Transcriptome Analysis Reveals the Symbiotic Mechanism of *Ustilago esculenta*-Induced Gall Formation of *Zizania latifolia*

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Zizania latifolia is a perennial aquatic vegetable, whose symbiosis with the fungus *Ustilago esculenta* (member of Basidiomycota, class *Ustilaginaceae*) results in the establishment of swollen gall formations. Here, we analyzed symbiotic relations of *Z. latifolia* and *U. esculenta*, using a triadimefon (TDF) treatment and transcriptome sequencing (RNA-seq). Specifically, accurately identify the whole growth cycle of *Z. latifolia*. Microstructure observations showed that the presence of *U. esculenta* could be clearly observed after gall formation but was absent after the TDF treatment. A total of 17,541 differentially expressed genes (DEGs) were identified, based on the transcriptome. According to gene ontology term and Kyoto Encyclopedia of Genes and Genomes pathway results, plant hormone signal transduction, and cell wall-loosening factors were all significantly enriched due to *U. esculenta* infecting *Z. latifolia*; relative expression levels of hormone-related genes were identified, of which downregulation of indole 3-acetic acid (IAA)-related DEGs was most pronounced in JB_D versus JB_B. The ultra-high performance liquid chromatography analysis revealed that IAA, zeatin+trans zeatin riboside, and gibberellin 3 were increased under *U. esculenta* infection. Based on our results, we proposed a hormone–cell wall loosening model to study the symbiotic mechanism of gall formation after *U. esculenta* infects *Z. latifolia*. Our study thus provides a new perspective for studying the physiological and molecular mechanisms of *U. esculenta* infection of *Z. latifolia* causing swollen gall

formations as well as a theoretical basis for enhancing future yields of cultivated *Z. latifolia*.

Keywords: gall formation, hormones, RNA-seq, symbiotic mechanism, *Ustilago esculenta*, *Zizania latifolia*

Zizania latifolia (Griseb.) Stapf, known as wild rice (Yang et al. 2014), is the only member of the wild *Oryza sativa* genus *Zizania* belonging to the Poaceae family that is native to Asia. In ancient China, *Z. latifolia* was one of the six most significant grain crops cultivated (Quan et al. 2009), being a perennial aquatic vegetable that can be grown in lakes and ponds, and which originated from China and southeastern Asia (Guo et al. 2007). This plant is an asexual aquatic vegetable with a unique flavor and texture that, due to the colonization of *Ustilago esculenta*, has since been cultivated in east and southeast Asia for more than 1,500 years (Chung and Tzeng 2004). The *Z. latifolia* rice plants are cultivated as a delicious and nutritious vegetable, containing sugar, protein, and minerals, along with providing some amino acids essential for the human body (Yan et al. 2018). Additionally, when used medicinally, this plant may also help the body relieve coughs, dampness, and heat and prevent poisoning induced by alcohol as well as possessing anti-atherogenic effects (Han et al. 2013; Moghadasian et al. 2019).

In its long use as an aquatic vegetable, all its parts are edible, including the swelling gall, rhizomes, and grains. Yet, after centuries of evolution, its use as a grain has completely disappeared in China, but it continues to be cultivated for its swollen gall. *Z. latifolia* is usually parasitized by *U. esculenta*, which stimulates the swelling of the gall under suitable environmental conditions (Guo et al. 2015; Qingsen 2006). The success of the swelling gall formation depends on the degree of *U. esculenta* infection of *Z. latifolia*, whose cultivation globally has led to the evolution of diverse white varieties of this vegetable plant, driven primarily by the extent of *U. esculenta* colonization. This infection of *Z. latifolia* that stimulates swollen galls to form is called normal ‘Jiaobai’ in China (You et al. 2011). When the fungus infects the host plant, it causes the latter’s expansion by enlarging the host cell size and number so that the growing formation swells into juicy galls; however, an *U. esculenta* infection can completely inhibit inflorescence growth and seed production in *Z. latifolia* (Thrower and Chan 1980). The internal tissues of formed edible galls, containing the cells expansion, give the appearance of a white gall whose inside is full of fungal hyphae; hence, the reason it is called

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“white *Z. latifolia*.” Previous studies have shown that the symbiosis of *Z. latifolia* and *U. esculenta* not only produces edible gall but also improves host photosynthesis and growth (Yan et al. 2013). Through microscopic observation of the gall formation of *Z. latifolia*, it was found that the inside contained a large amount of hyphae and spores of *U. esculenta* (Li et al. 2019; Zhang et al. 2014). These plant-fungi results may be due to the outcome of a series of internal changes caused by the defense response of *Z. latifolia*.

Fungal infection and host defense reaction are primary events involved in plant-fungus interactions (Baker et al. 1997). These interactions typically produce a range of responses, such as disease symptoms, formation of specialized structures, and changes in biological and morphological reactions in host plant cells (Doehlemann et al. 2008; Hahn and Mendgen 2001). Many plants interact with a fungus in a beneficial direction, such as in rhizobium symbiosis (Sanders 2011), or in a harmful way, such as that well-documented for some fungal diseases and necrosis of crops (Dean et al. 2012). Many fungi must obtain nutrients from their environment, and, in both plant-fungus symbiosis and the majority of pathogenic fungi, they penetrate their host without damaging the plant cell walls or membranes. In our study, we focused on the positive side of endophytic fungi living in plants. Furthermore, in soybean, by adopting RNA-seq methods, it was found possible to confirm the gene co-networks underpinning the potential defense against the rust pathogen *Phakopsora pachyrhizi* (Hossain et al. 2018; Wang et al. 2017). During plant-fungus interactions, the infecting pathogens stimulate host cell responses, which are translated into phytohormone signals, leading to the production of metabolites and the expression of various key genes in host plants (Horst et al. 2008; Rabe et al. 2013).

Plant responses to swelling galls involve a complex network of signaling mechanisms, which coordinate to regulate many complex changes, including cell expansion and biochemical processes (Bhargava and Sawant 2013; Chaves et al. 2003), but whose responses could vary widely depending on plant species (Ahmad and Prasad 2012). Hormone regulation is known to have a central role in plant responses to swollen gall formations because the kinds and levels of hormones govern diverse eco-physiological processes in plants, for which the fast-acting signaling transduction pathway can induce marked changes and plant expansion (Wilkinson et al. 2012). Both indole 3-acetic acid (IAA) and cytoberberin (CTK) are cell-responsive hormones that play a crucial role in how plants sense the presence of swelling galls in them (Davies et al. 2005; Mizoi et al. 2012). In recent years, for many plant-fungi relationships, the stimulated symptoms will often reveal an imbalance of hormones, as seen in the swelling galls stimulated by microbes via production of IAA and CTK (Doonan and Sablowski 2010; Morrison et al. 2015). The formation of a swelling gall might have evolved from a process that mainly involved the ability of IAA or CTK to inhibit cellular defense responses (Gelvin 2003). This would be a typical interaction in which *U. esculenta* infects *Z. latifolia* and these interactions cause hormonal changes in the plant body, leading to gall formation and swelling expansion. For plant-fungi symbiosis, this hormone production tends to elicit changes in the root system that are often required to facilitate these ecological interactions (Hirsch et al. 1997). However, some pathogenic bacteria whose infection does not produce host cell deformations can also prompt the plant to generate and secrete phytohormones, indicating a likely role for such molecular mechanisms in biological processes other than cell deformation per se. In China and Southeast Asia, swollen galls are harvested as an aquatic vegetable, for placement on the dining table, it being edible and rich nutritious food. Interestingly, *Z. latifolia* is possibly the only cultivated food that arises from a plant-fungi interaction (Zhang et al. 2012). Even though many

researchers have tried to survey plant-fungi interactions at both their hormonal and physiological levels, significant differences in host plant responses to *U. esculenta* as compared with other fungi have yet to be comprehensively investigated or interpreted (Yan et al. 2013; Zhang et al. 2014). Therefore, the symbiosis mechanisms responsible for the formation of plant-fungus swollen galls and their further growth remain to be uncovered, which, if done, could explain how the phytohormone response arises and regulates this process.

Triadimefon (TDF) (CAS: 43121-43-3) is widely used in agriculture and medicine as a fungicide, as it can prevent fungal colonization and inhibit the formation of galls and hyphae. Exogenous spraying of TDF has been studied in many fields for a long time. Such studies report that spraying TDF at the eight-leaf stage of *Z. latifolia* plants can inhibit water-driven swelling of the gall and, within a certain range, it has a discernible dose effect. By observing stem tissue slices, it was recently found that TDF can clearly inhibit *U. esculenta* growth and formation and that *Z. latifolia* gall formation is closely related to the distribution of *U. esculenta* in the host (Li 2016). To further search for related genes participating in the *U. esculenta* infection of *Z. latifolia* and causing gall formations that are closely related, transcriptome analysis is warranted. Previous studies have revealed that, when *Z. latifolia* plants were sprayed with TDF, swollen gall formations are less likely to occur, while the hereditary shape of male *Z. latifolia* can differ from normal *Z. latifolia* (Yan et al. 2013). Treatment with TDF was able to solve the problem of genetic variation. In preliminary work for this study, we sprayed it directly onto *Z. latifolia*, and these results showed no *U. esculenta*-colonized plants and no swelling gall formation in them. Thus, using TDF may help in the necessary further investigations of *Z. latifolia* gall information at the molecular level.

Currently, the study of *Z. latifolia* molecular biology is still in the early stage. Transcriptome methods have been successfully used to explore a wide variety of plant development processes and pathogen-infection interactions (Guo et al. 2015). Although some researchers (Guo et al. 2015) have tried to explain the detailed signal pathways related to the gall-formation mechanism in *Z. latifolia*, many questions remain unanswered. In this study, we analyzed the swelling formation of gall in *Z. latifolia* infected with *U. esculenta*. Ultrastructure, paraffin sections, and scanning observations were all made to look for the distribution and abundance of *U. esculenta*. TDF was used to eliminate the effect of *U. esculenta* infection on hosts, and transcriptome sequencing was carried out before and after in normal (control) and TDF-treated plants, so we could analyze the transcriptome spectrum of differentially expressed genes (DEGs) and detect the full gene expression spectrum. In this way, these study results may provide new, timely insights into the gall formation mechanism of *Z. latifolia*.

RESULTS

Morphological observations and period identification on the whole life cycle of *Z. latifolia*.

The phases of *Z. latifolia* development and growth had not yet been determined but discerning these was crucial for using an accurate period in the experiment and its TDF treatment for subsequently experiments. So, we observed and photographed the *Z. latifolia* at 10-day intervals starting 20 days after transplanting. The photos were divided into a front view, cross section, and vertical section (Fig. 1). In the 150 days after setting, we took photos every three days; a total of 27 groups of photos were taken in the entire process. The before gall formation period was divided as follows: i) three leaves (including three leaves) and 28 days after transplanting; ii) the fifth-leaf stage, 79 days after the transplanting of *Z. latifolia*; iii) the

seventh-leaf stage, 110 days after the determination of *Z. latifolia*; and iv) 153 days after transplanting *Z. latifolia*. Periods after gall formation were i) 156 days after transplanting, ii) 159 days after transplanting, iii) 162 days after transplanting, iv) 165 days after transplanting, and v) 168 days after transplanting. The observation of the periods laid a foundation for further before and after gall formation study of *Z. latifolia*.

Microscopic observations of *U. esculenta* in different sections reveal its invasion process of *Z. latifolia*.

In the normal growth of *Z. latifolia*, the formation of swelling galls was evident (Fig. 2A). However, treating *Z. latifolia* with TDF prevented the formations of swollen galls (Fig. 2B). We observed the samples of before and after gall formation in untreated (CK) and TDF plants. It was found that no swelling gall had formed early in plant development but in the late period of the CK group, the *Z. latifolia* stem continued to swell in size (Fig. 2C). Optical microscopy (aniline blue staining) showed that the hyphae could only be observed after gall formation in CK, based on examinations of ordinary sections and paraffin sections (Fig. 2D and E). The infection processes of *U. esculenta* in the gall of CK and TDF treatment were also observed using scanning electron and ultrastructure microscopy methods (Fig. 2F and G). As shown in Figure 2F and G, no difference was apparent between JB_A and JB_C before any swelling galls formed. In JB_B, however, we find many round spores. These sporophytes were clustered together and attached to the cell wall. The fungal cell wall can be clearly seen in the ultrastructure images, closely attached to the plant cell wall, where it may communicate with the plant body.

Analysis of different hormone responses to *U. esculenta* infection.

To detect the levels of hormonal response to *U. esculenta* infection, ultra-high performance liquid chromatography (UHPLC) was used to measure IAA, ABA (abscisic acid), Z+ZR (zeatin and trans zeatin riboside), and GA₃ (gibberellin 3) contents (Fig. 3) in *Z. latifolia* before and after gall formation in both CK and TDF groups. As Figure 3A shows, the Z content was high in JB-B, but it decreased sharply, by 8.21%, from JB-B to JB-D, with no significant change from JB-A to JB-C. To our surprise,

we also found that GA₃ and IAA contents exceeded the others, suggesting the contents of IAA, ABA, and GA₃ may be driven by the *U. esculenta* infection-plant symbiosis, which could have stimulated the plant to produce a large amount of growth hormones to cause the characteristic swollen gall formation. Both Z and ZR belong to cytokine (CTK) and are usually considered as CTK together. We found that the content of JB-B in these ratios was always higher than that of JB-D, while there was no significant change in JB-A and JB-C. According to Figure 3, we found that there was a significant difference in IAA, GA₃, and Z+ZR in JB-B versus JB-D. These results indicated that *U. esculenta* infection of *Z. latifolia* could stimulate the host to produce a large amount of the growth-promoting hormones that fuel gall formation and expansion.

Identification of DEGs by RNA-seq.

To study the differences in RNA-seq between *U. esculenta*-infected and noninfected *Z. latifolia* plants before and after gall formation, three biological replicates were used per group, for a total of 12 complementary DNA (cDNA) samples (Table 1; Supplementary Table S1), a total of 84.42 Gb of clean reads was generated. A total of 17,541 DEGs were optimized by known structures (Fig. 4A). The significant Pearson correlations (Supplementary Fig. S1) obtained between the FPKM (fragments per kilobase per million reads) distributions of biological replicates of all samples confirmed the high reproducibility of our sequencing data. As Figure 4 shows, there were respectively 3,122, 2,672, 6,704, and 5,042 DEGs, identified by the DESeq R package, based on FPKM data in JB_B versus JB_A, JB_D versus JB_B, JB_D versus JB_C, and JB_C versus JB_A, respectively. Venn and Upset plot diagrams were made based on the up- and down-regulation data in Figure 4C and D, from which it emerged that the four sample groups did not share all upregulation or down-regulation DEGs in common, suggesting that the gall before and after formation and TDF reached the expected effect in *Z. latifolia*.

Gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses for all DEGs.

To identify the similarities and differences between the pairings of JB_B and JB_A and JB_D and JB_B in transcriptomes

Fig. 1. Observations on the growth and development period of *Zizania latifolia* plants. We confirmed the before and after morphological changes in swelling gall formation of *Z. latifolia*. DAT = days after transplanting.

Fig. 2. Sample pictures, ordinary sections, and paraffin sections were observed in different samples. **A**, Normally grown *Zizania latifolia* with swollen gall formations. Arrows point to swelling gall formation (GF) and young gall formation (YGF). **B**, Triadimefon (TDF)-treated *Z. latifolia* with no formation of swollen gall. Arrows point to gall fibrous root (FR), rhizome (RH), and young plants (YP). **C**, JB-A shows control (CK), before swollen gall formation in *Z. latifolia*; JB-B shows CK, after swollen gall formation in *Z. latifolia*; JB-C shows TDF treatment, before swollen gall formation in *Z. latifolia*; JB-D shows TDF treatment after swollen gall formation in *Z. latifolia*. Bar = 1 cm. **D**, The *Ustilago esculenta* was observed by aniline blue stain in different samples. The arrows indicate the presence of clustered *U. esculenta*. Bar = 100 μ m. **E**, Paraffin sectioning, showing the clear observation of cell size and fungus using optical microscope. Bar = 100 μ m. **F**, Different samples were observed by scanning electron microscope. P points to the plant, S to the sporophore of *U. esculenta*. Bar = 5 μ m. **G**, Transmission electron microscopy images from before and after swollen gall formations of *Z. latifolia*. PCW = plant cell wall, H = hyphae, FCW = fungal cell wall. Bar = 500 nm (panels 1 and 3) and 1 μ m (panels 2 and 4).

under *U. esculenta* infection, their corresponding DEGs were subjected to GO classification and KEGG functional enrichment analysis. In comparing the distribution of these DEGs and all top-10 ranked genes, they were allocated to three categories: biological process, cellular component, and molecular function (Supplementary Fig. S2; Supplementary Table S3). The most common GO terms obtained were 'plant-type cell wall,' 'DNA-binding transcription factor activity,' and 'extracellular region'. Therefore, the corresponding genes of

these important terms might play a central role in defending against *U. esculenta* infection (or perhaps tolerating it). Many different genes associated with cell wall-loosening factors (e.g., *EXPA*, *EXPB*, *XTHs*, *FLAs*) had also been altered. The KEGG enriched top-20 pathways of these two pairings are shown in Figure 5B and D. For plant hormone signal transduction (Ko04075), 48 and 37 DEGs were identified in the JB_B versus JB_A and JB_D versus JB_B pairings, respectively (Supplementary Fig. S5; Supplementary Table S4).

Fig. 3. Content of hormones in *Zizania latifolia* plants before and after gall formation under the untreated and triadimefon (TDF) treatments. The hormones determined were **A**, indole 3-acetic acid (IAA), **B**, Z+ZR (zeatin and trans zeatin riboside), **C**, GA₃ (gibberellin 3), and **D**, ABA (abscisic acid). Values represent the mean $\pm$ standard error ($n = 3$). Lower case letters above bars indicate significant differences at $P < 0.05$, according to Duncan's multiple range tests.

Table 1. Quality assessment of raw RNA-seq data

Sample	Clean_reads (Million)	Clean_bases (Gb)	Valid_bases (%)	Q30 (%)	GC (%)
Sample_JB_A1	48.05	7.02	95.46%	95.80%	52.92%
Sample_JB_A2	48.13	7.03	95.54%	96.03%	52.61%
Sample_JB_A3	47.97	6.99	95.03%	95.45%	52.86%
Sample_JB_B1	48.00	7.00	95.33%	95.70%	52.80%
Sample_JB_B2	48.87	7.11	95.29%	96.06%	52.59%
Sample_JB_B3	48.57	7.07	95.27%	95.94%	52.70%
Sample_JB_C1	47.97	6.98	95.04%	95.75%	53.52%
Sample_JB_C2	48.77	7.10	95.22%	95.97%	53.18%
Sample_JB_C3	48.25	7.04	95.39%	95.91%	53.00%
Sample_JB_D1	48.67	7.06	94.86%	96.09%	52.17%
Sample_JB_D2	48.04	6.97	94.61%	95.72%	52.16%
Sample_JB_D3	48.72	7.05	94.42%	95.75%	52.23%

The divergent expression patterns of hormone metabolism-related DEGs between the CK and TDF groups suggest that plant hormones play a key role in governing responses to *U. esculenta* infection in *Z. latifolia* hosts.

Regulation overview, abiotic stress enrichment, and transcription factor (TF) analyses of all DEGs.

Regulatory pathways in JB_D versus JB_B were studied with the MapMan tool for annotation analyses (Fig. 6; Supplementary Table S2), and a regulation overview enrichment of DEGs that were up-regulated and functionally enriched in TFs included the homeobox TF family and receptor kinases (Fig. 6A). Some pathways, including those of ‘protein modification,’ and ‘calcium regulation,’ were also up-regulated or down-regulated (Supplementary Table S2 A) in comparing the response of JB_D versus JB_B. Most DEGs linked to plant hormones were associated with upregulation of cytokinin, ethylene (ETH), and IAA, with only gene expression for ABA undergoing a down-regulation (Fig. 6A; Supplementary Fig. S3; Supplementary Table S3A). Through abiotic stress analysis (Fig. 6B), the relevant genes could be divided into signal transduction, pathogenesis-related (PR) protein, TFs, hormones ABA, salicylic acid (SA), jasmonic acid (JA), and ETH, thus further supporting our inference of the crucial participation of these

pathways in the symbiotic environment, for the regulated sensing and promotion of responses to *U. esculenta* infection in *Z. latifolia*. A more detailed list of all the DEGs corresponding to MapMan functional categories is provided (Supplementary Table S3). The TFs involved were primary regulators of DEGs expression and perform significant functions in the transcriptional symbiosis of plant-fungi genes after infection by *U. esculenta*. Going further, we described TFs in detail and found that homologous box (HB) and basic helix-loop-helix (bHLH) had more differentially upregulated expression (Fig. 6C). Many TFs were significantly altered, as inferred by changes in expression of their corresponding genes. Among them, the auxin (AUX) response factor (ARF) family is an important participant, for which the *ARF10* and *ARF21* genes were respectively down-regulated by 4.45-fold and 5.01-fold (Fig. 7; Supplementary Table S6) in JB_D versus JB_B. We show that the expression levels of 308 putative TF-relative genes can be divided into 42 TF families in the JB_D versus JB_B pairing (Supplementary Table S3C). These TFs might be involved in hormone regulation, cell differentiation, and expansion, as well as expression patterns in different organisms after they undergo *U. esculenta* infection. In sum, these TFs seem to be linked to stimulating the response in *Z. latifolia* to gall formation, according to the annotation information. In the

Fig. 4. Numerical values of differentially expressed gene (DEG) analysis. **A**, For the RNA-seq data set, the number of all genes and the number of up- or downregulated genes in four comparison groups. The numbers of upregulated genes are in red, the downregulated genes in blue. **B**, Venn and Upset plot diagrams of the DEGs in the different group comparisons. The numbers indicate unique and common DEGs in three replicates for the different comparisons. **C** and **D**, Venn and Upset plot diagrams of the upregulated and downregulated genes in different comparisons.

comparison of JB_D versus JB_B, the majority of DEGs were down-regulated and functioned in the enrichment of TFs including ARF, bHLH, receptor kinases, and HB. Receptor kinases are involved in a variety of plant responses, including hormone sensing and reacting to pathogens, making them key players in defense responses.

Changes in expression of plant hormone signal transduction pathway-related genes after *U. esculenta* infection.

The expression of genes related to plant hormone signal transduction indicated meaningfully dynamic changes that occurred during the *U. esculenta* infection process. These DEGs were related to IAA, CTK, GA, ABA, ETH, JA, and SA hormones (Fig. 7; Table 2) and were observed as follows in the four pairing: 37 for JB_D versus JB_B, with all DEGs associated with plant hormone signal transduction (Supplementary Fig. S5).

The IAA-related pathways had the greatest response to plant phytoplasma infections. In the IAA signal transduction pathway, nine genes were up-regulated in JB_D versus JB_B, including four AUX-responsive proteins (AUX/IAA), three AUX transporter-like proteins (AUX influx carrier family), one AUX-responsive GH3 gene family (GH3), and one ARF. By

contrast, three AUX/IAA (*Zlat_10001127*, *Zlat_10020059*, *Zlat_10033997*) proteins, and an AUX transporter-like protein (AUX1, *Zlat_10014750*) were respectively down-regulated by -1.26 , -1.11 , -1.52 , and -2.29 -fold, in the JB_D versus JB_B pairing. Most of the downregulated expression levels indicated that IAA signaling was obviously induced by *U. esculenta* infection. We suspect that AUX may result from gall formations caused by the growth substances produced by the infection of *U. esculenta*. In the CTK-signaling transduction pathway, two DEGs (*Zlat_10010976*, *Zlat_10018529*) were found upregulated (1.31-fold and 1.22-fold, respectively), including the two-component response regulator ARR (B-ARR). In the GA-signaling pathway, an encoding TF (phytochrome-interacting factor 4, *Zlat_10039510*) was significantly down-regulated for JB_D versus JB_B. Interestingly, ten ABA-related DEGs were up-regulated with respect to the JB_D versus JB_B signal transduction pathways (Fig. 7). The ABA receptor protein phosphatase 2C (PP2C), serine/threonine-protein kinase (SnRK2), and ABA-insensitive (ABF) TF family each had upregulated DEGs in the signal transduction pathway and they participate in the negative regulation of ABA signaling. For example, PP2C (*Zlat_10022265*) was up-regulated by 4.96-fold in JB_D versus JB_B. Upregulation of these key DEGs in the ABA pathway indicated that ABA expression might be increased

Fig. 5. Classification and enrichment analyses of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. **A** and **C**, The histograms of the KEGG pathways classification for the differentially expressed genes (DEGs) of JB_B versus JB_A and JB_D versus JB_B, respectively. **B** and **D**, Bubble diagrams of top 20 enriched KEGG pathways for the DEGs of JB_B versus JB_A and JB_D versus JB_B, respectively. Dot size represents the number of genes, and its color the q-value range.

after the TDF treatment causes cell senescence. Two genes involved in JA signaling were also down-regulated (*JAR1*, *J-aminio synthetase*) for JB_D versus JB_B. From seven SA-related genes identified here, six were related to the transduction

factor TGA and one to the regulatory nonexpresser of PR genes 1 (*NPR1*). Generally, most of the DEGs were up-regulated. A few SA-related DEGs were nonetheless down-regulated, such as one associated with the regulatory protein NPR1 genes (upregulated

(Continued)

Fig. 6. Regulation overview of differentially expressed genes (DEGs) and transcriptional responses, visualized using Mapman software, for JB_D versus JB_B. **A**, Regulation overview of enrichment among DEGs comparing untreated (CK) and triadimefon (TDF) treatments after swollen gall formation in *Zizania latifolia* plants. **B**, Biotic stress enrichment among the DEGs. **C**, Main transcription factor family enrichment pathway among the DEGs. Transcriptional changes occurring after gall formation in *Z. latifolia* groups of CK and TDF. Genes in blue or red correspond to those significantly upregulated or downregulated in *Ustilago esculenta*-infected gall samples relative to DEGs. Individual genes are represented by small squares. The vertical color scale displays the transformed log₂-fold changes.

by 2.16-fold). In sum, most of the genes involved in IAA, CTK, and SA underwent downregulation in their expression, corresponding to downregulated hormone contents after *U. esculenta* infection. Conversely, most of the gene expression patterns involving SA, JA, and ABA metabolism and signal transduction were up-regulated by *U. esculenta* infection of in *Z. latifolia* (Fig. 7; Table 2).

Expression of plant hormone biosynthesis genes and their quantification by quantitative real time PCR (qRT-PCR).

A set of 25 DEGs (Fig. 8) were selected for real time qPCR analysis to confirm their functioning in the *Z. latifolia* response to *U. esculenta* infection. Comparing the transcriptome data with qRT-PCR results yielded a relatively strong correlation ($R^2 = 0.9723$) (Supplementary Fig. S4), thus validating the robustness of the RNA-seq analysis in this study. Of the candidate DEGs, we chose 25 related to hormones, 10 related to IAA, four to CTK, three to ABA, three to SA, three to JA, and two to GA. Among these 25 DEGs, the upregulation of 20 and the downregulation of five were confirmed by the qRT-PCR in JB_D versus JB_B, which agreed with the RNA-seq analysis (Fig. 8).

The ARF genes are central components of plant AUX signal transduction. To better understand how IAA and CTK may participate in and affect swollen gall formations, we identified genes encoding IAA and CTK signal transduction at the host gall tip and regulated their expression during gall formation. These candidates included 10 IAA genes (*ZLAA23*, *ZLAA2*, *Os11g0169200*, *ZLSAUR39*, *ZLPLC2*, *ZLYUC11*, *ZLAA9*, *ZLAA10*, *ZLARF21*, *ZLAA50*) and four CTK (*ZLCKX8*,

ZLBPA1, *ZLRR1*, *ZLRR4*) hormone-related genes. We detected that *ZLAA23*, *ZLAA2*, *Os11g0169200*, *ZLSAUR39*, *ZLPLC2*, *ZLYUC11*, *ZLAA10*, *ZLARF21*, and *ZLAA50* were not obviously affected by *U. esculenta* infection before gall formation. Yet, during or after *U. esculenta* infection, their expression levels were significantly induced (Fig. 8). Importantly, the expression of *SAUR39* and *PLC2* (Fig. 8; Supplementary Table S6) were down-regulated by the greatest magnitude (21.06- and 10.42-fold, respectively). Similarly, *ZLCKX8*, *ZLBPA1*, and *ZLRR4* were expressed consistently in the CK group before gall formation, but, with *U. esculenta* present, its expression level was obviously up-regulated in *Z. latifolia*.

Interaction network constructed based on the plant hormones and cell wall-related genes.

We selected many of those genes found related to plant hormones and cell walls to construct their interaction network (Fig. 9). In this figure, the outermost layer is related to cell-wall genes. Interestingly, we found that most of the genes were, in fact, related to cell wall softening-factor genes, while those most clearly expressed internally are AUX-related genes (Fig. 9A), a relationship that was particularly strong. Meanwhile, we selected genes for GO and KEGG annotation analysis. Most of them are located in plant hormone signal transduction and cell wall biogenesis, according to the KEGG annotation; in the GO analysis, we found that most of the genes are in the nucleus that controls gene expression (Fig. 9B).

We discerned significant differences in the expression of related DEGs in the cell walls of *U. esculenta*-infected *Z. latifolia* (Fig. 6B). Among these DEGS, the β -expansin gene (*EXPB11*) had the strongest downregulation in JB_D versus

Fig. 6. (Continued from previous page)

JB_B, but many similar genes were also down-regulated (*EXPB3*, *EXPB6*, *EXPB2*, and *EXPA8*, among others). Meanwhile, xyloglucan endotransglucosylase/hydrolase (XTH)-related genes were down-regulated, namely *XTH32*, *XTH28*, *XTH31*, and *XTH8*. Fasciclin-like arabinogalactan (FLA) proteins are an important component of cell-wall genes (Supplementary Table S7), for which large numbers of FLA-type genes (i.e., *FLA1*, *FLA2*, and others) were down-regulated in the transcriptome (Supplementary Fig. S2). AUX is considered pivotal to controlling the invasion of *U. esculenta*, given that AUX/IAA and ARF were expressed most strongly in the gene expression pattern analysis, with AUX1 upstream initiating AUX expression only after *U. esculenta* infection. During infection by *U. esculenta*, CTK, a hormone capable of promoting cell proliferation, can act as the second messenger of IAA signaling while also promoting gall expansion (Fig. 9).

Moreover, IAA transport enhances levels of GA by promoting its TF and upstream genes. Therefore, positive or negative interactions between IAA and CTKs, GA, ABA, JA, and SA can be observed during the gall formation process of *U. esculenta* infection, which implicates IAA as the cornerstone of this regulatory network (Fig. 9A). In this way, we surmised that cell wall-loosening factors clustered around the hormones, with AUX positioned at the center and other hormones functioning to regulate the expansion of the gall in *U. esculenta*-infected *Z. latifolia*.

DISCUSSION

Many fungi interact with their plant hosts in beneficial ways, such as rhizobium symbiosis (Sanders 2011), or they interact with plant hosts in harmful ways, such as in the case of fungal

Fig. 7. Expression of genes involved in hormones for JB_D versus JB_B. Red indicates the upregulated genes, blue the downregulated genes, by heatmap. Their values were log₂-fold change. AUX1 = auxin transporter-like protein; TIR1 = protein transport inhibitor response 1; AUX/IAA = AUX responsive protein indole 3-acetic acid; ARF = AUX response factor; GH3 = AUX-responsive GH3 gene family GH3; SAUR = AUX-responsive protein SAUR; CYCD3 = cyclin D3, plant; JAR1 = jasmonic acid-amido synthetase JAR1; COI1 = coronatine-insensitive protein 1; JAZ = protein TIFY; MYC2 = transcription factor MYC; PYR/PYL = abscissic acid receptor PYR/PYL family; PP2C = protein phosphatase 2C; TF = phytochrome-interacting factor 4; SnRK2 = serine/threonine-protein kinase SRK2; ABF = abscissic acid-insensitive; AHP = histidine-containing phosphotransfer protein; A/B-ARR = two-component response regulator ARR; GID1 = gibberellin receptor GID1; GID2 = gibberellin receptor GID2; DELLA = DELLA protein RG; NPR1 = nonexpresser of PR genes 1; TGA = transcription factor TGA; PR-1 = pathogenesis-related protein 1.

Table 2. Identities and analysis of differentially expressed genes enriched in plant hormone signal transaction pathways of JB_D vs. JB_B

Hormone category/ gene ID	Log ₂ fold change	P value	Up/down	Gene symbol	Pathway position	Description
Salicylic acid						
Zlat_10015168	3.038	0.036601284	Up	TGAL5	TGA	Os05g0443900, partial (<i>Oryza sativa japonica</i> group)
Zlat_10021635	1.404	0.026299925	Up	TGAL3	TGA	Predicted: transcription factor (TF) HBP-1b(c38)-like (<i>Oryza brachyantha</i>)
Zlat_10021667	-2.160	1.98147E-18	Down	TGAL10	TGA	Os08g0176900, partial (<i>Oryza sativa japonica</i> group)
Zlat_10031473	3.569	3.70428E-11	Up	TGAL1	TGA	Hypothetical protein BRADI_2g52860v3 (<i>Brachypodium distachyon</i>)
Zlat_10032525	1.860	2.10235E-10	Up	TGAL1	TGA	Predicted: TF HBP-1b(c1)-like isoform X2 (<i>Oryza brachyantha</i>)
Zlat_10037899	2.407	0.002668809	Up	TGAL4	TGA	Predicted: TF TGA6-like (<i>Oryza brachyantha</i>)
Zlat_10044810	2.444	2.12905E-11	Up	NH5.2	TF	Hypothetical protein PAHAL_8G025500 (<i>Panicum hallii</i>)
Jasmonic acid (JA)						
Zlat_10002850	-1.121	0.010941287	Down	GH3.5	SnRK2	Predicted: JA-amido synthetase JAR2-like (<i>Oryza brachyantha</i>)
Zlat_10006334	-1.258	2.99678E-08	Down	GH3.5	SnRK2	Predicted: JA-amido synthetase JAR1 (<i>Oryza brachyantha</i>)
Indole 3-acetic acid (IAA)						
Zlat_10000330	1.072	7.92464E-08	Up	Os01g0856500	SnRK2	Predicted: auxin (AUX) transporter-like protein 1 (<i>Oryza brachyantha</i>)
Zlat_10000572	1.070	0.035011956	Up	IAA27	PYR/PYL	Predicted protein (<i>Hordeum vulgare</i> subsp. <i>vulgare</i>)
Zlat_10001127	-1.261	0.001443811	Down	IAA23	PP2C	AUX-responsive protein IAA23 (<i>Setaria italica</i>)
Zlat_10005792	3.108	2.25714E-09	Up	ARF11	PP2C	RecName: Full = AUX response factor 11; AltName: Full = OsARF5
Zlat_10014750	-2.970	2.88514E-57	Down	Os11g0169200	PP2C	Predicted: putative AUX transporter-like protein 4 (<i>Oryza brachyantha</i>)
Zlat_10015270	1.248	0.002458758	Up	IAA6	PP2C	Predicted: AUX-responsive protein IAA6-like isoform X3 (<i>Oryza brachyantha</i>)
Zlat_10015482	2.632	7.39457E-05	Up	LAX2	NPR1	Predicted: AUX transporter-like protein 2 (<i>Oryza brachyantha</i>)
Zlat_10015922	1.354	0.009884568	Up	IAA7	JAR1	AUX-responsive protein IAA7 (<i>Dichanthelium oligosanthos</i>)
Zlat_10020059	-1.110	3.44535E-14	Down	IAA2	JAR1	Hypothetical protein OsI_00719 (<i>Oryza sativa indica</i> Group)
Zlat_10032090	1.085	1.12277E-06	Up	IAA6	GH3	Predicted: AUX-responsive protein IAA6-like isoform X3 (<i>Oryza brachyantha</i>)
Zlat_10032626	1.090	2.93836E-15	Up	Os10g0147400	CYCD3	LAX protein (<i>Phyllostachys edulis</i>)
Zlat_10033997	-1.522	5.01385E-29	Down	IAA30	BK11	AUX3 protein (<i>Phyllostachys edulis</i>)
Zlat_10043397	1.079	1.8115E-05	Up	GH3.4	B-ARR	Hypothetical protein OsI_20501 (<i>Oryza sativa indica</i> group)
Gibberellin (GA)						
Zlat_10039510	-1.993	0.010678803	Down		B-ARR	Hypothetical protein OsI_12756 (<i>Oryza sativa indica</i> group)
Cytoberberin						
Zlat_10010976	1.309	0.002744161	Up	RR25	AUX1	Predicted: two-component response regulator ORR25-like (<i>Oryza brachyantha</i>)
Zlat_10018529	1.222	0.006836765	Up	RR22	AUX1	Two-component response regulator ORR22 (<i>Oryza sativa japonica</i> group)
Brassinosteroid						
Zlat_10018497	1.073	0.000815632	Up	CYCD3-2	AUX1	Cyclin-D3-2-like (<i>Aegilops tauschii</i> subsp. <i>tauschii</i>)
Zlat_10043001		0.016310208	Up	BKI1	AUX1	Probable BRI1 kinase inhibitor 1 (<i>Aegilops tauschii</i> subsp. <i>tauschii</i>)
Abscisic acid (ABA)						
Zlat_10005614	-1.173	6.27066E-05	Down	PYL4	AUX/IAA	ABA receptor 7 (<i>Oryza sativa indica</i> group)
Zlat_10014121	1.239	1.83641E-07	Up	Os03g0268600	AUX/IAA	Predicted: probable protein phosphatase 2C 30 (<i>Oryza brachyantha</i>)
Zlat_10015895	1.596	7.49921E-06	Up	TRAB1	AUX/IAA	ABSCISIC ACID-INSENSITIVE 5-like protein 5 (<i>Zea mays</i>)
Zlat_10022265	4.958	1.22449E-05	Up	Os04g0167900	AUX/IAA	Predicted: probable protein phosphatase 2C 37 (<i>Oryza brachyantha</i>)
Zlat_10023618	1.085	6.1928E-10	Up	Os05g0537400	AUX/IAA	Predicted: probable protein phosphatase 2C 50, partial (<i>Oryza brachyantha</i>)
Zlat_10029966	1.930	0.000882927	Up	Os01g0656200	AUX/IAA	RecName: Full = Probable protein phosphatase 2C 8

(Continued on next page)

Table 2. (Continued from previous page)

Hormone category/ gene ID	Log ₂ fold change	P value	Up/down	Gene symbol	Pathway position	Description
Zlat_10032372	-1.019	2.24929E-05	Down		AUX/IAA	Hypothetical protein BRADI_2g24120v3 (<i>Brachypodium distachyon</i>)
Zlat_10037460	-1.523	0.015159773	Down	SAPK4	ARF	Os05g0433100 (<i>Oryza sativa japonica</i> group)
Zlat_10039544	1.077	8.18272E-09	Up	SAPK10	ABF	Serine/threonine-protein kinase SAPK10 isoform X2 (<i>Oryza sativa japonica</i> group)
Zlat_10046325	1.041	0.002060567	Up	SAPK7	ABF	Serine/threonine-protein kinase SAPK7 (<i>Oryza sativa japonica</i> group)

diseases (Dean et al. 2012). Fungal infection and host defense responses are crucial events involved in plant-fungus interactions (Baker et al. 1997). Yet it is currently not fully understood how fungi alter their physiology to adapt to host environments in plant-fungus interactions. The molecular mechanism by which a plant attacks a pathogen is a complex process. *Z. latifolia* is susceptible to infection with *U. esculenta*, which stimulates the emergence and enlargement of a swollen gall under appropriate conditions (Bruce et al. 2011). Therefore, the current study provided an RNA-seq profile of TDF treatment response to infection from *U. esculenta*, which causes gall formation. Interestingly, the results showed no swollen gall formations were observable in the JB_D group while, in the JB_B group, swelling galls did form because of the phytoplasma infection (Fig. 2C). The DEG analysis of *Z. latifolia* gall formation indicated that several significant biological processes were altered by the *U. esculenta* infection, which provides insight into the molecular interactions between *Z. latifolia* and *U. esculenta*.

***Z. latifolia* response to *U. esculenta* infection growth stage and microscopic observation.**

In previous studies, researchers have coarsely classified the growth stages of *Z. latifolia* (Yan et al. 2013), but their exact timing and duration (phases) were not determined. To resolve this matter, we tracked in detail the entire growth and development life cycle of *Z. latifolia*, photographing and observing each ontogenetic phase, which laid a solid foundation for studying *Z. latifolia* as well as for further research (Fig. 1). In the current observation of *Z. latifolia* morphological traits, we learned that the *U. esculenta* generally attached to the cell wall around it and engaged with these cells (Fig. 2). From the scanning sections, we saw massive sporophytes located in the plant cell wall, and further observations of the cell wall shows that the fungal ultrastructure is bound to it; being closely adhered to the cell wall enabled an intimate interaction between plant and fungi. *U. esculenta* may soften cell walls and change related genes. When *U. esculenta* interacts with plants to produce a series of metabolic reactions, plant hormone metabolism is most affected.

***Z. latifolia* RNA-seq analysis reveal DEGs induced by *U. esculenta*.**

Many catalytically active DEGs in the infected galls were abundantly expressed throughout the gall formation process, which may be related to survival and gall cell proliferation of pathogenic fungi in host plant tissues (Kämper et al. 2006). In our study, we paid close attention to changes in the plant-type cell wall and signaling pathway, finding that *U. esculenta* can cause plants to recognize and respond to it as a host plant stimulus (Supplementary Fig. S2); accordingly, this would suggest that *U. esculenta* could play a significant role in protecting plants from other fungal infections (Li et al. 2007). Secondary metabolites, such as lignin biosynthesis, via the phenylpropanoid biosynthesis pathway, can promote plant resistance to pathogens (Rubery and Fosket 1969). We found that most of the MYB TF was up-regulated in the JB_B group (Fig. 6)

and that MYB could regulate the pathways of lignin biosynthesis and phenylpropanoid biosynthesis. This may provide opportunities for the emergence and expansion of cell-wall resistance in multiple plant tissues.

TFs play a central role in plant defense (Singh et al. 2002), which involves a number of transcriptional regulations triggered by endophytic fungi in a host to produce a series of responses to attacking endophytic fungi. TF is a protein that, by binding to specific DNA sequences, regulates genes in a coordinated manner, thereby directing cell division, development, and death. In our study, most TFs were significantly up- or down-regulated (Supplementary Table S2C) in response to *U. esculenta* infection. Transcriptome and qPCR expression analyses of selected DEGs indicated that these TFs could regulate transcriptional changes in response to *U. esculenta* infection. Further research should focus on molecular mechanisms of these TFs to better understand the different regulation mechanisms of IAA-related DEGs conferring a coordinated plant response to *U. esculenta*.

Expression levels of DEGs involved in plant hormone signal transduction changed significantly after *U. esculenta* infection.

Plant hormones play a role in a complex network that regulates plant growth, gall formation, and plant responses to environmental signals (Denancé et al. 2013; Jaillais and Chory 2010; Pieterse et al. 2009). The role of plant-derived hormones in phytoplasma infection resistance has been widely reviewed (De Vleeschauwer et al. 2010; Roberts et al. 2011). Meanwhile, pathogens have evolved complex mechanisms to regulate hormone metabolism and signal transduction in their hosts, thereby promoting their ability to overcome plant defense mechanisms (Dodds and Rathjen 2010). Some hormones, such as IAA and ABA, figure prominently in regulating plant growth and responding to abiotic stresses, but they have recently been shown to play an important role in plant-fungal interactions also (Fu and Wang 2011; Ton et al. 2009). Similarly, in this study, we also observed that *U. esculenta* infection affected gene expression levels related to IAA, CTK, and GA signaling and metabolism. Therefore, during the *U. esculenta* infection period, all plant hormone pathways were linked to each other in a large and complex network, and *Z. latifolia* appears to activate all regulators of IAA, CTK, GA, and ABA in response to endogenous symbiosis with the fungus. Future research on this interaction should focus on detailed comparisons of JB_D versus JB_B-mediated endophytic fungal infection of plants. The majority of genes involved in plant hormone signal transduction of IAA, CTK, and GA were down-regulated and ABA, SA, and ETH were up-regulated at JB_D versus JB_B. We speculate that *U. esculenta* targets the host plant hormone system during its main invasion phase and that these downregulated DEGs may be a host protective response with some stimulants secreted. Those DEGs related to IAA were down-regulated, which may be linked to the higher content of IAA present when broom symptoms are present in pathogenic infections (Zafari et al. 2012).

In the current study, most genes involved in IAA metabolism and signal pathway were down-regulated (e.g., SAUR39, PLC2) in JB_D versus JB_B plants of *Z. latifolia*. Moreover, expression of the SAUR39 gene was rapidly augmented under the action of an AUX-responsive protein, and SAUR39 is thought to play a significant role in the local regulation of AUX polar transport in plant responses against *U. esculenta* infection (Kant et al. 2009). The ARF genes are central components of plant AUX metabolism and signal transduction. The IAA content was greatly increased in the JB_B group (Figs. 3 and 8; Supplementary Table S6), which exhibited the highest expression change among the IAA-related genes. Therefore, the gene for SAUR39 and ARF TFs could be key components for regulating how plants respond to *U. esculenta* infection.

From the KEGG enrichment pathways, we found that ‘phytohormone signal transduction pathway’ and ‘phenylpropanoid

biosynthesis pathway’ had the highest concentrations of KEGG pathways (Fig. 5). In the process of fungal infection, plants usually synthesize flavonoids and lignin by using phenylpropanoid compounds (Dixon et al. 2002). In many plant species, including *Arabidopsis* and *Oryza sativa*, upregulation of AUX/IAA and ARF genes after gall swelling occurs downstream of IAA signal transduction (Bridge et al. 2012; Song et al. 2009). Moreover, previous work has shown that symptomatic *Arabidopsis* plants infected by bacteria had detectable increases in their IAA content (Dolzblasz et al. 2018); similarly, the IAA content was substantially increased in *Z. latifolia* in the JB_B group (Fig. 3A). The ARF gene is a crucial component of an alternative pathway leading to IAA metabolism and signaling transduction (Bargmann and Estelle 2014). AUX positively controls the plants’ gall expansion, growth, and development, whereas IAA pathways can commonly regulate endophytic fungi quite effectively (Khan et al. 2012; Mehmood et al. 2018).

Fig. 8. Expression analysis of hormone-related genes in different groups. The experiment included three biological replicates and used SPSS for the analysis. We chose the following number of hormone-related differentially expressed genes: 10 related to indole 3-acetic acid, four to cytoberberin, three to abscisic acid, three to salicylic acid, three to jasmonic acid, and two to gibberellin. In all panels, the x axis represents the four sample groups: A (JB_A), B (JB_B), C (JB_C), and D (JB_D).

Most of the DEGs related to both IAA and CTK pathways had down-regulated expression in JB_D versus JB_B, resulting in greater JA and SA contents at the initial stage of *U. esculenta* infection in the JB_B group. It seems the combination of DEGs

associated with IAA and CTK hormones has been changed to either foster a synergistic effect against *U. esculenta* infection or is simply a part of the evolved symbiotic relationship between *Z. latifolia* and *U. esculenta*. The hormone ABA

Fig. 9. The interaction network was constructed by plant hormone and cell wall–relative genes in *Zizania latifolia*. **A**, We used interaction network maps to draw the interaction networks among six key plant hormone–related genes (indole 3-acetic acid [IAA], cytoberberin [CTK], salicylic acid [SA], gibberellin [GA], jasmonic acid [JA], abscisic acid [ABA]) and cell-wall wall loosening–related genes (IAA in red, CTK in blue, SA in light blue, cell wall in green, ABA in purple, GA in gray, JA in yellow), finding that cell wall–loosening factors clustered around the hormones. Auxin is the center and other hormones regulated the expansion of gall formations in *Z. latifolia*. **B**, The major genes were analyzed by gene ontology and Kyoto Encyclopedia of Genes and Genomes. The heatmap in each group is represented in the left half.

integrates diverse stress signals to regulate downstream stress response of plants (Hauser et al. 2011; Tuteja 2007). In our study, the DEGs involved in the ABA signal transduction pathway (including *ABF*, *PP2C*, *PYL/PYL*, and *SNRK2*) were significantly down-regulated in the JB_D versus JB_B comparison (Fig. 7; Supplementary Table S6). *PP2C* is a feedback regulation gene in ABA signaling and transcriptionally regulates expression of the ABA-responsive gene (Wang et al. 2019). Therefore, downregulation of the *PP2C* gene indicated that ABA signaling might have been reduced in the plant after its infection with *U. esculenta*. In addition, plant immune signals become activated to inhibit diseases caused by the response effects of ABA signaling (Kim 2012). This result may indicate activation of ABA signaling during gall formation of *U. esculenta* postinfection, but, when the JB_B immune system is recognized, the ABA content undergoes a relative decline.

U. esculenta response to the host is to soften cell walls and further produce large amounts of hormones to promote gall swelling.

A hormone regulation model with plant hormone signal transduction and cell wall-loosening genes functional during *U. esculenta* infection in *Z. latifolia* is depicted in Figures 9 and 10. We devised a working model (Fig. 10) in which IAA plays a significant role in regulating *U. esculenta* infection in *Z. latifolia* plants. On the one hand, *U. esculenta* infection

reduces the IAA level and upregulates *AUX1*, *AUX/IAA*, and *ARF* gene family expression. CTK synthesis is negatively regulated by IAA, and this process depends on the inhibition of B-ARR (Müller and Leyser 2011). One of the dominating responses was enhanced CTK signaling, which may be due to B-ARR-related gene upregulation. Elevated CTK-related gene expression levels and content generate a range of reactions, including cell division, shoot initiation, and gall enlargement. The other response to *PYL/PYL* downregulation is decreased ABA synthesis, followed by upregulation of the *XTHs*, *FLAs*, *EXPA*, and *EXPB* required for the cell wall-loosening factor (Figs. 9 and 10). On the other hand, *U. esculenta* infection stimulates IAA transport and synthesis, which diminishes GA catabolism. However, an increased GA3 content in *Z. latifolia*, possibly by downregulation of TF genes, was also observed in our study, and ABA synthesis was accordingly reduced in response. Additionally, *U. esculenta* infection leads to the upregulation of *ABF*, resulting in the rapid reduction of ABA transduction via SnRK2 activity, which are negative signals for the formation of swelling galls in *Z. latifolia* (Fig. 10). It seems reasonable, then, to infer that host transcriptome hormonal adjustments and cell-wall loosening in this model constitute a key fine-tuning process for the coexistence of *U. esculenta* and *Z. latifolia*. The former can loosen the latter's cell wall, thereby gaining access to the interior of host cells, which stimulates a series of hormone responses in symbiosis with *Z. latifolia*, utilizing in vivo activation of swelling gall formation.

MATERIALS AND METHODS

Plant materials and growth conditions.

The variety used in the experiments was the single-season *Zizania latifolia* cv. Dabieshan No.1, purchased from Yuexi County (Yuexi County Longjing Ecological Agriculture Development Co., Ltd., Anhui, China). Photographs (front view, cross-section, and vertical section) were taken of each plant growth stage to confirm the sampling and treatment periods for experimentation (Fig. 1). Samples, which included the swelling stem, were immediately frozen, using liquid nitrogen flash-freezing, and were stored at -80°C for later use in the relative gene expression research and experiments.

For transcriptome assembly, to test and quantify the effect of swelling gall formation, TDF was used because it prevents and inhibits fungal activity in *Z. latifolia*. The exogenous application of TDF (80 mg per liter) was carried out twice, before and after gall formation, and the spray was applied at intervals of 5 days. Given the known development of *Z. latifolia*, these plants were treated twice, 148 (on September 15, 2018) and 159 (on September 26, 2018) days after transplanting. For subsequent experiments, gall samples were collected at 5 days before and 5 days after gall formation, with minimum gall diameters of approximately 5 cm. Some swelling galls were harvested at each timepoint, for which their sampling was done in triplicate, using freeze tube storage. All samples were placed in cryopreservation tubes and were immediately stored in liquid nitrogen in a refrigerator at -80°C for RNA extraction and several index determinations. Using a 2×2 factorial design, we harvested four groups of samples: CK (control: no treatment) versus TDF-treated *Z. latifolia* plants, sampled either before or after the swollen gall formation occurred in them. The four groups were called JB-A (CK, before swelling gall formation of *Z. latifolia*), JB-B (CK, after swelling gall formation of *Z. latifolia*), JB-C (TDF treatment, before swelling gall formation of *Z. latifolia*), JB-D (TDF treatment, after swelling gall formation of *Z. latifolia*). All experiments were repeated in triplicate.

Fig. 10. Proposed hormone-cell wall loosening model of *Zizania latifolia* plants infected with *Ustilago esculenta*. The fungus *U. esculenta* infects the cells via the cell wall, softening it, eliciting changes in the activity of cell wall-loosening-related genes (*ZIEXPA*, *ZIFLAs*, *ZIXTHs*, and *ZIEXPB*). Dashed lines indicate that further research is needed. Italics represent genes, the red arrows indicate upregulation or increased content, and the blue arrows indicate downregulation or decreased content.

Microscopic observations before and after gall formation in the CK and TDF plants.

To study the interaction relationships of the above four experimental groups, their respective swollen gall samples were stained using aniline blue (CAS number 28631-66-5; Solarbio) according to an improved method (Faoro et al. 2008). Fresh stem tip samples of *Z. latifolia* tissue were sliced and fixed with a fluid containing the Carnot fixative (glacial acetic acid and alcohol, volume ratio of 1:3) and were put into a 2-ml centrifuge tube, were stored in refrigerator at 4°C for 24 h. To it was added the right amount of 10% KOH solution, after which it was subjected to a 85°C high-temperature treatment lasting 1 h; it was washed several times during the KOH solution and, after processing, was used for subsequent dyeing observation. The slices were transferred into a Petri dish containing aniline blue dye and were stained on a horizontal shaker for 5 to 10 min. After decolorization with 75% alcohol, the slices were observed and photographed under a fluorescence microscope (Olympus SZX10). Then, paraffin sections were made and stained with aniline blue for observation under an optical microscope (Nikon Eclipse E100).

To investigate the effect of exogenous TDF on gall swelling and formation at the biological level, the ultrastructure of the gall tip cell was observed using electron microscopy with the following method (Chen et al. 2019). Specifically, the slices were observed under a transmission electron microscope, set to an accelerating voltage of 80 kv (H-7650, Hitachi). In parallel, a scanning electron microscope (Hitachi S-4800) was used to observe the *U. esculenta* spores (Waikhom et al. 2013).

Determination of the plant endogenous hormone contents.

The swollen galls were analyzed before and after gall formation in CK and TDF. The extraction, purification, and determination of endogenous IAA, Z+ZR, GA₃, and ABA by UHPLC followed methodologies described before (Li et al. 2019; Yuan et al. 2008). For these determinations, we used the mixed labeling method to distinguish the respective content of these five hormones in the plants.

Hormone contents were measured by UHPLC, four samples were collected, and the extraction, purification, and measurement protocols were already described by Li et al. (2019). To analyze the hormone contents, a Thermo Fisher Scientific UHPLC UltiMate 3000 was used, equipped with a vacuum degasser, quantitative pump, automatic sampler, thermostatic column chamber, and fluorescence detector. A BETASIL C18 column (Thermo Fisher Scientific; 4.6 × 250 mm, 5 mm) and fluorescence detection wavelength of Eml 1/4 254 nm were both use. Each sample (10 ml) was injected automatically into the UHPLC system, at a flow rate of 1 ml min⁻¹. Quantifications were made by comparing the peak area of a detected compound with the known dose of that hormone.

cDNA library construction and in novo assembly.

As for the microscopic observations and analyses described above, RNA-seq analysis was also performed before and after the gall formation of CK and TDF plants, from which a total of 12 gall-tip samples were taken. To simplify their description and results reporting, we followed the same protocol used for naming the early four groups in naming their three replicates, i.e., JB_A, JB_B, JB_C, and JB_D, respectively. Total RNA was extracted from 12 gall samples before and after gall formation in CK and TDF by using the mir-Vana miRNA isolation kit (mirVana miRNA ISolation kit; Ambion) following manufacturer protocol. The group-specific libraries were constructed with the TruSeq Stranded mRNA LTSample prep kit (Illumina), according to manufacturer instructions. These cDNA libraries were then sequenced on the Illumina

sequencing platform (Illumina HiSeq 2500) to generate 125-bp/150-bp paired-end reads. Then, transcriptome sequencing and raw reads were processed by Trimmomatic (Bolger et al. 2014). To obtain clear reads, those reads containing ploy-N and any low-quality reads were first removed. The ensuing clean reads were then mapped onto the reference genome (in the RiceRelatives genomic database), using the 'HISAT2' alignment software program (Kim et al. 2015). A small proportion of *U. esculenta* was filtered out directly by comparisons with the *Z. latifolia* genome.

Analysis of DEGs, cluster analysis, and GO and KEGG enrichments.

The FPKM (Roberts et al. 2011) value of all genes was calculated by the Cufflinks tool (Trapnell et al. 2010), and the read counts for all genes were obtained using the 'htseq-count' tool (Anders et al. 2015). For transcriptional-level quantifications, the FPKM (Kim et al. 2015) and read counts (protein coding) values for all transcripts were calculated by Bowtie2 (Langmead and Salzberg 2012) and eXpress (Roberts and Pachter 2013). The DEGs were identified using the DESeq package in R (Anders and Huber 2013), which relied on its function estimation size factor and nbinom test; a *P* value < 0.05 and absolute fold change > 2 or < 0.5 were the threshold criteria for designating a gene having significant differential expression among the group pairings. Hierarchical clustering analysis of DEGs was analyzed to further explore patterns of gene expression. Based on the hypergeometric distribution, GO enrichment and KEGG (Kanehisa et al. 2008) pathway enrichment analysis of identified DEGs were respectively implemented in the R program (version: 3.6.1). The read portions were reassembled by StringTie (Pertea et al. 2015), after which the reference genome and known annotated genes were aligned, using the Cuffcompare software program, for both gene structure extensions and new transcript identifications.

Functional annotation and KEGG enrichment pathway analysis.

Identified DEGs were characterized by GO and KEGG enrichment (KEGG database) to characterize biological functions and significantly enriched metabolic pathways or signal transduction pathways. Based on Wallenius' noncentral hypergeometric distribution (Young et al. 2010), DEGs in our study were submitted to GO enrichment analysis, using the 'GO-seq' R package, for their enrichment analysis. The statistical evidence for significant enrichment of DEGs in the KEGG pathway was detected by KOBAS 3.0 (Mao et al. 2005) online. Finally, the MapMan graphing tool was used to depict an overview of the metabolic and regulatory pathways.

qRT-PCR analysis.

To explore the expression patterns of genes participating in gall formation, 10 IAA, four ABA, three CTK, three SA, three JA, two GA-related genes were analyzed in CK and TDF treatment, using real-time qPCR and improved methodology (Chen et al. 2018). Total RNA samples were isolated from different stages with the RNA-prep Pure plant kit (Tiangen). The DNase-treated RNA was extracted and reverse transcribed into cDNA by the Prime Script RT reagent kit (TaKaRa), after which real-time qPCR was implemented with the TB Green Premix Ex Taq II (TaKaRa). The relative expression level of the housekeeping *Actin* gene served as an internal reference for standardizing the data. Three biological replicates were performed per sample, and the relative expression levels were calculated as 2^{-ΔΔC_t} (Livak and Schmittgen 2001). The real-time qPCR primer sequences used for this can be found in Supplementary Table S5. These results are displayed as the

mean $\pm$ standard deviation (biological replicates, $n = 3$). The relative expression data were statistically analyzed using SPSS software. Every sample consisted of three biological replicates.

Statistical analysis.

Our experimental work used a completely randomized block design. The data were analyzed in SPSS software, by applying analysis of variance and using Duncan's multiple range test. $P < 0.05$ was considered significant.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

KEGG database: <http://www.genome.jp/kegg>
KOBAS 3.0 analysis tool: <http://kobas.cbi.pku.edu.cn>
MapMan graphing tool: <https://mapman.gabipd.org>
RiceRelatives genomic database: <http://ibi.zju.edu.cn/ricerelativesgd>

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