of differentially expressed TFs

In order to predict the TFs mediating the responses of male *Z. latifolia* to *U. esculenta* infection, the PlantTFDB was used to analyse DEGs in CK-3 hr vs. TR-3 hr, CK-12 hr vs. TR-12 hr and CK-24 hr vs. TR-24 hr comparisons. A total of 139 TF genes were identified in the three groups, of which the number of TF genes in the CK-3 hr vs. TR-3 hr comparison was the largest (110),

followed by CK-24 hr vs. TR-24 hr (28), and there was only one DEG in the CK-12 hr vs. TR-12 hr comparison (Figure S3 in Supplementary Materials). DEGs at 3 hr after inoculation (CK-3 hr vs. TR-3 hr) were mainly enriched in WRKY (22 members; 19 upregulated, 3 downregulated), NAC (NAM, ATAF1/2, CUC2; 15 members; 11 upregulated, 4 downregulated), basic helix-loop-helix (bHLH; 8 members; 7 upregulated, 1 downregulated), basic leucine zipper (bZIP; 3 members; 1 upregulated, 2 downregulated), MYB (v-myb avian myeloblastosis viral oncogene homologue; 10 members; 6 upregulated, 4 downregulated), MYB-related (6 members; 2 upregulated, 4 downregulated) and other TF families (Figure S3 in Supplementary Materials). At 12 hr after inoculation (CK-12 hr vs. TR-12 hr), there was only one differential TF gene, belonging to the NAC family, which was downregulated. At 24 hr after inoculation (CK-24 hr vs. TR-24 hr), DEGs belonged to bHLH (7 members; 2 upregulated, 5 downregulated), MYB (5 members; 1 upregulated, 4 downregulated), NAC (2 members; downregulated), MYB-related (1 member; downregulated), B3 (1 member; downregulated) and other TF families (Figure S3 in Supplementary Materials).

qPCR verification of DEGs

Twenty DEGs were selected, specific primers were designed and the reliability of transcriptome data was assessed by qPCR. The results showed that expression of 19 differential genes was consistent with the transcriptome data, with *Zlat_10020427* being the only exception (Figures 2A and 2B). This indicates that the transcriptome data were reliable and could be used for subsequent experimental analysis. Meanwhile, further study is needed to ascertain whether the *Zlat_10020427* gene plays an important role in the response of *Z. latifolia* to *U. esculenta* infection.

Mining candidate genes of male Z. latifolia involved in the response to U. esculenta infection

Gene coexpression network analysis

WGCNA was used to construct a gene coexpression network, and seven characteristic modules were identified by correlation analysis between gene expression modules and *U. esculenta* infection. The MEturquoise module was the most interesting module, in which expression patterns for the CK-3 hr vs. TR-3 hr comparison were quite different, while expression patterns for CK-12 hr vs. TR-12 hr and CK-24 hr vs. TR-24 hr comparisons were quite similar (Figure 3A). Therefore, 3 hr after inoculation was identified as an important node for male *Z. latifolia* responses to *U. esculenta* infection in the early stages, and the MEturquoise module could be used as the target module for subsequent analysis (Figure 4A). In order to further explore the functions of target module genes, KEGG enrichment analysis of the MEturquoise module was performed, which showed that genes were mainly enriched in the 'plant–pathogen interaction' (49) and 'MAPK signal pathway-plant' (28) pathways (Figure 3B). These two pathways are directly related to plant defences; hence, we speculated that they may play an important role in the responses of male *Z. latifolia* to *U. esculenta* infection.

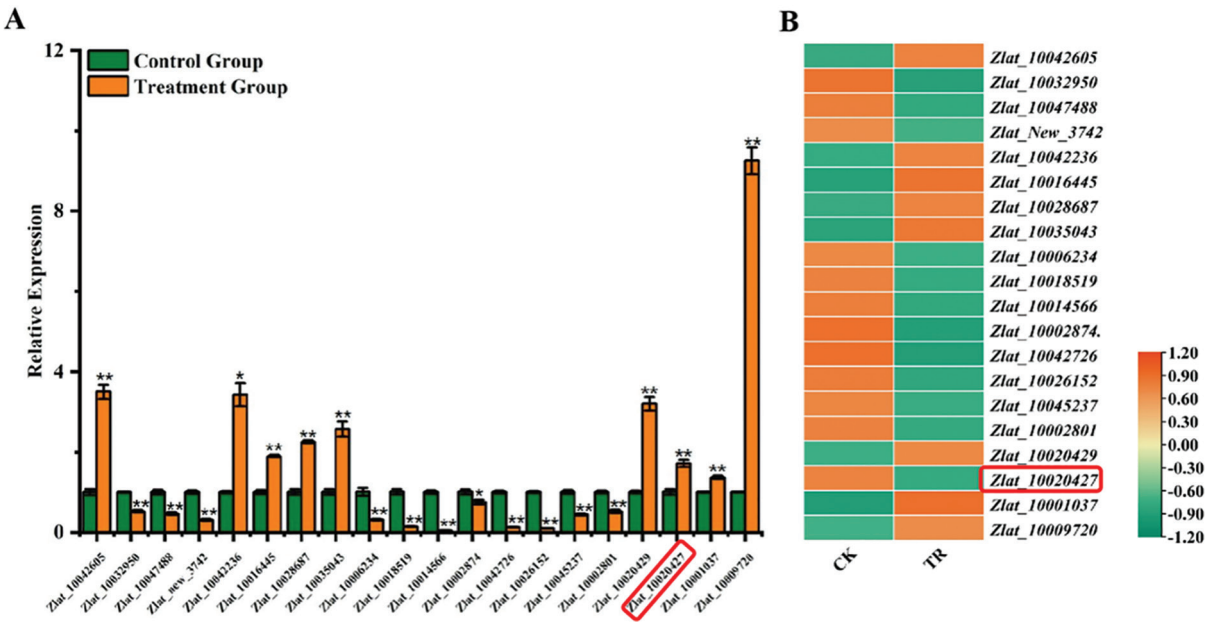

Figure 2. qPCR verification of DEGs. (A) qPCR analysis of DEGs. Orange represents the treatment group and green represents the control group. (B) Differential gene expression heatmap of transcriptome sequencing data. Orange indicates upregulation whereas green indicates downregulation, and the darker the colour, the more obvious the greater the up or downregulation. DEGs, differentially expressed genes; qPCR, quantitative PCR.

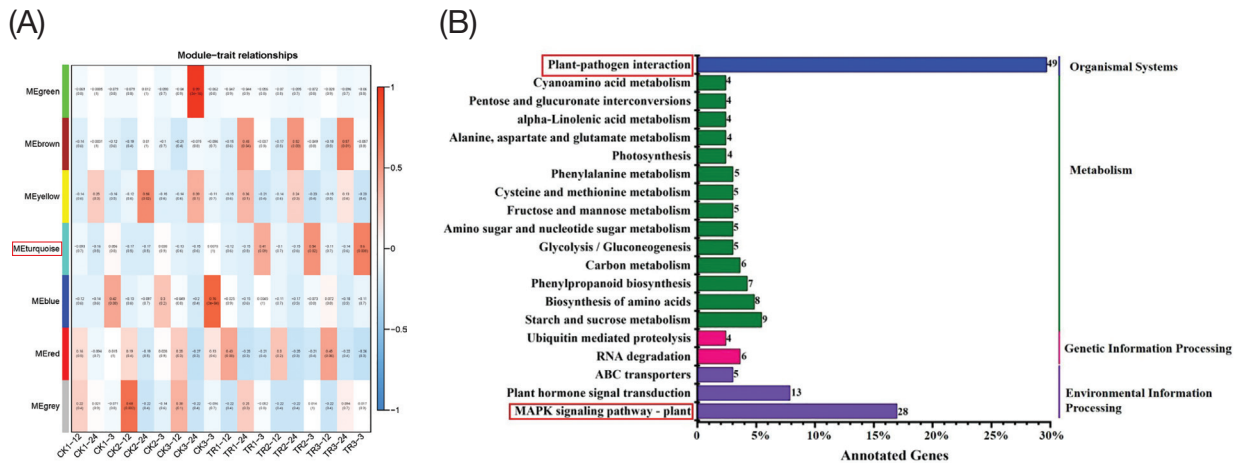

Figure 3. Correlation heatmap of the gene expression network module and different processes (A), and KEGG pathway analysis results of MEturquoise modules (B). (A) The abscissa represents samples, and the ordinate represents modules. The number of each block represents the correlation between modules and samples; the closer the value is to 1, the stronger the positive correlation between modules and samples; the closer the value is to –1, the stronger the negative correlation. The number in brackets represents the significance *p*-value; the lower the number, the stronger the significance. The darker the colour of the square (the redder), the stronger the correlation; the lighter the colour, the weaker the correlation. KEGG, Kyoto Encyclopedia of Genes and Genomes.

Analysis of DEGs related to 'plant–pathogen interaction' and 'MAPK signalling pathway-plant' pathways

DEGs in the 'plant–pathogen interaction' and 'MAPK signalling pathway-plant' pathways were analysed at 3 hr after inoculation. A total of 17 DEGs were identified, consisting of 2 LRR receptor kinase *FLS2* (flagellin sensing 2) genes, 5 RLK receptor kinase *BAK1* genes, 6 WRKY TFs (WRKY33), 2 *MPK3* genes, 1 receptor kinase *FRK1* gene and a single 1-aminocyclopropane-1-carboxylic acid synthase *ACS6* gene, all of which were upregulated (Figure 4). Regarding 'MAPK signalling pathway-plant' pathways, six *WRKY33* genes were differentially expressed (Figure 4). We speculated that *WRKY33* might be key genes of male *Z. latifolia* involved in response to *U. esculenta* infection. Regarding the five receptor kinase *BAK1* genes, *BAK1* and *FLS2* are known to form a dimer that activates the immune response, promotes ROS production and induces allergic reactions in plants, causing callose deposition and thus strengthening the cell wall. Additionally, the *BAK1-FLS2* dimer stimulates *WRKY33* and *MPK3/6* gene expression and upregulates downstream related resistance genes. Additionally, *MPK3/6* inhibits the ubiquitination-mediated degradation of *ACS6* through phosphorylation modification, and promotes ethylene (ET) synthesis, thereby supporting the response of male *Z. latifolia* to *U. esculenta* infection (Figure 4).

Real-time expression

In order to further explore the expression of genes related to 'plant–pathogen interaction' and 'MAPK signalling pathway-plant' pathways, total RNA from male *Z. latifolia* leaves at 3 hr after inoculation was extracted and reverse-transcribed into cDNA. Seventeen DEGs in the above pathways were selected, including two *FLS2* genes (*Zlat_10042236* and *Zlat_10029338*), five *BAK1* genes (*Zlat_10028687*, *Zlat_10016445*, *Zlat_10007178*, *Zlat_10004780* and *Zlat_10023424*), two *MPK3* genes (*Zlat_10030446* and *Zlat_10024926*), six *WRKY33* genes (*Zlat_10008237*, *Zlat_10002195*, *Zlat_10020312*, *Zlat_10028790*, *Zlat_10045823* and *Zlat_10004358*), one *FRK1* gene (*Zlat_10008367*) and one *ACS6* gene (*Zlat_10003815*). Specific primers were designed to measure expression of these genes by qPCR. The results showed that except for one gene (*Zlat_10003815*), expression of the other 16 genes was upregulated (Figure 5A), consistent with the results of transcriptome analysis (Figure 5B). Relevant information for these 16 candidate genes is listed in Table S2 in Supplementary Materials. These genes might be key players in the male *Z. latifolia* responses to *U. esculenta* infection, and their functions should be investigated in the future.

DISCUSSION

Analysis of DEGs

Pathogens invading plants are attacked by the plant immune system. Firstly, signal networks such as the MAPK cascade can help plants identify foreign invaders and quickly trigger immune responses to prevent infection (Cheng et al., 2012). Secondly, plant hormones also play an important role in helping plants respond to adverse environments (Verma et al., 2016). For example, gibberellin (GA), abscisic acid (ABA), indole acetic acid (IAA) and cytokinin hormones are related to plant defence signal pathways, and play an important role in regulating plant defence response to pathogen infection (Bari and Jones, 2009). In this

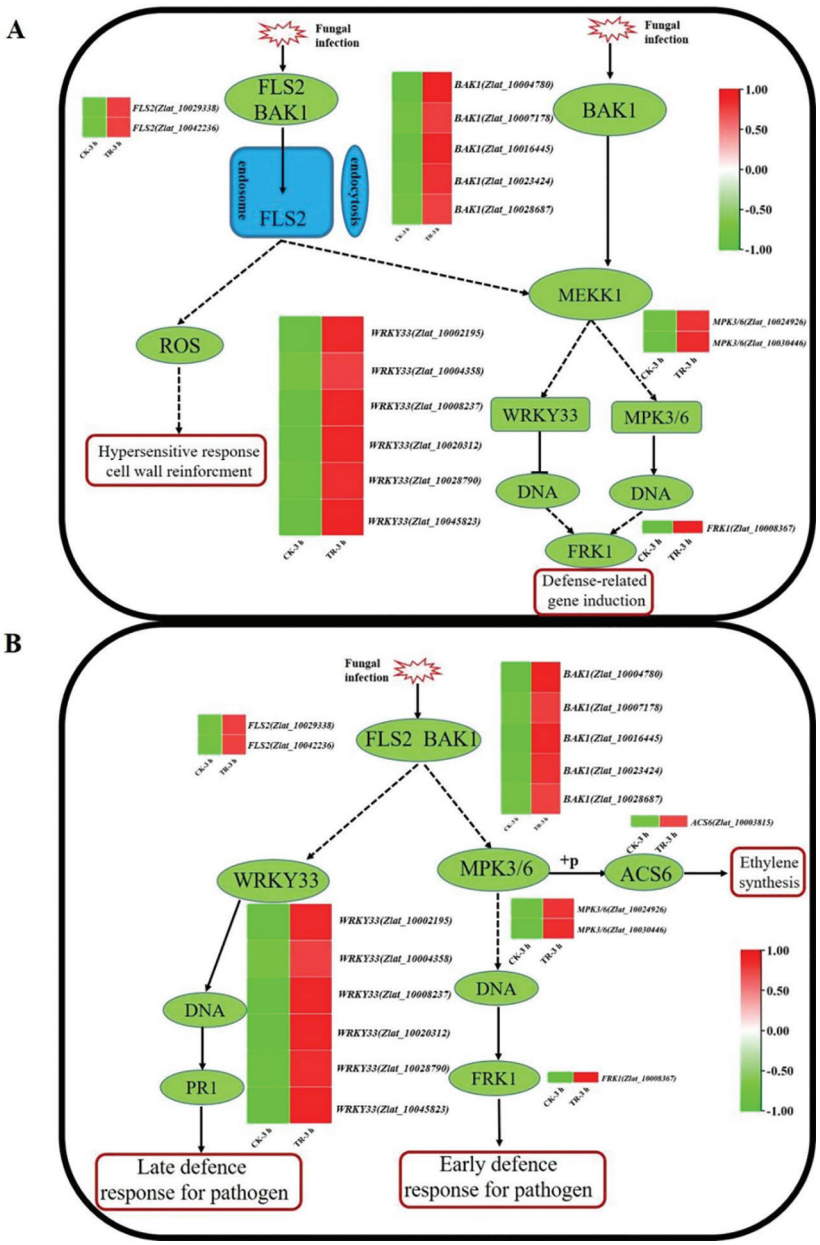

Figure 4. Analysis of important pathways in CK-3 hr vs. TR-3 hr. (A) Analysis of 'plant–pathogen interaction' pathways. (B) Analysis of 'MAPK signalling pathway-plant' pathways. Red indicates the treatment group (TR-3 hr) whereas green the control group (CK-3 hr).

study, DEGs were mainly associated with the metabolic pathways of auxins, cytokinins, gibberellins, abscisic acid, brassinosteroids and jasmonic acid. Among them, brassinosteroid pathways accounted for the most (12) DEGs (9 upregulated, 3 downregulated), followed by auxin (2 upregulated, 5 downregulated) and jasmonic acid pathway (6 upregulated, 1 downregulated), but further investigation is needed to ascertain whether these genes participate in the response process. Additionally, phenylpropane derivatives participate in the biosynthesis of lignin or flavonoids, and can also enhance the resistance of plants to pathogens (Dong and Lin, 2021). In the present study, 1,226 DEGs were identified at different timepoints after inoculation of *Z. latifolia* with *U. esculenta*. KEGG enrichment analysis revealed that most DEGs appeared at 3 hr after inoculation, most were largely enriched in 'plant–pathogen interaction' and 'MAPK signalling pathway-plant' pathways, and most were significantly upregulated. This indicates that male *Z. latifolia* plants might have already begun to respond to *U. esculenta* infection at 3 hr after inoculation, by activating the expression of related stress resistance genes.

Changes in differentially expressed TFs

TFs are important participants in plant responses to biological stress that can enhance the ability of plants to resist insect attack and pathogen infection (Amorim et al., 2017). As regulatory proteins, TFs play an important role in transcription reprogramming (Bordenave et al.,

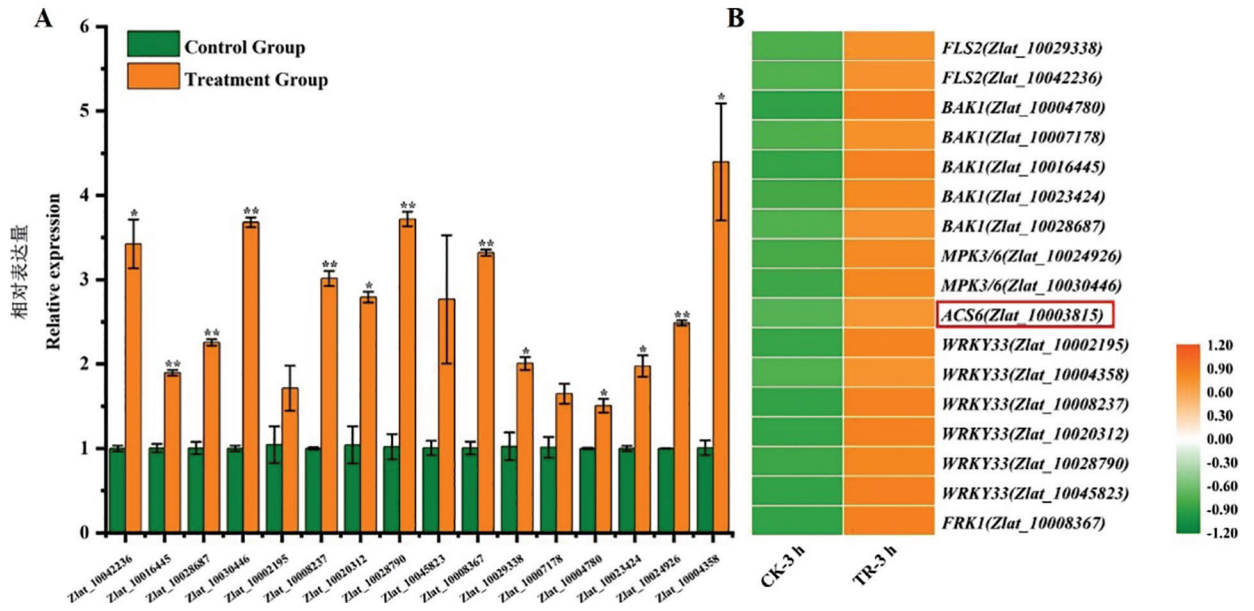

Figure 5. Analysis of differential gene expression in 'plant–pathogen interaction' and 'MAPK signalling pathway-plant' pathways. (A) qPCR verification of DEGs. Orange indicates the treatment group whereas green indicates the control group. (B) Differential gene expression heatmap of transcriptome sequencing data. Orange indicates upregulation whereas green indicates downregulation, and the darker the colour, the greater the up or downregulation. qPCR, quantitative PCR.

2013). Members of WRKY, NAC, MYB and other TF families are involved in the regulation of plant response to biological stress (Feller et al., 2011; Amorim et al., 2017; Li et al., 2017). Cui et al. (2019) found that the *GmWRKY40* gene could positively regulate the response of soybean to *Phytophthora infestans* by regulating hydrogen peroxide accumulation and JA signalling. The *AtMYB30* gene is a positive regulator of *Arabidopsis* defence and related cell death responses (Raffaele and Rivas, 2013). Overexpression of the NAC family *ATAF1* gene can enhance the tolerance of plants to *Botrytis cinerea* and *Pseudomonas syringae* (Christianson et al., 2010). In the present study, 139 TF genes were identified, mainly enriched in 31 families including WRKY, MYB, NAC, bHLH and other TF families. Among them, TF genes enriched in the CK-3 hr vs. TR-3 hr comparison mainly belonged to the WRKY family. Studies have shown that the WRKY family is involved in the early regulation of plant resistance to pathogen infection, and members play a crucial role in plant responses to biological stress (Zheng et al., 2006; Chi et al., 2013; Fu et al., 2022).

Validation of transcriptome data

With the rapid development of sequencing technology, the cost of genome detection has been greatly reduced, and transcriptome sequencing has become a popular choice to study differential gene expression. However, RNA-seq still faces many challenges in terms of data processing and analysis. Studies have shown that only 85% of results are typically consistent with qPCR results. Through analysis of all inconsistent genes, it was found that they were usually small in length, with fewer exons, and relatively scarce in the transcriptome (Everaert et al., 2017). In the present study, when the transcriptome was verified by qPCR, 19 of 20 DEGs were consistent with the transcriptome sequencing results. Subsequently, screening criteria can be optimised, and highly expressed DEGs can be selected for further analysis.

Screening candidate genes of male Z. latifolia involved in the response to U. esculenta infection

During evolution, plants have evolved various defence mechanisms to resist infection by pathogens. These include the nonspecific pattern-triggered immunity (PTI) mechanism induced by pathogen-related molecular patterns (PAMPs) mediated by pattern recognition receptors (PRRs), and effector-triggered immunity (ETI), a specific defence mechanism induced by effector proteins secreted by pathogens (Liu, 2018; Zhang et al., 2018). Studies have shown that the MAPK pathway transmits extracellular signals to downstream response factors through receptors on the cell membrane via MAPKKK, MAPKK and MAPK, and plays an important role in PTI and ETI defence responses (Wang et al., 2019). In plant nonspecific defences, FLS2 combines with the receptor kinase BAK1 to activate immune responses (Chinchilla et al., 2007). In the present study, at 3 hr after inoculation with *U. esculenta*, both *FLS2* and *BAK1* genes were significantly upregulated in male *Z. latifolia*, implying involvement in the infection process of male *Z. latifolia* in response to *U. esculenta*. Studies have shown that *Arabidopsis*

FLG22 can upregulate MAPK3/4/6/11, thereby improving resistance to pathogens (Roux et al., 2011), consistent with the results of the present study. MPK3/MPK6 can enhance the stability of proteins and promote the production of ET by inhibiting the ubiquitination and degradation of ACS6, thereby enhancing the resistance of plants to pathogens (Joo et al., 2008; Han et al., 2010). MAPKs can also regulate transcription reprogramming by phosphorylating some WRKY TFs. When *Arabidopsis* is infected by pathogenic bacteria, WRKY33 forms a complex with MPK and MKS1, and phosphorylated WRKY33 promotes the synthesis of antitoxins, thereby improving the immune response of *Arabidopsis* (Qiu et al., 2008). In this study, 16 candidate genes were screened in 'plant–pathogen interaction' and 'MAPK signalling pathway-plant' pathways, and these candidate genes might participate in the responses of male *Z. latifolia* to *U. esculenta* infection. The functions of these candidate genes need to be studied in the future.

ACKNOWLEDGEMENTS

We would like to thank the native English speaking scientists of Elixigen Company (Huntington Beach, California) for editing our manuscript.

FUNDING

This research work was supported by grants from the Natural Science Foundation of Anhui Province (2008085MC80) and the Anhui Province Crop Variety Breeding Project. The funders had no role in the study design, data collection or analysis, decision to publish, or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

P.G. and D.G. conceived and designed the experiments. P.G., H.Z. and L.B. performed the experiments. P.G., H.Z. and L.B. analyzed the data. P.G., H.Z. and D.G. wrote the paper. Y.L., Y.Z., B.W. and X.H. participated in the design of the study. H.Z., X.H. and D.G. revised the manuscript. X.H. and D.G. – these authors contributed equally to this study.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest to this work.

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Received: November 1, 2022; accepted: March 7, 2023

SUPPLEMENTARY MATERIALS

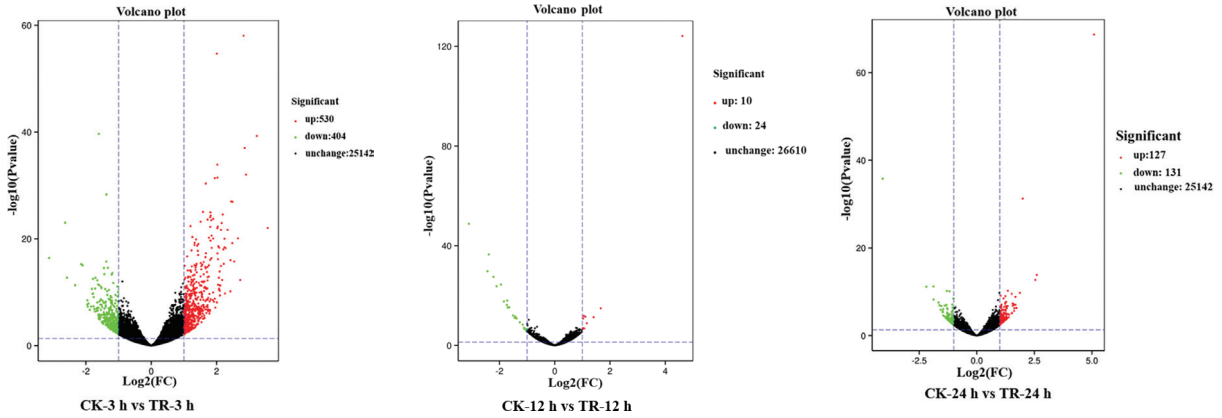

Figure S1. Volcano map of DEGs at 3 hr, 12 hr and 24 hr after inoculation with *U. esculenta*. DEGs, differentially expressed genes.

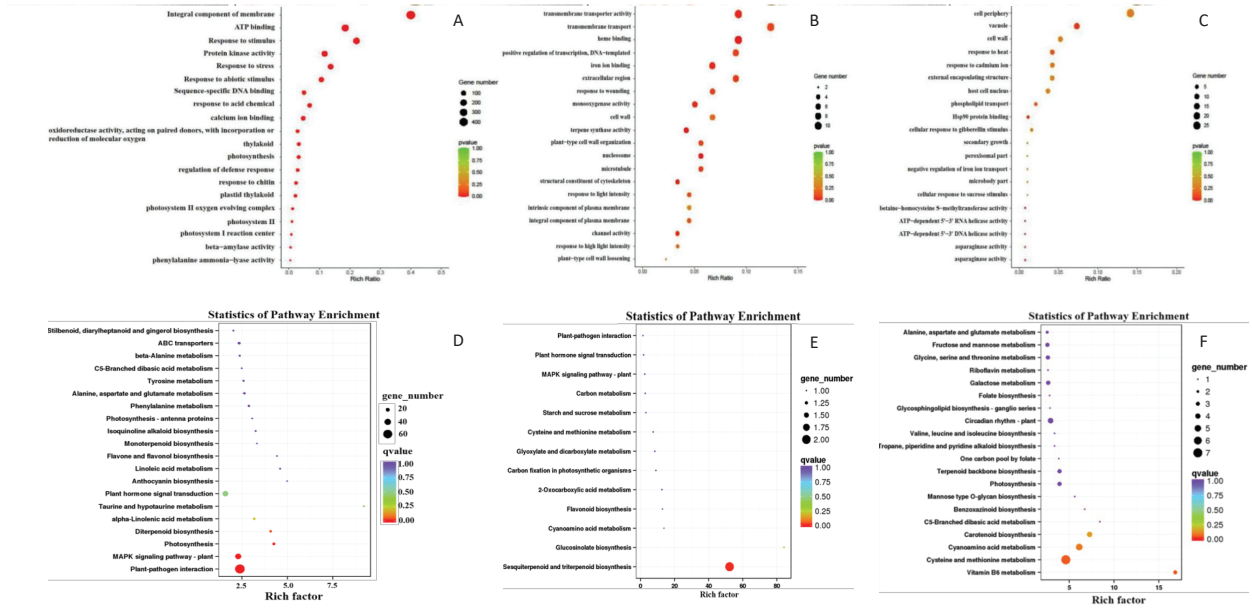

Figure S2. GO enrichment and KEGG pathway analyses of DEGs after inoculation. (A) GO enrichment at 3 hr after inoculation. (B) GO enrichment 12 hr after inoculation. (C) GO enrichment 24 hr after inoculation. (D) KEGG pathways at 3 hr after inoculation. (E) KEGG pathways at 12 hr after inoculation. (F) KEGG pathways at 24 hr after inoculation. DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

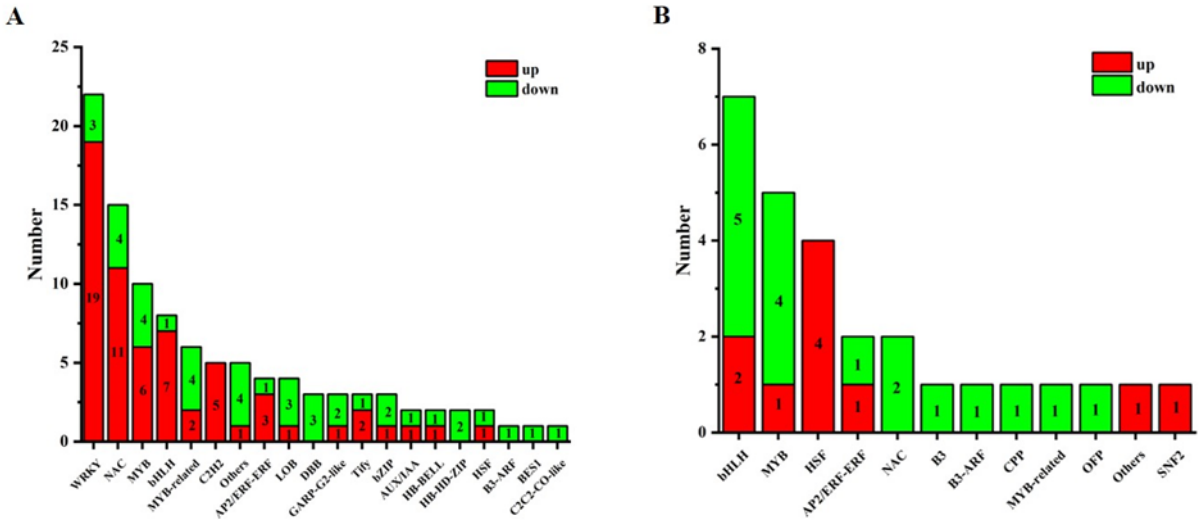

Figure S3. Statistics of differentially expressed transcription factor after inoculation. Red indicates upregulation whereas blue indicates downregulation, and the numbers indicates the number of transcription factors. (A) 3 hr after inoculation. (B) 24 hr after inoculation.

Table S1. Real-time fluorescence qPCR primers for transcriptome data validation.

Gene name	Forward primer (5′ – 3′)	Reverse primer (5′ – 3′)
Zlat_10042605	GCTTGTTCCTCCTCGTCATC	TT-GACGGCGAAGGGAGGTT
Zlat_10032950	CCAACACCAACCTCAACTACG	A-CGGAGATCCTGATGCCTAC
Zlat_10047488	CGTGGTATCGGAGACAGGGT	CG-GAAGAGAGCGAAGAGGTACACG
Zlat _New_3742	ACCAACACGACGACCAGACT	AAGAAGAGCACCGCCAATG
Zlat_10042236	AGTGGTGACTCTGGAATTGG	G-CCCATGAGTGTGTTGTGATCT
Zlat_10016445	CATGGAAGGAAGGCAGATAC	CAAAGCCACCCTCACCTATT
Zlat_10028687	GAGTCAAGACAGGGAGTAAAGG	CCTTCCACACAATAGCCATAAAG
Zlat_10035043	CGTGGATTGGGCAACCTT	CTTCTTGTTCTCCTCGCTCG
Zlat_10006234	GTGACGGCGACCAACTTCT	T-GAGTGCCCGTTGATGGTG
Zlat_10018519	GTGGAAGGGTATGGCAGTG	C-AGATTCGGGTTTGGTAGGC
Zlat_10014566	ATCACCGCCACCAACCT	T-CCTCCCTTCTTCACGCAC
Zlat_10002874	GGAGTATCTCCACCTACCTGTCT	CCGTCGTCGTATTCTTCGTCT
Zlat_10042726	TT-GGCTTCTTCCCTCTCCTCC	CGGTGAAGTCAGAGGCGTT
Zlat_10026152	GTGGGTGGACGAGAAGAAGT	CAGCCGATGAGGATGGAGT
Zlat_10045237	A-TGTGGCAGTCGTGTCCGT	A-CGTTGAACAGGGGCTCGT
Zlat_10002801	ACGACAGAGGAACTGAAGGACT	TCGTGTTGAGGATTTGGAGG
Zlat_10020429	ACGGGCTCACCTACACCAAC	T-ACAGCCGACGTGGTCGAT
Zlat_10020427	T-CCTACCCTGGTGTCTCCTTC	T-CCGTGGACACCTTGATGC
Zlat_10001037	T-ATTCACCTCCCACACTCAGC	CCATCACCACCTATCTTCAAGC
Zlat_10009720	CATCGCCTTCTCCCTCAT	GGTTCCAGGGTTGATTGC
ZlActin2	C-TAACCGGCCACGTGTATTT	AGAGCAGAGGCATTCCAAGT

qPCR, quantitative PCR.

Table S2. Information on candidate genes related to 'plant–pathogen interaction' and 'MAPK signalling pathway-plant' pathways in male *Z. latifolia* in response to *U. esculenta* infection.

Gene ID	Log2(FC)	Genetic traits	Homologous gene
Zlat_10029338	1.16	LRR receptor-like serine/threonine-protein kinase FLS2	*AT5G46330*
Zlat_10042236	1.30	LRR receptor-like serine/threonine-protein kinase FLS2	*AT5G46330*
Zlat_10004780	1.32	BAK1	*AT4G33430*
Zlat_10007178	1.10	BAK1	*AT4G33430*
Zlat_10016445	1.06	BAK1	*AT4G33430*
Zlat_10023424	1.48	BAK1	*AT4G33430*
Zlat_10028687	1.20	BAK1	*AT4G33430*
Zlat_10024926	1.02	Mitogen-activated protein kinase 3	*AT3G45640*
Zlat_10030446	1.49	Mitogen-activated protein kinase 3	*AT3G45640*
Zlat_10002195	1.24	WRKY33	*AT2G38470*
Zlat_10004358	2.72	WRKY33	*AT2G38470*
Zlat_10008237	2.02	WRKY33	*AT2G38470*
Zlat_10020312	1.60	WRKY33	*AT2G38470*
Zlat_10028790	1.85	WRKY33	*AT2G38470*
Zlat_10008367	1.52	WRKY33	*AT2G38470*
Zlat_10045823	1.59	Senescence-induced receptor-like serine/threonine-protein kinase (FRK1)	*AT2G19190*

BAK1, brassinosteroid insensitive 1-associated receptor kinase 1.