Molecular mechanism of the parasitic interaction between *Orobanche cumana* wallr. and sunflowers

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**ABSTRACT**

*Orobanche cumana* Wallr. is the most serious parasitic weed that threatens sunflower production in China, it infects sunflower roots and causes severe yield and economic losses. This study determined the effects of *O. cumana* infection on sunflower growth, physiological, biochemical, photosynthesis indexes and used RNA-Seq to investigate the potential regulatory factors involved in the parasitic interaction. Results showed that *O. cumana* infestation significantly inhibited sunflower height, fresh weight, chlorophyll contents, photosynthetic and the MDA content, SOD and POD activities were increased in different degrees, which might be related to sunflower resistance to *O. cumana* infestation. Additionally, six parasite-related genes were selected, which markedly enriched in plant hormone signal transduction, photosynthesis and phenylpropanoid metabolism pathway. Among these genes, *HsP90A, MYC2* and *HAO* were discovered for the first time in sunflowers and *O. cumana* parasitic interaction. Undoubtedly, the results lay a foundation for revealing the parasitic interactions molecular mechanism of *O. cumana* and sunflowers.

**Introduction**

Sunflower broomrape (*Orobanche cumana* Wallr.) belongs to the genus *Orobanche*, family Orobanchaceae. It is an obligate root holoparasitic weed that lacks roots and chlorophyll and completely depends on the host plant for water and essential nutrients. *O. cumana* is widely distributed in Europe, Asia and Africa (Moliner-Ruiz et al. 2015; Nabloussi et al. 2017). With the development of oil crops in China, *O. cumana* has widely spread in sunflower (*Helianthus annuus* L.) production areas in China, mainly distributed in the provinces of Jilin, Inner Mongolia, Hebei, Shanxi, Shaanxi, Heilongjiang, Xinjiang, Gansu and Ningxia. However, *O. cumana* parasitism leads to severe growth retardation of sunflowers and yield losses (Shi and Zhao 2020). It has been reported that almost all sunflowers are infested, with 40,000 ha experiencing serious damage in Spain (Parker 2009). In Turkey and Greece, broomrape has reduced sunflower production by more than 50% and 60%, respectively (Parker 2009). In Bayannur, Inner Mongolia, the infested area reached 0.13 million ha, the average parasitism rate in sunflower fields was more than 72%, and the parasitism rate of serious plots was 100%, resulting in crop failure and serious economic losses (Bai et al. 2013; Sun 2017).

Currently, the commonly used control measures include hand weeding, crop rotation, chemical control, trapping crops and breeding of resistant cultivars (Abang et al. 2007; Aly 2007). Because *O. cumana* causes serious harm to sunflowers during their underground stages of development; Moreover, the germination of *O. cumana* seeds is not at the same stage of sunflower development stages, that lead to the prevention and control is rather difficult. Hand weeding is time-consuming and laborious and also damages sunflower roots. Chemical control is currently the most commonly used method, but the effects of stem leaf treatment agents is not satisfactory because of the lack of chlorophyll in *O. cumana* and the lack of selective and effective herbicides (Sauerborn et al. 2002). At present, breeding for resistance has been indicated to be the most reliable way to control parasitic weeds (Škorić et al. 2010), but new physiological *O. cumana* races with increased virulence will be produced with the development of resistant cultivars (Encheva et al. 1994; Pérez-Vich et al. 2013). Therefore, it is necessary to understand the pathogenesis of *O. cumana* and the defence mechanism of sunflowers during their interaction, particularly to develop novel sources and effective strategies to control this parasitic weed.

Host resistance is critical to integrated parasitic weed control strategies. Resistance mechanisms of the host plant against *Orobanche* can operate at the preattachment, prehaustorial, and posthaustorial stages (Pérez-de-Luque et al. 2008, 2009; Yoder and Scholes 2010). Preamtachment resistance mechanisms are a result of germination inhibitors and the low-level secretion of germination stimulants (Höniges et al. 2008). The sesquiterpene lactones (STLs), dehydroxy lactone (DCL), tomentosin, costunolide, 8-epixanthatin and heliolactone have been identified from sunflower root exudate to be involved in the germination of *O. cumana* (Joel et al. 2011; Raupp and Spring 2013; Ueno et al. 2014). Prehaustorial resistance mechanisms include protein crosslinking in the host cell walls, the occlusion of vessels, the deposition of compounds such as callose and suberin and the accumulation of phenolic compounds.
(Labrousse et al. 2001; Echevarría-Zomeño et al. 2006; Pérez-de-Luque et al. 2008, 2006a, 2007). The posthaustorial resistance mechanism manifests as necrosis and death of the formed O. cumana tubercles after the parasite has developed a haustorium and established a vascular connection with the host, which is related to unsuccessful seedling penetration and the presence of a gel or gelatinous substances in the host vessels (Pérez-de-Luque et al. 2005b, 2006b; Martin-Sanz et al. 2020). In conclusion, host resistance to parasitic plants is a multicomponent process that involves very complex mechanisms, which makes resistance breeding difficult.

In recent years, the development of high-throughput transcriptome sequencing technology has become increasingly rapid (Yang et al. 2015; Yang et al. 2020). Under the stress of O. cumana, host plants initiate a series of physiological, biochemical and molecular changes, resulting in alterations in metabolism, substance biosynthesis and signal transduction pathways (Vincent et al. 2017; Yang et al. 2020). Studies have shown that the defence-related genes PAL, defensin, PR5, MYB, WRKY, SA-responsive genes and multiple immunity-related genes play important regulatory roles in sunflower resistance to broomrape (de Zélicourt et al. 2019). Several other resistance loci have been deployed to manage O. cumana infection in sunflowers, but the parasite rapidly overcomes such resistance (Moliner-Ruiz et al. 2015).

Therefore, it is very important to identify defence responses involved in conferring resistance to O. cumana, an essential step for the development of effective sunflower breeding programs. Currently, very limited information is available about the effects of O. cumana infestation on the growth of sunflowers and the molecular mechanism of parasitic interaction. Therefore, the objectives of this study were to: (i) assay certain relevant physiological and indices to evaluate the effects of O. cumana infestation on sunflower growth; (ii) identify and validate parasitism interaction-related genes from transcriptome analysis using quantitative real-time PCR (qPCR); and (iii) determine the metabolic and signaling pathways involved in the parasitism interaction response to O. cumana infestation. Undoubtedly, the results here lay a foundation for revealing the pathogenetic mechanism of O. cumana and the molecular mechanisms during parasitic interactions.

**Materials and methods**

**Plant materials and growth conditions**

Seeds of O. cumana were collected from heavily infested sunflower field in Altay, Xinjiang (86.66°N, 47.85°E). The seeds were mixture that collected from different O. cumana plants from one sunflower plot, which had been identified as race G (Yun et al. 2021). Seeds of both the susceptible sunflower cultivar SH363 and resistant cultivar TH33 were obtained from the Institute of Plant Protection, Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences, Hohhot, China. Sunflower seeds were sown in pots (11.5 cm×11 cm) filled with a mixture of soil and sand (3:1, v/v), and each pot was inoculated with 30 mg of O. cumana seeds. Sunflower seeds without O. cumana inoculation were used as controls, with three biological replicates for each treatment. All plants were grown in a greenhouse under an average 14 h light/10 h dark (20 °C/15 °C) photoperiod with 300 µmol m⁻²s⁻¹ light intensity. Plants were watered every 5 d with 100 ml of Hoagland Nutrient Solution per pot to maintain optimal moisture and nutrient levels in the soil.

**Determination of the growth and physiological parameters**

Based on the pre-experiment, to identify the key genes in the parasitism interaction stage between O. cumana and sunflowers, we obtained sunflower root samples at 10 and 20 days after inoculation with O. cumana for RNA-Seq analysis. The sunflower plant heights and fresh weights were recorded at 10, 15, 20, 25 and 30 days (sunflower 4, 6, 8, 10, 12 leaf stage) after inoculation. The symptoms of infection were observed and the parasitic number of O. cumana was counted. Due to its lack of chlorophyl, O. cumana is completely dependent on the host to provide carbohydrates. To determine whether O. cumana infection had a photoinhibitory effect on the host, the chlorophyl content and photosynthetic fluorescence of each sunflower treatment group were measured. A chlorophyl pigment mixture consisting of 200 mg of fresh leaves was extracted with 95% ethanol at 4 °C in the dark until the tissues were completely decolorized. Then, the extraction solvent was centrifuged at 4 °C and 10,000 rpm for 5 min. The OD values of the supernatant were determined spectrophotometrically at 649 and 665 nm, and 95% ethanol was used as a blank. The concentrations of Chl(a) (Ca), Chl(b) (Cb), and Chl(a+b) (C(a+b)) were calculated according to Equations 1, 2, and 3.

\[
\begin{align*}
C_a &= 13.95OD_{665} - 6.88OD_{649} \\
C_b &= 24.96OD_{649} - 7.32OD_{665} \\
C_{(a+b)} &= 6.63OD_{665} + 18.08OD_{649}
\end{align*}
\]

Three biological replicates were assessed for each treatment. Chlorophyl fluorescence parameters were measured on the center of the top two symmetrical sunflower leaves with a Dual PAM-100 system (Heinz Walz GmbH) at 10, 15, 20, 25, and 30 days after O. cumana inoculation. At least eight readings per treatment were recorded (Zhou and Leul 1998). The maximum PSII photochemical efficiency (Fv/Fm) and the actual PSII photochemical efficiency (Y(II)) were assayed after the plants were kept in the dark for 30 min.

**Determination of the effects of the sunflower root extract on the germination of O. cumana**

Sunflower roots (0.1 g) were placed in a tube and 1 mL of methanol was added. The tube was then centrifuged at 6400 rpm for 2 min after ultrasonic treatment for 30 min. The supernatant was absorbed and diluted 100-fold with distilled water for O. cumana seed germination. O. cumana seeds were placed in a tea bag and sterilized with 2% (v/v) sodium hypochlorite for 3 min, and then 75% ethanol was used for sonication for 3 minutes. Disinfectant residue on the seeds removed by rinsing with sterile water repeatedly an additional 3 times by air-drying on an ultraclean table for use. Then, the cleaned O. cumana seeds were spread on sterile water-moistened Whatman GF/A glass microfibre papers in 9 cm Petri
dishes and conditioned in the dark at 25 °C. After 5 days, the pretreated seeds were transferred to new Petri dishes containing 20 μL of sunflower root extract and 40 μL of sterile water and sealed with a sealing membrane. GR24 (10 mg L⁻¹), a synthetic strigolactone analogue, was used as a positive control, and sterile distilled water was used as a negative control. After 7 days, the germination rate of *O. cumana* seeds was calculated in stereoscopy microscope. When a radicle appeared, the seed was regarded as germinated. Each measurement consisted of three replicates.

**Determination of the MDA content and antioxidant enzyme activity**

Sunflower roots (0.1 g) were ground in phosphoric acid buffer in an ice bath and centrifuged at 8500 rpm, 4 °C for 10 min, and the supernatant was collected for reserve. The content of malondialdehyde (MDA) was determined by thiobarbituric acid (TBA) method, the activity of superoxide dismutase (SOD) was determined by nitrogen blue tetrazole (NBT) method and the activity of peroxidase (POD) was determined by guaiacol colorimetric method. Specific operation steps were performed according to the instructions of the kits provided by Comin Biotech, Suzhou, China. Three sample replicates were set for each treatment group, and three technical replicates were used.

**Total RNA extraction, library construction and sequencing**

Sunflower roots at 10 and 20 days after inoculation were sampled, immediately frozen in liquid nitrogen and then stored at -80 °C until use. Three biological replicates of each treatment were used for RNA extraction. Total RNA was extracted from 24 sunflower root samples with the addition of DNase I digestion. Eukaryotic mRNA was enriched with oligo(dT) magnetic beads and fragmented with fragmentation buffer. cDNA was synthesized with mRNA fragments and random primers. Subsequently, the purified cDNA was ligated to generate 3' adenine base overlaps after repair, and then sequencing adaptors were ligated to the fragments. Finally, the cDNA library was constructed through PCR amplification, and the quality and quantity were evaluated using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and ABI StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). After quality control (QC), the cDNA library was sequenced with an Illumina HiSeq (Illumina, San Diego, CA, USA).

**Analysis of the transcriptome and differentially expressed genes (DEGs)**

Quality control was strictly conducted on raw data. High-quality clean reads were aligned to the sunflower genome ([https://sunflowergenome.org/](https://sunflowergenome.org/)) (Kim et al. 2015). Gene expression levels were calculated as fragments per kilobase of exon model per million mapped reads (FPKM) (Trapnell et al. 2012). The DESeq2 algorithm was adopted to improve the differences in genetic testing and screening | log2 (fold change) | > 1 and q value (adjusted p value)< 0.001 were the parameters used to identify differentially expressed genes (DEGs) (Anders and Huber 2010). Expression differences among genes were compared between the susceptible cultivar SH363 and resistant cultivar TH33 10 d and 20 d after *O. cumana* inoculation. Functional annotation analysis of the DEGs was performed using the GO and KEGG databases. GO enrichment analysis divided the sequences into three categories, biological processes, cellular components and molecular functions, by GOSEQ software based on Wallenius’ noncentral hypergeometric distribution (Young et al. 2010). All the major biochemical metabolic pathways and signal transduction pathways that the DEGs were involved in were also mapped to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

**Quantitative real-time PCR analysis sequencing**

The RNA-Seq data were verified by qRT–PCR analysis. Total RNA was extracted from independent samples with an RNA-prep Pure Plant Kit (Tiangen Biotech, Beijing, China). A total of 800 ng of RNA was reverse-transcribed using FastKing gDNA Dispelling RT SuperMix (Tiangen Biotech, Beijing, China). All qPCR primers were designed using Oligo 7 software (Table 1). qPCR was conducted with Talent qPCR Premix (SYBR Green) (Tiangen Biotech, Beijing, China) in a 20 μl reaction volume containing 10 μl of qPCR Premix, 7.8 μl of ddH₂O, 0.6 μl of forward and reverse primer, and 1 μl of cDNA. U6 was regarded as a reference gene to normalize the expression level of the target genes. The 2⁻ΔΔCt method was used to calculate the gene expression levels. Each measurement had three biological replicates, and each biological replicate included three technical replicates.

**Statistical analysis**

The means and standard deviations of all experimental data were determined by three independent biological replicates. A t test was used to analyse the significant differences at the 5% probability level.

**Results**

**Symptoms of infected plants**

During the interaction with the susceptible sunflower cultivar SH363, *O. cumana* seeds can form a complete parasitic history. The *O. cumana* germination and attachment period was approximately 10 days after inoculation (Figure 1A).
Approximately 15 days after inoculation, slight swelling was observed at the attachment site of the sunflower root (Figure 1B). Additionally, *O. cumana* established a vascular connection with the sunflower root and developed a tubercle at 20 days after inoculation (Figure 1C), and the parasitic number of *O. cumana* increased with the increase of inoculation days (Figure 2). The *O. cumana* tubercle began to bud at 25 days after inoculation, and the number of inoculations increased continuously for 30 days (Figure 1D, E). However, during the resistant cultivar *TH33* interaction, *O. cumana* seeds could germinate, but no connection was established with the sunflower roots. The buds were brown and tended to die (Figure 1F, G). There was no *Orobanche cumana* parasites during the whole growth stages of resistant sunflower *TH33*.

**Germination assay of the sunflower root exudates on *O. cumana***

The germination assay results of the sunflower root exudates on *O. cumana* showed that the root extracts of both the susceptible and resistant cultivars induced the germination of the seeds of *O. cumana*, but there was a significant difference in the induced germination rates. The germination induction rates of the positive control GR24 and negative control sterile distilled water were 85.28 ± 2.76% and 0%, respectively. The germination induction rate of the susceptible cultivar *SH363* on *O. cumana* seeds was 80.54 ± 7.49%, which was close to that of positive control GR24 and 3.74 times that of the resistant cultivar *TH33*, whose germination induction rate was only 21.56 ± 3.70% (Figure 3).

**Effects of *O. cumana* on sunflower growth**

The measurement of biomass at different parasitic stages showed that compared with the uninoculated control treatment group, the plant heights and fresh weights of the two cultivars had no significant changes at the early stage of inoculation. Then, 15 days after inoculation, the plant heights of the susceptible cultivar significantly decreased to 7.99% compared with the control group, but the resistant cultivar plant heights had no significant change. After 20 days of inoculation, the fresh weights of both the susceptible and resistant sunflowers significantly decreased to 14.3% and 12.42%, respectively. However, the fresh weight of the resistant cultivar *TH33* inoculated with *O. cumana* increased by 9.65% after 30 days compared with that of the uninoculated control treatment group. These results indicated that *O. cumana* infection had different degrees of inhibition on the growth of the two sunflower cultivars during the process of parasitic interaction (Figure 4).

**Chlorophyl pigment content and photosynthesis assay**

The results of these analyses showed that the potential maximum photosynthetic capacity of the susceptible cultivar *SH363* began to decrease by 2.31% after the 15th day of inoculation, and the actual photosynthetic rate was significantly reduced by 18.82% after the 20th day of inoculation, which was consistent with the results of the above study on biomass. However, the potential maximum photosynthetic capacity and actual photosynthetic rate were not significantly different between the resistant cultivar *TH33* inoculated with *O. cumana* and the control treatment group without inoculation, and photosynthesis was not affected. The chlorophyl content determination results indicated that the susceptible

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**Figure 1.** Different growth stages of *Orobanche cumana*. (A) *O. cumana* germination and attachment at 10 days after co-cultivation with susceptible sunflower; (B) *O. cumana* establish a connection with the host root at 15 days; (C) tubercle formation at 20 days; (D) bud at 25 days; (E) the buds elongate and grow at 30 days. (F) *O. cumana* germination and attachment at 10 days after co-cultivation with resistant sunflower; (G) the buds were brown and tended to die at 20 days.

**Figure 2.** The parasitic number of *Orobanche cumana* on susceptible sunflower *SH363* in different growth stages. During the whole growth stages of sunflower, resistant sunflower *TH33* without *Orobanche cumana* parasites.
The cultivar was significantly inhibited to 50.18% after infection with *O. cumana* but no significant effect was observed on the resistant cultivar; these results were also consistent with the fluorescence results.

Malondialdehyde (MDA) content and superoxide dismutase (SOD) and peroxidase (POD) activity

The results of the enzymatic activity assays showed that compared with the control treatment group without inoculation, the MDA content and SOD and POD activity of the two cultivars were induced and enhanced to different degrees 20 days after inoculation. The MDA content increased by 66.7% in the susceptible cultivar and 58.9% in the resistant cultivar; SOD activity increased by 37.73% in the susceptible cultivar and 37.27% in the resistant cultivar; and POD activity increased by 95.7% in the susceptible cultivar and slightly increased (38.2%) in the resistant cultivar. The increase of MDA content and POD activity in susceptible cultivar was higher than that in resistant cultivar, indicating that sunflowers could respond to *O. cumana* infestation by increasing the MDA content and POD activity. *O. cumana* infestation caused a stronger degree of oxidative burst in the

Figure 3. Germination rates of *Orobanche cumana* seed included by different sunflower root extracts. GR24 was used as control.

Figure 4. Effects of *Orobanche cumana* on plant height(A), fresh weight(B), actual photosynthetic rate(C), potential maximum photosynthetic capacity(D) and chlorophyll content(E) of sunflower cultivars TH33 and SH363 at different growth stages. Each measurement had three replicates.
susceptible cultivar, which would cause certain oxidative stress in these sunflowers (Figure 5).

**Analysis of the transcriptome and differentially expressed genes**

In this study, transcriptome sequencing was performed on root samples from the resistant cultivar TH33 and the susceptible cultivar SH363 at 10 d and 20 d after inoculation with *O. cumana* or not, as in the case of the control. Each sample produced an average of 6.28 GB of data, and 85.67% of the reads were aligned against the sunflower genome, which are available at https://sunflowergenome.org/. A total of 136,156 new transcripts were finally detected, among which 66,047 were transcripts of new protein-coding genes, and the remaining 70,109 were long-chain noncoding RNAs. A total of 1005.3 million raw reads were obtained from 24 samples after RNA-Seq, ranging from 38.40 million reads to 48.44 million reads. The Q20 and Q30 of all samples were higher than 97.39% and 92.82%, respectively, indicating that the data were abundant and reliable and could be used for subsequent analysis in this study. Clean reads from each sample were compared to the reference genome after quality control. Among them, 80.74 to 92.66% of the clean reads were successfully mapped to the sunflower genome with a unique map ranging from 24.66 to 37.25% (Table 2). Sunflowers not inoculated with *O. cumana* were selected as the controls. Finally, 218 and 32 DEGs were detected in the resistant sunflower roots cocultured with *O. cumana* for 10 and 20 days, respectively, and 13 and 107 DEGs were detected in the susceptible sunflower roots, respectively. After coculture with *O. cumana* for 10 and 20 days, 52 and 181 DEGs in the sunflower roots were detected between the resistant and susceptible plants (Figure 6).

**GO and KEGG enrichment analysis**

The GO analysis showed that, among biological processes, there were many different genes involved in cellular processes and metabolic processes in the different treatment groups. In the cell composition category, genes involved in cells and cell components were the most enriched, and in the molecular function category, genes involved in adhesion and catalytic activity were the most enriched. KEGG analysis showed that transport and metabolism, signal transduction, translation, and the endocrine system were significantly affected by *O. cumana* infestation of sunflowers (Figure 7).

**Candidate parasitic-related gene selection and validation**

To validate the RNA-Seq results, we analysed the expression of 16 sunflower genes using qRT–PCR, and a strong correlation ($R^2 \geq 0.86$) was found. The results showed that the quantitative fluorescence expression trend of most genes was consistent with the transcriptome sequencing results,

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**Table 2. Alignment statistics results with reference genome for all sunflower samples.**

| Sample | Total Reads | Clean Reads(M) | Clean Bases(G) | Q20 (%) | Q30 (%) | GC (%) | Total Mapped(%) | Unique Mapped(%) |
|--------|-------------|----------------|----------------|---------|---------|--------|----------------|-----------------|
| SS-N10-1 | 39147492 | 44.0462 | 6.6069 | 97.75 | 93.41 | 46.43 | 86.18 | 36.73 |
| SS-N10-2 | 39587182 | 39.2309 | 5.8846 | 97.66 | 93.44 | 46.51 | 86.40 | 35.55 |
| SS-N10-3 | 48402626 | 39.4690 | 5.9204 | 97.57 | 93.24 | 46.62 | 86.17 | 36.94 |
| SS-P10-1 | 42915984 | 45.0712 | 6.7607 | 97.48 | 93.09 | 46.90 | 85.94 | 35.83 |
| SS-P10-2 | 42373602 | 40.3575 | 6.0536 | 97.41 | 92.90 | 46.01 | 83.26 | 32.81 |
| SS-P10-3 | 39147042 | 39.1475 | 5.8721 | 97.55 | 93.37 | 45.40 | 87.88 | 35.91 |
| RS-N10-1 | 42287042 | 39.1705 | 5.8782 | 97.54 | 93.15 | 46.25 | 85.20 | 36.46 |
| RS-N10-2 | 415819452 | 39.5872 | 5.9381 | 97.54 | 93.30 | 48.42 | 84.12 | 36.08 |
| RS-N10-3 | 39518080 | 48.4403 | 7.2660 | 97.63 | 93.63 | 47.29 | 90.33 | 33.61 |
| RS-N20-1 | 44022552 | 42.2870 | 6.3431 | 97.85 | 93.63 | 47.29 | 90.33 | 33.61 |
| RS-N20-2 | 43497826 | 41.6195 | 6.2429 | 97.39 | 92.82 | 46.07 | 85.82 | 37.25 |
| RS-N20-3 | 46254974 | 39.5181 | 5.9277 | 97.62 | 93.33 | 44.79 | 89.54 | 34.31 |
| RS-P20-1 | 38830236 | 38.8302 | 5.8245 | 98.07 | 94.38 | 48.36 | 82.36 | 35.40 |
| RS-P20-2 | 39203994 | 43.1708 | 6.4756 | 97.66 | 93.56 | 49.42 | 85.57 | 35.91 |
| RS-P20-3 | 39469042 | 41.2006 | 6.1801 | 97.70 | 93.59 | 47.51 | 86.61 | 36.57 |
| RS-P20-4 | 38830236 | 46.8017 | 7.2902 | 98.13 | 94.52 | 54.79 | 87.79 | 35.25 |
| RS-P20-5 | 43170764 | 38.4033 | 5.7605 | 98.30 | 94.93 | 56.31 | 85.59 | 34.04 |
| RS-P20-6 | 41000646 | 39.0333 | 5.8550 | 98.21 | 94.74 | 56.29 | 82.80 | 34.94 |
| RS-P20-7 | 38899442 | 39.2663 | 5.8699 | 97.42 | 92.99 | 47.47 | 84.62 | 35.64 |
| RS-P20-8 | 40571166 | 42.9160 | 6.4374 | 97.65 | 93.49 | 48.23 | 80.74 | 33.96 |
| RS-P20-9 | 40357482 | 42.3736 | 6.3560 | 97.92 | 94.27 | 49.12 | 81.73 | 34.87 |
| RS-P20-10 | 48601656 | 44.0226 | 6.6034 | 98.02 | 94.21 | 50.57 | 81.17 | 24.66 |
| RS-P20-11 | 38403270 | 43.4978 | 6.5247 | 98.01 | 94.19 | 48.23 | 88.11 | 29.52 |
| RS-P20-12 | 39033340 | 46.2530 | 6.9382 | 98.06 | 94.32 | 51.03 | 92.66 | 32.63 |
indicating that the gene expression data obtained by transcriptome sequencing were reliable (Figure 8).

Heat shock protein factor (HsP90A) is involved in hypersensitivity reactions and plays an important role in the plant PTI immune signaling system and early defence response. MYC2 is a core transcription factor in the plant jasmonic acid metabolic pathway, which is closely related to the plant hormone response and plant stress response. At the early parasitism stage, the susceptible cultivar had a clear defence response, resulting in high expression of the genes HsP90A and MYC2, which then decreased rapidly with increasing parasitism severity, indicating that these genes could not resist the influence of parasitism. However, there was no notable defence response in the resistant cultivar, but by the late parasitism stage, the HsP90A and MYC2 genes were highly expressed to resist parasitism. These results suggest that the rate, degree and efficiency of defence system activation during the parasitic interaction may be related to sunflower resistance. (S)-2-Hydroxy-acid oxidase (HAO) is a catalytic enzyme involved in the photosynthesis pathway of plants that can enhance the oxygenation activity of Rubisco and inhibit its carboxylation reaction, thus playing a feedback role to regulate the photosynthetic pathway of plants (https://www.uniprot.org/uniprot/A0A251T4T6).

The gene expression of HAO was upregulated in the susceptible cultivar after 20 d of infection by O. cumana, but there was no significant effect on the resistance interaction. These results indicated that the photosynthetic system of sunflowers was inhibited by O. cumana and that the photosynthesis pathway might be involved in the defence response of sunflowers to O. cumana. In the phenylpropanoid metabolic pathway, 4-coumarate coenzyme A ligase (4CL), a gene related to secondary metabolism, was highly expressed during the parasitic interaction with the resistant cultivar. Additionally, 4CL was highly expressed during the early-stage parasitism interaction with the susceptible cultivar, and the amount of induced expression decreased with parasitism severity after 20 d. Prephenate dehydratase (PDA) and cinnamyl alcohol dehydrogenase (CAD) are related to the phenylpropanoid and lignin synthesis pathways, respectively. They were found to be widely expressed in the resistant cultivar after infection with O. cumana, while their expression was unchanged in the susceptible species. These results indicate that the phenylpropanoid metabolic pathway and lignin synthesis pathway may be involved in the parasitic interaction between O. cumana and sunflowers (Figure 9).

**Discussion**

It has been reported that some resistant cultivars of legumes and sunflowers develop resistance by secreting very low levels of germination stimulants (Pérez-de-Luque et al. 2006a). In this study, the root exudates of the susceptible sunflower SH363 showed stronger induction rates on the germination of O. cumana seeds compared with resistant sunflower TH33, suggesting that stimulants could promote seed germination in the roots of different sunflower cultivars and that the differences in their types and contents might be related to the resistance of sunflowers. Studies have shown that sunflower broomrape inhibits the growth of susceptible sunflower cultivars by competing with the host for water and nutrients. In our study, the pot experiment results showed that the bud tube appeared brown and tended to die after O. cumana seed germination during the resistance interaction between O. cumana and sunflowers (Figure 1F, G). This result is supported by Echevarría-Zomeño et al. (2006), who used histochemical techniques and found that the formation of resistance may be related to cell wall embolization and protein cross-linking of host cells. However, O. cumana could undergo a complete parasitic process during the susceptible interaction, including seed germination and contact with the host root after coculture for 10 days (Figure 1A); nodule formation after coculture for 20 days (Figure 1C); and nodule germination and growth after 30 days (Figure 1D), which was unearthed in 45-60 days. In some susceptible interactions, the xylem vessels of parasitic plants are directly connected to the vascular tissues of parasitic plants.
Figure 7. GO analysis and KEGG pathway enrichment analysis of differentially expressed genes (DEGs) at sunflower.
Figure 8. The validation of RNA-seq results of 16 differentially expressed genes (DEGs) with qPCR.
Figure 8 Continued
host plants, thus transporting water and nutrients from the host to the parasitic plant (Labrousse et al. 2001; Echevarria-Zomeño et al. 2006), which may also explain the significant decreases in the plant height and fresh weight of the susceptible cultivar SH363 during the susceptible interaction in our study (Figure 4A, B). These results are similar to those of Mauromical et al., who found that Orobanche aegyptiaca can significantly inhibit tomato growth because it absorbs water and nutrients and affects chlorophyll synthesis and photosynthetic efficiency in tomatoes (Mor et al. 2010).

Compared with the resistant cultivar, broomrape infection decreased chlorophyll levels in susceptible sunflower leaves. Previous studies have shown that light is essential for the biosynthesis of chlorophyll and that broomrape infection observably influences the contents of chlorophyll (Yang et al. 2020); these findings strongly correlate with the observations in this study. Chlorophyll is crucial for photosynthesis by its absorption and transfer of light. As expected, a weaker photosynthetic capability was observed in susceptible sunflower leaves during broomrape infection.

MDA is a product of lipid peroxidation and is considered a marker of oxidative damage (Mor et al. 2010). When plants are stressed, a large amount of reactive oxygen species will be produced, which will damage the stability of the plant cell lipid membrane. However, SOD and POD in plants are considered the main antioxidant substances that can remove reactive oxygen species, which can endow plants with a certain tolerance to stress. Studies have shown that callose deposition and protein cross-linking are closely related to peroxidase activity (Bradley et al. 1992). In our study, compared with the control groups, the MDA contents and SOD and POD activities of the two cultivars were induced and enhanced to different degrees 20 days after inoculation with O. cumana, and the degree of enhancement in the resistant cultivar was higher than that in the susceptible cultivar. The results indicated that the sunflowers could respond to O. cumana infestation by increasing the content of MDA and the activity of SOD and POD. O. cumana infestation caused a stronger oxidative burst in the susceptible cultivar, which would cause a certain degree of oxidative stress in sunflowers. A series of physiological and biochemical changes in sunflowers were caused by O. cumana infection, and these changes may directly affect sunflower cultivar infection resistance.

The plant defence response, peroxidase, suberization, lignification and production of phenolic compounds are well-known defensive responses to several biotic and abiotic stresses, including other genera of parasitic plants (Labrousse et al. 2001; Pérez-de-Luque et al. 2006a; Šestacova et al. 2016). During the O. cumana and sunflower parasitic interaction process, large changes in gene expression occurred on both sides. In this regard, large-scale gene expression analysis is important to unveil the molecular mechanisms of the pathogenesis of O. cumana and defence mechanisms of sunflowers. Six parasite-related genes were selected from the sunflower transcriptome analysis, and their expression patterns were favored by qPCR validation, indicating that they are presumably involved in parasitic interactions. Among these six parasite-related genes, HsP90A, MYC2 and HAO were discovered for the first time to be involved in the parasitic interaction between sunflowers and O. cumana. The plant defence response-related genes HsP90A and MYC2 were dramatically highly expressed in the inoculated roots of resistant sunflower after inoculation, while in susceptible sunflower cultivars, their expression was high in the early parasitism period and then decreased with increasing parasitism severity. These results suggest that the rate, degree and efficiency of defence system activation during parasitic interaction may be related to sunflower resistance. HAO is a catalytic enzyme in the photorespiration pathway of plants that can enhance the oxygenation activity of Rubisco and inhibit its carboxylation reaction, thus playing a feedback role to regulate the photosynthetic pathway of plants (https://www.uniprot.org/uniprot/A0A251T4T6). The gene expression of HAO was upregulated in the susceptible cultivar 20 d after O. cumana infection, but there was no significant effect on the resistance interaction (Figure 9C). These results were consistent with the above actual

![Figure 9](image-url) The expression levels of differentially expressed genes (DEGs) related with parasitic interaction in sunflower and Orobanche cumana.
photosynthetic rate (Figure 4C) results, indicating that the photosynthetic system in these sunflowers was inhibited by *O. cumana* and that the photorespiration pathway might be involved in the sunflower defence response. In the phenylpropanoid metabolism pathway, three genes, 4CL, CAD, and PDA, showed dramatically higher expression in the resistant sunflower cultivar in the later stage of parasitism interaction than in the susceptible sunflower cultivar. These results indicate that the phenylpropanoid secondary metabolic pathway regulated by the genes 4CL, CAD and PDA may confer different sunflower cultivars with different degrees of resistance to *O. cumana*. Anterola A M et al. (Anterola and Lewis 2003) showed that reducing the expression of 4CL in plants reduces the resistance of plants to pathogen infestation, which highly supports our speculations. However, in the future, more investigative work is required to elucidate the actual functions of these genes during sunflower resistance to *O. cumana*.

The parasitic interaction mechanism between *O. cumana* and sunflowers is a combination of a cultivar of mechanisms, including multiple signaling pathways, an *O. cumana* germination stimulation regulatory mechanism, the recognition of invasive plants, the accumulation of defence-related genes and the synthesis of lignin. In addition, the rate, degree and efficiency of defence system activation during the parasitic interaction may be related to sunflower resistance.

**Conclusions**

In conclusion, plants can change the related physiological and biochemical metabolic pathways by regulating gene expression under *O. cumana* stress. Determination of the physiological and biochemical indices of sunflowers under *O. cumana* stress further validates sunflower transcriptome sequencing. *O. cumana* infestation distinctly restricted the development of sunflowers and decreased their photosynthesis. In sunflowers, six parasite-related genes were identified, and sixteen differential genes were validated. Furthermore, sunflowers might guard against the infestation of *O. cumana* by increasing their antioxidiant activity. Accordingly, the inadequate defence response of susceptible sunflower cultivars compared with resistant sunflower cultivars may be related to the rate, degree and efficiency of defence system activation during parasitic interaction. In summary, this study will help to explore the parasitic interaction molecular mechanism between *O. cumana* and sunflowers and provide important genetic resources for sunflower resistance breeding and *O. cumana* control.

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