Research Article

**De novo Transcriptome Reveals Gene Changes in the Development of the Endosperm Chalazal Haustorium in *Taxillus chinensis* (DC.) Danser**

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Loranthus (*Taxillus chinensis*) is a facultative, hemiparasite and stem parasitic plant that attacks other plants for living. Transcriptome sequencing and bioinformatics analysis were applied in this study to identify the gene expression profiles of fresh seeds (CK), baby (FB), and adult haustoria tissues (FD). We assembled 160,571 loranthus genes, of which 64,926, 35,417, and 47,249 were aligned to NR, GO, and KEGG pathway databases, respectively. We identified 14,295, 15,921, and 16,402 genes in CK, FB, and FD, respectively. We next identified 5,480 differentially expressed genes (DEGs) in the process, of which 258, 174, 81, and 94 were encoding ribosomal proteins (RP), transcription factors (TF), ubiquitin, and disease resistance proteins, respectively. Some DEGs were identified to be upregulated along with the haustoria development (e.g., 68 RP and 26 ubiquitin genes). Notably, 36 RP DEGs peak at FB; 10 ER, 5 WRKY, 6 bHLH, and 4 MYB TF genes upregulated only in FD. Further, we identified 4 out of 32 microRNA genes dysregulated in the loranthus haustoria development. This is the first haustoria transcriptome of loranthus, and our findings will improve our understanding of the molecular mechanism of haustoria.

1. Introduction

*Taxillus chinensis* (DC.) Danser, also called loranthus or “San Ji Sheng” (in Chinese), is a member of Loranthaceae family and mainly distributed in the southern and southwestern areas of China. It has a long history of being used in the Chinese traditional medicine, mainly because its stems and leaves can be used for the treatment of rheumatoid arthralgia, threat of abortion, and hypertension [1, 2]. Loranthus is a parasitic plant that attacks other plants, such as Aceraceae, Anacardiaceae, Euphorbiaceae, Fabaceae, Fagaceae, Juglandaceae, Moraceae, Rosaceae, and Rutaceae [2]. The successful parasitism is a key process for the plants to obtain water and nutrients from the host plants via specialized feeding structures called haustoria.

In plants, approximately 4,500 parasitic species belonging to 28 families, representing 1% of the dicotyledonous angiosperm species, have been reported [3]. Depending on the attachment site in the host plants, parasitic plants can be classified in to two groups—stem and root parasites. Also, according to the degree of host dependency, parasites can be facultative or obligate. Facultative parasites can live autotrophically but latter cannot, such as *Triphysaria* spp. and *Pitheirospermum* spp., while obligate parasites have to parasitize a host in order to complete their life cycles, for example, *Viscum* spp., *Cuscuta* spp., *Orobanche* spp., and *Striga* spp.. Further, parasitic plants can be classified as hemiparasites or holoparasites based on whether they have retained or completely lost the photosynthetic activity [3]. Based on these characteristics, loranthus is a facultative, hemiparasite, and stem parasite.

It has been reported that after seed germination, most parasitic plants will develop a functional haustoria depending on a second chemical signal also derived from the host exudate, such as 2,6-dimethoxy-p-benzoquinone (DMBQ), phenolic acids, and flavonoids (a haustoria-inducing factors (HIFs)) [4]. Some studies have shown the mechanisms of haustoria development in parasitic plants. For example, a
single-electron reducing quinone oxidoreductase (TvPirin) is required to trigger the haustoria development in the roots of Triphysaria versicolor [5]. The seeds of Santalum album, an aggressive root hemiparasite, can germinate in sand or in vitro on Murashige and Skoog medium after a pretreatment of 2–8 mM GA3 for 12 h and then develop the haustoria within one month without the need for induction by HIFs [6–8]. Many is unknown about haustoria development in loranthus.

Transcriptome sequencing has been used to identify differentially expressed genes in the process of parasitism of Cuscuta pentagona, including genes encoding plant hormone (e.g., auxin, gibberellin, and strigolactones), transporters, and genes associated with cell wall modifications [9]. Also, it has been used to show that genes involved in cell wall metabolism, protein metabolism, and mitochondrial electron transport, genes related to auxin signaling and genes encoding nodulin-like proteins, were important for the haustoria development in Santalum album [4]. Transcriptome analysis also found that genes related to protein turnover, detoxification of reactive oxygen species, and fungal pathogenesis are abundant in the haustoria of Golovinomyces orontii [10]. Recently, small RNA sequencing characterized that some dodders’ (Cuscuta spp.) microRNAs (miRNAs) could target the host (Arabidopsis thaliana) genes and further improve the parasitism [11].

In the present study, we constructed a transcriptome profile of haustoria development and identified genes encoding ribosomal proteins (RPs), transcription factors (TFs), ubiquitin, and disease-resistant proteins (DRPs) which might be involved in the loranthus haustoria development. Our results provide a valuable resource for further exploration and a basis towards understanding the molecular mechanisms of the haustoria development and underlying host-parasite interaction in angiosperms.

2. Materials and Methods

2.1. Plant Material. Fifty seeds of Taxillus chinensis (DC.) Danser were collected from the experimental field of Guangxi Botanical Garden of Medicinal Plants in China, confirmed by senior botanists and deposited in the herbarium of Guangxi Botanical Garden of Medical Plants (accession number: s0001794). Then, the seeds were peeled, washed with sterile water, placed on a germination dish, and incubated under the environment of 25°C temperature and 80% moisture. Every day, the seeds were lighted under 2000 Lx for 10 h. Three fresh seeds were collected as control (CK). After 10 days of incubation, three seeds with protruding seed-type radicle and tiny suction device were randomly collected (FB). After 20 days of incubation, the loranthus haustoria was formed and elongated, and the true leaves began to grow. Three of them were collected as adult haustoria (FD).

2.2. Total RNA Extraction and Transcriptome Sequencing. Total RNA was extracted using TRIZol reagent, as described [1, 12]. After the quality and quantity were determined by Agilent 2100 Bioanalyzer, total RNA (1 µg) of each sample was used to construct the cDNA library using the TruSeq RNA Library Preparation Kit v2 protocol (Illumina), as described [13]. Then, cDNA libraries were quality controlled by the Agilent 2100 Bioanalyzer and qRT-PCR, followed by sequencing on the Illumina HiSeq2500 platform with paired-end 100 strategy.

2.3. De Novo of the Transcriptome. Raw data were cleaned using trim_galore (v0.5.0) and quality controlled using FASTQC (v0.11.7). Next, we used Trinity (v2.8.4) to de novo assemble the loranthus haustoria transcriptome with default parameters, as previously described [1].

2.4. Transcriptome Annotation. After the likely proteins were extracted from the assembled transcriptome using TransDecoder, they were annotated using Trinotate (v3.1.1). In this step, likely proteins were searched against the UniProtKB/Swiss-Prot database to identify known proteins, functional PFAM domains were identified using HMMER [14], signal peptides were predicted using SignalP [15], transmembrane domains were predicted using TMHMM Sever v2.0 [16], and rRNA transcripts were predicted using RNAMMER [17]. Then, EggNOG database (v4.1) [18] was searched against to identify proteins in EuKaryotic Orthologous Groups (KOG), Clusters of Orthologous Groups (COGs), and nonsupervised orthologous groups (NOGs).

Next, we annotated the assembled loranthus genes using KEGG pathway Gene Ontology (GO) databases. BLAST software was used to map the assembled genes to the NR database and the hits with e-value of $>1 \times 10^{-5}$ were filtered. Remaining genes were processed to retrieve GO annotation in terms of biological process, cellular component, and molecular function by BLAST2GO [19]. Using the enzyme commission numbers produced by BLAST2GO, we mapped the assembled transcriptome to KEGG pathway database and obtained the pathway annotation.

2.5. Noncoding Gene and miRNA Annotation. Unannotated loranthus genes were processed by the Coding Potential Calculator (CPC, v2) with default parameters to identify potential long noncoding genes [20]. Then, all the plant mature microRNAs (miRNAs) were mapped to these noncoding genes to identify loranthus miRNAs using SOAP2 with maximal two mismatches [21]. Then, MIREAP was used to predict the miRNA precursor sequences, and psRobot was used to predict the target genes of miRNAs [22].

2.6. Gene Expression Profile and Differential Expression Analysis. Bowtie2 and RSEM tools were used to align clean reads to the assembled transcriptome and to profile the gene expression for each sample, respectively, [23]. Transcripts-per-million (TPM) reads method was for normalization, and lowly expressed genes (TPM < 5) were filtered. Then, differential expressed genes (DEGs) were identified using edgeR [24] with a strict criteria: log2 fold change (Log2FC) > 1 or $<-1$ and false discovery rate (FDR) of <0.05.

2.7. Functional Analysis. p value calculated using Fisher’s exact test and q value calculated by the R package “qvalue” were used to identify enriched GO terms and KEGG pathways.
pathways (p value of <0.05 and q value of <0.05). Human or other animal-related GO terms and pathways were filtered.

2.8. qRT-PCR. We randomly selected 9 genes for qRT-PCR validation, and 18S rRNA was used as internal control. Forward and reverse primers were predicted using Primer3 and synthesized at BGI-Shenzhen. The procedure of qRT-PCR experiment was same as our previous study [1]. The expression of genes was shown in $\Delta \Delta CT$. $\Delta \Delta CT$ was used to present the difference of gene expression between two samples. Then, we used relative normalized expression (RNE) to show the gene expression changes: $RNE = 2^{-\Delta \Delta \delta}$, p values were calculated using the multiple t tests function in Prism GraphPad 8.0.

3. Results

3.1. Plants, Sequencing, and De Novo Analysis. Compared to CK, the green colors of FB and FD seeds were darker (Figure 1(a)). In addition, FB seeds produced seed-type radicle and tiny suction device. FD seeds formed and elongated the haustoria, and their true leaves began to grow. We generated a total of ~322.92 million reads (average: ~35.88 million reads) for these samples. After data cleaning, ~321.92 million reads were obtained, and Trinity assembled 160,571 loranthus genes that can produce 266,379 transcripts (Table 1). The size of the loranthus haustoria transcriptome was ~110 Mb, the GC percentage was 42.83%, the N50 was 1,191 bp, which revealed that 50% of the assembled loranthus genes were >1,191 bp, and the average gene length was 685.36 bp. Furthermore, gene length distribution (Figure 1(b)) showed 106,251 (66.17%) genes between 200 bp to 500 bp and 8,199 (5.11%) genes longer than 3000 bp.

3.2. Annotation of Coding and Noncoding Genes. We next aligned the assembled genes to public databases, including NCBI nonredundant (NR), UniProt/SwissProt, GO, and KEGG pathway (Figure 2(a)). It was shown that the 64,926 genes aligned to NR and that the top five species that are aligned by loranthus genes were Vitis vinifera (grape, 34,147 transcripts), Theobroma cacao (cacao tree, 5,456 transcripts), Nelumbo nucifera (lotus, 5,323 transcripts), Ziziphus jujube (jujube, 4,757 transcripts), and Citrus sinensis (orange, 3,967 transcripts) (Figure 2(b)). Top two GO terms involved by the assembled genes were "metabolic process" (23,049 genes) and "cellular process" (22,002 genes) (Figure 2(c)) while the top KEGG pathway involved with the loranthus
genes was “metabolic pathway” (ko01100, 11,714 genes). Notably, we found 1,757 genes related to the pathway of “plant-pathogen interaction” (ko04626).

Then, TransDecoder predicted 96,665 proteins encoded by the loranthus genes and 78,196 (derived from 56,472 genes) were aligned to UniProtKB/SwissProt database (Figure 2(a)). Next, we identified 65,702 functional Pfam domains, 5,986 signal peptides, and 20,910 transmembrane regions in the likely proteins using HMMER, SignalP, and TMHMM, respectively (Figure 2(a)). Next, we aligned the loranthus genes to EggNOG database and found that the top three categories were “signal transduction mechanisms” (7,922 genes), “post-translational modification, protein turnover, and chaperones” (6,568 genes), and “translation, ribosomal structure and biogenesis” (5,693 genes) (Figure 2(d)).

RNAMMER predicted 19 genes that can produce ribosomal RNAs in the assembled genes. Next, CPC identified 99,817 potential long noncoding genes in the unannotated

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**Figure 2:** Annotation of the assembled transcriptome. (a) Number of genes aligned to databases. (b) Number of genes aligned to different species. (c) GO annotation of the assembled transcriptome. (d) COG annotation.
genes, of which 32 were predicted to encode microRNAs from 19 microRNA families (Supplementary Table S1). Interestingly, we found that 3,457 protein coding genes might be specific to loranthus according to the CPC label.

3.3. Gene Expression Profile and Differential Expression Analysis. After lowly expressed genes (TPM < 5) were filtered, we identified 14,295, 15,921, and 16,402 genes in CK, FB, and FD, respectively, and found 12,888 genes commonly detected in all three samples. Next, we performed DEG analysis to identify genes involved in the loranthus haustoria development. Using edgeR, we identified 3,749 and 4,139 DEGs in FB and FD, respectively, compared to CK (Supplementary Table S2). The upregulation of ubiquitin genes (Figure 3(e)) was notable that most of the dysregulated TFs were shared (Figure 3(b)) by FB and FD. Next, we analyzed the expression changes of some TF subfamilies (Table 2, Figure 3(c)), including ethylene-responsive (ER), MYB, WRKY, and bHLH. Compared to CK, 9 ER, 5 MYB, 6 WRKY, and 11 bHLH TF genes were upregulated in both FB and FD (Figure 3(c)). Some TF genes were specifically upregulated in FB or FD. For example, 1 ER, 4 WRKY, and 5 bHLH TF genes were upregulated only in FB, while 10 ER, 5 WRKY, 6 bHLH, and 4 MYB TF genes were upregulated only in FD. This indicates that these TFs might be functionally translated as required for different stages. No TF genes were upregulated along with the loranthus haustoria development; however, we found some key TF genes started their upregulation from FB, including 9 ER, 4 WRKY, and 4 MYB (Supplementary Table S2).

3.4.1. Ribosomal Protein. Among the 2,576 RP genes, 255 were differentially expressed during the loranthus haustoria development and formation (Supplementary Table S2), such as ribosomal protein (RP), transcription factor (TF), ubiquitin, heat shock protein (HSP), auxin, and disease-resistant protein (DRP) (Table 2).

3.4.2. Transcription Factor. We identified 863 TF genes in the loranthus haustoria, of which 174 were dysregulated in the developmental process. We found that most of the dysregulated TFs were shared (Figure 3(b)) by FB and FD. Next, we analyzed the expression changes of some TF subfamilies (Table 2, Figure 3(c)), including ethylene-responsive (ER), MYB, WRKY, and bHLH. Compared to CK, 9 ER, 5 MYB, 6 WRKY, and 11 bHLH TF genes were upregulated in both FB and FD (Figure 3(c)). Some TF genes were specifically upregulated in FB or FD. For example, 1 ER, 4 WRKY, and 5 bHLH TF genes were upregulated only in FB, while 10 ER, 5 WRKY, 6 bHLH, and 4 MYB TF genes were upregulated only in FD. This indicates that these TFs might be functionally translated as required for different stages. No TF genes were dysregulated in the process (Figure 3(a)). We next predicted the target genes for these miRNAs and found that they had no common target genes except mir156a and mir156c (Figure 4(b)). The dysregulation of miRNA host genes might explain the change of their target genes, such as TRINITY_DN3353_c2_g1, TRINITY_DN2184_c0_g1,

| Gene_family | FB_vs_CK | FD_vs_CK | FD_vs_FB |
|-------------|----------|----------|----------|
| Ribosomal protein | 123/7 | 200/5 | 184/38 |
| TF | 80/46 | 101/41 | 32/12 |
| TF_bHLH | 15/4 | 17/2 | 1/3 |
| TF_ER | 9/10 | 18/7 | 13/1 |
| TF_MYB | 7/7 | 12/8 | 5/2 |
| TF_WRKY | 10/5 | 11/3 | 5/1 |
| Ubiquitin | 38/17 | 48/11 | 27/15 |
| Disease-resistant protein | 71/6 | 67/1 | 7/8 |
Figure 3: Continued.
3.6. qRT-PCR Validation. We used qRT-PCR to validate the expression changes of 9 randomly selected genes in the loranthus haustoria development, and 18S rRNA was used as internal control. The primer sequences of these genes can be found in Supplementary Table S3. The comparison of RNA-Seq and qRT-PCR results can be found in Table 3. Overall, 22 (81.48%) out of 27 events were agreed by both RNA-Seq and qRT-PCR. The expression patterns of 6 genes, including TRINITY_DN10066_c0_g1, TRINITY_DN3842_c0_g2, TRINITY_DN6353_c3_g1, TRINITY_DN6903_c0_g1, TRINITY_DN7338_c0_g1, and TRINITY_DN759_c0_g2, were consistent in RNA-Seq and qRT-PCR. High agreement of gene expression patterns in RNA-Seq and qRT-PCR results can be found in Table 3. Over 27 events were agreed by both RNA-Seq and qRT-PCR. The expression patterns of 6 genes, including TRINITY_DN10066_c0_g1, TRINITY_DN3842_c0_g2, TRINITY_DN6353_c3_g1, TRINITY_DN6903_c0_g1, TRINITY_DN7338_c0_g1, and TRINITY_DN759_c0_g2, were consistent in RNA-Seq and qRT-PCR. High agreement of gene expression patterns in RNA-Seq
and qRT-PCR indicates that the genes identified in this study might be functional during the loranthus haustoria development, which requires future functional experiments.

4. Discussion

Some studies have shown TFs’ function in both parasitic plants and their hosts during the infection. For example, the upregulation of AtWRKY is important for the seeding site establishment of plant-parasitic nematodes [25]. Nearly one-half of the mobile mRNAs transferred from tomato or pumpkin to their parasitic plant Cuscuta pentagona were regulatory genes such as TFs and calmodulin proteins [26]. These evidences suggest that endogenous or exogenous TFs are important for the interaction of parasitic plants. We identified the dysregulation of bHLH, ER, MYB, and WRKY TFs (Table 2, Figure 3(c)), which may function in the formation and development of endosperm chalazal haustorium in Taxi-llus chinensis. These TFs have been reported to be inducible by the various environmental stresses, such as cold, drought, pathogen infection, and wounding, and be functional in the plant defense [27].

In parasitic plants, RP genes might play a key role in the survival and development. During the evolution of Epifagus virginiana, although some RP genes are deleted, the E. virginiana plastid genomes are still transcribed and translated due to the fulfilled function by the nuclear components [28]. In addition, RPs have shown higher level of accumulation in resistant sunflower plants after the sunflower broomrape infection [29]. We found 258 out of 2,576 RP genes differentially expressed during the loranthus haustoria developmental and most are upregulated (Figure 3(a), Supplementary Table S2). We assume that both host and parasitic plants have RP genes elevated during the early phase of parasitism.

Some studies have uncovered the functions of ubiquitin proteins in the parasitism in plants and animals. For example, a unique ubiquitin carboxyl extension protein (grUBCEP12) is secreted by the plant-parasitic nematode...
**Table 3: qRT-PCR validation.**

| Gene_id            | Log2FC FB_vs_CK | Log2FC FD_vs_CK | Log2FC FD_vs_FB | UniProtID     | Description                                      |
|--------------------|-----------------|-----------------|-----------------|---------------|--------------------------------------------------|
| TRINITY_DN10066_c0_g1 | 10.61           | 8.82E-29        | 1.54            | RL37A_OSTOS   | 60S ribosomal protein L37a                       |
| TRINITY_DN12554_c1_g1 | 0.00            | 1               | -1.27           | RL403_CHLRE   | Ubiquitin-60S ribosomal protein L40              |
| TRINITY_DN2024_c3_g1 | 1.43            | 0.0009          | 1.25            | WRKY4_ARATH   | Probable WRKY transcription factor 4             |
| TRINITY_DN2307_c2_g1 | -0.12           | 0.8821          | 1.33            | RAP24_ARATH   | Ethylene-responsive transcription factor RAP2-4   |
| TRINITY_DN3842_c0_g2 | 12.76           | 2.04E-45        | 1.31            | EF1A_PODCU    | Elongation factor 1-alpha                        |
| TRINITY_DN6353_c3_g1 | 3.87            | 1.94E-17        | 1.40            | DRL21_ARATH   | Putative disease resistance protein At3g14660    |
| TRINITY_DN6903_c0_g1 | -3.23           | 3.88E-14        | -1.04           | AIL7_ARATH    | AP2-like ethylene-responsive transcription factor AIL7 |
| TRINITY_DN7338_c0_g1 | 0.18            | 0.8210          | 1.72            | ERF78_ARATH   | Ethylene-responsive transcription factor 4       |
| TRINITY_DN759_c0_g2  | 10.89           | 4.42E-30        | 1.09            | EF3_CANAL     | Elongation factor 3                              |

*RNE < 0 represents the downregulation.*
**Globodera rostochiensis** can promote successful plant parasitism through suppressing the plant’s defense through the suppression of plant immunity and can further generate within root tissue the feeding cells essential for nematode development [30]. Rhiannon reported that E2 and E3 ubiquitin proteins secreted by the parasitic nematode *Trichinella spiralis* have the capacity of modifying the host skeletal muscle cells [31]. In this study, we identified 66, 176, and 540 genes encoding E1, E2, and E3 ubiquitin enzymes, respectively. Among them, 81 were differentially expressed (Table 2, Figure 3(d)), including 8 E2 and 29 E3 ubiquitin genes (Supplementary Table S2). Based on these evidences, we assume that the secretion of ubiquitin genes and proteins by loranthus has positive efforts in the parasitism. While further experiments are required to study the functions of ubiquitin genes and proteins in the parasitism of loranthus.

A recent study reported that *Arabidopsis thaliana* miRNAs are targeted by miRNAs produced by *Cuscuta campestris* during the parasitism, resulting in mRNA cleavage, secondary siRNA production, and decreased mRNA accumulation [11]. Here, we predicted 32 miRNA host genes in the loranthus haustoria (Supplementary Table S1) and identified the dysregulation of miR156c, miR156d, miR166d, and miR396a (Figure 4). Due to the limited information of loranthus genes, we only found a few genes targeted by these four miRNAs (Figure 4). Further experiments are required to identify the mature miRNA sequences and their function in the loranthus haustoria development.

### 5. Conclusions

In conclusion, we studied the transcriptome profiles of the loranthus haustoria development. We assembled 160,571 loranthus genes and annotated them by aligning them to NR, GO, KEGG, UniProt/Swiss-Prot, Pfam, and EggNOG databases. After lowly expressed genes were filtered, we identified 18,360 genes in the loranthus haustoria, of which 3,749 and 4,139 were dysregulated in FB and FD, respectively, compared to CK. Some important gene families were found to be related to the loranthus haustoria development, such as transcription factor, ubiquitin, ribosomal protein, and disease-resistant protein. Further, 32 miRNA host genes were identified and the dysregulation of 4 miRNA host genes might be one of the reasons for some genes which are dysregulated as well in the process. This is the first time to report the transcriptome of loranthus haustoria. It will provide valuable resources to other studies. More importantly, the findings of this study will improve our understanding of parasitism and contribute to the breeding program of loranthus.

### Data Availability

The raw sequencing data can be accessed from the NCBI Sequence Read Archive (SRA) platform (https://trace.ncbi.nlm.nih.gov/Traces/sra/) under the accession number SRA896707. The assembled transcriptome of loranthus haustoria can be accessed in the TSA database of NCBI under the accession number GHNL00000000.

### Conflicts of Interest

The authors declare that there is no conflict of interest.

### Authors’ Contributions

SW, JF, and LP conceived and designed the experiments. SW, LH, HL, and XJ performed the experiments. SW, YW, and LW analyzed the data. SW and JF wrote the manuscript. LP revised the manuscript. All the authors have read and approved the final version of manuscript.

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### Supplementary Materials

**Supplementary 1.** Table S1. miRNA host genes and sequences.

**Supplementary 2.** Table S2: dysregulated genes identified in FB and FD compared to CK.

**Supplementary 3.** Table S3: forward and reverse primers used in the qRT-PCR experiment.

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