

Transcriptome Analysis Reveals the Symbiotic Mechanism of *Ustilago esculenta* induced Gall formation of *Zizania latifolia*

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Research article

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Abstract

Background: *Zizania latifolia* is a perennial aquatic vegetable due to the establishment of symbiosis between *Z. latifolia* and *U. esculenta* results in the swelling gall formation. However, the mechanism of symbiosis for the gall formation in *U. esculenta* infected *Z. latifolia* is still unclear. To understand the mechanism of symbiosis for the gall formation in *U. esculenta* infected *Z. latifolia*, we analyzed *Z. latifolia* and *U. esculenta* symbiotic circumstances using Triadimefon (TDF) treatment and RNA-seq technique. **Results:** In this present study, 4 different samples were induced under gall before and after formation of CK and TDF treatment in *Z. latifolia*. Whole growth cycles were recorded and microstructure observation showed that the presence of *U. esculenta* could be clearly observed after the gall formation, after TDF treatment were not found *U. esculenta*. A total of 17541 differentially expressed genes (DEGs) were identified based on transcriptome. Meanwhile, GO term and KEGG pathway analyses, plant hormone metabolism, signal transduction and cell wall loosening factors were significantly enriched due to *U. esculenta* infected *Z. latifolia*, and relative expression levels of hormone-related genes were identified, among which IAA-relative DEGs up-regulation was the most obvious in JB_B by qRT-PCR. As revealed by UHPLC analysis, the IAA, GA, Z and ZR content were increase while *U. esculenta* infected and ratio of these hormones to ABA also increased. **Conclusion:** In this study, we come up with the hormone-cell wall loosening model to study symbiotic mechanism of gall formation after *U. esculenta* infected *Z. latifolia*. Meanwhile, our study provides a new perspective for studying the physiological and molecular mechanisms of *U. esculenta* infection *Z. latifolia* that causes swelling gall formation and theoretical basis for further increasing the future yield of *Z. latifolia*.

Background

Zizania latifolia (Griseb.) Stapf, known as wild rice [1], is the only member of the wild *Oryza sativa* genus *Zizania* native to Asia belong to Gramineae. *Z. latifolia* (*Zizania latifolia*) was one of the 6 significant grain crops in ancient China [2]. *Z. latifolia* is perennial aquatic vegetable grown in lakes, ponds originated from China and southeastern Asia [3]. It has been documented to be an asexual aquatic vegetable with unique flavor and texture that has been cultivated in east and southeast Asia for more than 1500 years due to the colonization of *U. esculenta* [4]. *Z. latifolia* are cultivated as a delicious and nutritious vegetable, containing many sugar, protein and minerals along with some human body essential amino acids [5]. Additionally, it can also regulate the body relieve cough, damp and heat and prevent poisoning induced by alcohol as a medicinal plant.

It is used as an aquatic vegetable, with both the swelling gall, rhizomes and grain being edible. However, after long-time evolution, its use as a grain has completely disappeared in China, though it continues to be cultivated for its swelling gall. *Z. latifolia* is usually parasitized by *U. esculenta*, which stimulates enlargement of the swelling gall under suitable conditions [6, 7]. The success of the swelling gall formation depends on the *U. esculenta* infection. It has been documented that be eaten as a delicious aquatic vegetable for evolution over the years to form a variety of white varieties all over the world, due to the colonization of *U. esculenta*, belong to *Ustilaginaceae* of basidiomycetes. The fungus infection within *Z. latifolia* and stimulus swelling gall formation called normal Jiaobai in China [8]. When the fungus infection

the host plant it causes it to expansion and cells size and number increasing formation swell into juicy galls. However, due to *U. esculenta* infection can completely inhibit inflorescence growth and seed production in *Z. latifolia* [9]. The internal tissues of the formation edible galls, containing cell expansion, appear white gall and inner are full of fungal hyphae therefore called white *Z. latifolia*. Previous studies have shown that the symbiosis of *Z. latifolia* and *U. esculenta* can not only produce delicious gall, but also improve the photosynthesis and growth [10]. By microscopic observation the gall formation of *Z. latifolia*, was found that the inside contained a large amount of hyphae and spores of *U. esculenta* [11, 12]. Plant-fungi results may be due to 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 [13]. Meanwhile, these interactions typically produce a range of responses, such as disease symptoms, specialized structures formation, and changes in biological and morphological reactions in host plant cells [14, 15]. Many plant-fungus interact in a beneficial direction, as in rhizobium symbiosis [16] or in a harmful way, such as some fungal diseases and necrosis [17]. Many funguses to obtain nutrients and environment, plant-fungus symbiotic and majority of pathogenic fungi penetrate their host without damage the plant cell walls and membranes. In our study, we focused on the positive side of endophytic fungi in the plant. Furthermore, in soybean, by adopting RNA-seq methods, it is possible to confirm the gene co-networks potential defense *Phakopsora pachyrhizi* [18]. During plant–fungus interactions, the infection pathogens stimulate cell responses, transform in phytohormone signals, production of metabolites and the expression of various plant genes [19, 20].

Plant responses to swelling gall formation involve a complex network of signaling mechanisms, regulating many complex changes including cell expansion and biochemical processes [21, 22], and responses could be different depending on plant species [23]. Hormone regulation is known to have a central role in plant responses to swelling gall formation. Because it regulates diverse processes in plants, signaling transaction pathway can induce some levels change and plant expansion [24]. IAA and CTK are cell responsive hormones that play a crucial role in swelling gall formation sensing [25, 26]. In recent years, many plant-fungi often stimulated symptoms revealing imbalance of hormone, as seen in swelling galls stimulated by microbes via in IAA and CTK production [27, 28]. The formation of swelling gall might have evolved from a process that primally involved the ability of IAA or CTK to inhibit cellular defense responses [29]. This would be a typical interaction in which *U. esculenta* infects *Z. latifolia* and interactions cause hormonal changes in the body, leading to gall formation and swelling expansion. For symbiosis of plant fungi, the hormone production is accordance with changes in the root that are often required in these interactions [30]. However, some pathogenic bacteria that do without infection cell deformations can also generate and secrete phytohormone, indicating a role of these molecular mechanism in biological processes other than cell deformation. In china and southeast Asia, swelling galls are harvested as aquatic vegetable and are considered to place on table of people, as a delicious and rich nutritious food. Based on some reference we know that *Z. latifolia* is possibly the only cultivated food that results from plant-fungi interaction [31, 32]. Even though many researchers have tried to survey plant-fungi interaction at the hormone and physiological levels, the significant differences in plant response to *U. esculenta* compared to other fungi have yet to be comprehensive interpretation [10, 11]. Therefore, the symbiosis mechanisms referred to

plant-fungus regulation swelling gall formation and further growth remain to be indicated to explain how phytohormone response and regulate this process.

Triadimefon (TDF, CAS: 43121-43-3) is widely used in agriculture and medicine. TDF can obviously prevent and inhibit the formation of fungi. Exogenous spraying has been studied in many fields for a long time. Studies have reported by spraying TDF 8 leaf stage *Z. latifolia* plants can inhibit water swelling gall enlargement, and within a certain range with dose effect. Through the stem tissue slice observation found that TDF can obviously inhibit the growth of *U. esculenta* growth and formation, *Z. latifolia* gall formation is closely related to the distribution of *U. esculenta* (*Ustilago esculenta*) [33]. To further search for related genes for research *U. esculenta* infection *Z. latifolia* and gall formation are closely related. Previous studies revealed that *Z. latifolia* was sprayed with TDF, it may lead to the no swelling gall formation. Therefore, it was found that the hereditary shape of male *Z. latifolia* was different from that of normal *Z. latifolia* [10]. TDF treatment was able to solve the problem of genetic variation. We sprayed *Z. latifolia* directly, and the results showed that there was no *U. esculenta* and no swelling gall formation. Thus, it is essential to explore the situation of *Z. latifolia* gall information from the molecular level.

At present, the study on the molecular biology of *Z. latifolia* is still in the initial stage. The transcriptome methods have been successfully used to research a wide variety of plant development and pathogen infection interaction [6]. Although some researcher has tried to explain the detailed signal pathways related to mechanism of *Z. latifolia*, many questions remain unanswered. In this study, we analyzed the swelling formation of *Z. latifolia* infected with *U. esculenta*. Ultrastructure, paraffin sections and scanning observation were made to look for the distribution and content of *U. esculenta*. TDF was used to eliminate the infection effect of *U. esculenta*, and transcriptome sequencing was carried out before and after normal *Z. latifolia* and TDF treatment, so as to analyze the transcriptome spectrum of differentially regulated genes and detect gene expression spectrum. In this study, we can provide a new insight into the gall formation mechanism of *Z. latifolia*.

Methods

Plant materials and growth conditions

The variety was single season *Zizania latifolia* (Griseb.) Stapf, cv. 'Dabieshan No.1' and were selected as the experimental material. *Z. latifolia* were purchased from Yuexi County (Yuexi County Longjing Ecological Agriculture Development Co., Ltd., Anhui, China). We took photographs (Front view, cross section and vertical section) of each growth period to confirm the sampling and treatment periods for subsequent experiment (Fig. 1). Samples including swelling steam, were immediately freeze using liquid nitrogen flash freezing and stored in at -80°C for relative expression research and subsequent experiments.

For transcriptome assembly, to testify the effect swelling gall formation, Triadimefon (TDF, can obviously prevent and inhibit the formation of fungi) was used. The exogenous application of TDF (80 mg L^{-1}) was carried out twice before and after gall formation and they were sprayed at intervals of 5 days. Combined with the development of *Z. latifolia*, these plants were treated twice: 148 (September 15, 2018) and 159

(September 26, 2018) days after transplanting. For subsequent experiments, gall samples were collected at -5 and 5 day after gall formation, with gall diameters about 5cm, respectively. Some swelling galls were harvested at appropriate time point, and each sampling was selected in triplicate using freeze tube storage. All samples were placed in cryopreservation tubes and immediately followed by liquid nitrogen storage in a refrigerator at -80°C for RNA extraction and some index determination. We harvest four groups of samples in CK (no treatment) and TDF treatment after swelling gall formation of *Z. latifolia*. Including 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 experiment was repeated in triplicate.

Microscopic observation of before and after gall formation in CK and TDF

To study the interaction relationships of before and after gall formation in CK and TDF, the swelling gall samples were stained using aniline blue (CAS: 28631-66-5, Solarbio) according to an improved method [62]. Sliced a samples of fresh *Z. latifolia*, under fixed fluid containing the Carnot fixative (glacial acetic acid and alcohol volume ratio of 1:3) 2 mL centrifuge tube, in the 4 °C refrigerator fixed 24 h. Then add right amount 10% KOH solution to the centrifugal tube, 85°C high temperature treatment 1 h, changed several times during the KOH solution, after processing the used for subsequent dyeing observation. The slices were transferred into a petri dish containing aniline blue dye, and stained on a horizontal shaker for 5-10 min. After decolorization with 75% alcohol, the slices were observed and photographed using fluorescence microscope (Olympus, Japan, SZX10). Then we made paraffin sections and stained them with aniline blue and observed them with an optical microscope (Nikon Eclipse E100, Japan).

To investigate the effect of exogenous TDF on swelling gall at the biological level, the ultrastructure of gall tip cell was observed using electronic microscope according to the method [63]. The slices were observed under a transmission electron microscope with an accelerating voltage of 80kv (H-7650, Hitachi, Tokyo, Japan). Meanwhile, we also used scanning electron microscope (Hitachi S-4800) to observe the *U. esculenta* [64].

Determination of plant endogenous hormone contents

The swelling galls were analyzed at before and after gall formation in CK and TDF. The extraction, purification, and determination of endogenous IAA (Indole-3-acetic acid, CAS: 87-51-4) level, Z (Zeatin, CAS: 13114-27-7), ZR (Trans-Zeatin-riboside, CAS: 6025-53-2), GA₃ (Gibberellin, CAS: 77-06-5) and ABA (Absciscic acid, CAS: 21293-29-8) by Ultra high performance liquid chromatography (UHPLC) were described [12, 65]. We used mixed labeling method to determine the content of these five hormones in plants.

The content of hormone was measured by UHPLC. Effects before and after the formation of gall in CK and TDF, (fresh weight per 1.0g) after freezing in liquid nitrogen. The extraction, purification, and measurement protocols were described by Li [12]. Hormone analysis was performed using Thermo Fisher Scientific UHPLC UltiMate 3000 (Thermo Fisher Scientific, USA), equipped with 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), fluorescence detection wavelength was Eml ¼ 254 nm.

Each sample (10ml) was injected automatically at a flow rate of 1ml min⁻¹. Quantification was made by comparing the peak area with the known hormone dose of hormone.

cDNA library construction and in novo assembly

According to the above microscopic observation and analysis, RNA-seq analysis was performed before and after the gall formation of CK and TDF, and a total of 12 gall tip samples were taken. To simplify the description, we specify three replicates of JB_A, JB_B, JB_C, and JB_D, respectively. Total RNA was extracted from 12 gall samples of before and after gall formation in CK and TDF using the mir-Vana miRNA Isolation Kit (mirVana™ miRNA ISOLation Kit, Ambion-1561) following the protocol of manufacturer. The libraries were constructed using TruSeq Stranded mRNA LTSample Prep Kit (Illumina, San Diego, CA, USA) according to the instructions of manufacturer. Then these cDNA libraries were sequenced on the Illumina sequencing platform (Illumina HiSeq™ 2500) and 125bp/150bp paired-end reads were generated. The transcriptome sequencing and raw reads were processed by Trimmomatic [66]. The reads containing poly-N and low-quality reads were removed to get a clean read. The clean reads were then mapped to the reference genome using hisat2 [67].

Analysis of DEGs, cluster analysis, GO and KEGG enrichment

FPKM [42] value of all gene was calculated by cufflinks [68], and the read counts for all gene were obtained using htseq-count [69]. For transcriptional level quantization, the FPKM [67] and read counts (protein coding) values for all transcript were calculated by bowtie2 [70] and eXpress [71]. DEGs were identified using the DESeq [72] R-packet function estimation size factor and nbinom test. *P* value < 0.05, fold Change >2 or <0.5 as the threshold for significantly differential expression. Hierarchical clustering analysis of DEGs was analyzed to explore gene expression patterns. Based on the hypergeometric distribution, GO enrichment and KEGG [73] pathway enrichment analysis of DEGs were respectively indicated by R program. The read portion were reassembled by StringTie [74]. The reference genome and known annotated genes were then aligned using cuffcompare software for gene structure extension and new transcript identification.

Functional annotation and KEGG enrichment pathway analysis

DEGs are characterized by GO and KEGG enrichment (<http://www.genome.jp/kegg/>) to characterize biological functions and significantly enrich metabolic pathways or signal transduction pathways. Based on Wallenius's non-central hyper-geometric distribution [75], DEGs were submitted to GO enrichment analysis by the GO-seq R package for enrichment analysis. The statistical enrichment of DEGs in the KEGG pathway was detected by the KOBAS 3.0 [76] website (<http://kobas.cbi.pku.edu.cn>). Then, the MapMan tool (<https://mapman.gabipd.org>) was used to display a graphical overview of the metabolic and regulatory pathways.

qRT-PCR analysis

To explore the expression patterns to analysis of 10 IAA, 4 ABA, 3 CTK, 3 SA, 3 JA, 2 GA were performed CK and TDF treatment in before and after swelling gall formation of *Z. latifolia* using real-time qPCR according to an improved method [77]. Total RNA samples were isolated from different *Z. latifolia* stages using RNA-prep Pure plant kit (Tiangen, DP432). The DNase-treated RNA was extracted and reverse transcription of cDNA by the Prime Script™ RT Reagent Kit (TaKaRa, RR047A). Quantitative real-time PCR was then implemented with the TB Green™ Premix Ex Taq™ II (TaKaRa, RR820A). Using the relative expression level of *Actin* gene was analyzed as an internal reference for data standardization. Three biological replicates were performed for all sample, and the relative expression was analyzed as $2^{-\Delta\Delta C_t}$ [78]. The Real time qPCR primer sequences are shown in Table S5. The result is displayed as the mean $\pm$ SD (n = 3 biological replicates). The statistical of relative expressed data was analyzed using SPSS software. Every sample had three biological replicates.

Statistical analysis

The experimental design was a completely randomized block design. Statistical analysis was performed on the data using SPSS software to variance (ANOVA) analysis, and Duncan's multiple range test was used, $P < 0.05$ was considered significant.

Results

Observation on growth and development whole period of *Z. latifolia*

The development and growth period of *Z. latifolia* had not been determined yet, and it is a pretty important parameter to find a reasonable period for us to accurately grasp the experiment and conduct experimental treatment. Therefore, we observed and photographed the *Z. latifolia* at 10 days intervals for 20 days after transplanting. The photos were divided into front view, cross section and vertical section (Fig. 1). In the 150 days after setting, we took photos every 3 days, and a total of 27 group of photos were taken in the whole process. The period was divided into three leaves (including three leaves) and 28 days after transplanting. At the fifth leaf stage, 79 days after the transplanting of *Z. latifolia*. At the 7th leaf, 110 days after the determination of *Z. latifolia*; 153 days after transplanting *Z. latifolia*, before gall formation; After gall formation first period, 156 days after transplanting; After gall formation second period, 159 days after transplanting; After gall formation third period, 162 days after transplanting; After gall formation fourth period, 165 days after transplanting; After gall formation fifth period, 168 days after transplanting.

Microscopic observation of *U. esculenta* in different section reveals the invasion process of *Z. latifolia*

In the growth of *Z. latifolia*, we found that normally grown can formation swelling gall (Fig. 2A). However, TDF treat *Z. latifolia* without formation of swelling gall (Fig. 2B). We observed the samples of before and after gall formation in CK and TDF. It was found that no swelling gall was formed in the before, but in the late period of untreated, the *Z. latifolia* had continues to expand (Fig. 2C). Optical microscope observation find that aniline blue staining showed that hyphae could only be observed at after gall formation in CK using ordinary section and paraffin section (Fig. 2D, E). The infection processes of *U. esculenta* in the gall of CK and TDF treatment were observed using scanning electron and ultrastructure (Fig. 2F, G). By shown in

Fig. 2F, G no difference between JB_A and JB_C was observed at swelling gall formation before. In JB_B, we find many spores showing round. The sporophytes are clustered together and attached to the cell wall around. The fungal cell wall (FCW) can be clearly find in the ultrastructure, and it is closely attached to the plant cell wall and may communicate with the plant body.

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

To detection the levels of hormone response to *U. esculenta* infection, UHPLC was used to measure IAA, ABA, Z, ZR and GA₃ contents (Fig. 8, S3) in *Z. latifolia* gall formation before and after CK and TDF group. As shown in Fig. 8A, Z content was high in JB-B, decreased sharply by 8.21% from JB-B to JB-D and then no significant change from JB-A to JB-C. To our surprise, we also found that GA₃ and IAA contents were most than others, indicating the contents of IAA, ABA and GA₃ may be due to the symbiosis of *U. esculenta* infection plant, which stimulates the plant to produce a large amount of growth hormone and causes the swelling gall formation. ABA and ZR content were relatively low during the JB-B. Both Z and ZR belong to cytokinin (CTK) and are usually considered as CTK together. Meanwhile, we compared the ratio of each hormone to ABA content and divided them into 7 groups including IAA/ ABA (Fig. 8B), Z+ZR/ABA (Fig. 8C), GA₃/ABA (Fig. 8D), IAA+ GA₃/ABA (Fig. 8E), IAA+Z+ZR/ABA (Fig. 8F), Z+ZR+ GA₃/ABA (Fig. 8G) and IAA+ GA₃+Z+ZR/ABA (Fig. 8H). 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. The results indicated that the *U. esculenta* infection *Z. latifolia* could stimulate the host to produce a large amount of growth-promoting hormone and cause the gall formation and expansion.

Overview of sequencing and transcript identification

To study the differences in RNA-seq between *U. esculenta* infection *Z. latifolia* and non-infected *Z. latifolia* in gall formation before and after, three biological replicates were performed on 12 cDNA samples. After removing the low copy and quality sequences using the generic Perl script (Table 1), a total of 84.42 Gb of clean reads was generated. The quality score of more than 95.45% reads and more is equal or greater than Q30, accounting for 52.16-53.52% of the GC content as shown in Table 1. An average of 88.42% of reads were mapped to the reference genome (<http://ibi.zju.edu.cn/ricerelativesgd>), of which 86.26% were located at unique locations (Table 1). A total of 17,541 DEGs were optimized by known structures (Fig. 3A). Pearson's significant correlation (Fig. S1) between the FPKM distributions of biological replicates of all samples confirmed the high reproducibility of the sequencing data.

Identification of different expressed genes

By shown in Figure 3, in JB_B vs. JB_A, JB_D vs. JB_B, JB_D vs. JB_C and JB_C vs. JB_A, there were 3122, 2672, 6704 and 5042 DEGs identified by the DESeq R package based on FPKM data respectively (Fig. 3). Principal component analysis (PCA) indicated that replicate samples within each sample group clustered together. Venn and upset plot diagram show that among them, 1430, 2852, 1293 and 2795 DEGs were up-regulated, while 1242, 2191, 1829 and 3909 DEGs were down-regulated in JB_B vs. JB_A, JB_D vs. JB_B, JB_D vs. JB_C and JB_C vs. JB_A, respectively (Fig. 3A). Through the comparison between the four libraries,

3542 unique and 477 common DEGs were identified, among which JB_B pairs JB_A, JB_D pairs JB_B, JB_D pairs JB_C, JB_C pairs JB_A were 713, 537, 1726 and 566 unique DEGs respectively (Fig. S9). Venn and upset plot diagram were made based on the up-regulation and down-regulation data in Fig C and Fig D, from which we could find that the 4 groups of data were not up-regulation or down-regulation common DEGs, suggesting that the gall before and after formation and TDF reached the expected effect in *Z. latifolia*.

GO and KEGG enrichment analyses for all DEGs

To identify the similarities and differences between JB_B and JB_A and JB_D and JB_B in *U. esculenta* infection transcriptomes, DEGs were used for GO classification and KEGG functional enrichment analysis. The comparison of the distribution of DEGs and all genes at top 10 in JB_B vs. JB_A and JB_D vs. JB_B were allocated to three categories of the biological process (BP), cellular component (CC) and molecular function (MF) (Fig. S2; Table S3). For the DEGs in JB_B vs. JB_A, the significant GO terms were mostly enriched in adjusting of transcription, DNA-templated, cell wall organization and positive regulation of transcription, in BP category. The DEGs were mainly distributed in extracellular region, membrane anchoring components and plant cell wall in the CC category. The top three GO terms in the MF category were "DNA-binding transcription factor activity", "sequence-specific DNA binding" and "heme binding" (Table S3A). In the DEGs of JB_D vs. JB_B, 'sinapoylglucose-choline O-sinapoyltransferase activity' and 'cellulose synthase activity' term was remarkable in the BP category, the 'plasma membrane', 'extracellular region' and 'plasmodesma' terms in the CC category. Meanwhile, 'zinc ion transmembrane transport' and 'adventitious root development' term in the MF category was most significant. The most common GO terms were 'cell wall', 'DNA-binding transcription factor activity' and 'extracellular region' (Table S3B). Therefore, the corresponding genes of these important terms might play a central role in the fight against *U. esculenta* infection. In the figure 4A, C, we found that the most KEGG classification level was in the environmental information and classification level2 was signal transduction processing in JB_B vs. JB_A and JB_D vs. JB_B. The gene number is 80 and 105 JB_B vs. JB_A and JB_D vs. JB_B (Fig. 4, Table S4).

The KEGG enrichment top 20 pathway in the two groups were shown in figure 4B, D. In JB_B vs. JB_A, DEGs are dominating enriched expressed in plant hormone signal transduction, phenylpropanoid biosynthesis, starch and sucrose metabolism. Similarly, in JB_D vs. JB_B, plant hormone signal transduction was also enriched most. In plant hormone signal transduction (Ko04075), 48 and 37 DEGs were identified in the JB_B vs. JB_A and JB_D vs. JB_B, respectively. Among 48 DEGs in comparison of JB_D vs. JB_B, 22 genes were overlapped with those in JB_B vs. JB_A, and 26 genes belonging to signal transduction response regulator were unique (Fig. 7A). The different expression patterns of hormone metabolism related DEGs between CK and TDF suggest that plant hormones play a central role in regulating the against response to *U. esculenta* infection in *Z. latifolia*.

Metabolism and regulatory pathways analyses of all DEGs

Regulatory pathways in JB_D vs. JB_B were studied by MapMan tool to annotation analyses (Fig. 5, Table S2), and regulation overview enrichment of DEGs were up-regulated and functionally enriched in

transcription factors (TFs) including homeobox transcription factor family and receptor kinases (Fig. 5A). Some pathways including protein modification, calcium regulation and protein degradation were also up-regulated or down-regulated in response of JB_D vs. JB_B to *U. esculenta* infection. Most DEGs linked to plant hormones associated with down-regulation of cytokinin, ethylene and abscisic acid, and only IAA mostly up-regulation expression (Fig. 5A, Table S3A). Through biological stress analysis (Fig. 5B), the relevant genes are divided into signal transduction, PR protein, TFs, hormones ABA, SA, JA and ethylene, further supporting the crucial of these pathways in symbiotic environment regulated sensing and promotion response to *U. esculenta* infection in *Z. latifolia*. A more detailed list of all DEGs corresponding to MapMan functional categories was provided (Fig. 5A, Table S3B). TFs were primary regulators of DEGs expression and perform significant functions in the transcriptional symbiosis of plant-fungi genes after *U. esculenta*. Meanwhile, we described TFs in detail and found that HB, bHLH and AP2-EREBP had more differentially up-regulated expression (Fig. 5A). We showed the expressions of 308 putative TF genes, which can be divided into 42 TF families in JB_D vs. JB_B (Table S3C). Both the B3 and the homeobox transcription factor family have obvious high-expressed TFs, including *Zlat_10033978* and *Zlat_10028938*. B3 transcription factor family was main auxin response factors family that regulate auxin response. HB (homeobox transcription factor family) was a DNA binding motif within TF proteins. These TF might be involved in hormone regulate, cell differentiation and expansion, as well as expression patterns in patterning of different organisms after *U. esculenta* infection. These TFs seem to be in connection with stimulate gall formation response according to the annotation information.

Changes in plant hormone signal transduction and metabolism related gene expression after *U. esculenta* infection

The expression of genes related to plant hormone metabolism and signal transduction indicated meaningfully dynamic changes during the *U. esculenta* infection process. DEGs were related to IAA, CTK, GA, ABA, Eth, BR, JA and SA (Fig. 6; Table 2) were observed from 37 to JB_D vs. JB_B. At JB_B vs. JB_A 48 DEGs, 76 DEGs in JB_D vs. JB_C and 53 DEGs in JB_C vs. JB_A (Fig. 7B).

The IAA-related pathways had the most response to plant phytoplasma infections. In the IAA signal transduction pathway, 9 genes were up-regulated in JB_D vs. JB_B, including 4 auxin-responsive proteins (AUX/IAA), 3 AUX1 family proteins (auxin influx carrier family), 1 auxin responsive GH3 gene family (GH3) and 1 auxin response factor (ARF). On the contrary, 3 AUX/IAA (*Zlat_10001127*, *Zlat_10020059*, *Zlat_10033997*) and an AUX1 (*Zlat_10014750*) were down-regulated by -1.26, -1.11, -1.52 and -2.29-fold, respectively, at JB_D vs. JB_B. Most of the up-regulation expression which indicated that IAA signaling was obviously affected by *U. esculenta* infection. We suspect that auxin may be caused by the gall formation caused by the growth substances produced by the infection of *U. esculenta*. In the CTK signaling transduction pathway, 2 DEGs (*Zlat_10010976*, *Zlat_10018529*) were found to be Up-regulated, including two-component response regulator ARR-B family (B-ARR) was up-regulated 1.31-fold and 1.22-fold. In the GA signaling pathway, encoding TF (phytochrome-interacting factor 4, *Zlat_10039510*), was significantly down-regulated at JB_D vs. JB_B, which indicated that GA signaling was less affected by *U. esculenta* infection. The 8 DEGs were associated with ABA signal transduction pathway, 6 were up-regulation expression and 2 were down-regulation expression (Fig. 7C). Interestingly, 6 ABA-related DEGs were up-

regulated on JB_D vs. JB_B signal transduction pathways (Fig. 7C). The ABA receptor PP2C, SnRK2 and ABF transcription factor family were up-regulated DEGs in the signal transduction pathway and participate in the negative regulation of ABA signaling. For example, PP2C (*Zlat_10022265*) was up-regulated by 4.96-fold in JB_D vs. JB_B. Up-regulation of these key DEGs in ABA pathway indicated that ABA expression might be increase after TDF treatment causes the cell senescence. On the contrary, ABA lower content after *U. esculenta* infection and down-regulation of gene expression were characterized by cell proliferation and development in JB_D vs. JB_B. Two genes involved in JA signaling were down-regulated (JAR1, Jasmonic acid amino synthetase) at JB_D vs. JB_B. From 7 SA related genes were identified, with 6 of them related TGA transaction factor and 1 related to regulatory protein NPR1. Most of the DEGs were up-regulated. For example, the largest change in SA-relative gene expression was for TGAL1 (*Zlat_10031473*), which was up-regulated by 3.57-fold at 3 JB_D vs. JB_B. A few SA-related DEGs were down-regulated, such as one associated with the regulatory protein NPR1 genes, and down-regulated by 2.16-fold. In summary, most of the genes involved in IAA, CTK, and SA are down-regulated, and the hormone content after *U. esculenta* infection. However, most of the gene expression patterns involving SA, JA, ABA metabolism and signal transduction are up-regulated by *U. esculenta* infection (Fig. 6, 7; Table 2).

Expression of plant hormone biosynthesis genes and quantitative by qRT-PCR

A set of 25 DEGs (Fig. 9) were selected for quantitative real time PCR analysis to confirm their function of response *U. esculenta* infection in 17 JB_B vs. JB_A and 15 JB_D vs. JB_B. As shown in Fig. S4, comparison of transcriptome data with qRT-PCR results showed a relatively high correlation ($R^2 = 0.9723$), verifying accountable RNA-seq analysis in the present research. We chose 25 of DEGs including 10 (IAA), 4 (CTK), 3 (ABA), 3 (SA), 3 (JA) and 2 (GA). Among 25 DEGs, 20 DEGs up-regulation and 5 down-regulation were confirmed by qPCR in JB_D vs. JB_B accordance with results of RNA-seq analysis (Fig. 9).

Many phytohormone play a significant role in regulating plant development and modulating diverse biological processes such as IAA, CTK, GA and so on. The auxin response factor (ARF) genes are central components of plant auxin signal transaction. To understand how IAA and CTK participate in and affect swollen gall formation, we identified genes encoding IAA and CTK signal transduction relative genes at the host gall tip and regulated their expression during gall formation. These candidates included 10 IAA genes (*ZLIAA23*, *ZLIAA2*, *Os11g0169200*, *ZLSAUR39*, *ZLPLC2*, *ZLYUC11*, *ZLIAA9*, *ZLIAA10*, *ZLARF21* and *ZLIAA50*) and 4 CTK (*ZLCKX8*, *ZLBPA1*, *ZLRR1* and *ZLRR4*) hormone related genes. We detected that *ZLIAA23*, *ZLIAA2*, *Os11g0169200*, *ZLSAUR39*, *ZLPLC2*, *ZLYUC11*, *ZLIAA10*, *ZLARF21* and *ZLIAA50* were not obviously affected by *U. esculenta* infection before gall formation, but at *U. esculenta* infection, its expression was significantly induced (Fig. 9). Similarly, *ZLCKX8*, *ZLBPA1* and *ZLRR4* expressed consistently in control before gall formation, but at *U. esculenta*, its expression level was obviously up-regulated in *Z. latifolia*.

Discussion

Many fungi interact with plant-host in beneficial ways, such as rhizobium symbiosis [16], or interact with plant-host in harmful ways, such as fungal diseases [17]. Fungi infection and host defense are the crucial

events involved in plant–fungus interactions [13]. It is currently not fully understood how fungi alter their physiology to adapt to host environments in plant–fungus interactions. However, the molecular mechanism by which plants attack pathogens is a complex process. *Z. latifolia* is caused by infection with *U. esculenta* which stimulates enlargement of the swollen gall enlargement under appropriate conditions [34]. The mechanism of interaction between endophytic fungi and host relationship is still unclear. Most of these reports are concentrated only on the relationship between *U. esculenta* and *Z. latifolia* and no specific molecular mechanisms have been observed. It may be difficult to use male *Z. latifolia* as the same species though the genetic relationship between male *Z. latifolia* and normal *Z. latifolia* is different. Therefore, to study the effects of this endophytic fungus we used new approach that application of TDF to prevent the infection of *U. esculenta* has important research value compared with that of *Z. latifolia* and to explain how the host respond to *U. esculenta* infection. To the best of our knowledge, the current study is the first to provide an RNA-seq profile of TDF treatment response to *U. esculenta* infection. Therefore, 4 key sample of *U. esculenta* infection and no infection were selected for transcriptome analysis in our study. Interestingly, the results showed that no swelling gall formation were observed at the JB_D, while JB_B were swelling gall formation of phytoplasma infection (Fig. 2C). DEG analysis of *Z. latifolia* gall formation indicated that some significant biological processes were affected by *U. esculenta* infection, which gave insights into the molecular interaction between the *Z. latifolia* and *U. esculenta*.

***Z. latifolia* response to *U. esculenta* infection comprised growth stage and observed**

In previous studies, people have only made a rough classified growth stage of *Z. latifolia* [10], but it has not yet reached an exact time division. Therefore, it is difficult to make an accurate judgment for conducting experiments. We tracked the whole growth and development life cycle of *Z. latifolia*, and photographed and observed each developmental period, which laid a solid foundation for the research on *Z. latifolia* for further research (Fig. 1). In current study, by observing the morphology of the *Z. latifolia*, we learned that the *U. esculenta* generally attached to the cell wall around and produces some exchanges with the cells (Fig. 2), but the communication content needs further study and exploration. From the scanning section, we can see that massive sporophytes are located in the plant cell wall, and further observation in the cell wall shows that the ultrastructure is bound to the cell wall, closely adhered to it and conveys the interaction between plant and fungi. *U. esculenta* may soften cell walls and change related genes. When *U. esculenta* interact with plants to produce a series of metabolic reactions, among which plant hormone metabolism is the most obvious.

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

We identified a total of 17541 DEGs in swollen gall before and after formation of CK and TDF treatment, among which 2672 DEGs were involve *U. esculenta* infection in JB_D vs. JB_B (Fig. 3). Many catalytically active DEGs in infected gall were abundantly expressed throughout the formation of gall formation, which may be related to the survival and gall cell proliferation of pathogenic fungi in host tissues [35]. Among 25 DEGs (Table S6) validated by qRT-PCR, 10 IAA-relative, 4 CTK-relative, 3 ABA-relative, 3 SA-relative, 3 JA-relative and 2 GA-relative were uniquely activated in JB_D vs. JB_B and annotated plant–fungi interaction, including ARF, AUX/IAA, B-ARR, PP2C, JAR, NPR, HB and bHLH transcription factor or gene, suggesting

different defense regulatory mechanisms response to *U. esculenta* infection and gall expanse, GO and KEGG enrichment analysis indicated that most DEGs were classified into response to an endophytic fungal infection pathways including plant hormone signal transduction (Ko04075), phenylpropanoid biosynthesis (Ko00940), starch and sucrose metabolism (Ko00500) consistent with the results of Wang [36]. In our study, we paid close attention to the changes in biological processes and found that *U. esculenta* can cause plants to respond to stimulus (Fig. 4), which means that *U. esculenta* plays a significant role in protecting plants from fungal infections [37]. Secondary metabolites, such as lignin biosynthesis via the phenylpropanoid biosynthesis pathway, promote plant resistance to pathogens [38]. We found that most of MYB transcription factor was up-regulated in JB_B group (Fig. 5), and MYB could regulate lignin biosynthesis pathway, phenylpropanoid biosynthesis and plant hormone synthesis. In this study, the DEGs in this critical pathway, as well as some enzyme-related genes in the metabolic and the defense system process, showed a significant increase in the expression of infected plants (Fig. 4), indicating that infected plants do not fail to respond plant-fungi infection. Although it provides opportunities for the penetration and expansion of plant resistance in plant tissues.

RNA-seq networks play a central role in plant immunity [39], which involves a number of transcriptional regulations triggered by endophytic fungi in a host to produce a series of responses to endophytic fungi attacking the host. TF is a protein that controls the rate of transcription of genetic information from DNA to mRNA. By binding to specific DNA sequences, it regulates genes in a coordinated manner, thereby directing cell division, development and death. In the current study, most of TFs were significantly up-regulated or down-regulated in response to *U. esculenta* infection. In comparison of JB_D vs. JB_B, the masses of DEGs were down-regulated and function of enrichment in TFs including ARF, bHLH, Receptor kinases and HB. Receptor kinases are involved in a variety of plant responses, including hormone sensing and response to pathogens as significant players in defense responses. The 25 selected DEGs were uniquely activated in JB_D vs. JB_B and annotated plant hormone, including IAA, CTK and GA-related gene, which is consistent with previous results (Fig. 9). Transcriptome and qPCR expression analysis of selected DEGs indicated that TFs regulate transcriptional changes in response to *U. esculenta* infection. It reported significant change of 33 HB TFs in JB_D vs. JB_B response to *U. esculenta* infection (Fig. 5C). Further researches should focus on molecular mechanism of these transcription factors to better understand the different regulation mechanisms of IAA-related DEGs conferring response to *U. esculenta*.

The expression level of DEGs involved in plant hormone signal transduction changed significantly after *U. esculenta* infection

Phytohormones are central regulators of plant development and response cell expanse [40]. The role of plant derived hormones in phytoplasma infection resistance has been reviewed widely [41, 42]. *U. esculenta* infection transformed the expression of DEGs involved in plant hormone signal transduction and metabolism, including the up-regulation or down-regulation of DEGs related to 8 hormones including IAA, CTK, ABA, BR, GA, ET, JA, and SA in *Z. latifolia*. Majority of the genes involved in plant hormone signal transduction and metabolism of IAA, CTK, and GA were down-regulated at JB_D vs. JB_B. However, some of the DEGs related to ABA, SA and Eth were up-regulated at JB_D vs. JB_B. We speculate that *U. esculenta* is targeted to the host plant hormone system during its main invasion phase, and that the down-regulated

DEGs may be the host protective response and some stimulant secreted. DEGs related to IAA were down-regulated, which may have a connection with the higher content of IAA present when broom symptoms are present in *Pathogenic* infection [43].

In the current study, majority of genes involved in IAA metabolism and signal pathway were down-regulated from JB_D vs. JB_B. Expression of SAUR39 and PLC2 (Fig. 9; Table S6), the most obvious down-regulation were 21.06-fold and 10.42-fold. Moreover, the expression of SAUR39 gene is rapidly increased under the action of auxin-responsive protein, and SAUR39 is thought to play a significant role in the local regulation of auxin polar transport to responses against *U. esculenta* infection [44]. Many transcription factors (Fig. 5C) are significantly altered with changes in gene expression. Among them, the ARF family plays an important part, in which the ARF10 and ARF21 genes are down-regulated by 4.45-fold and 5.01-fold (Fig. 9, Table S6) respectively in JB_D vs. JB_B. The auxin response factor (ARF) genes are central components of plant auxin metabolism and signal transduction. IAA content and IAA-relative ratio were greatly increased at JB_B (Fig. 9, Table S6), with the highest expression change among the IAA-related genes. Therefore, the SAUR39 gene and ARF transcription factors may be a key part regulating to response *U. esculenta* infection.

From KEGG enrichment pathway, we found that phytohormone signal transduction pathway (Ko04075) and phenylpropanoid biosynthesis pathway (Ko00940) had the highest concentration of KEGG (Fig. 4). In the process of fungal infection, plants usually synthesize flavonoids and lignin using phenylpropanoid compounds [45]. AUX/IAA and ARF are key transduction factors for this metabolic pathway. In many species, including *Arabidopsis* and *Oryza sativa*, up-regulation of AUX/IAA and ARF genes after swelling gall expansion is downstream of IAA signal transduction [46, 47]. In this study, majority of genes related to IAA metabolism and signal transduction were down-regulated during the process of *U. esculenta* infection, such as ARF10, ARF21, IAA30, IAA2 and IAA23 genes (Table 2). Their gene expression levels significant different from JB_D vs. JB_B, which indicated that AUX/IAA and ARF were crucial gene family involved in *U. esculenta* infection in *Z. latifolia*. Moreover, previous reports showed that symptomatic *Arabidopsis* infected by bacteria were detected to contain increase IAA content [48], and that IAA content was substantially increased in the *Z. latifolia* at JB_B (Fig. 8A). In addition, the ARF gene is a crucial component of an alternative pathway leading to IAA metabolism and signaling transduction [49]. This indicated that the down-regulated expression of ARF21 gene (*Zlat_10012761*) in *Z. latifolia* infection is 5.01-fold, which may be one of the reasons for the increased IAA content (Table S6). **Auxin positively control plant gall expanse, growth and development, whereas IAA pathways** commonly regulate well **endophytic fungi** [50, 51]. Most of DEGs related to both IAA and CTK pathways were down-regulated expression in JB_D vs. JB_B, resulting in increases of JA and SA contents at the initial stage of *U. esculenta* infection in JB_B. It appears that the combination of DEGs associated with IAA and CTK has been changed to either synergistic *U. esculenta* infection or as part of the symbiotic relationship between *Z. latifolia* and the *U. esculenta*.

ABA integrates diverse stress signals to regulate downstream stress response [52, 53]. In this study, expression levels of DEGs contacted with ABA signaling transduction pathway, such as abscisic acid PP2C gene, were down-regulated from JB_D vs. JB_B *U. esculenta* infection. PP2C is a feedback regulation gene in ABA signaling and is transcriptional regulated in ABA responsive gene expression [54]. Therefore, down-regulation of the PP2C gene indicated that ABA signaling might be reduced after infection with *U.*

esculenta. In addition, plant immune signals were activated to inhibit diseases caused by the response effects of ABA signaling [55]. In the current study, DEGs involved in the ABA signal transduction pathway, including ABF, PP2C, PYL/PYL and SNRK2, were significantly down-regulation in JB_D vs. JB_B (Fig. 7C; Table S6). This might indicate that the activation of *Z. latifolia* signal inhibits ABA signaling during the gall formation of the *U. esculenta* after infection, but when the JB_B immune system is recognized, the ABA content is relatively reduced.

Plant hormones play a role in a complex network that regulates plant growth, gall formation and response to environmental signals [56, 57]. Meanwhile, pathogens have evolved complex mechanisms to regulate hormone metabolism and signal transduction, thereby promoting their ability to overcome plant defense mechanisms [58]. Some hormones, such as IAA and ABA, play a role in regulating plant growth and responding to abiotic stresses, but have recently played an important role in plant-fungal interactions [59, 60]. In this study, we also observed that *U. esculenta* infection affected gene expression related to IAA, CTK and GA signaling and metabolism. Therefore, during *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. Future research should focus on detailed comparisons of JB_D vs. JB_B mediated endophytic plant-fungi infection.

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

A hormone regulation model, secondary metabolism and cell wall loosening genes during *U. esculenta* infection in *Z. latifolia* is shown in Fig. 11. It was found that there were significant differences in the expression of related DEGs in the cell walls of *U. esculenta* infection *Z. latifolia* (Fig. 5B). We found that in differentially expressed genes β - expansin gene (EXPB11) down-regulation most obviously and many of these genes are also down-regulated (EXPB3, EXPB6, EXPB2, EXPA8 and so on) in JB_D vs. JB_B. Meanwhile, xyloglucan endotransglu-cosylase/hydrolase (XTH) relative gene was down-regulation such as XTH32, XTH28, XTH31 and XTH8. Fasciclin-like arabinogalactan proteins (FLA) was an important component of cell wall genes (Table S7) that large amounts of FLA gene (FLA1, FLA2 et al.) are down-regulated in the transcriptome(Fig. S2). Auxin is considered to be central to the control of the invasion of *U. esculenta*, because AUX/IAA and ARF are expressed most obviously in the gene expression pattern, with AUX1 upstream initiating auxin expression after *U. esculenta* infection as central bases. During *U. esculenta* infection, CTK, as a hormone that can promote cell proliferation, can act as the second messenger of IAA signaling, together with promoting gall expansion (Fig. 10). CTK synthesis is negatively regulated by IAA, and its process depends on inhibition of B-ARR. CTK synthesis is negatively regulated by IAA, and the process depends on the inhibition B-ARR [61]. Moreover, IAA transport enhances GA levels by promoting TF and upstream DELLA. Therefore, positive or negative interactions between IAA and CTKs, GA, ABA, JA, and SA can be observed during the transformation of *U. esculenta* infection, while IAA is located at the center of the regulatory network (Fig. 11).

Here, we came up with a model (Fig. 11) in which IAA plays a significant role in regulating *U. esculenta* infection in *Z. latifolia*. On the one hand, *U. esculenta* infection reduced the IAA level and up-regulated AUX1,

AUX/IAA and ARF gene family expression. One of the dominating responses was enhanced CTK signaling, may be due to B-ARR relative gene up-regulation. Increased CTK-relative gene expression levels and content were generated a range of reactions including cell division, shoot initiation and gall enlargement. The other response to PYR/PYL down-regulation and PP2C up-regulation were increased ABA synthesis, followed by up-regulation of XTHs, FLAs, EXPA and EXPB required for cell wall loosening factor (Fig. 6). On the other hand, *U. esculenta* infection stimulus IAA transport and synthetic, which caused in loss of GA catabolism. However, increased GA3 content in *Z. latifolia*, possibly because of down-regulation of TF gene were observed in our study and ABA synthesis was decreased in response. In addition, *U. esculenta* infection up-regulation of ABF resulted in the rapid reduction of ABA transduction through SnRK2, which are negative signals for formation of swelling gall in *Z. latifolia*. It seems reasonable to indicated that host transcriptome hormone and cell wall loosening model is a key fine turning process for co-existence. *U. esculenta* can make the cell wall loosening of *Z. latifolia* for enter into the internal of cell, and produce a series of hormone responses in symbiosis with *Z. latifolia*, utilizing in novo activation the swelling gall formation.

Conclusions

In the current study, we conducted comparative RNA-seq analysis to explain the molecular mechanism and DEGs expression of *U. esculenta* infection *Z. latifolia* symbiotic mechanism in gall formation. It is of great research value to use TDF spraying for the first time to eliminate the influence of *U. esculenta* on *Z. latifolia*. Whole growth cycles were recorded and microstructure observation showed that the presence of *U. esculenta* could be clearly observed after the gall formation, after TDF treatment were not found *U. esculenta*. Meanwhile, the content of hormone IAA, CTK and GA in JB_B was significantly higher than other three groups. RNA-seq analysis indicated that a total of 43703 unigenes and 17541 DEGs were identified. According to FPKM analysis and the number of DEGs at each of the four points. Some key genes and TFs such as ARF, AUX/IAA, PP2C and SAUR39 in different plant hormone metabolism, signal transduction and cell wall loosening factors were up or down-regulated. Our study provides a new perspective for studying the physiological and molecular mechanisms of *U. esculenta* infection *Z. latifolia* that causes swelling gall formation. This research will provide hormone-cell wall loosening model. To further understand the symbiotic regulation mechanism between *Z. latifolia* and *U. esculenta* which provide a solid foundation and theoretical basis for further increasing the future yield of *Z. latifolia*.

Abbreviations

TDF: Triadimefon; CK: Control Check; IAA: Indole-3-acetic acid; Z: Zeatin; ZR: Trans Zeatin riboside; GA₃: Gibberellin 3; ABA: Absciscic acid; CTK: Cytoberberin; *U. esculenta*: *Ustilago esculenta*; *Z. latifolia*: *Zizania latifolia*; DEGs: Different expressed genes; BP: Biological process; CC: Cellular component; MF: Molecular function; TF: Transcription factor; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; FPKM: Fragments per kilobase of exon per million fragments mapped; PCA: Principal component analysis; PPI: Protein Protein Interaction network; ARF: Auxin response factor.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SZ and JL designed the research, analyzed the data, and wrote the manuscript. JH, LY and GC performed the data integration analyses. CW and SZ provide some resources. YY, ZL, YG, SJ and XF performed assist in completing the experiment. All authors discussed the data and reviewed and commented on the manuscript. all authors have read and approved the manuscript.

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Tables

Table 1. Quality assessment of raw RNA-seq data.

Sample	clean_reads (Million)	clean_bases (Gigabytes)	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%

Table 2. Identities and analysis of DEGs enriched in plant hormone signal transaction pathways of JB_D VS JB_B.

Gene ID	Log ₂ FC	pval	Up/Down	Gene symbol	Hormone category	Pathway position	Description
Zlat_10015168	3.038	0.036601284	Up	TGAL5	SA	TGA	Os05g0443900, partial [Oryza sativa Japonica Group]
Zlat_10021635	1.404	0.026299925	Up	TGAL3	SA	TGA	PREDICTED: transcription factor HBP-1b(c38)-like [Oryza brachyantha]
Zlat_10021667	-2.160	1.98147E-18	Down	TGAL10	SA	TGA	Os08g0176900, partial [Oryza sativa Japonica Group]
Zlat_10031473	3.569	3.70428E-11	Up	TGAL1	SA	TGA	hypothetical protein BRADI_2g52860v3 [Brachypodium distachyon]
Zlat_10032525	1.860	2.10235E-10	Up	TGAL1	SA	TGA	PREDICTED: transcription factor HBP-1b(c1)-like isoform X2 [Oryza brachyantha]
Zlat_10037899	2.407	0.002668809	Up	TGAL4	SA	TGA	PREDICTED: transcription factor TGA6-like [Oryza brachyantha]
Zlat_10044810	2.444	2.12905E-11	Up	NH5.2	SA	TF	hypothetical protein PAHAL_8G025500 [Panicum hallii]
Zlat_10002850	-1.121	0.010941287	Down	GH3.5	JA	SnRK2	PREDICTED: jasmonic acid-amido synthetase JAR2-like [Oryza brachyantha]
Zlat_10006334	-1.258	2.99678E-08	Down	GH3.5	JA	SnRK2	PREDICTED: jasmonic acid-amido synthetase JAR1 [Oryza brachyantha]
Zlat_10000330	1.072	7.92464E-08	Up	Os01g0856500	IAA	SnRK2	PREDICTED: auxin transporter-like protein 1 [Oryza brachyantha]
Zlat_10000572	1.070	0.035011956	Up	IAA27	IAA	PYR/PYL	predicted protein [Hordeum vulgare subsp. vulgare]

Gene ID	Log ₂ FC	pval	Up/Down	Gene symbol	Hormone category	Pathway position	Description
Zlat_10001127	-1.261	0.001443811	Down	IAA23	IAA	PP2C	auxin-responsive protein IAA23 [Setaria italica]

Zlat_10005792	3.108	2.25714E-09	Up	ARF11	IAA	PP2C	RecName: Full=Auxin response factor 11; AltName: Full=OsARF5
Zlat_10014750	-2.970	2.88514E-57	Down	Os11g0169200	IAA	PP2C	PREDICTED: putative auxin transporter-like protein 4 [Oryza brachyantha]
Zlat_10015270	1.248	0.002458758	Up	IAA6	IAA	PP2C	PREDICTED: auxin-responsive protein IAA6-like isoform X3 [Oryza brachyantha]
Zlat_10015482	2.632	7.39457E-05	Up	LAX2	IAA	NPR1	PREDICTED: auxin transporter- like protein 2 [Oryza brachyantha]
Zlat_10015922	1.354	0.009884568	Up	IAA7	IAA	JAR1	Auxin-responsive protein IAA7 [Dichanthelium oligosanthes]
Zlat_10020059	-1.110	3.44535E-14	Down	IAA2	IAA	JAR1	hypothetical protein OsI_00719 [Oryza sativa Indica Group]
Zlat_10032090	1.085	1.12277E-06	Up	IAA6	IAA	GH3	PREDICTED: auxin-responsive protein IAA6-like isoform X3 [Oryza brachyantha]
Zlat_10032626	1.090	2.93836E-15	Up	Os10g0147400	IAA	CYCD3	LAX protein [Phyllostachys edulis]
Zlat_10033997	-1.522	5.01385E-29	Down	IAA30	IAA	BK11	AUX3 protein [Phyllostachys edulis]
Zlat_10043397	1.079	1.8115E-05	Up	GH3.4	IAA	B-ARR	hypothetical protein OsI_20501 [Oryza sativa Indica Group]
Zlat_10039510	-1.993	0.010678803	Down		GA	B-ARR	hypothetical protein OsI_12756 [Oryza sativa Indica Group]
Zlat_10010976	1.309	0.002744161	Up	RR25	CTK	AUX1	PREDICTED: two- component response regulator ORR25- like [Oryza brachyantha]
Gene ID	Log ₂ FC	pval	Up/Down	Gene symbol	Hormone category	Pathway position	Description
Zlat_10018529	1.222	0.006836765	Up	RR22	CTK	AUX1	two-component response regulator ORR22 [Oryza sativa Japonica Group]

Zlat_10018497	1.073	0.000815632	Up	CYCD3-2	Bra	AUX1	cyclin-D3-2-like [Aegilops tauschii subsp. tauschii]
Zlat_10043001		0.016310208	Up	BKI1	Bra	AUX1	probable BRI1 kinase inhibitor 1 [Aegilops tauschii subsp. tauschii]
Zlat_10005614	-1.173	6.27066E-05	Down	PYL4	ABA	AUX/IAA	ABA receptor 7 [Oryza sativa Indica Group]
Zlat_10014121	1.239	1.83641E-07	Up	Os03g0268600	ABA	AUX/IAA	PREDICTED: probable protein phosphatase 2C 30 [Oryza brachyantha]
Zlat_10015895	1.596	7.49921E-06	Up	TRAB1	ABA	AUX/IAA	ABSCISIC ACID-INSENSITIVE 5-like protein 5 [Zea mays]
Zlat_10022265	4.958	1.22449E-05	Up	Os04g0167900	ABA	AUX/IAA	PREDICTED: probable protein phosphatase 2C 37 [Oryza brachyantha]
Zlat_10023618	1.085	6.1928E-10	Up	Os05g0537400	ABA	AUX/IAA	PREDICTED: probable protein phosphatase 2C 50, partial [Oryza brachyantha]
Zlat_10029966	1.930	0.000882927	Up	Os01g0656200	ABA	AUX/IAA	RecName: Full=Probable protein phosphatase 2C 8
Zlat_10032372	-1.019	2.24929E-05	Down		ABA	AUX/IAA	hypothetical protein BRADI_2g24120v3 [Brachypodium distachyon]
Zlat_10037460	-1.523	0.015159773	Down	SAPK4	ABA	ARF	Os05g0433100 [Oryza sativa Japonica Group]
Zlat_10039544	1.077	8.18272E-09	Up	SAPK10	ABA	ABF	serine/threonine-protein kinase SAPK10 isoform X2 [Oryza sativa Japonica Group]
Zlat_10046325	1.041	0.002060567	Up	SAPK7	ABA	ABF	serine/threonine-protein kinase SAPK7 [Oryza sativa Japonica Group]

Figures

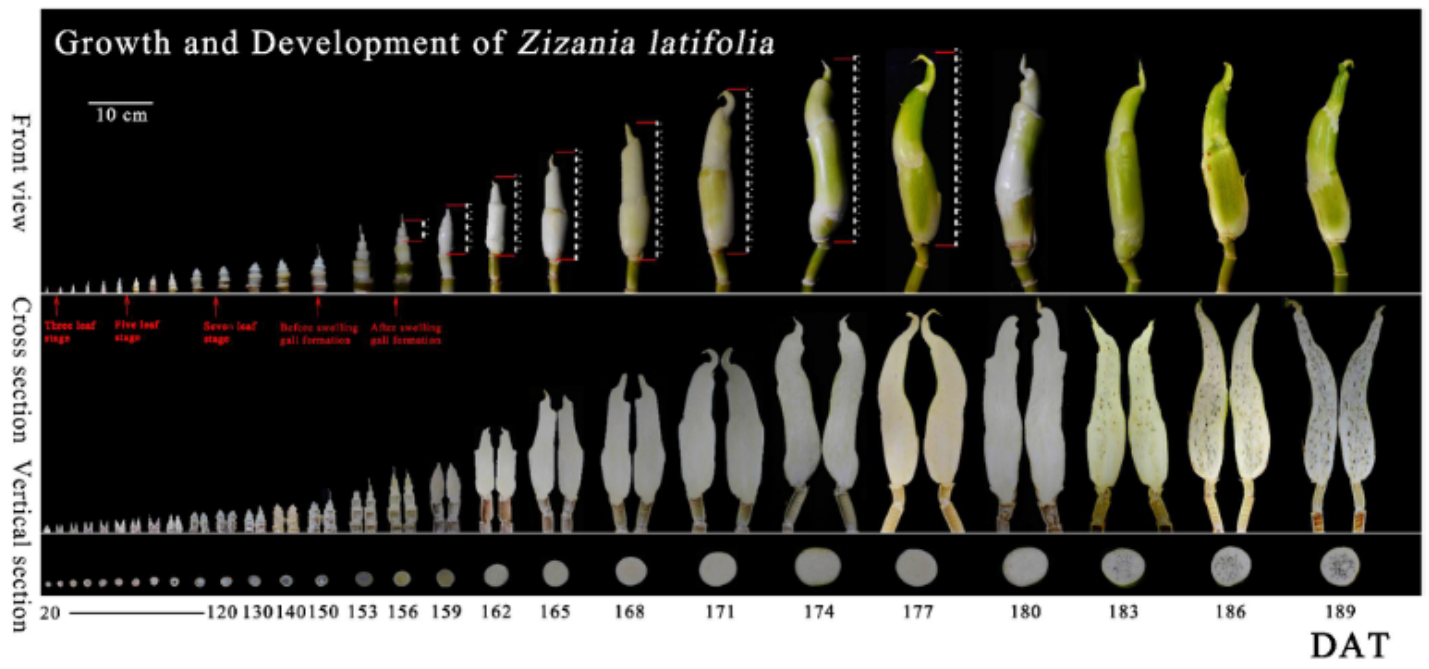


Figure 1

Observation on growth and development period of *Zizania latifolia*. We confirmed before and after in swelling gall formation of *Zizania latifolia*. DAT means day after transplanting.

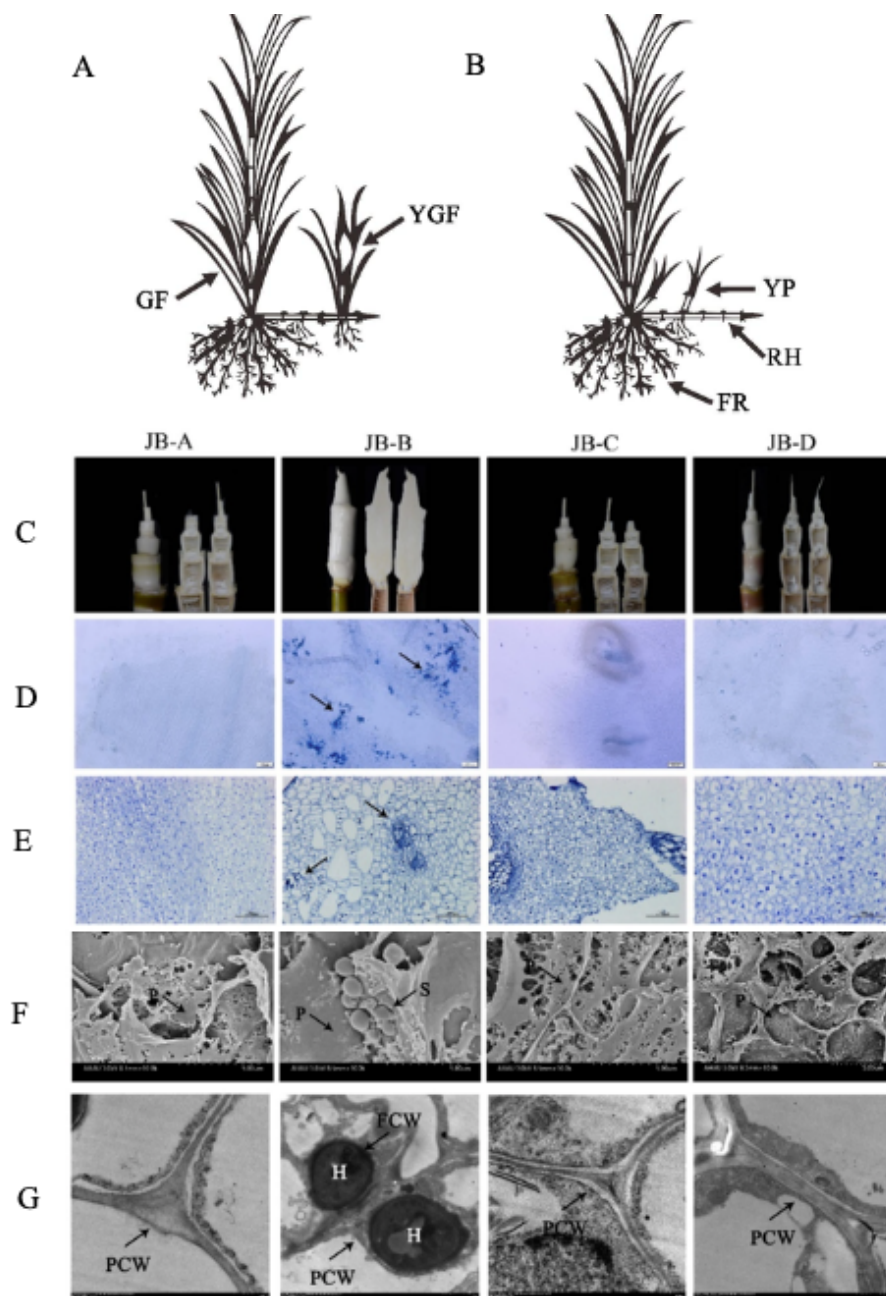


Figure 2

Sample pictures, ordinary sections and paraffin sections were stained with aniline blue to observe the situation of *Ustilago esculenta* in swelling gall of *Zizania latifolia*. (A) Normally grown *Zizania latifolia* swelling gall formation. Information is shown in the figure gall formation (GF), young gall formation (YGF). (B) TDF treat *Zizania latifolia* no formation of swelling gall. Information is shown in the figure gall fibrous root (FR), Rhizome (RH) and young plants (YP). (C) In the picture, JB-A: CK, before swelling gall formation of *Zizania latifolia*; JB-B: CK, after swelling gall formation of *Zizania latifolia*; JB-C: TDF treatment, before swelling gall formation of *Zizania latifolia*; JB-D: TDF treatment, after swelling gall formation of *Zizania latifolia*. (D) The *Ustilago esculenta* was observed by different slice. The arrows indicate the presence of clustered *Ustilago esculenta*. (E) Paraffin section, clear observation of cell size and content of *Ustilago esculenta*. (F) TDF and CK group were observed by scanning section. P in the figure represent plant, S in the

figure represent sporophore of *Ustilago esculenta*. (G) transmission electron microscope in before and after swelling gall formation of *Zizania latifolia*. PCW: plant cell wall; H: hyphae; FCW: fungal cell wall.

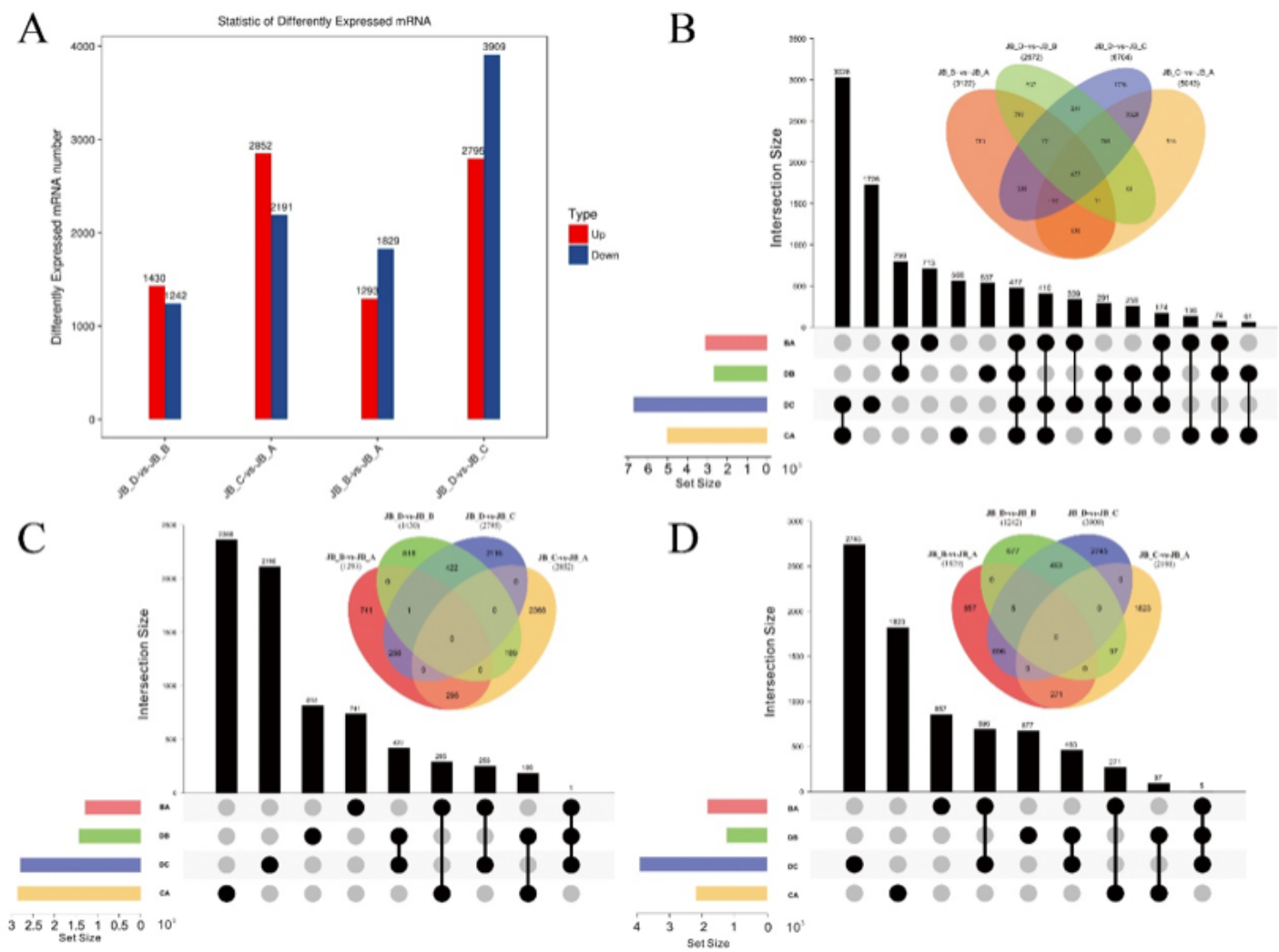


Figure 3

Transcriptome data differential gene number and Venn diagram. (A) For RNA-seq database, the number of all genes and the number of up-down-regulated genes in different period and treatment. Up-regulation number are shown in red, while the number of down-regulated genes is shown in blue. (B) Venn and Upset plot diagram of the DEGs in different comparisons. The numbers indicate unique and common DEGs in three replicates for the different comparisons. (C) Venn and Upset plot diagram of the up-regulation genes in different comparisons. (D) Venn and Upset plot diagram of the down-regulation genes in different comparisons.

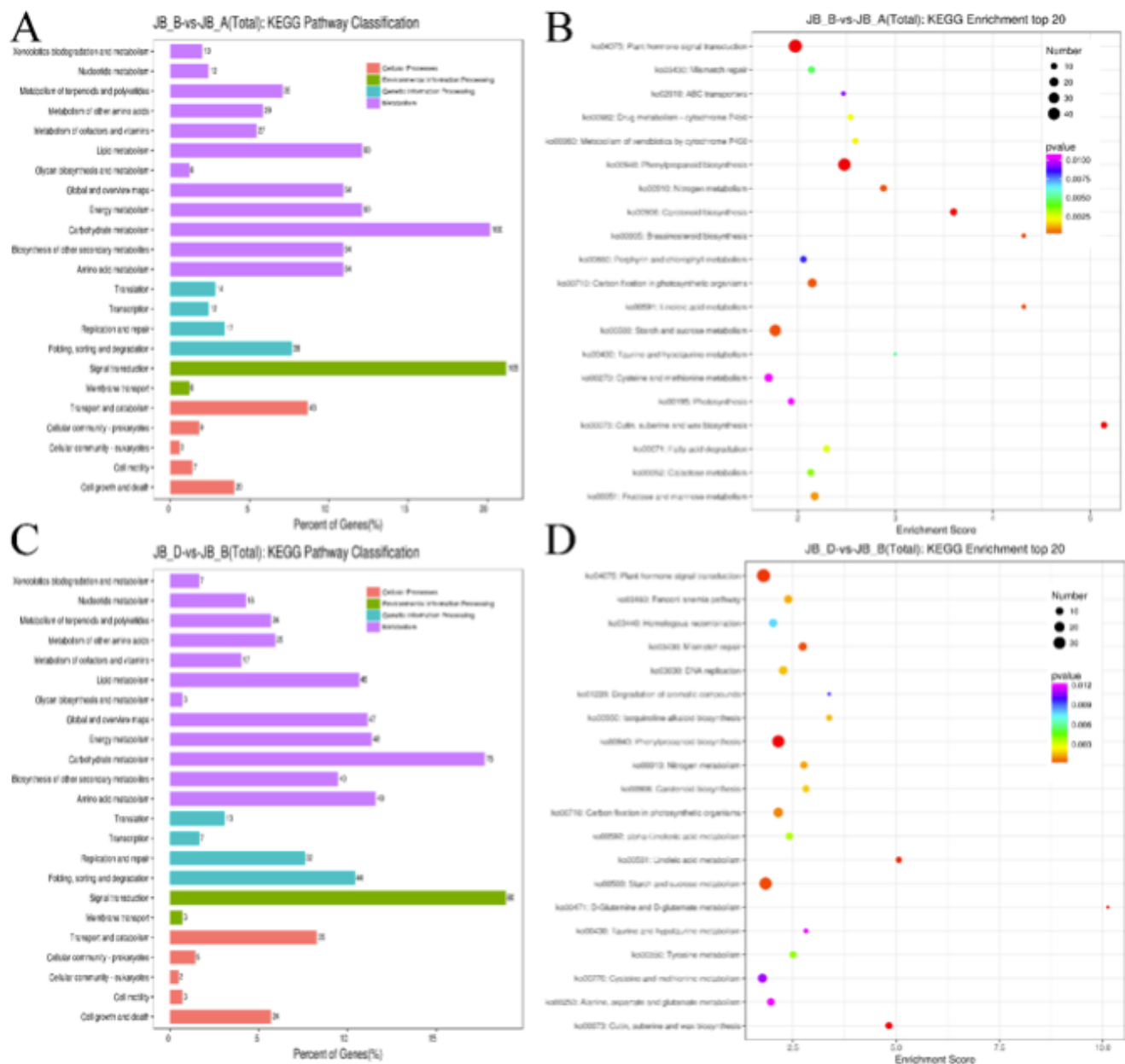


Figure 4

KEGG pathways classification and KEGG enrichment were analyzed. Scatter plot of the KEGG pathways for the DEGs of JB_B vs. JB_A (A, B) and JB_D vs. JB_B (C, D), respectively. The dot size represents the number of genes, and the color represents the q-value range.

infected gall samples relative to DEGs. Individual genes are represented by small squares. The scale bar displays transformed log2 fold changes.

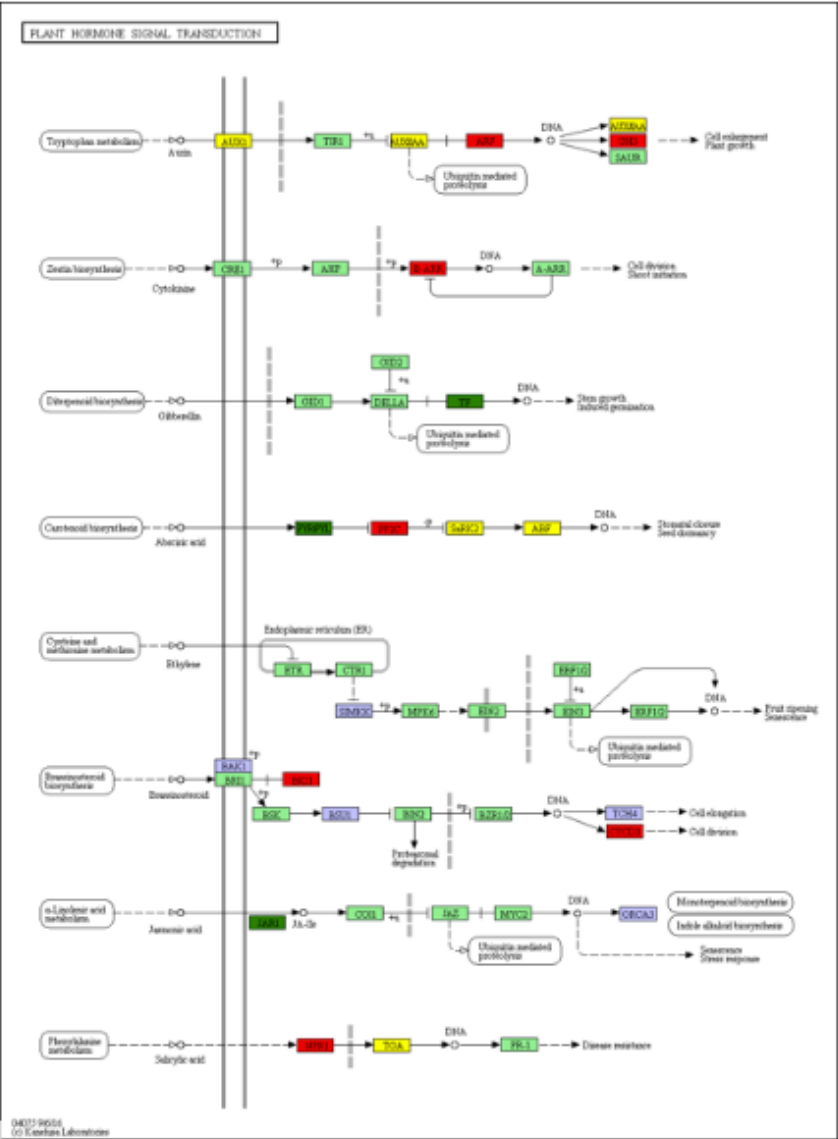


Figure 6

Expression of genes involved in hormone in JB_D vs. JB_B. Red indicates up-regulated genes, green indicates down-regulated genes, and yellow indicates that the corresponding genes are both up-regulated and down-regulated.

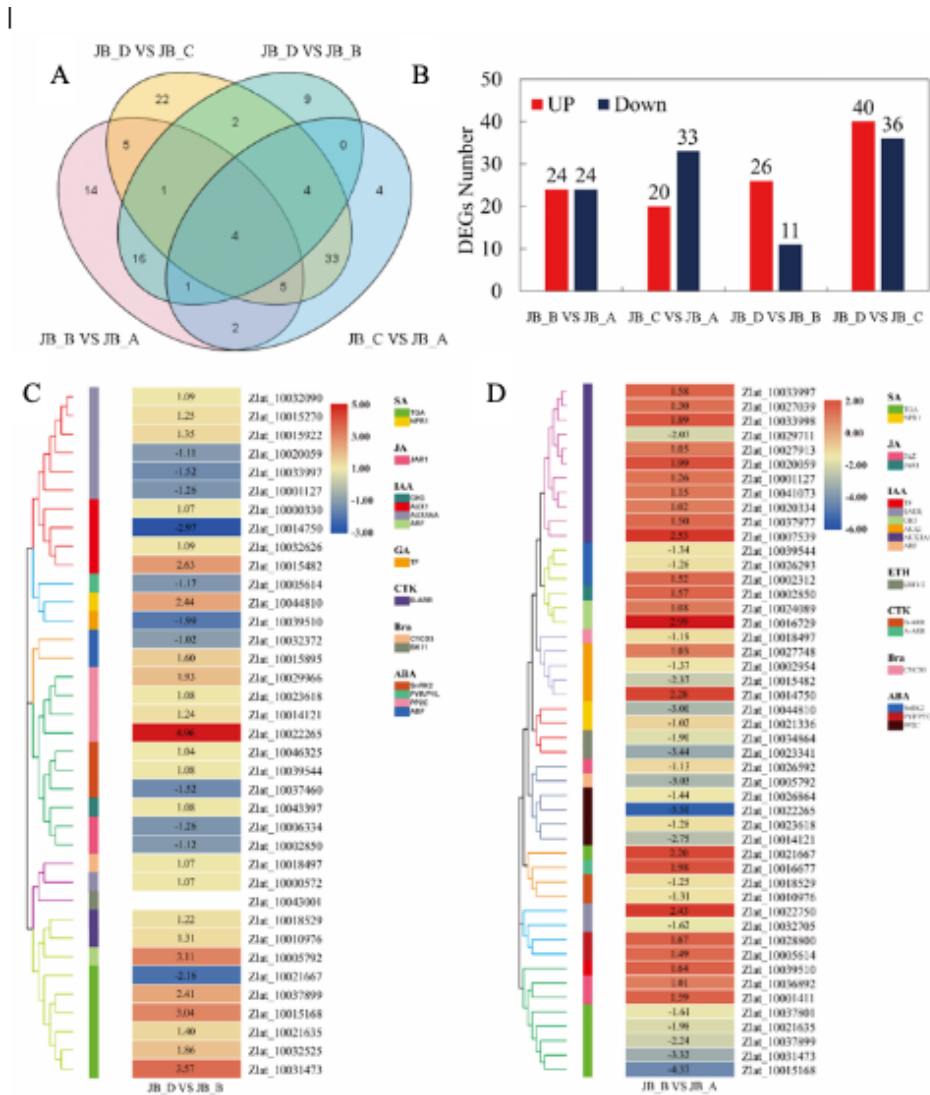


Figure 7

From this diagram we analyzed all the genes in the plant signal transduction pathway. (A) Venn diagram of the DEGs in different comparisons. The numbers indicate unique and common DEGs in three replicates for the different comparisons. (B) For RNA-seq database, the number of all genes and the number of up-down-regulated genes in plant signal transduction. (C) All genes involved in plant hormone signal transduction in JB_D VS JB_B were analyzed by heat map and cluster analysis, and the values were log2FC. (D) Means that JB_B VS JB_A is the same as C diagram.

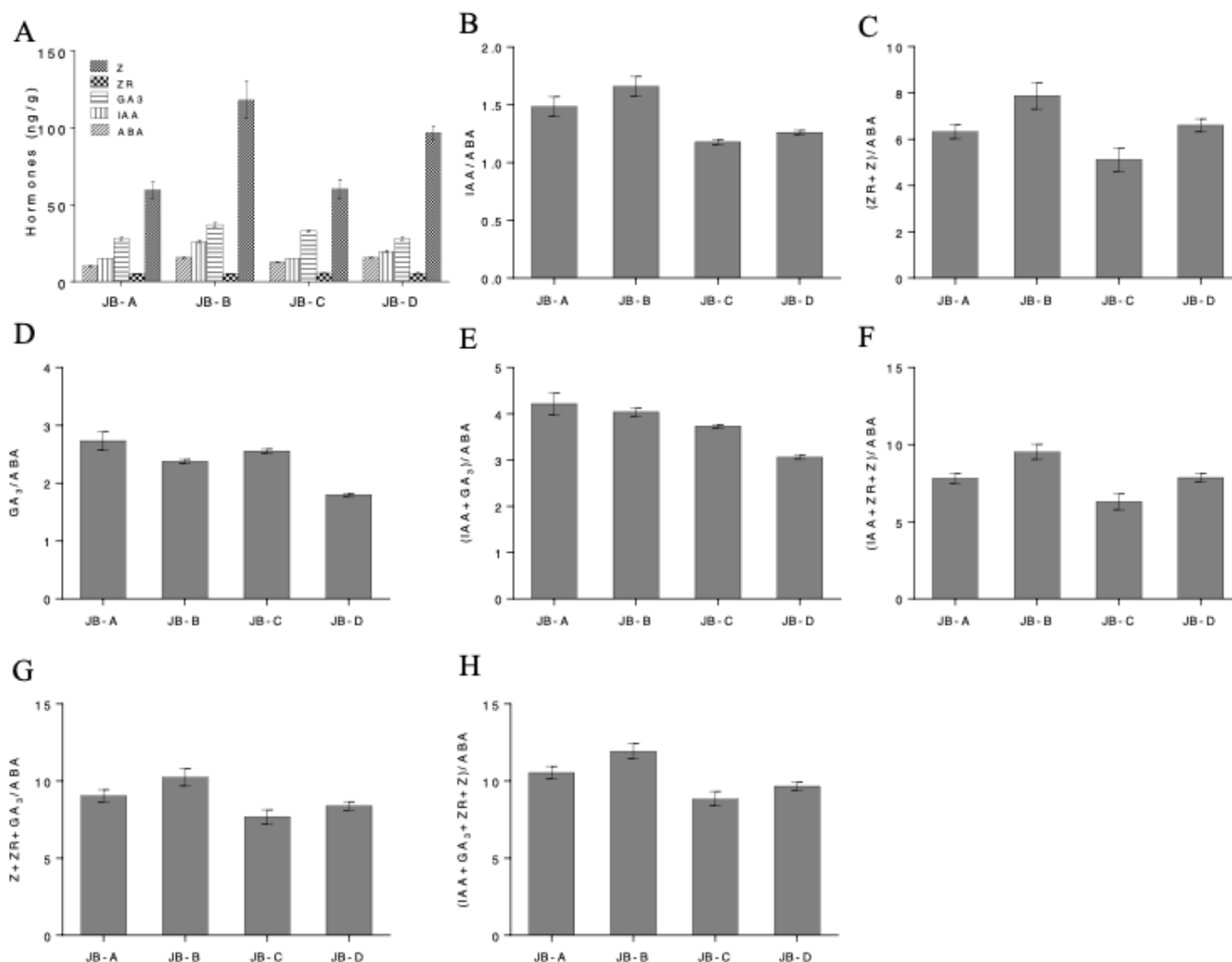


Figure 8

Content of hormones in *Z. latifolia* gall formation before and after CK and TDF group. Determination of hormone including: IAA (Indole-3-acetic acid, CAS: 87-51-4), Z (Zeatin, CAS: 13114-27-7), ZR (trans-Zeatin-riboside, CAS: 6025-53-2), GA3 (Gibberellin, CAS: 77-06-5) and ABA (Absciscic acid, CAS: 21293-29-8). Values represent the mean $\pm$ S.E. (n = 3). (A) The content of five hormones before and after *Z. latifolia* was determined by UHPLC in different treatment; (B) The relationship between IAA and ABA content was analyzed; (C) The relationship between Z+ZR and ABA content was analyzed; (D) The relationship between GA3 and ABA content was analyzed; (E) The relationship between IAA+GA3 and ABA content was analyzed; (F) The relationship between IAA+Z+ZR and ABA content was analyzed; (G) The relationship between Z+ZR+ GA3 and ABA content was analyzed; (H) The relationship between IAA+Z+ZR+ GA3 and ABA content was analyzed.

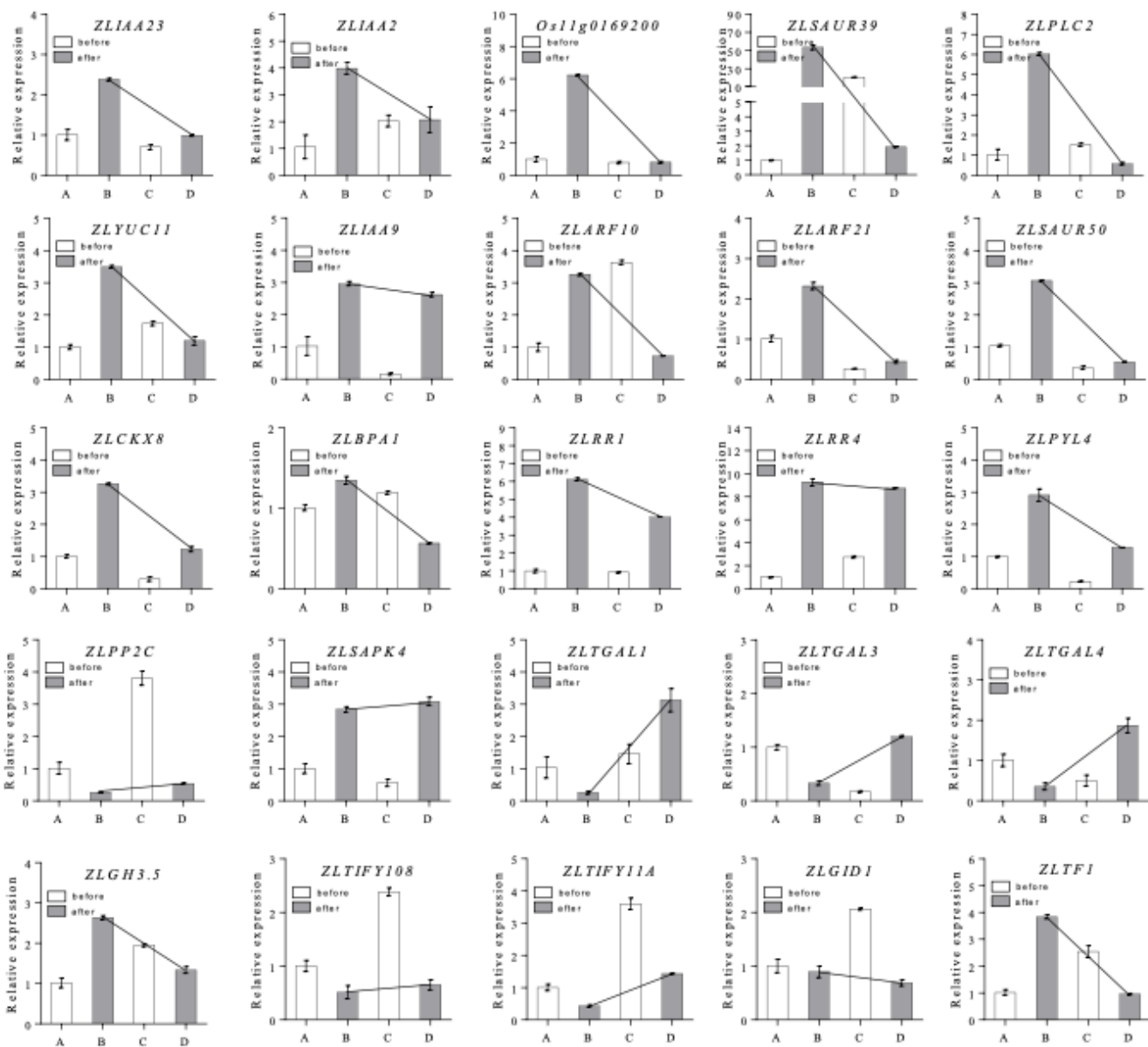


Figure 9

Expression analysis of hormone-related genes in swollen gall before and after formation of CK and TDF treatment in *Z. latifolia*. The experiment included three biological replicates and using SPSS analysis. We chose number of 10 (IAA), 4 (CTK), 3 (ABA), 3 (SA), 3 (JA) and 2 (GA). The X-coordinates of all the graphs represent A (JB_A), B (JB_B), C (JB_C), and D (JB_D).

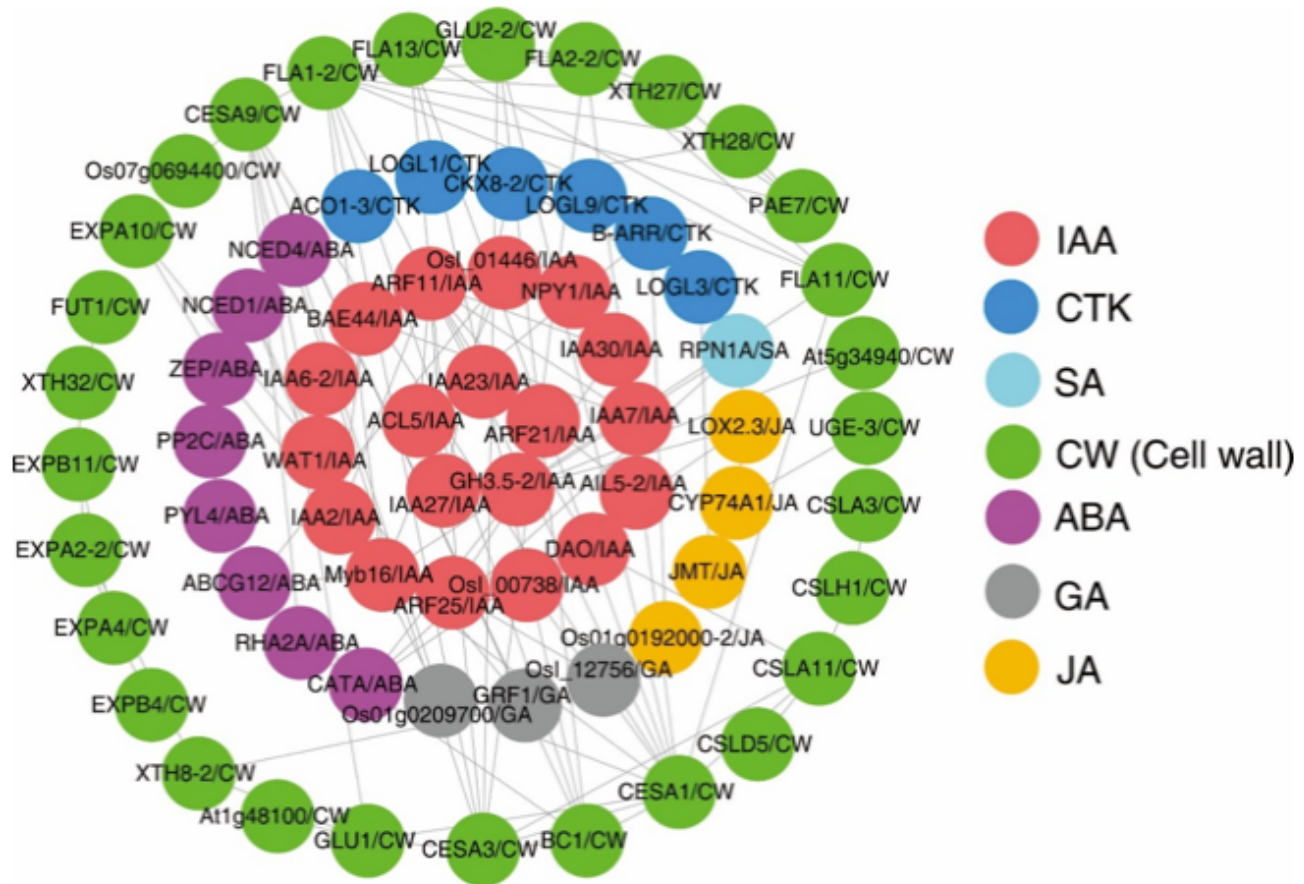


Figure 10

The interaction network was constructed by plant hormone and cell wall in *Zizania latifolia*. We used PPI (Protein Protein Interaction network) maps to map the interaction networks between six hormones (IAA, CTK, SA, GA, JA, ABA) and cell wall-related genes (IAA in red, CTK in blue, SA in light blue, CW in green, ABA in purple, GA in gray and JA in yellow), and found that cell wall loosening factors clustered around the hormones. Auxin is the center and other hormones regulate the expansion of gall in *Z. latifolia*.

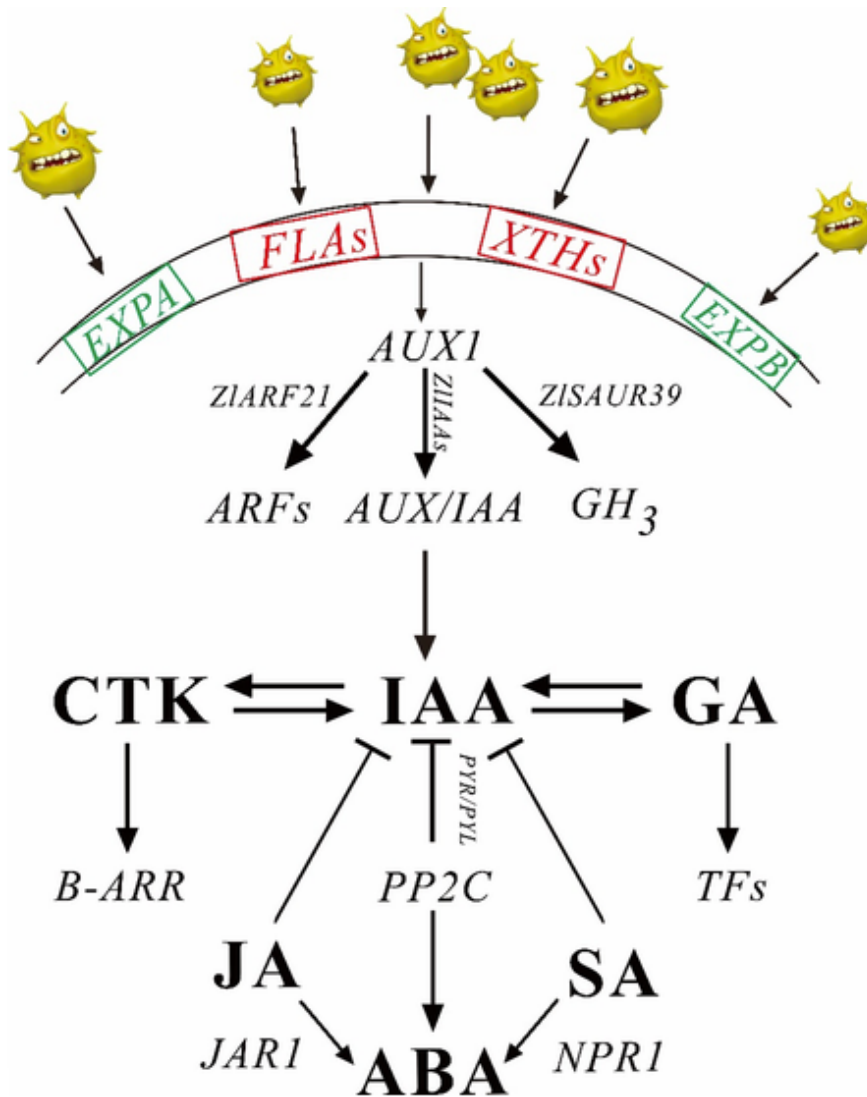


Figure 11

We established a hormone-cell wall loosening model of *Z. latifolia* infected with *U. esculenta*. The *U. esculenta* infects the cells from cell-wall and softens the cell wall, cell wall loosening relative genes change (ZIEXPA, ZIFLAs, ZIXTHs and ZIEXPB). Then *U. esculenta* and symbiosis with the host to produce plant hormones, and stimulates the swelling gall formation.

Supplementary Files

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

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