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The Analysis of Short-Term Differential Expression of Transcription Factor Family Genes in Diploid and Tetraploid Rice (*Oryza sativa* L.) Varieties during Blast Fungus Infection

Minghong Xu [1,†], Dayong Li [2,†], Zitian Leng [1], Keyan Liu [1], Chenxi Wang [1], Yingkai Wang [1], Weilong Meng [1], Lintian Yu [1], Chunying Zhang [1,3], Jian Ma [1,3,*] and Ningning Wang [1,*]

[1] Faculty of Agronomy, Jilin Agricultural University, Changchun 130000, China; 13756579606@163.com (M.X.); 18943776363@163.com (Z.L.); lky1556629498820 23@163.com (K.L.); 15947157832@163.com (C.W.); wangyingkai99@163.com (Y.W.); mengweilong0212@163.com (W.M.); ylt258036@mails.jlau.edu.cn (L.Y.); zhangchunying@jlau.edu.cn (C.Z.)
[2] College of Plant Protection, Jilin Agricultural University, Changchun 130000, China; lidayong@jlau.edu.cn
[3] Jilin Provincial Laboratory of Crop Germplasm Resources, Changchun 130000, China
* Correspondence: majian197916@jlau.edu.cn (J.M.); ningningw@jlau.edu.cn (N.W.)
† These authors contributed equally to this work.

Abstract: The necessity to understand plant adaptations to environmental stressors is underscored by the role of polyploidy in species evolution. This study focuses on the superior stress resistance exhibited by autotetraploid rice, which arises from chromosome doubling, in comparison to its diploid donor. We provide a quantitative analysis that highlights the differing susceptibilities of diploid (GFD-2X) and autotetraploid (GFD-4X) rice to rice blast disease, with GFD-2X being significantly more susceptible. Our investigation centers on transcription factors (TFs), which are crucial in regulating biological stress responses, by analyzing their expression in the face of a pathogen attack. This study uncovers variations in the number and expression timing of differentially expressed TF genes, providing a quantitative view of GFD-4X's resistance. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses confirm the role of specific pathways, including "response to stimulus" and the "MAPK signaling pathway," in resistance mechanisms. An extensive analysis of protein–protein interaction networks further clarifies the complex role of TFs during stress responses. The rationale for our experimental approach is rooted in the imperative to decipher the molecular basis of disease resistance across different ploidies, which has implications for crop enhancement. The conclusion from our research is that autotetraploid rice has a unique and more effective defense response regulation system, facilitated by transcription factors, when faced with rice blast disease. This finding provides a foundation for future genetic strategies aimed at improving crop resistance.

Keywords: diploid rice; autotetraploid rice; rice blast stress; transcription factors

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1. Introduction

Whole genome duplication (WGD) or polyploidy is a common occurrence in the evolution of plant species, playing a pivotal role in speciation and adaptation to various environmental conditions [1,2]. The induction of plant polyploidy generally occurs through two methods: one is through the natural process, while the other is artificially induced. Based on the origins of their chromosomes, the resultant polyploids can be segregated into homologous polyploids and allopolyploids [3]. It should be noted that homologous polyploids lack interspecific hybridization, whereas the hybridization of different species characterizes allopolyploids.

Chromosome doubling can induce alterations in plant chromosome numbers, repetitive DNA sequences, genome size, and gene expression. These changes can enhance the genetic diversity of species and potentially lead to the emergence of novel traits [1]. Polyploid

plants typically exhibit increased size, elevated nutrient content, and a high concentration of secondary metabolites [4]. Additionally, they possess certain advantages in resisting biotic and abiotic stresses [5]. In studies investigating wheat resistance to Fusarium crown rot (FCR), it was discovered that the phenylalanine ammonia lyase (PAL) gene, involved in salicylic acid (SA) and lignin biosynthesis in hexaploid wheat, was induced to express under FCR infection. This may explain why hexaploidy wheat exhibits more excellent resistance to FCR than its diploid and tetraploid progenitors [6]. When different ploidy levels of Arabidopsis were inoculated with microorganisms, the expression level of defense-related genes in diploid Arabidopsis increased. At the same time, polyploid Arabidopsis resisted pathogen infections by continuously activating their defensive responses [7]. In a previous investigation, autotetraploid rice demonstrated superior tolerance to short-term salt stress, and the expression levels of phytohormones and genes related to oxidative enzyme activities were significantly higher in tetraploid rice than in diploid rice [8]. When diploid and autotetraploid citrus were exposed to drought stress, it was observed that autotetraploid citrus exhibited enhanced drought tolerance compared to its diploid donor. It was further noted that tetraploid citrus improved its drought stress tolerance by co-regulating photosynthesis, phosphorylation, and phytohormone accumulation [9].

Autotetraploid rice, a novel germplasm, is derived through chromosome doubling in diploid rice [10]. This type of rice exhibits numerous superior characteristics, including broader and lengthier leaves, denser grains [11], elevated content of lipids, amino acid derivatives, and phenolic acids [12], heightened hybrid vigor [13], and substantial advantages in adaptation and stress tolerance [10]. Nevertheless, its low fruiting rate and yield have significantly hindered the extensive application of polyploid rice [14]. Rice blast, caused by the fungus *Magnaporthe oryzae* (*M. oryzae*), is the most debilitating fungal disease affecting cultivated rice [15]. This disease has a broad impact, and the pathogen can infect various parts of rice, including seedlings, leaves, spikes, nodes, and grains, resulting in eye-shaped necrotic lesions [16–18]. Studies reveal that rice blast disease leads to a 10–30% annual reduction in the rice year [19,20], severely impacting the growth, development, and productivity of rice. Consequently, additional comprehensive investigations are imperative to elucidate the mechanisms underlying disease resistance in autotetraploid organisms relative to their progenitor diploid counterparts. This necessitates a more detailed examination of the potential disparities in the response mechanisms to fungal incursion and the ensuing initiation of defense responses among rice plants of different ploidies following pathogen inoculation.

Plants, in response to the continuous stimulation of the external environment, rely on the development of intricate sensing and signal transduction mechanisms to combat various stresses [21]. Transcription factors (TFs), a unique class of protein molecules [22], serve as the primary regulators controlling a wide array of biological processes. These processes include plant growth and development, response to stimuli, and the activation of defense responses [23]. Transcription factors can specifically bind to short DNA sequences in the gene promoter and enhancer regions, thereby regulating gene expression [24,25]. In the face of abiotic and biotic stresses, transcription factors can transform external signals into intracellular signals. This transformation triggers gene expression cascades and specific hormone signaling pathways, activating defense-related targets [21].

In the previous reports, it has been demonstrated that the ERF [26], NAC [27], WRKY [28], and bZIP [29] families participated in the regulation of a myriad of biological processes. The crucial roles of specific transcription factors in controlling rice disease resistance have been extensively studied, which holds significant implications for crop improvement. For instance, *OsERF83* was expressed in rice leaves in response to biotic stress from rice blast, and transgenic rice lines overexpressing *OsERF83* exhibit enhanced disease resistance [30]. Conversely, *OsERF922* acted as a negative regulator role in plant disease resistance, with its overexpression in rice lines leading to increased susceptibility to *M. oryzae* infection compared to knockout lines [31]. Transgenic rice lines overexpressing *OsNAC6* not only bolstered their tolerance to salt stress and post-flooding dehydration stress but also improved their resistance to rice blast stress [32]. Similarly, rice with tran-

scriptional up-regulation and over-expression of *OsNAC111* under blast stress showed increased resistance to rice blast [33]. *OsWRKY13* played a pivotal role in mediating rice resistance by regulating the expression of defense-related genes in salicylic acid and jasmonic acid (JA) dependent signaling pathways, making it an essential gene for resistance to biotic stresses [34,35]. However, overexpression of *OsWRKY62.1* and *OsWRKY76.1* genes renders rice more susceptible to disease, while RNAi and gene knockout rice plants exhibited higher resistance [36]. The bZIP family transcription factor *OsTGAP1* was implicated in the regulation of rice terpenoid phytoalexin biosynthesis through its binding to the promoters of the genes coding for two essential terpene synthases, *OsKSL4* and *OsCPS4* [37].

Compared with previous studies, we found that the adaptation mechanisms of diploid and autotetraploid rice to biotic and abiotic stresses under the conditions of rice blast fungus infection were significantly different. The expression levels of TFs in rice of different ploidy under rice blast fungus infection and the mechanisms by which TFs regulate disease resistance in polyploid rice still need to be further investigated. Therefore, the present study comprehensively analyzed the TF genes expressed in diploid and its autotetraploid rice at different time points without stress treatment and rice blast infestation, aiming to (1) characterize the temporal expression patterns of TF genes in both diploid and autotetraploid rice under blast infection; (2) compare the differential expression of TF genes between diploid and autotetraploid rice; (3) elucidate the potential roles of transcription factors during the progression of rice blast disease in infected plants. This will lay the foundation for the next in-depth study of gene function.

2. Materials and Methods

2.1. The Materials of Plants

The rice materials utilized in this study consisted of diploid rice (GFD-2X) and tetraploid rice (GFD-4X). GFD-4X was an autotetraploid rice variant derived from a natural mutation of field-screened GFD-2X, which underwent six generations of self-pollination. To initiate germination, fully developed GFD-2X and GFD-4X rice seeds were placed on wet germinating paper for 2 to 3 days. Subsequently, seeds exhibiting satisfactory germination and similar growth were selected for sowing. The rice seedlings were cultivated under controlled conditions of 28 °C temperature, a photoperiod of 16 h of light, and 8 h of dark condition.

2.2. Fungal Culture and Treatment

The specific rice blast strain used in this study was obtained from the College of Plant Protection, Jilin Agricultural University. The *Magnaporthe oryzae* strain "2018–0211", isolated from the Jilin Province of China in 2018, was confirmed as a strain with high virulence, suitable for use in screening assays for blast resistance in rice varieties. Firstly, the prepared Tomato Oatmeal Agar (TOA) medium was sterilized and spread onto the plate, the test strain was transferred to the medium, and then placed into a constant temperature incubator at 28 °C for 5–7 d. When the colony area reached 1/2 of the plate area, 2 mL of sterile water was added to the plate, and the bacterial filaments were scraped off by a sterilized cotton swab and mixed well, and then, 400 mL of the aqueous solution with the bacterial filaments was added to the new TOA medium, blow-dried, and sealed (the above operation should be carried out on a clean bench). The plates were then incubated in an incubator at 28 °C for 2–3 d until a layer of white visible hairy mycelium just formed on the surface of the medium. After that, all the aerial mycelium on the surface was scraped off with a toothpick, and then, the plates were sealed with two layers of gauze and irradiated with a black light for 72 h in the incubator at 28 °C to promote the spore production of *M. oryzae*.

Inoculation of the rice blast fungus was performed when the rice reached the three-leaf stage. Before inoculation, a spore suspension of blast fungus should be prepared. The specific steps are as follows: add 0.1% Tween-20 Solution to the plate, gently scrape the plate and wash the spores on the surface, then filter with double gauze, and then use a microscope (OM) to detect the concentration of the spore suspension and adjust the concentration to

1×10^5 spores/mL. The diploid and autotetraploid rice were inoculated with fungi by spray inoculation. The spore suspension prepared was sprayed onto rice uniformly in a hand-sprayer. Subsequently, the inoculated rice seedlings were placed in a dark environment with a relative humidity exceeding 85% and a temperature of 25 °C for 24 h. Following this dark incubation, the seedlings were transferred to a light culture condition [38]. The aboveground parts of GFD-2X and GFD-4X seedlings, subjected to *M. oryzae* treatment for 36 h and 72 h, as well as aboveground parts of GFD-2X and GFD-4X seedlings not treated with *M. oryzae* (0 h), were collected. These samples were promptly frozen in liquid nitrogen and stored at −80 °C for subsequent transcriptome sequencing analysis.

2.3. RNA Isolation and RNA-seq

Total RNA was extracted using TRIzol reagent (Life Technologies Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The RNA was then treated with RNase-free DNase I (Life Technologies Invitrogen, Carlsbad, CA, USA) to eliminate possible genomic DNA contamination before being reverse-transcribed with the Super-Script RNase H- Reverse Transcriptase (Life Technologies Invitrogen, Carlsbad, CA, USA). cDNA library preparation and subsequent sequencing were conducted using the Illumina RNA-sequencing (RNA-seq) platform (Illumina, San Diego, CA, USA).

After library construction, preliminary quantification was performed using a Qubit 3.0 fluorescence quantifier. The insert fragments of the library were analyzed using the Qsep400 high-throughput analysis system to ensure the quality of the library. The quality-qualified libraries were sequenced using the Illumina NovaSeq6000 sequencing platform. Raw data generated from sequencing were filtered to obtain clean data by removing reads containing adapters and low-quality reads [8,39]. The clean reads were aligned to the reference genome (Oryza_sativa.MSU_v7.0.genome.fa) using HISAT2 software (version 2.2.1). Gene expression levels were calculated by the FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) method.

2.4. The Analysis of DEGs

As this experiment included three biological replicates, analysis of variance was conducted using the DESeq2 software package (version 1.30.1). DEGs were identified based on a significant p-value < 0.05 and $|\log2 \text{ fold change}| > 1$ [40]. Heatmaps illustrating gene expression levels and Venn diagrams displaying DEGs were generated using TB tools software (version 1.120). Volcano plots were generated using the bioinformatics analysis tool (https://www.bioinformatics.com.cn/ (accessed on 10 August 2023)). Upset and Di Venn analyses were performed using https://www.bioladder.cn/web/#/chart/16 (accessed on 22 August 2023) and https://divenn.tch.harvard.edu/ (accessed on 22 August 2023), respectively. Gene Ontology analysis was employed to examine the enrichment of DETF genes. Protein–protein interaction networks (PPIs) were constructed using the String database (https://cn.string-db.org/ (accessed on 30 August 2023)), where nodes represented known and predicted proteins and edges represented interaction relationships between proteins.

2.5. qRT-PCR Validation

To validate the RNA-seq data, real-time quantitative PCR (*qRT-PCR*) was performed on six randomly selected genes using the SYBR Green I PCR master mix kit (TaKaRa, Tokyo, Japan), and the results of the *qRT-PCR* analyses are as shown in Supplementary Figure S1. The experiment contained three biological replicates. The primers used are visible in Supplementary Table S1.

2.6. Statistical Analysis

Statistical analyses were performed using t-tests; the results were expressed as mean ± standard deviation of three biological replicates, and the statistical significance threshold was p-value < 0.05.

3. Results

3.1. Overview of TFs in GFD-2X and GFD-4X Transcriptomes across Various Time Points of Rice Blast Stress

We performed the comparative analysis of the transcriptome data for GFD-2X and GFD-4X under blast stress at different time points. Among them, the GFD-2X and GFD-4X rice samples not treated with *M. oryzae* at the start (0 h) were labeled as 2X_0h and 4X_0h, respectively. The GFD-2X and GFD-4X rice samples treated with *M. oryzae* for 36 h were labeled as 2X_36h and 4X_36h, respectively. Similarly, the GFD-2X and GFD-4X rice samples treated with *M. oryzae* for 72 h were labeled as 2X_72h and 4X_72h, respectively. For RNA-seq, the total number of pairs of end-reads in the clean data was greater than 20.33 million, the total number of bases in the clean data was greater than 6 billion, the GC content in the clean data was greater than 52.63%, and the Q30 was greater than 94.15% (Supplementary Table S2). We focused on the research of transcription factors and the results showed that all expressed TFs belonged to 56 different families in rice, which were then clustered using the standard of read counts > 30 (Figure 1a). The results demonstrated that 1257 TFs were implicated in the expression of GFD-2X and GFD-4X. Notably, the genes from the bHLH, bZIP, WRKY, MYB, and ERF families constituted the most significant proportion of these TFs (Supplementary Table S3). The results of the clustering analysis revealed that diploid and tetraploid rice samples from different periods clustered together. However, the rice samples infected for 36 h and 72 h showed distinct clustering patterns from the uninfected rice samples at 0 h. Simultaneously, at various time points under the stress of rice blast disease, we identified a higher number of genes with a low expression in tetraploid rice compared to diploid rice. The Venn diagram analysis revealed that GFD-2X and GFD-4X expressed 55 TF families at various time points during rice blast stress (Figure 1b). In GFD-2X, we identified a comprehensive total of 1067 TF genes participating in co-expression both in the absence of blast treatment and under blast stress conditions at 36 h and 72 h. Similarly, in GFD-4X, we observed 1045 TF genes involved in co-expression without blast treatment and under blast stress conditions of 36 h and 72h (Figure 1c). At 0 h, 1106 TF genes were co-expressed in both GFD-4X and GFD-2X, with 40 TF genes specifically expressed in GFD-4X and 43 TF genes specifically expressed in GFD-2X. Following a 36 h blast stress, 1078 TF genes were co-expressed in both GFD-4X and GFD-2X, with 46 TF genes specifically expressed in GFD-4X and 56 TF genes specifically expressed in GFD-2X. After a 72 h blast stress, 1068 TF genes were co-expressed in both GFD-4X and GFD-2X, with 44 TF genes specifically expressed in GFD-4X and 59 TF genes specifically expressed in GFD-2X (Figure 1d).

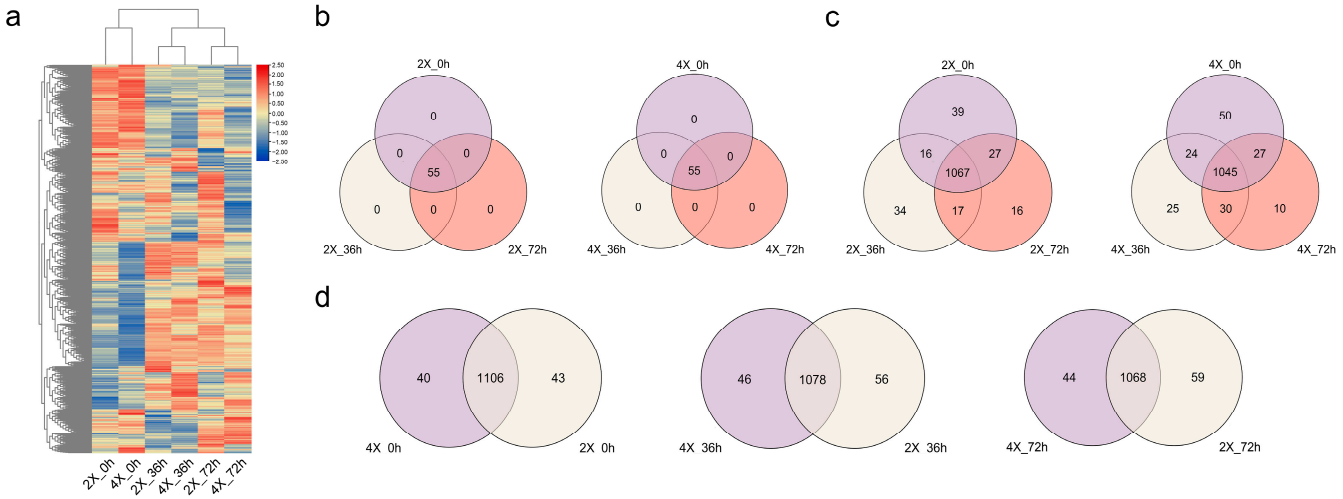

Figure 1. Expression analysis of 56 transcription factor families and their respective family members in GFD-2X and GFD-4X under varying time points of rice blast stress. (**a**) The heatmap depicted the

expression levels of genes related to the 56 transcription factor families. (**b**) Venn diagrams illustrated the number of transcription factor families expressed in GFD-2X and GFD-4X. (**c**) Venn diagrams showed the number of transcription factor genes expressed in GFD-2X and GFD-4X. (**d**) Comparative analysis of the transcription factor genes expressed in GFD-4X and GFD-2X.

3.2. Differential Expression Analysis of Genes Belonging to 56 Transcription Factor Families in GFD-2X and GFD-4X under Blast Fungus Infection

To investigate deeper into the gene expression alterations in diploid and tetraploid rice following infection with the rice blast fungus, we analyzed the differentially expressed transcription factor (DETF) genes. At the initial time point (0 h), a total of 89 DETF genes were identified between diploid and tetraploid rice samples (4X_0h vs. 2X_0h), with 74 genes exhibiting up-regulation (6.2%) and 15 genes displaying down-regulation (1.2%). Following 36 h of blast stress, a total of 92 DETF genes were identified in diploid and tetraploid rice samples (4X_36h vs. 2X_36h), with 12 genes being up-regulated (1.0%) and 80 genes being down-regulated (6.7%). After 72 h of blast stress, a total of 189 DETF genes were identified in diploid and tetraploid rice samples (4X_72h vs. 2X_72h), with 41 genes exhibiting up-regulation (3.5%) and 148 genes showing down-regulation (12.6%) (Figure 2a).

The analysis of Di Venn revealed that 18 DETF genes were consistently co-expressed in the comparisons of 4X_0h vs. 2X_0h, 4X_36h vs. 2X_36h, and 4X_72h vs. 2X_72h. Additionally, 49 DETF genes were found to be specifically expressed in 4X_0h vs. 2X_0h comparison, 29 DETF genes were specifically expressed in 4X_36h vs. 2X_36h comparison, and 110 DETF genes were specifically expressed in the 4X_72h vs. 2X_72h comparison (Figure 2b). The co-expressed and specifically expressed DETF genes were listed in Supplementary Table S4. The GO enrichment analysis of DETF genes between GFD-2X and GFD-4X groups showed that the "regulation of transcription, DNA-templated", "response to stimulus", and "regulation of transcription from RNA polymerase II promoter" were the top three biological processes with the highest gene enrichment in the 4X_0h vs. 2X_0h comparison. Among these, the "response to stimulus" was found to be related to the biotic stress pathway. Furthermore, the "response to fungus" and "response to gibberellin" were also enriched and expressed in this comparison.

In the 4X_36h vs. 2X_36h comparison, the top three biological processes with the highest gene enrichment were the "regulation of transcription, DNA-templated", "response to stimulus", and "ethylene-activated signaling pathway". Among these, the "response to stimulus" was associated with the biotic stress pathway, while the "ethylene-activated signaling pathway" was related to hormones. Additionally, the "response to fungus," "response to salicylic acid", and "response to jasmonic acid" were also enriched and expressed.

In the 4X_72h vs. 2X_72h comparison, the most enriched biological processes were the "regulation of transcription, DNA-templated", "positive regulation of transcription, DNA-templated", "response to stimulus", and "regulation of transcription from RNA polymerase II promoter". The term "response to stimulus" was found to be related to biological stress pathways. Furthermore, the "response to fungus", "ethylene-activated signaling pathway", "response to ethylene", "negative regulation of gibberellic acid mediated signaling pathway", "brassinosteroid mediated signaling pathway", "response to jasmonic acid", "gibberellic acid mediated signaling pathway," and "response to abscisic acid" were also enriched and expressed (Figure 2c). In summary, at 72 h of rice infection, diploid and tetraploid rice showed more significant enrichment of differentially expressed variant genes in the pathways related to biotic stress and hormones.

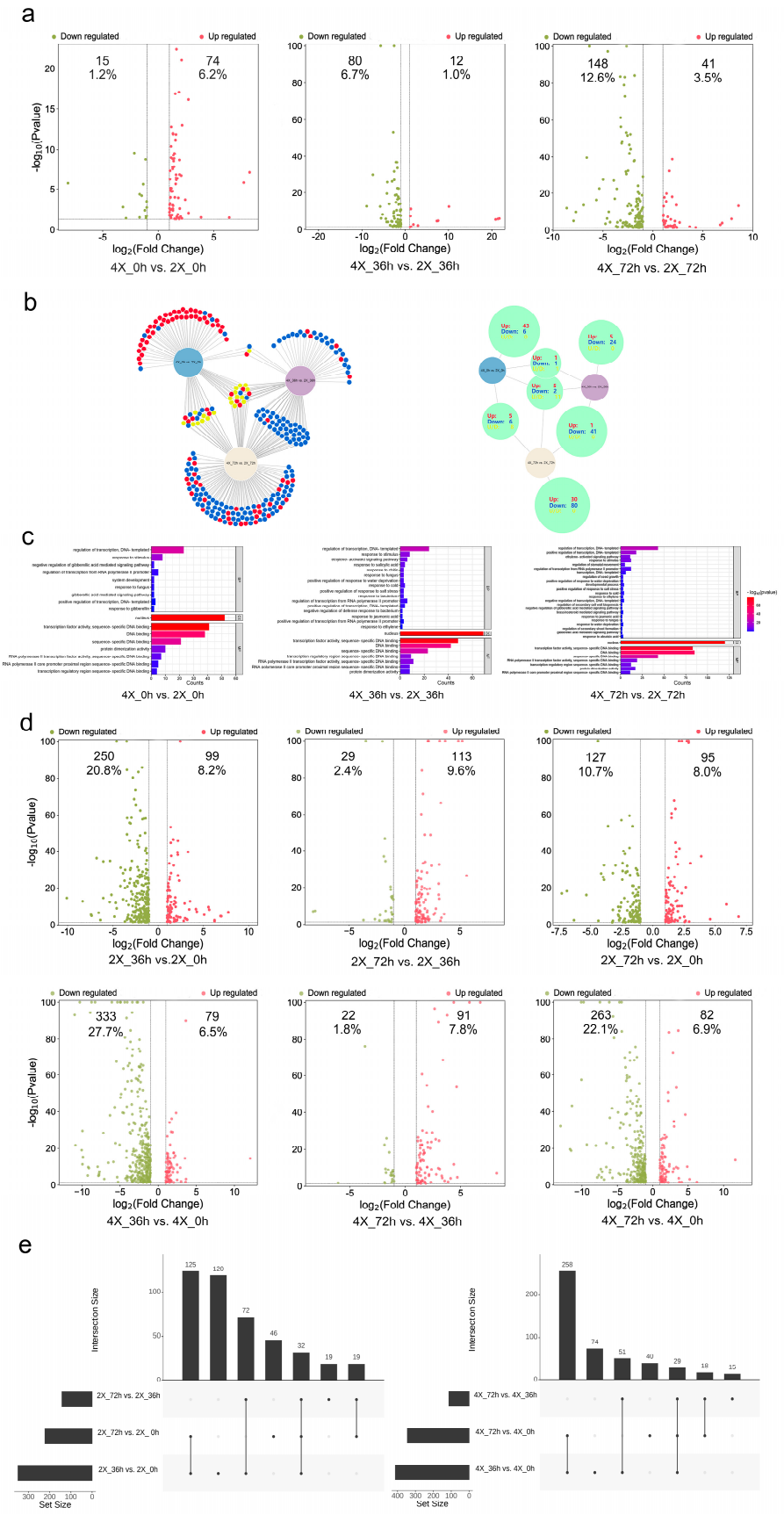

Figure 2. Analysis of differentially expressed transcription factor (DETF) genes of GFD-2X and GFD-4X under blast fungus treatment and at different time points. (**a**) Volcano plots illustrated DETF

gene expression compared between GFD-2x and GFD-4x. (**b**) Di Venn diagram analysis of DETF genes between GFD-2X and GFD-4X groups, with red circles indicating up-regulation, blue circles showing down-regulation, and yellow circles indicating up-regulation or down-regulation. (**c**) GO enrichment analysis highlighted the functional enrichment of DETF genes between GFD-2X and GFD-4X groups. (**d**) Volcano plots illustrated DETF gene expression in GFD-2X and GFD-4X groups. (**e**) The upset map showed the expression patterns of DETF genes in GFD-2X and GFD-4X groups.

In the GFD-2X group, when exposed to rice blast stress for 36 h (2X_36h vs. 2X_0h), a total of 349 DETF genes were identified, with 99 genes being up-regulated (8.2%) and 250 genes being down-regulated (20.8%). Similarly, when subjected to rice blast stress for 36–72 h (2X_72h vs. 2X_36h), 142 DETF genes were found, with 113 genes being up-regulated (9.6%) and 29 genes being down-regulated (2.4%). Lastly, under rice blast stress for 72 h (2X_72h vs. 2X_0h), 222 DETF genes were detected, with 95 genes being up-regulated (8.0%) and 127 genes being down-regulated (10.7%) (Figure 2d). In the GFD-4X, when subjected to rice blast stress for 36 h (4X_36h vs. 4X_0h), a total of 412 DETF genes were identified, with 79 genes being up-regulated (6.5%) and 333 genes being down-regulated (27.7%). Similarly, when exposed to rice blast stress for 36–72 h (4X_72h vs. 4X_36h), 113 DETF genes were found, with 91 genes being up-regulated (7.8%) and 22 genes being down-regulated (1.8%). Furthermore, under rice blast stress for 72 h (4X_72h vs. 4X_0h), 345 DETF genes were detected, with 82 genes being up-regulated (6.9%) and 263 genes being down-regulated (22.1%) (Figure 2d).

The Upset plot results demonstrated that 32 DETF genes were co-expressed in 2X_36h vs. 2X_0h, 2X_72h vs. 2X_36h, and 2X_72h vs. 2X_0h, while 29 DETF genes were co-expressed in 4X_36h vs. 4X_0h, 4X_72h vs. 4X_36h, and 4X_72h vs. 4X_0h (Figure 2e).

3.3. Functional Analysis of Differentially Expressed Genes within Transcription Factor Families in GFD-2X and GFD-4X under Blast Fungus Infection

We conducted a comparative analysis of DETF genes in diploid and autotetraploid rice varieties induced by rice blast stress at different time points. The Venn diagram illustrates the co-expression and specific expression pattern of DETF genes in GFD-2X and GFD-4X rice varieties caused by 0 h, 36 h, and 72 h stress. The number of related transcription factor families and their corresponding members are indicated below the Venn diagram. After subjecting the rice plants to blast stress for 36 h, we found that 266 DETF genes were co-expressed in 4X_36h vs. 4X_0h and 2X_36h vs. 2X_0h, while 146 DETF genes exhibited specific expression in 4X_36h vs. 4X_0h. Additionally, 83 DETF genes showed specific expression in 2X_36h vs. 2X_0h (Figure 3a).

Under rice blast stress for 36–72 h, a total of 60 DETF genes were found to be co-expressed in 4X_72h vs. 4X_36h and 2X_72h vs. 2X_36h, while 53 DETF genes exhibited specific expression in 4X_72h vs. 4X_36h. Additionally, 82 DETF genes showed specific expression in 2X_72h vs. 2X_36h (Figure 3b).

Furthermore, when the rice plants were subjected to blast stress for 72 h, we observed that 156 DETF genes were co-expressed in 4X_72h vs. 4X_0h and 2X_72h vs. 2X_0h, while 189 DETF genes exhibited specific expression in 4X_72h vs. 4X_0h. Additionally, 66 DETF genes showed specific expression in 2X_72h vs. 2X_0h (Figure 3c).

We also performed GO and KEGG enrichment analyses of the GFD-2X and GFD-4X co-expressed DETF genes, as well as the specifically expressed DETF genes identified from the Venn diagram comparisons. The results of GO enrichment analysis revealed that the specifically expressed DETF genes in the 4X_36h vs. 4X_0h comparison were significantly enriched in biotic stress pathways associated with "response to stimulus". Similarly, the specifically expressed DETF genes in the 2X_36h vs. 2X_0h comparison showed significant enrichment in the biotic stress pathways related to "response to stimulus". The DETF genes co-expressed in 4X_36h vs. 4X_0h and 2X_36h vs. 2X_0h comparisons were enriched in hormone-related signaling pathways (ethylene-activated, auxin-activated, negative regulation of gibberellic acid mediated, and response to jasmonic acid). Additionally, these

genes were associated with the biotic stress response pathway, specifically "response to stimulus" and "response to fungus" (Figure 4a).

The specific DETF expressed in the 4X_72h vs. 4X_36h comparison is associated with the "brassinosteroid mediated signaling pathway". In the 2X_72h vs. 2X_36h comparison, the specific DETF genes are enriched in both "response to stimulus" and "defense response" biotic stress pathways. Additionally, hormone-related signaling pathways are associated with ethylene and gibberellins. The biotic stress pathway enriched by the co-expressed DETF genes in the 4X_72h vs. 4X_36h and 2X_72h vs. 2X_36h comparisons is a "response to stimulus" (Figure 4c). The DETF genes specifically expressed in the 4X_72h vs. 4X_0h comparison was enriched in "response to stimulus" and "response to fungus." Additionally, hormone-related signaling pathways associated with ethylene, gibberellins, auxin activation signals, and brassinosteroids are enriched (Figure 4e).

Furthermore, the KEGG enrichment analysis revealed that the DETF genes in the 4X_36h vs. 4X_0h and 2X_36h vs. 2X_0h comparisons were involved in "Plant hormone signal transduction" and the "MAPK signaling pathway". Specifically, the DETF genes in the 4X_36h vs. 4X_0h comparisons were also associated with the "MAPK signaling pathway" (Figure 4b). The DETF genes in the 4X_72h vs. 4X_36h comparison were involved in "Plant hormone signal transduction". In contrast, the DETF genes in the 2X_72h vs. 2X_36h comparison were involved in the "MAPK signaling pathway" and "Plant-pathogen interaction" (Figure 4d). Furthermore, the 4X_72h vs. 4X_0h DETF genes were also involved in "Plant hormone signal transduction" and the "MAPK signaling pathway" (Figure 4f).

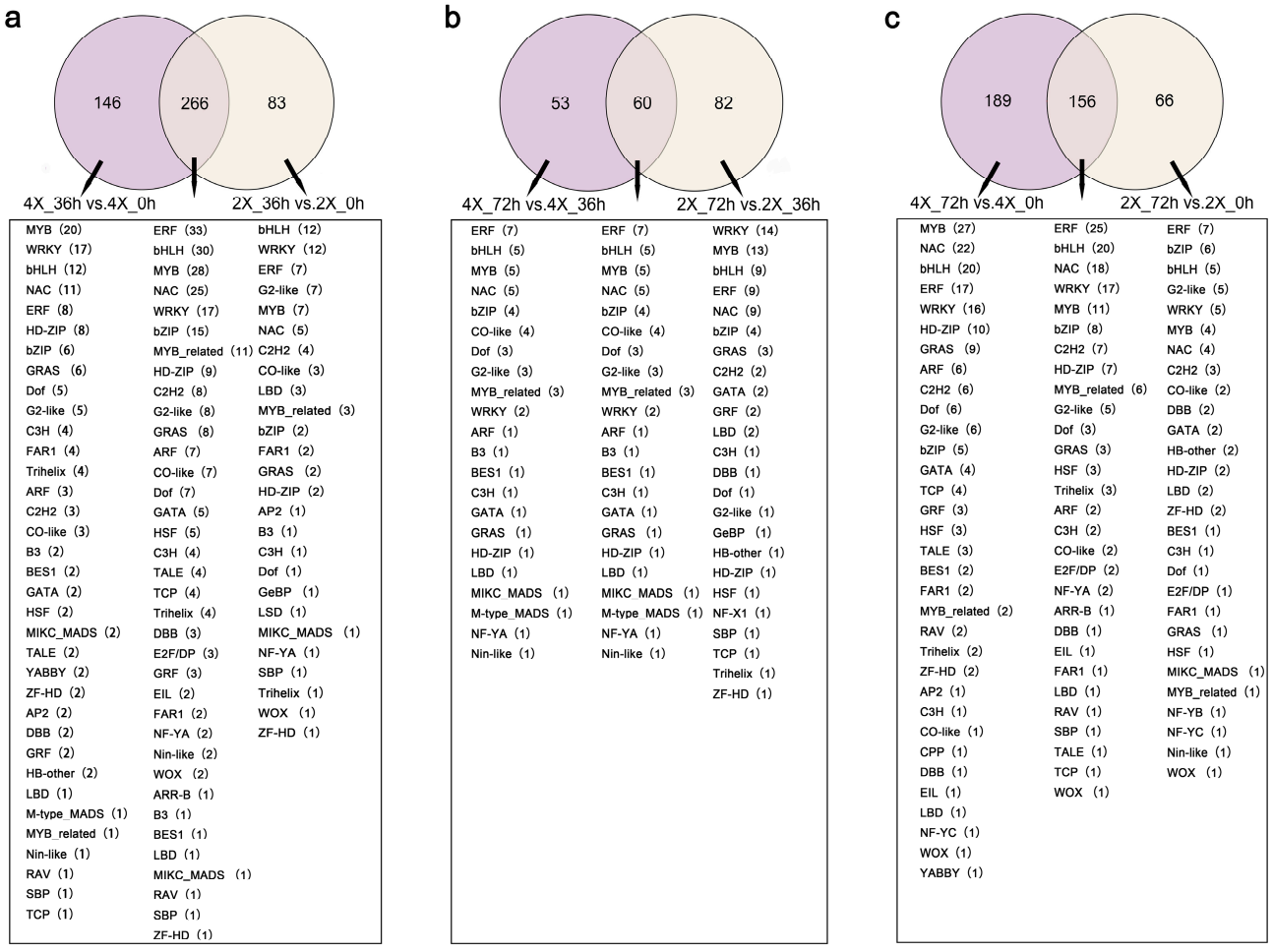

Figure 3. Analysis of the DETF gene compared GFD-4X and GFD-2X under rice blast stress at different time points. (**a**) Venn diagram comparison at 36 h of rice blast stress. (**b**) Venn diagram comparison under 36–72 h of rice blast stress. (**c**) Venn diagram comparison at 72 h of rice blast stress.

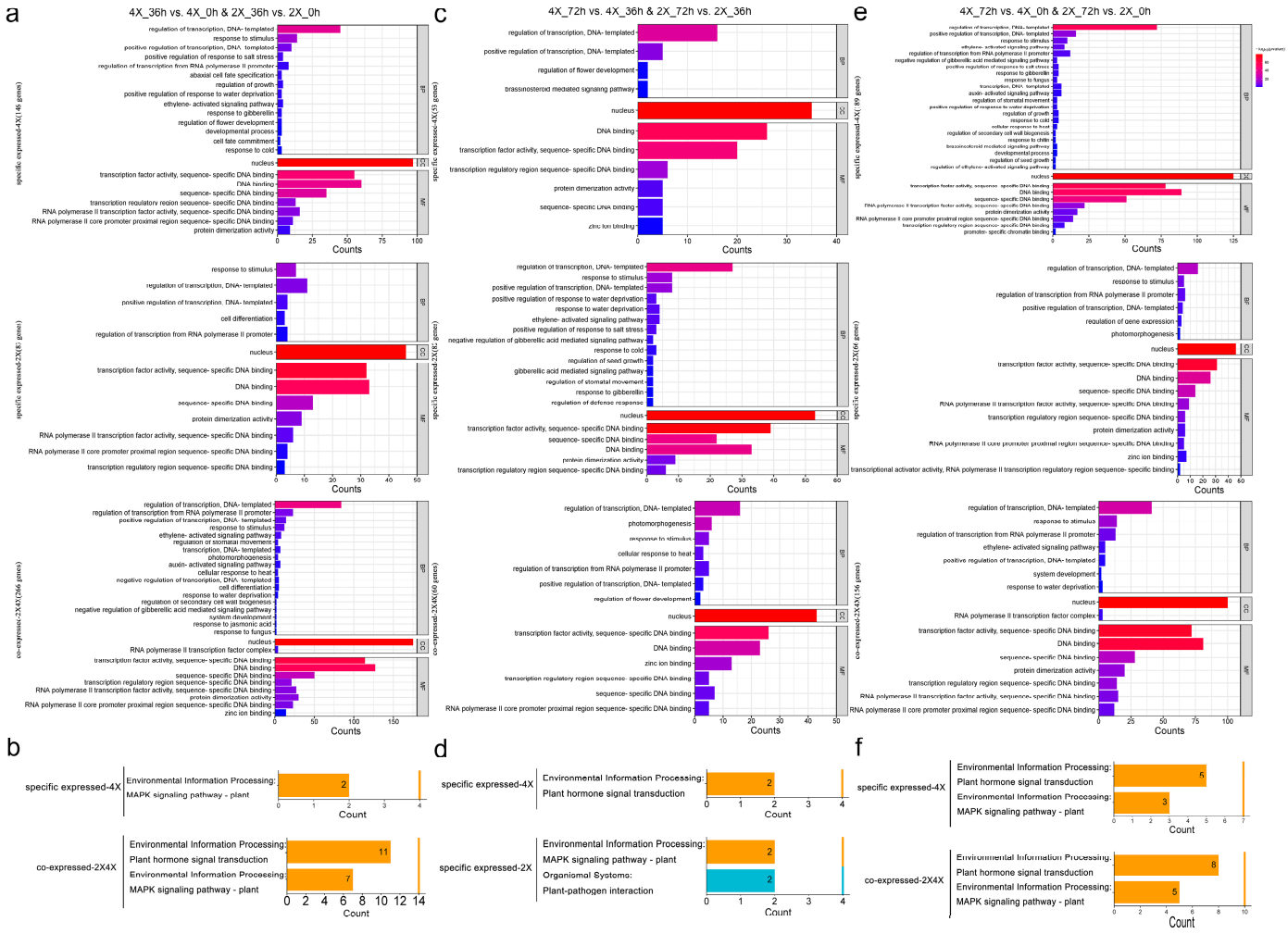

Figure 4. Enrichment analysis of the DETF gene compared GFD-4X and GFD-2X under rice blast stress at different times. (**a**) GO enrichment analysis, (**b**) KEGG enrichment analysis of GFD-2X and GFD-4X DETF genes at 36 h of rice blast stress. (**c**) GO enrichment analysis, (**d**) KEGG enrichment analysis of GFD-2X and GFD-4X DETF genes under 36–72 h of rice blast stress. (**e**) GO enrichment analysis, (**f**) KEGG enrichment analysis of GFD-2X and GFD-4X DETF genes at 72 h of rice blast stress.

It is worth noting that the "plant-pathogen interaction" pathway was only enriched in diploid rice under stress between 36 and 72 h and not in autotetraploid rice. However, overall, autotetraploid rice exhibited a significantly higher number of enriched pathways at different time points of stress than diploid rice.

3.4. Analysis of DETF Genes and Their Protein-Interaction Networks Involved in Biotic Stress Response and Phytohormone Response Induced by Rice Blast Disease

To investigate the functions of these DETF genes, we examined their involvement in biotic stress responses and phytohormone responses in diploid and autotetraploid rice varieties when exposed to rice blast stress. We detected the expression levels of these DETF genes at various time points during the stress period. Figure 5 provides a detailed illustration of the specific DETF genes implicated in the biotic stress response.

Under 36 h of rice blast stress, the genes *Os08g0198900*, *Os03g0335200*, and *Os11g0117500* were up-regulated in GFD-2X, while the *Os01g0186000* gene was up-regulated in GFD-4X. Additionally, the *Os08g0499300* gene showed up-regulation in both GFD-2X and GFD-4X. During the 36–72 h stress period, nine genes from the WRKY family and the bHLH family, the genes *Os05g0474800*, *Os08g0386200*, *Os01g0821600*, *Os12g0116400*, *Os01g0821300*, *Os03g0798500*, *Os05g0537100*, *Os02g0181300*, and *Os03g0135700*, were induced and up-regulated by rice blast in GFD-2X. Among the five genes from the WRKY family, *Os11g0117400*, *Os12g0116600*, and *Os12g0116700* were up-regulated in GFD-2X and GFD-4X, while *Os08g0499300* and *Os03g0758950* genes were down-regulated in both GFD-2X and GFD-4X. Moreover, *Os08g0198900* and *Os01g0186000* were up-regulated in GFD-2X, *Os01g0185900* and *Os02g0770500* were up-regulated in GFD-4X, and *Os07g0111400*, *Os11g0117400*, and *Os12g0116700* were up-regulated in both GFD-2X and GFD-4X after 72 h of stress. Notably, the expression of *Os08g0386200* was up-regulated in GFD-2X but down-regulated in GFD-4X (Figure 5a).

Phytohormones such as ethylene (ETH), gibberellin (GA), auxin (IAA), JA, and brassinosteroids (BRs) act as signals to trigger and mediate a variety of plant immune responses [17]. In this study, we investigated the induction of genes related to ETH and GA signaling pathways in GFD-2X and GFD-4X after inoculation with blast fungus. Under rice blast stress for 36 h, we observed up-regulation of the ETH signaling-related genes *Os09g0287000* (ERF family) and GA signaling-related genes *Os02g0220400* (GATA family) in GFD-4X. Additionally, the ETH signaling-related genes *Os03g0183000* and *Os09g0572000* (ERF family) were up-regulated in both GFD-2X and GFD-4X. Notably, the expression of *Os01g0797600* was up-regulated in GFD-2x but down-regulated in GFD-4X.

Under rice blast stress for 36–72 h, we observed that the ETH signaling-related genes *Os07g0674800*, *Os02g0764700*, and *Os08g0474000* (ERF family) and the GA signaling-related genes *Os02g0181300* and *Os01g0826400* (WRKY family) were exclusively present and up-regulated in GFD-2X. In contrast, the ETH signaling-related genes *Os09g0286600* and *Os09g0287000* (ERF family) and the GA signaling-related gene *Os02g0220400* (GATA family) were up-regulated in GFD-4X under 72 h stress conditions. Notably, the expression levels of *Os09g0287000* and *Os02g0220400* genes increased compared to the 36 h stress period. Additionally, the ETH signaling-related genes *Os03g0183000* and *Os09g0572000* (ERF family) were up-regulated in both GFD-2X and GFD-4X, with a higher gene expression level of *Os09g0572000* compared to the 36 h stress period (Figure 5b).

To explore the interactions between DETF genes enriched in GFD-2X and GFD-4X involved in biotic stress pathways and hormone pathway-related DETF genes, we utilized the STRING database to construct protein interaction networks for these genes. The protein interaction network associated with TF genes in the biotic stress pathway comprised 23 nodes and 37 edges. Notably, we observed a significant presence of WRKY family genes, including WRKY71, WRKY76, WRKY45-1, WRKY21, WRKY4, and WRKY77. Among them, WRKY71, WRKY76, and WRKY21 exhibited strong interactions with several TFs (Figure 5c). Furthermore, we constructed a protein interaction network of DETF genes involved in plant hormone responses, which consisted of nine nodes and nine edges. With this network, we identified BZR1 TFs of the BES1 family, ILI4 TFs of the bHLH family, and EIL2 TFs of the EIL family. Notably, there was an interaction between BZR1 TFs and ILI4 TFs, as well as interactions among four proteins: EIL2, Q6H7H6, B7F8P7, and Q6YVT1. Additionally, we observed interactions between WRKY71 of the WRKY family and WRKY24 and WRKY51, respectively (Figure 5d). Interestingly, WRKY71 demonstrated strong interactions with various TFs involved in different biological processes.

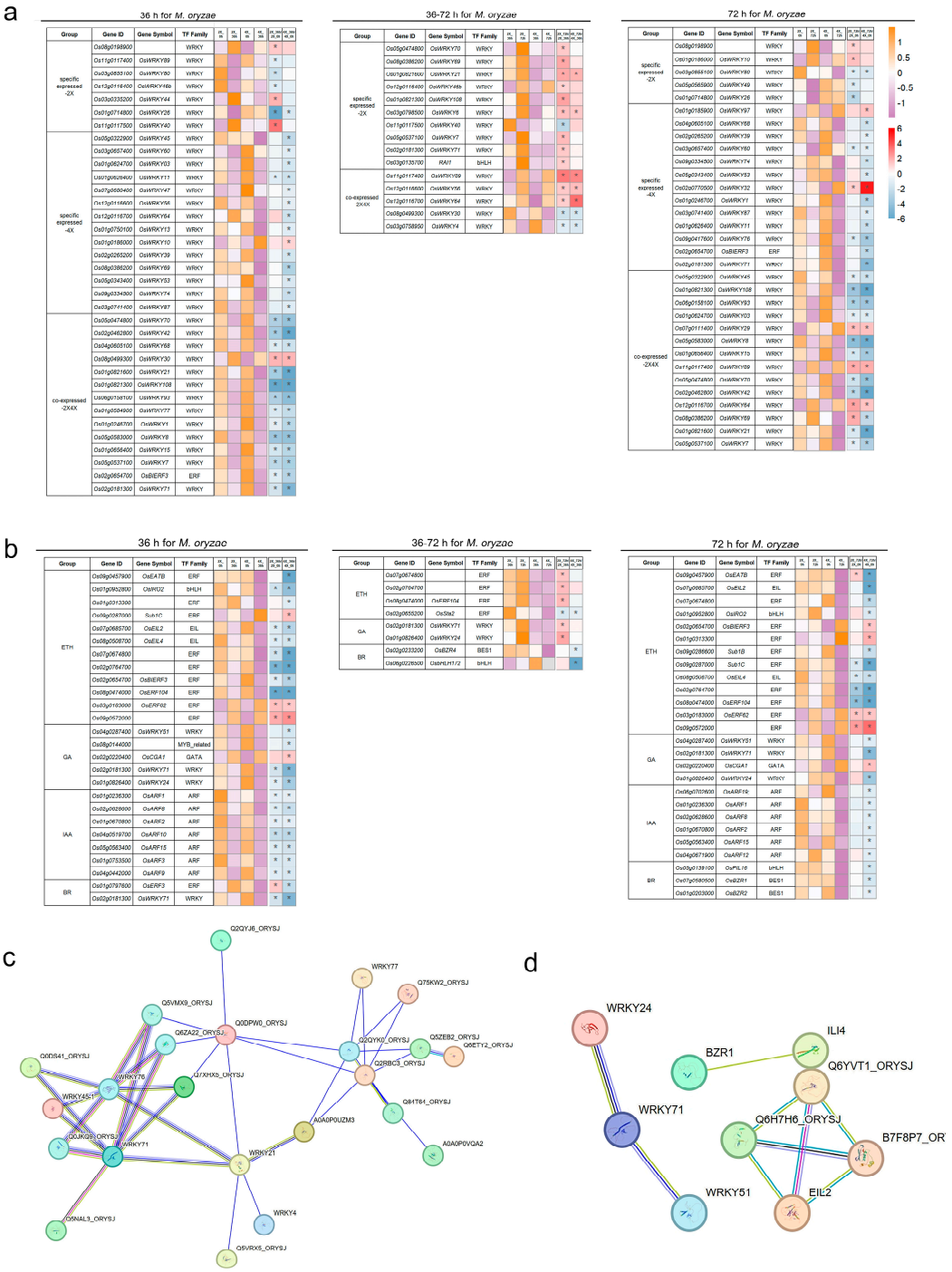

Figure 5. Gene expression levels and predicted protein interaction networks associated with biological stress and hormone pathways were compared between GFD-4X and GFD-2X under different durations of rice blast stress. (**a**) Expression levels of TF genes in the biotic stress-related pathway. (**b**) Expression levels of TF genes in the hormone signaling pathway. (**c**) Protein interaction networks of genes involved in biotic stress pathways. (**d**) Protein interaction networks of genes involved in hormone signaling pathways. * Indicated the level of significance of differences ($p < 0.05$) (Student's *t* test).

3.5. Identification of Co-Expressed DETF Genes in Diploid and Tetraploid Rice under Blast Stress at Different Time Points

To further investigate the shared and unique genes in diploid and tetraploid rice under rice blast stress, we integrated specific transcription factors identified in diploid rice at

different time points based on our previous analysis. The analysis revealed the expression of 83 genes under 36 h of stress, 82 genes under 36–72 h of stress, and 66 genes under 72 h of stress (Figure 3). The Venn diagram analysis demonstrated the co-expression of two DETF genes from the NAC and ERF families in 2X_36h vs. 2X_0h, 2X_72h vs. 2X_36h, and 2X_72h vs. 2X_0h comparisons. Notably, the heatmap analysis indicated that these two genes exhibited up-regulation, specifically during the 36–72 h period of stress in diploid rice. In contrast, in GFD-2X, only 45 DETF genes were specifically expressed during the 36 h rice blast stress, 59 DETF genes were specifically expressed during the 36–72 h stress, and 41 DETF genes were specifically expressed during 72 h stress (Figure 6a,d).

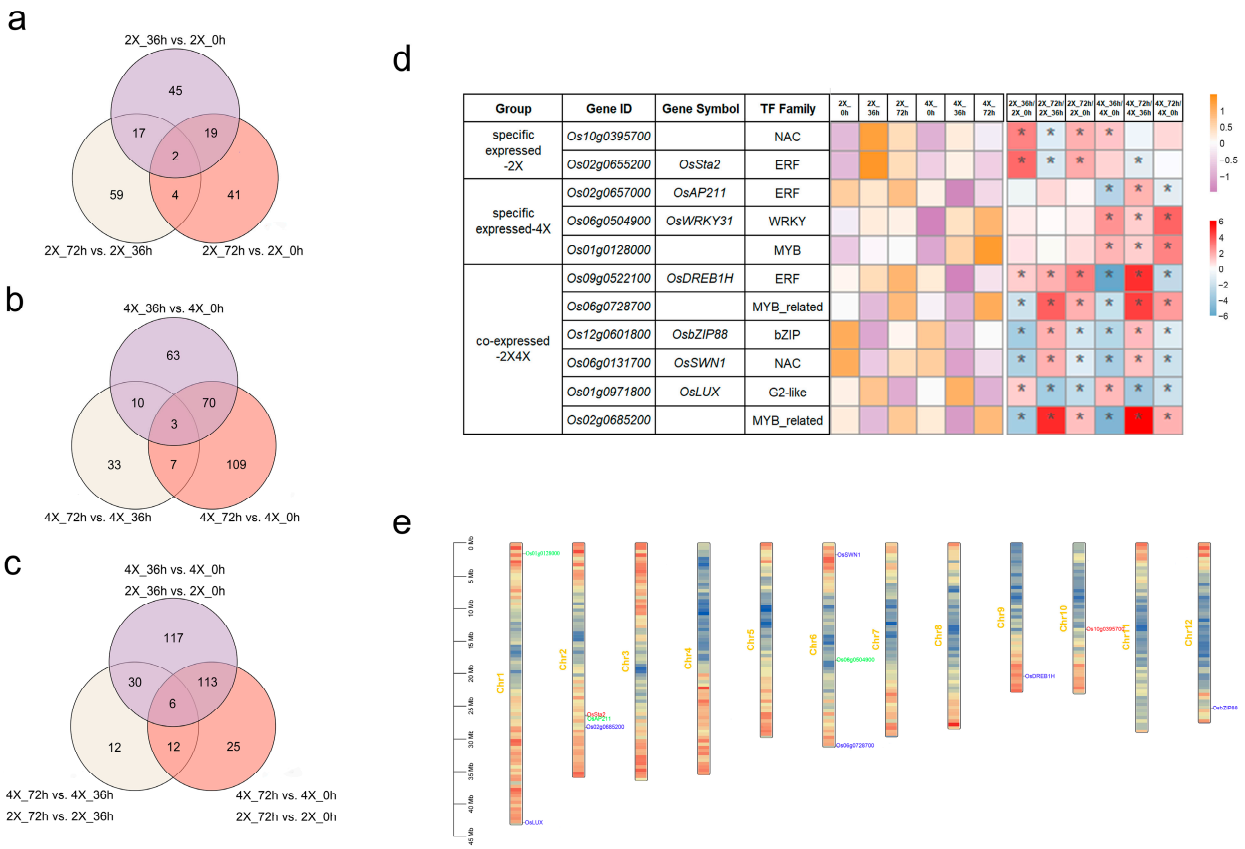

Figure 6. Co-expression and chromosomal localization of DETF genes in GFD-2X and GFD-4X under different blast stress durations. (**a**) Venn diagram illustrated the co-expression of DETF genes in GFD-2X, (**b**) showed the co-expression of DETF genes in GFD-4X, and (**c**) illustrated the co-expression of DETF genes in GFD-2X and GFD-4X. (**d**) Heatmap displaying the expression patterns of all co-expressed DETF genes. (**e**) Chromosomal localization of DETF genes on the 12 rice chromosomes. The color bars represented the chromosomes, the different colors in the color bar indicate the enrichment of genes at the chromosome location, and the left scale indicated the gene position. DETF genes co-expressed in GFD-2X at different blast stress durations were depicted in red, DETF genes co-expressed in GFD-4X were displayed in green, and DETF genes co-expressed in GFD-2X and GFD-4X were shown in blue. * Indicated the level of significance of differences ($p < 0.05$) (Student's *t* test).

Furthermore, we identified stress-specific TF genes expressed by GFD-4X at different time points (Figure 3). Specifically, there were 146 genes expressed under 36 h of stress, 53 genes expressed under 36–72 h of stress, and 189 genes expressed under 72 h of stress. The Venn diagram analysis revealed the co-expression of three DETF genes from the ERF, WRKY, and MYB families in the 4X_36h vs. 4X_0h, 4X_72h vs. 4X_36h, and 4X_72h vs. 4X_0h comparisons, as depicted in the heatmap. In contrast, in GFD-4X, only 63 DETF genes were specifically expressed under blast stress at 36 h, 33 DETF genes were

specifically expressed during the 36–72 h period, and 109 DETF genes were specifically expressed under 72 h of stress (Figure 6b,d).

Simultaneously, we identified TF genes expressed by GFD-2X and GFD-4X under different stress durations (Figure 3). Specifically, there were 266 genes expressed under 36 h of stress, 60 genes expressed under 36–72 h of stress, and 156 genes expressed under 72 h of stress. The Venn diagram analysis revealed the co-expression of six DETF genes from the ERF, MYB_related, bZIP, NAC, and G2-like families in the 4X_36h vs. 4X_0h and 2X_36h vs. 2X_0h, 4X_72h vs. 4X_36h and 2X_72h vs. 2X_36h, and 4X_72h vs. 4X_0h and 2X_72h vs. 2X_0h comparisons. Furthermore, the heatmap analysis indicated that only the *Os09g0522100* gene exhibited differential expression between GFD-4X and GFD-2X. Interestingly, among GFD-2X and GFD-4X, only 117 DETF genes were specifically expressed at 36 h of rice blast stress, 12 DETF genes were specifically expressed during the 36–72 h period, and 25 DETF genes were specifically expressed at 72 h of stress (Figure 6c,d). Additionally, eleven co-expressed DETF genes were localized to specific regions on the rice chromosome (Figure 6e).

4. Discussion

Rice blast represents the most pervasive and detrimental disease commonly cultivated rice [41]. To elucidate the mechanism underlying the response to rice blast stress, we conducted a comparative analysis of TFs expressed in diploid and autotetraploid rice under non-stress conditions and at varying time points following stress treatment. Our study confirmed notable differences in the expression of TF genes in both GFD-2X and GFD-4X under the induction of rice blast. Furthermore, we identified the TF genes uniquely expressed and co-expressed in GFD-2X and GFD-4X at different time points of rice blast stress. These findings hold substantial promise for advancing research on resistance to rice blast stress.

Upon analyzing the collective expression of TFs in GFD-2X and GFD-4X, we observed that the TFs expressed by GFD-2X and GFD-4X under 36 h of stress clustered with those expressed under 72 h of stress, illustrating a consistent clustering pattern (Figure 1a). We further analyzed and identified differentially expressed TF genes in GFD-2X and GFD-4X. Our findings revealed a decrease in TF gene expression in GFD-2X and GFD-4X by 20.8% and 27.7%, respectively, at 36 h compared to 0 h. This could be attributed to the response to stimulus triggered by the early stage of rice blast infection. As the duration of pathogen infestation increased, the TF genes expressed by GFD-2X and GFD-4X were significantly up-regulated at 72 h of stress compared to 36 h of stress. Moreover, some TF genes were found to be actively expressed to counteract rice blast stress (Figure 2d). This suggested that these TFs may play a crucial role in the defense mechanism of rice against blast stress.

Our comparative analysis of DETF genes in rice subjected to rice blast stress revealed distinct expression patterns. Specifically, under 36 h of rice blast stress, we identified 495 DEGs across 45 TF families in GFD-2X and GFD-4X. Under prolonged stress from 36 to 72 h, 195 genes from 37 TF families exhibited differentially expression. After 72 h of stress, 411 genes from 43 TF families showed differential expression. Notably, the NF-X1 family was uniquely expressed under 36–72 h of stress, while the CPP, NF-YC, WOX, and NF-YB families were differentially expressed only under 72 h of stress conditions. These observations suggested that the TF families respond variably to different durations of fungal infestation, indicating diverse regulatory mechanisms.

In the GO enrichment analysis of these DETF genes, most biological processes were associated with transcription, response to biotic stresses, and hormone signaling. Several abiotic stress-related signaling pathways were also identified, including "positive regulation of response to salt stress", "positive regulation of response to water deprivation", "response to cold", and "cellular response to heat" (Figure 4). It is worth noting that the DETFs in tetraploid rice were not enriched for biotic stress-related signaling pathways during 36–72 h of rice blast stress, suggesting that TF genes regulating rice disease resistance may not play a role in this process.

Intriguingly, upon GO enrichment of rice DETF genes induced by rice blast stress, we found that many WRKY family TF genes regulated the processes of "response to stimulus" and "response to fungus". To further explore the biological processes regulated by other TF families under rice blast stress conditions, we performed a re-enriched analysis on four TF families other than the WRKY family prevalent in each set of two-by-two comparisons. We found that the MYB family was primarily involved in the regulation of stomatal movement and response to cold, the bHLH family mainly regulated transcription and growth, the NAC family positively regulated response to salt and water deprivation, and the ERF family was enriched for the ethylene-activated signaling pathway (Supplementary Figure S2). These findings provide valuable insights into the complex regulatory networks involved in rice's response to blast stress.

To explore the essential TF genes implicated in the regulation of rice disease resistance, we conducted an in-depth analysis of the protein interactions associated with the biotic stress pathway and hormone pathway-related DETF genes, as identified by GO enrichment. We further probed the functions and roles of several core proteins, namely WRKY71, WRKY76, and WRKY21. Previous studies have established that WRKY proteins primarily participate in plant responses to biotic stressors [41]. Notably, Liu et al. (2015) utilized a yeast one-hybrid approach to demonstrate that WRKY71 can bind to the promoter of GF14b and modulate its expression. GF14b has been shown to positively regulate rice spike blight resistance while concurrently negatively regulating rice leaf blight resistance [42]. WRKY76, a protein identified in rice, is a transcriptional repressor that bolsters rice resistance to cold stress. On the contrary, overexpression of *OsWRKY76* can increase rice susceptibility to blast. In our study, *OsWRKY76* expressed GFD-4X exclusively under 72 h of GFD-4X stress and was down-regulated more than three-fold. This down-regulation might have amplified the tolerance of GFD-4X to rice blast stress [43].

WRKY21 has been found to interact with WRKY71, WRKY76, WRKY4, Q0DPW0, Q5VRX5, and A0A0P0UZM3 under rice blast stress conditions. Previous studies have primarily focused on the role of WRKY21 in Pi accumulation in rice [44]. Based on these findings, we hypothesized that WRKY21 may be pivotal in enhancing rice resistance to blasts under a complex regulatory network. Further investigations are warranted to validate this supposition and elucidate the underlying mechanisms.

Furthermore, our study revealed that several DETF genes were not represented in the protein interactions network, owing to their lack of interaction with other proteins. However, these TF genes are crucial in disease resistance in diploid and homologous tetraploid rice. For instance, *OsWRKY89* was found to be instrumental in responding to biotic stresses in a previous investigation, and overexpression of this gene bolstered rice resistance to blasts [45]. In our study, *OsWRKY89* was significantly up-regulated under GFD-2X and GFD-4X stresses for 36–72 h, demonstrating a more than two-fold increase under 72 h of stress. Therefore, *OsWRKY89* appears to contribute to the rice's tolerance to blast.

Previous research identified *OsWRKY30* as rapidly induced by *M. oryzae* and regulated by SA or JA [46]. The constitutive expression of the rice WRKY30 gene at the same locus led to the activated expression of jasmonic acid synthesis-related genes LOX and AOS2, as well as disease process-related proteins PR3 and PR10. This supported the results that increased endogenous jasmonic acid content and enhanced rice resistance to rice blast [47]. In this study, the expression of *OsWRKY30* was up-regulated under 36 h of GFD-2X and GFD-4X stress. We hypothesized that this elevated expression level contributes to enhanced disease resistance in rice. Berri S et al. demonstrated that *OsWRKY64* was differentially expressed in rice leaves induced by blasts [48]. Consistent with this, our study found that *OsWRKY64* expression was up-regulated at different time points under GFD-2X and GFD-4X stresses, supporting the findings of previous studies.

Co-expressed DETF genes were present in GFD-2X and GFD-4X at different time points of rice blast stress (Figure 6). Among them, *Os06g0728700* gene and *Os02g0685200* gene had been previously reported to contribute to brown planthopper-induced resistance

potentially [49]. Our findings demonstrated that these two genes were up-regulated under rice blast stress, leading us to speculate that the *Os06g0728700* gene and the *Os02g0685200* gene play a positive regulatory role in rice disease resistance (Figure 6c,d).

Moreover, specific transcription factors in diploid and its homologous tetraploid rice respond differently to rice blast stress. For instance, the *OsDREB1H* gene was up-regulated in GFD-2X and down-regulated in GFD-4X under 36 h and 72 h of rice blast stress, which may be attributed to the chromosomal doubling, resulting in varied expression levels of transcription factor-related genes in different ploidy rice (Figure 6d).

In addition, we found that three genes were co-expressed in autotetraploid rice obtained by chromosome doubling at different times of stress in our study, where both *OsWRKY31* and *Os01g0128000* showed up-regulated (Figure 6b,d). Previous research has indicated that overexpression of *OsWRKY31* bolsters rice resistance to blast and triggers the expression of defense-related genes [50,51]. *Os01g0128000*, a member of the MYB family, regulates numerous processes such as plant growth, development, differentiation, metabolism, defense, and responses to biotic and abiotic stresses [52]. Furthermore, *Os01g0128000* is involved in Pi starvation signaling and GA biosynthesis and is a gene controlling the growth and development of the rice root system [53,54]. Therefore, these two TF genes are key genes involved in the regulation of disease resistance in tetraploid rice. The above results suggest that transcription factors play an important role in participating in the regulation of disease resistance in polyploid rice, while changes in ploidy level may be the main reason for the enhancement of rice tolerance to biotic stresses.

Interestingly, chromosomal localization of the 11 DETF genes revealed that the *OsSta2* gene and *OsAP211* gene are situated on chromosome 2, and their coding regions (CDS) are closely located. *OsSta2*, a co-expressed DETF gene in diploid rice, is now recognized to play a crucial role in determining rice yield and improving its salt tolerance [55]. *OsAP211*, one of the DETF genes co-expressed in tetraploid rice, is upregulated by salt stress [56]. Our study suggests that both genes have equivalent functions in rice disease resistance (Figure 6e).

5. Conclusions

In conclusion, this study represents a comprehensive investigation into the differential expression profiles of TFs in diploid and autotetraploid rice under varying durations of rice blast stress. It was demonstrated that diploid rice and homozygous tetraploid rice obtained by chromosome doubling have different response mechanisms to rice blast stress and that tetraploid rice is better able to withstand biotic stresses, and therefore, the change in ploidy level may have enhanced the disease resistance of rice. In addition, we identified different DETF genes specifically expressed in diploid or tetraploid rice at different stress time points and 11 DETF genes co-expressed in both ploidy forms. The identification and analysis of these DETF genes provide valuable insights into the molecular mechanisms underlying the differential responses of diploid and tetraploid rice to rice blast stress, thereby establishing the foundation for subsequent comprehensive research endeavors.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy13123007/s1, Table S1: List of primers of qPCR; Table S2: RNA-seq data of diploid and autotetraploid rice under different time points of rice blast stress; Table S3: Expression of 56 transcription factor family members of GFD-2X and GFD-4X at other times of rice blast stress. Table S4: The co-expressed and specifically expressed DETF genes in 4X_0h vs. 2X_0h, 4X_36h vs. 2X_36h, and 4X_72h vs. 2X_72h. Figure S1: (a) The results of six genes expression amplified by qRT-PCR. * and ** indicate the level of significance of differences between treatment and control groups ($p < 0.05$ and $p < 0.01$) (Student's t test). (b) Comparison of RNA-seq results and qRT-PCR analysis of gene expression levels; Figure S2: GO enrichment analysis of several TF families with the highest abundance of specific expression and co-expression of GFD-2X and GFD-4X in each set of two-by-two comparisons at different time points of rice blast stress.

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Conflicts of Interest: The authors declare no conflict of interest.

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