The Critical Role of Polyketide Synthase Gene On The Swainsonine Biosynthesis in The Fungus *Metarhizium Anisopliae*

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Abstract

Swainsonine (SW) is the principal toxic ingredient of locoweeds, and is produced by fungi including *Metarhizium anisopliae*, *Slafractonia leguminicola*, and *Alternaria oxytropis*. While the SW biosynthesis pathway of fungi and the catalytic enzyme genes that regulate synthesis are not cleanly. In this study, we used homologous recombination (HR) to knock out and interfere with the polyketide synthase gene (*pks*) of *M. anisopliae* to determine its effect on the SW biosynthesis pathway. The concentration of SW was measured in the fermentation broth of *M. anisopliae* at 1 d, 2 d, 3 d, 4 d, 5 d, 6 d or 7 d using LC-MS. The gene for the *pks* gene was detected by RT-qPCR. Day 5 of *M. anisopliae* gave the highest content of SW and the highest expression of the *pks* gene. To determine the role of the *pks* gene in the SW biosynthesis pathway of *M. anisopliae*, we used PEG-mediated homologous recombination (HR) to transform a wild-type strain (WT) with a Benomyl (*ben*)-resistant fragment to knock out the *pks* gene producing a mutant-type strain (MT) and used PEG-mediated RNAi to transform a wild-type strain (WT) with a Benomyl (*ben*)-resistant plasmid to interfere with the *pks* gene. A complemented-type (CT) strain was produced by adding a complementation vector that contains the geneticin (G418) resistance gene as a marker. The content of SW didn't detected in MT strain, and returned to the original level in the CT strain, while the content of SW was significantly decreased in RNAi strain. We suggest that mutation and RNAi in the *pks* gene affect the cell wall formation of *M. anisopliae*, while the colony diameters, phenotypes, and growth rates did not change significantly, and no obvious changes in other cellular organelles were noted. These results indicate that the *pks* gene plays a crucial role in the SW biosynthesis of *M. anisopliae*, which provides an important theoretical basis for illuminating the SW biosynthesis and solving locoism in livestock.

Introduction

In 1909, Marsh's [1] first study confirmed that animal locoweeds were related to the consumption of certain *Oxytropis* and *Astragalus* plants. In 1936, Fraps [2] discovered its main toxic components from the poisonous plant *Astragalus earlei*. Colegate et al. [3] isolated an indolizidine alkaloid, swainsonine (SW) (Supplementary Fig. 1) for the first time from *Swainsona canescens*. SW was subsequently isolated from locoweed plants, and shown to be the primary toxic component of locoweeds [4, 5], It is also proved that SW is the main toxic component of locoweeds, and it is the only toxin characterized by chronic nervous system dysfunction caused by animals eating these plants [6, 7]. Subsequent studies found that the SW can be produced by *Alternaria oxytropis* [8–10], *Metarhizium* spp. [6, 11], and *Slafractonia leguminicola* [12, 13], and many other fungi, but the SW biosynthesis pathway of fungi and the catalytic enzyme genes that regulate synthesis have not been completely characterized.

Studies have found that by adding different precursor substances to the medium to observe SW production, the results show that L-pipecolic acid can significantly increase the content of SW [14], and it is determined that it is an important precursor substance for SW biosynthesis. Lu et al. [15] supposed the saccharopine dehydrogenase (SDH), saccharopine oxidase (FAD2) and pyrroline-5-carboxylate reductase (P5CR) is related catalytic enzyme in the SW biosynthesis pathway of L-Lysine to L-pipecolic acid by

whole-genome sequencing on *A. oxytropis*. Some scholars have studied the SW biosynthesis related gene SDH in *A. oxytropis* [16, 17] and found that this gene has an impact on SW biosynthesis and the accumulation of L-pipecolic acid, a precursor substance. Recently, Cook et al. [18] published a comparison of the swainsonine biosynthetic gene cluster SWN, among many swainsonine-producing fungi. All the swainsonine-producing fungi of *Metarhizium* sp. contained a common gene cluster "*SWN*", which included $swnH_1$, $swnH_2$, *swnK*, *swnN*, *swnR*, *swnA* and *swnT*. Fungi that do not produce swainsonine do not contain the SWN cluster. These genes encode catalytic enzymes involved in the L-pipecolic acid to SW biosynthesis. On this basis, [18] successfully constructed a *swnK* knockout vector for the entomopathogen *M. robertsii*, and obtained a *swnK* mutant strain by HR. The LC-MS analysis showed that SW was eliminated in the fermentation broth of the mutant strain and the content of SW returned to normal after complementation with the *swnK* gene, indicating that the *swnK* gene is essential for SW biosynthesis. Alhawatema et al. [19] conducted RNAi studies on the *swnK* homologous gene *pks* of *S. leguminicola* and found that *pks* gene also plays an important role in SW biosynthesis. Subsequently, Luo et al. [20] studied other genes in the SWN gene cluster of *M. robertsii* and found that the biosynthesis of SW is a multi-branch synthetic pathway, not a linear biosynthetic pathway. Noor et al. [21] identify other swainsonine-producing fungi by PCR detect the *swnK*-KS nucleotide sequence of fungi.

M. anisopliae is a broad-spectrum insect pathological fungus, can produce SW [22, 23]. At present, the catalytic enzyme related to SW biosynthesis in *M. anisopliae* is not completely clear. In our earlier studies, we inferred that *pks* gene could play an important role in the fungus swainsonine biosynthesis [15]. Therefore, in order to study the role of *pks* gene in SW biosynthesis of *M. anisopliae*. Using HR to knock out the *pks* gene of *M. anisopliae*, the mutation of the *pks* gene caused *M. anisopliae* to no longer produce SW, and the SW content in the complementary strain (CT) was similar to that in the wild-type strain. And the specific RNAi plasmid was introduced into *M. anisopliae*, the content of SW was also significantly reduced. In addition, the mutation or interference of the *pks* gene damages the cell wall of *M. anisopliae*. This change is expected to become one of the features for identifying *pks* gene mutations in swainsonine-producing fungi.

Results And Discussions

Detection of SW and RT-qPCR analysis of pks gene of M. anisopliae **in different periods.** To detect the SW content at different time points, the same volume of *M. anisopliae* fermentation broth and the dried mycelium were concentrated, and the level of SW was detected using a LC-MS. The standard curve was drawn according to the calculated regression equation: Y=486.14665X+34.36851 (r=0.99049) (Supplementary Fig. 2A). From the linear regression equation of mass concentration-peak area of SW, the SW content of *M. anisopliae* was calculated at 1, 2, 3, 4, 5, 6 and 7 days of growth, respectively. Day 5 of *M. anisopliae* gave the highest content of SW (Supplementary Fig. 2B).

RT-qPCR was conducted for *pks* gene to determine their relative expression at 1d, 2d, 3d, 4d, 5d, 6d and 7d. The *pks* gene expression level of each strain is similar to the content of SW, day 5 of fermentation gave the highest expression of *pks* gene (Supplementary Fig. 3).

Sensitivity Screening of M. anisopliae. *M. anisopliae* was inoculated into SDA media with different concentrations of benomyl or G418. After 10 days, *M. anisopliae* is more sensitive to 40 μg/mL benomyl and 500 μg/mL G418 (Supplementary Fig. 4A, B). After the *pks* gene MT is obtained, the MT is the most sensitive of to 2 mg/mL G418 (Supplementary Fig. 4C).

Production of MT, CT and RNAi strain. To determine the role of the *pks* gene in the SW biosynthesis pathway of *M. anisopliae*, homologous recombination was used to knock out *pks* and RNAi interference vector was used to interfer *pks* gene. The resulting transformant grew on SDA media containing 40 μg/mL benomyl. Subsequently, the primer L8/R8, L9/R9, L10/R10, L11/R11, L12/R12 and ITS1/ITS4 (Supplementary Table 1 and Supplementary Fig. 5) set was used to identify the genomic DNA of the transformant (MT, CT and RNAi strain) using electrophoresis and sequencing (Fig. 1). To verify the status of MT, a complement was produced by transforming the wild-type *PKS* gene and G418 gene of pSilent-Dual1 plasmid in pUC19 into the MT. The complement transformant was grown on SDA medium containing 2 mg/mL G418 and was identified as above.

Phenotypic observation and growth rate determination of WT, MT, CT and RNAi strain. The WT, MT, CT and RNAi strain isolates were grown on SDA plates (with or without 100 μg/mL Congo red) for 10 d (Fig. 2) to compare growth. The colony diameters, phenotypes, dry weight of mycelium and growth rates did not change significantly (Fig. 2, 3), the hyphae of the MT and the RNAi strain were turned obviously red on SDA plates (with 100 μg/mL Congo red). Transmission electron microscopy showed that the cell wall periphery of the MT and RNAi strain was in a diffuse state, while the cell wall boundaries of the WT and CT were obvious, while other organelles in the cells did not change significantly (Fig. 2), such mutation and RNAi in the *pks* gene affect the cell wall formation of *M. anisopliae*.

LC-MS detection of SW and RT-qPCR analysis of pks gene of MT, CT WT and RNAi strain. By detecting the swainsonine content and *pks* gene expression of MT, CT WT and RNAi strain, it was found that the MT did not produce SW, and the swainsonine content of the RNAi strain was significantly reduced, which was 0.018±0.005 μg/mL, the SW content of the CT is basically the same as that of the wild-type strain, which are 0.581±0.084 μg/mL and 0.625±0.056 μg/mL (Fig. 4A), respectively. The standard curve was drawn according to the calculated regression equation: Y=89.15243X+18.12349 (r = 0.99493) (Fig. 4B). The *pks* gene expression level of each strain is similar to the content of SW (Fig. 5).

Cook et al. [18] and Luo et al. [20] proved that the *swnK* gene is necessary for the SW biosynthesis of *M. robertsii*, and Noor et al. [21] proved the swnK-KS nucleotide sequence of fungi was used to identifying other swainsonine-producing fungi by PCR. Therefore, we supposed that the *pks* gene that is homologous to the *swnK* gene in *M. anisopliae* is necessary for SW biosynthesis, and Alhawatema et al. [19] proved after interfering with the *pks* gene SW content is lower significantly in *S. leguminicola*, which is consistent with the significant reduction in SW content after the *pks* gene of *M. anisopliae* was interfered in this study. However, after the *pks* gene was knocked out, *M. anisopliae* no longer produced swainsonine, indicating that the *pks* gene it is necessary for SW biosynthesis of *M. anisopliae*.

A large number of studies have shown that polyketide synthase is widely present in bacteria, fungi, actinomycetes and plants, and it can catalyze the production of substances with anti-infection, anti-fungal, anti-tumor, immunosuppressive and other biological activities [24–26]. In addition, some researchers have found that the biosynthesis of fungal cell walls is also related to the *pks* gene [27]. In this study, WT, MT, CT, and RNAi strain of *M. anisopliae* were inoculated on the media (with 100 μg/mL Congo red), found that the mutation of the *pks* gene caused the permeability of the strain to change, and the strain was stained red, verifying that the *pks* gene is related to the formatiom of the fungal cell wall.

In recent years, researchers have studied the cultivation conditions of *M. anisopliae* and found that *M. anisopliae* does not require high carbon sources, but high-quality nitrogen sources are indispensable, and yeast extract and peptone are relatively commonly used nitrogen sources. Source [28–32], therefore, the potato agar medium most commonly used by fungi is not suitable for its growth, while SDA media is more in line with the requirement of strains for higher nitrogen sources, which is added peptone to it. During the culture process, Li and Holdom [33] used the Sigmoid function to construct a liquid fermentation kinetic model of *M. anisopliae* IMI330189, and used Origin7.5 software to fit the growth of the mycelium and the substrate during the fermentation process. It is found that the strain consumes the highest rate of total sugar and total nitrogen in the culture medium at about 10 hours of growth, and its growth rate reaches the maximum at the communication between the first day and the second day, which is consistent with the significant increase on the second day in the dry weight of mycelium measured in this experiment.

Materials And Methods

Strain and fermentation culture. *M. anisopliae*, obtained from Xi'an Jin Berry Biological Technology Co. Ltd., Shaanxi, China, was inoculated onto Sabouraud medium (SDA) [34] and cultured at 28°C for 10 days. Conidium (1.0×10^7) of *M. anisopliae* were transferred into 300 mL SDA liquid culture media (without agar) and fermented at 28°C, 180 rpm, for 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, or 7 d. The mycelium and the fermentation broth were filtered through three layers of Miracloth (EMD Millipore Corp, Billerica, MA, USA) to collect the mycelium. The mycelium was dried at room temperature, dried mycelium and the cultures stored at 4°C for use. then the mycelium is extracted with methanol [35]. The cultures and the extract were analyzed using HRLC-MS LC-30A+Triple TOF 5600+ (AB SCIEX) using the methods of Luo et al. [20]. SW concentration was tested three times for each strain.

RT-qPCR analysis of pks gene of M. anisopliae. Fungal RNA was extracted using the E.Z.N.A. Fungal RNA Kit (Omega). RNA was reverse transcribed into DNA by using PrimeScript^{TM}RT reagent Kit (Takara). Primers for amplification of *pks*, and 5.8S rRNA are shown in Supplementary Table 2. The genes were amplified using RT-qPCR with the following conditions: 1 cycle of 95°C for 10 min; 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 90 s, and an extension at 72°C for 32 s. Cultures were tested using RT-qPCR at 1 d, 2 d, 3 d, 4 d, 5 d, 6 d and 7 d.

Sensitivity Screening of M. anisopliae. *M. anisopliae* was inoculated into SDA media with 0 µg/mL, 100 µg/mL, 300 µg/mL, 600 µg/mL, and 1,000 µg/mL of benomyl or G418, the growth of the colony was observed after 10 days. After the *pks* gene MT is obtained, sensitivity of the MT was observed after 10 days to G418.

Identification of pks gene of M. anisopliae. Fungal DNA was extracted using the CTAB method. Primers for amplification of *pks* (Supplementary Table 2) were designed from the *pks* sequence from Cook et al. [18], GeneBank KID61008 of *M. anisopliae* ARSEF 549. The *pks* gene was amplified from *M. anisopliae* DNA using L1/R1 primers.

Vector construction. The upstream and downstream fragments of the *pks* gene and the benomyl (fungicide) resistance gene (*ben*) were inserted into pUC19 (Takara) digested with *Eco*R I/*Bam*H I (Takara) using the In-Fusion® HD Cloning System (Takara) to construct a knockout construct targeting the *pks* gene (Supplementary Fig. 6A, 7A, 8A and Supplementary Table 3).

The primers Ben-F and Ben-R (Supplementary Table 3) were used to amplify the *ben* resistance gene from pBARGPE1-BenA (Wuhan Jingxiu Scientific Biotechnology Co., Ltd., China) as a template. The primers F2/R2 and F3/R3 (Supplementary Table 3) were used to amplify the upstream target fragment (*pks*-I) and the downstream target fragment (*pks*-II), respectively, of the *pks* gene from the genomic DNA of *M. anisopliae*. The *pks*-I, *ben*, *pks*-II and the double-cut pUC19 vector were ligated using In-Fusion cloning. The *pks* gene fragment was amplified using primers L6/R6 (Supplementary Table 3) from the genomic DNA of *M. anisopliae*. The *G418* gene was amplified using primers L7/R7 (Supplementary Table 3) from pSilent-Dual1. To produce a complementation vector, the *pks* gene and G418 gene were inserted the MCS of pUC19 vector using In-Fusion cloning (Supplementary Fig. 6B, 7B, 8B). The *pks*-1 and *pks*-2 fragment were amplified using primers L4/R4, L5/R5 respectively (Supplementary Table 3) from the genomic DNA of *M. anisopliae*. To produce a RNAi vector, the *pks*-1 and *pks*-2 fragment were inserted the MCS1 and MCS2 of pSilent-1 vector respectively using In-Fusion cloning (Supplementary Fig. 6C, 7C, 8C).

Preparation of protoplasts. Conidium (1.0×10^7) of *M. anisopliae* grown on SDA media were transferred into each 300 mL flask of SDA liquid culture media (without agar), and incubated at 28°C, 180 rpm for 3 d. The resulting mycelia were filtered through sterile miracloth. To the collected hyphae were added different concentrations of enzymatic hydrolysate (Sigma Aldrich) prepared with 1.2 M KCl, and hydrolyzed at 30°C, 100 rpm 3 h. The optimal combination of enzymes and conditions were determined based on protoplast yield. Yield from different enzymes, including 1% snail enzyme, 1% cellulase, and 1% lysing enzymes, and combinations of the enzymes were also tested. The enzymatically digested mixtures were filtered through a layer of sterile miracloth and two layers of filter paper into a sterile 50 mL centrifuge tube, and the protoplasts were washed extensively with 1.2 M KCl and centrifuged at 4000 rpm for 6 min at room temperature. After discarding the supernatant, 10 mL of STC Buffer (0.6 M Sorbitol; 10 mM Tris-HCl; 10 mM $CaCl_2$, pH 6.5) was added and the protoplasts were gently resuspended. The mixture was centrifuged at 4000 rpm for 6 minutes. After discarding the supernatant, 1 mL of STC Buffer was

added. The protoplasts were then centrifuged at room temperature at 3500 rpm for 6 min, which was repeated. Finally, protoplasts were adjusted to 2-5×10^7/mL for subsequent experiments.

PEG mediated DNA transformation. Transformation of the protoplasts were done as in Proctor et al. [36] Approximately 5-10 μg of the linearized *pks* knockout vector and RNAi vector were added to 50 mL centrifuge tube containing 2-5×10^7/mL protoplasts, and allowed to stand at room temperature for 20 min without shaking, respectively. Then 1-1.25 mL of 40% PTC (40% PEG 8000, 20% sucrose, 50 mM $CaCl_2$, 10 mM Tris-HCl) was added to the tube (mixed thoroughly by inversion), and let stand at room temperature for 20 min without shaking. Thereafter, 5 mL of TB_3 (0.3% Yeast Extract, 0.3% acid hydrolyzed casein, 20% sucrose) containing 50 g/mL ampicillin (Sigma Aldrich) was added and shaken at room temperature overnight. The overnight protoplasts were centrifuged at 4000 rpm for 6 min, the supernatant was discarded, and about 1 mL of the remaining liquid was used to suspend the remainder. The regenerated protoplasts were added to 10 mL of Bottom Agar (0.3% Yeast Extract, 0.3% acid hydrolyzed casein, 20% sucrose, 1% Agar) containing 20 μg/mL benomyl. After incubation at 30°C for 10 hours, Top Agar (0.3% Yeast Extract, 0.3% acid hydrolyzed casein, 20% sucrose, 1.5% Agar) containing 40 μg/mL benomyl was added. After 3-5 d, a single colony transformant grew on the plate, which was transferred to SDA medium containing 40 μg/mL benomyl. The wild type *M. anisopliae* was used as a control. The *pks* gene mutant strain of *M. anisopliae* was named MT. The transformation of the complement vector was the same as described above, and 2 mg/mL of G418 was used for screening of the complement (CT).

RT-qPCR identification of MT, CT and RNAi strain. Fungal RNA was extracted using the E.Z.N.A. Fungal RNA Kit (Omega). RNA was reverse transcribed into DNA by using PrimeScript^{TM}RT reagent Kit (Takara). Primer L8/R8, L9/R9, L10/R10, L11/R11, L12/R12 and ITS1/ITS4 for amplification of MT, CT and RNAi strain are shown in Supplementary Table 1 and the schematic diagram is shown in Supplementary Fig. 5. The genes were amplified using RT-qPCR with the following conditions: 1 cycle of 95°C for 10 min; 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 90 s, and an extension at 72°C for 32 s. Cultures were tested using RT-qPCR at MT, CT and RNAi strain.

Phenotypic observation and growth rate determination MT, CT, WT and RNAi strain. Conidium (1.0×10^7) of MT, CT, WT and RNAi strain were inoculated into the same position on the SDA medium (with or without 100 μg/mL Congo red) and grown at 28°C for 10 d, after which they were measured for diameter and photographed.

SW content detection of WT, MT, CT and RNAi strain in M. anisopliae. The WT, MT, CT and RNAi strain were inoculated into SDA medium cultured at 28°C for 10 days. Conidium (1.0×10^7) of each strain were transferred into 300 mL flasks of SDA (without agar) culture medium and grown at 28°C, 180 rpm, for 5 d. The mycelium and the fermentation broth were filtered through three layers of Miracloth (EMD Millipore Corp, Billerica, MA, USA) to collect the mycelium. The mycelium was dried at room temperature, dried mycelium and the cultures stored at 4°C for use, and then the mycelium is extracted with methanol [35].

The cultures and the extract were analyzed using HRLCMS LC-30A+Triple TOF 5600+ (AB SCIEX) using the methods of [20]. SW concentration was tested three times for each strain.

Statistical analysis. In this study, each measurement was tested three times. Statistical analysis was performed on the measured data using SPSS 20.0 software. The results were expressed as mean ± SEM. One-way ANOVA was performed on each sample, *P<0.05, indicating a significant difference between the two groups, **P<0.01, indicating that the difference between the two groups is highly significant. Results from the cultures were used for determining the optimal time periods for swainsonine production. The mass concentration peak area for SW was compared using linear regression. The colony diameters were measured by ruler. RT-qPCT data were analyzed using the $2^{-\triangle\triangle}$CT method.

Conclusions

In this study, we demonstrated that the mutation of the *pks* gene will cause *M. anisopliae* to no longer produce SW, and the recovery of SW concentration to normal levels in the CT. This suggests that the *pks* gene plays an important role in the SW biosynthesis of *M. anisopliae*. In addition, the *pks* gene in *M. anisopliae* is closely related to the *pks* gene of *A. oxytropis* (Fig. 6) [37]. Thus it have a good reference value to SW biosynthesis and its catalytic enzyme gene in the endophytic fungus of locoweed and will contribute to solve locoism in the future.

Declarations

Acknowledgements

Not Applicable.

Authors' Contributions

H.L., B.Z. and C.M. contributed to the conception of the focus for the study. L.S., E.H., Y.Z., Z.G., K.W., and Y.Z. performed the experiments. J.W. and C.M. analyzed the data. L.S. and E.H. contributed to the compilation of all sections, figure and table design, and wrote the first draft of the manuscript. All authors contributed to revision, read and approved the submitted version of the manuscript.

Competing Interests

The authors declare no competing interests.

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Figures

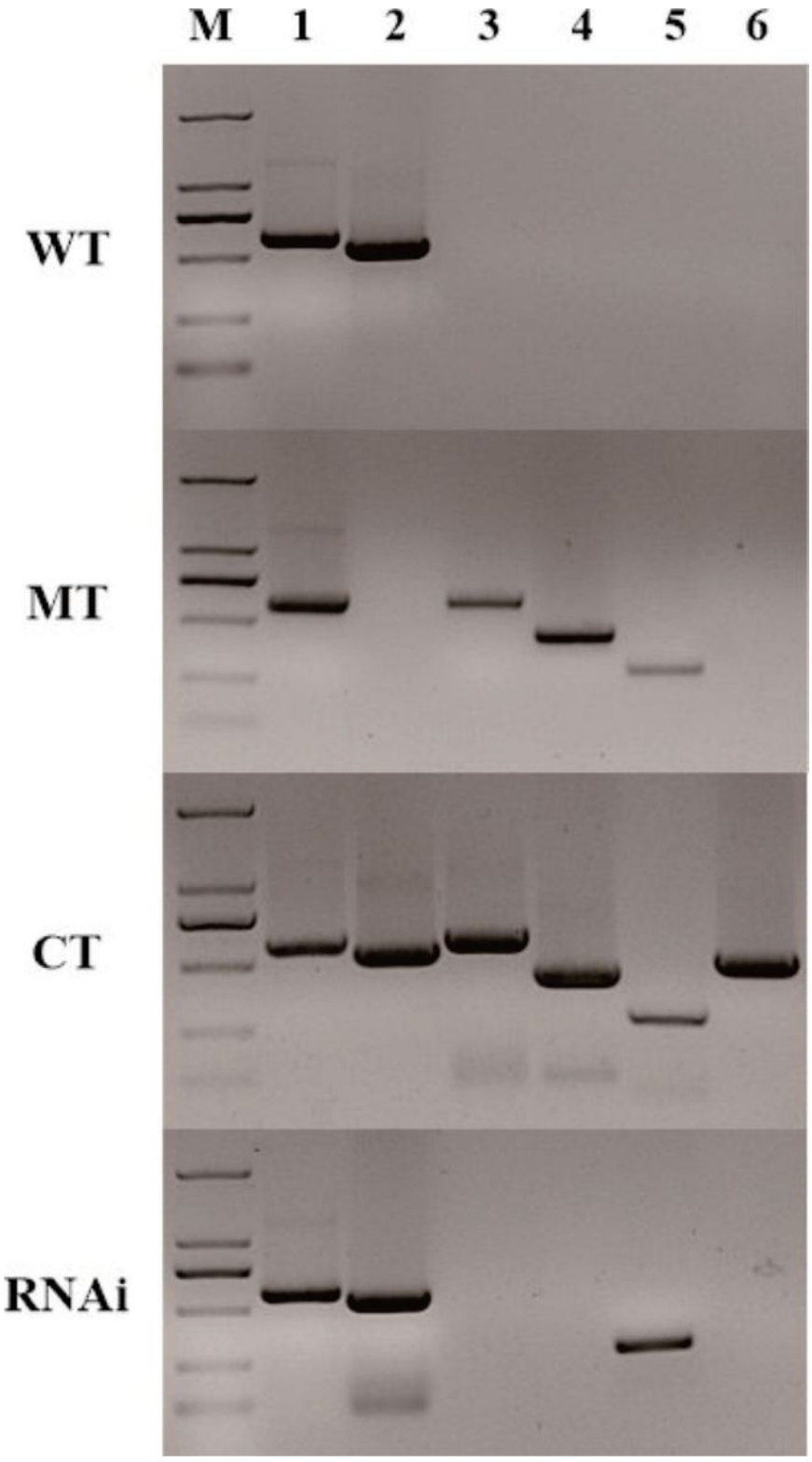

Figure 1

RT-qPCR identification of MT, CT and RNAi strain. Lane 1: Conserved sequence fragment of ITS of *M. anisopliae*; Lane 2: *Pks* gene starting sequence; Lane 3: the junction of the upstream homologous arm of the *pks* gene and the ben gene; Lane 4: the junction of the ben gene and the downstream homologous arm of the *pks* gene; Lane 5: *Ben* gene fragment; Lane 6: *G418* gene fragment.

Marker：2 000 bp, 1 000 bp, 750 bp (Highlight), 500 bp, 250 bp and 100 bp.

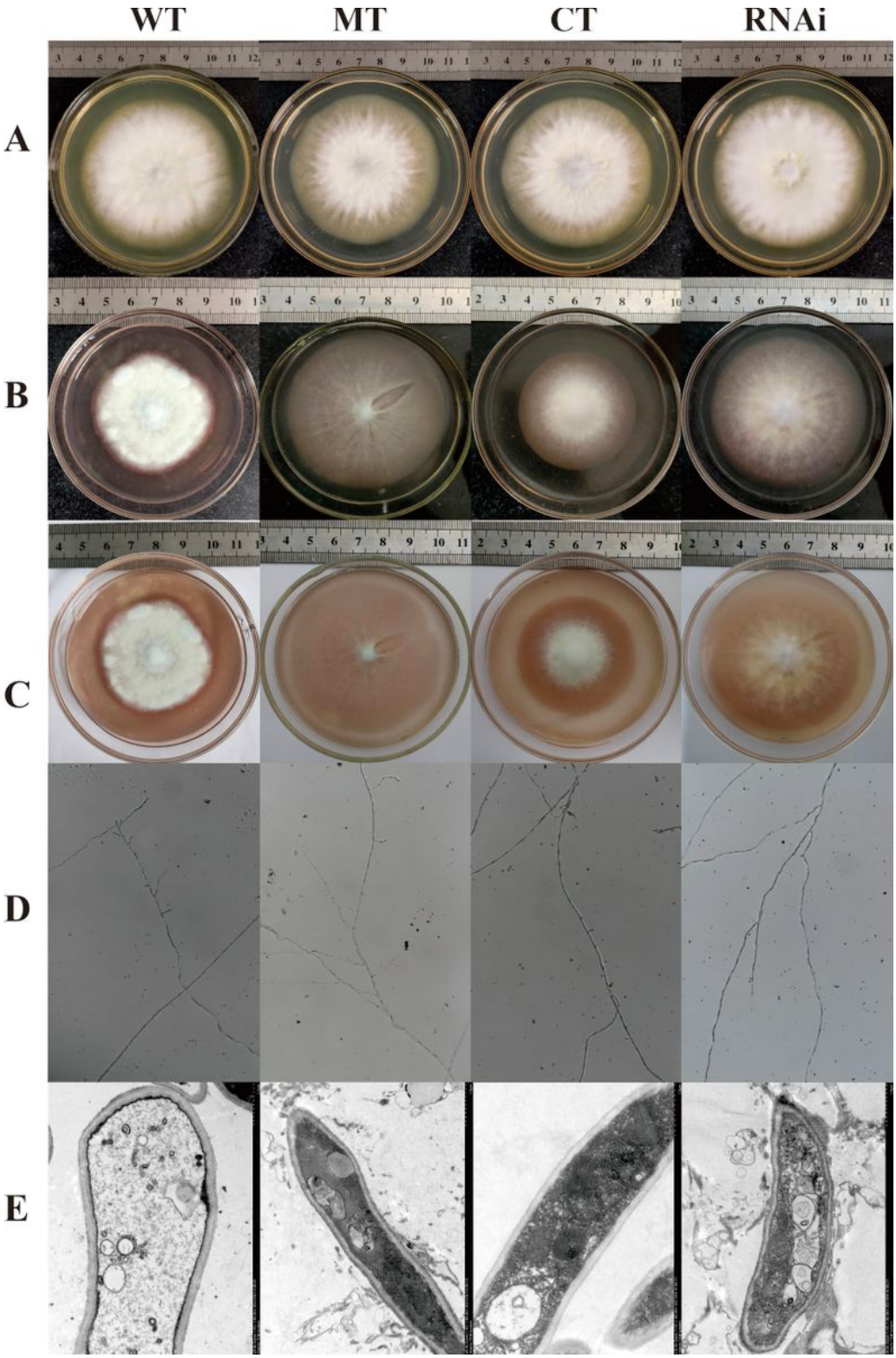

Figure 2

Observation of colonies and hyphae of WT, MT, CT and RNAi strain of *M. anisopliae*. A: Colony morphology on SDA media; B (black background) and C (white background): Colony morphology on SDA medium containing Congo red (100 μg/mL); D: Hyphae morphology under light microscope (400×); E: Hyphae morphology under electron microscope.

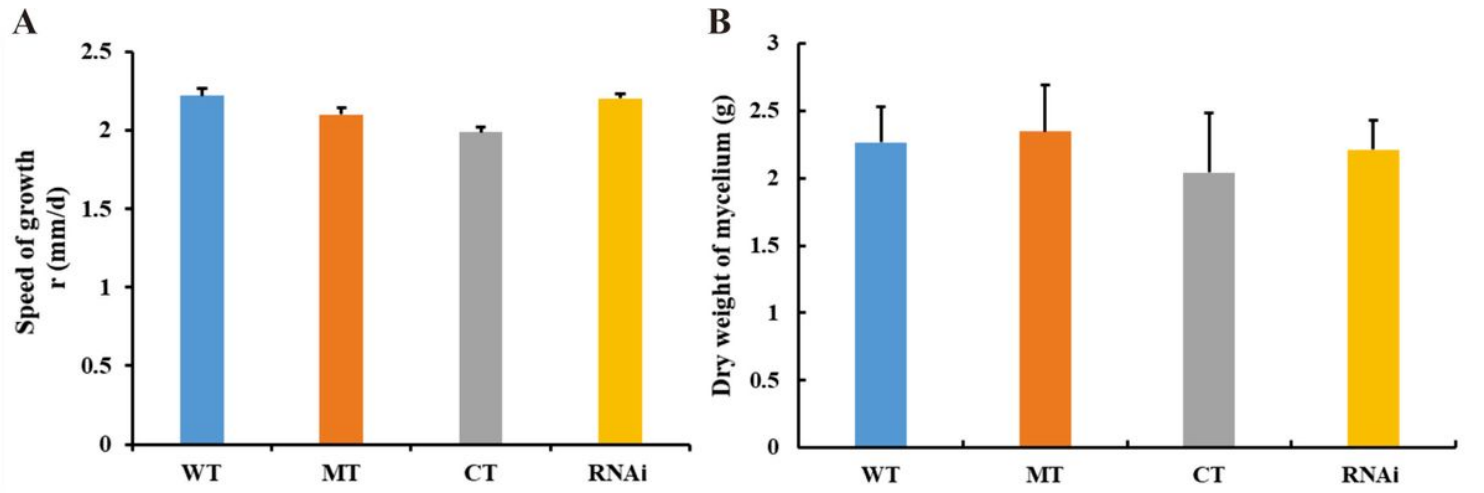

Figure 3

Statistical results of colony growth rate (A) and dry weight of mycelium (B) of WT, MT, CT and RNAi strain of *M. anisopliae*. Error bars represent the standard error of the mean (n = 3)

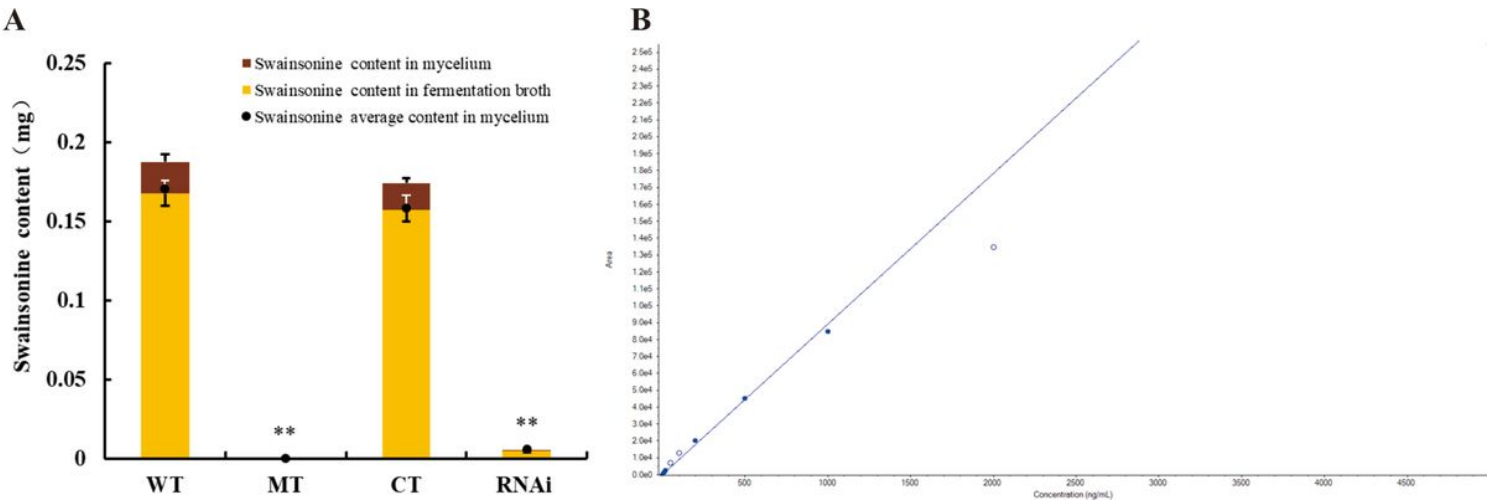

Figure 4

The content of swainsonine in the fermentation broth and mycelium of WT, MT, CT and RNAi strain (A). B: The standard curve was drawn according to the calculated regression equation: y=89.15243x+18.12349 (r = 0.99493). Error bars represent the standard error of the mean (n = 3), *P< 0.05; **P<0.01.

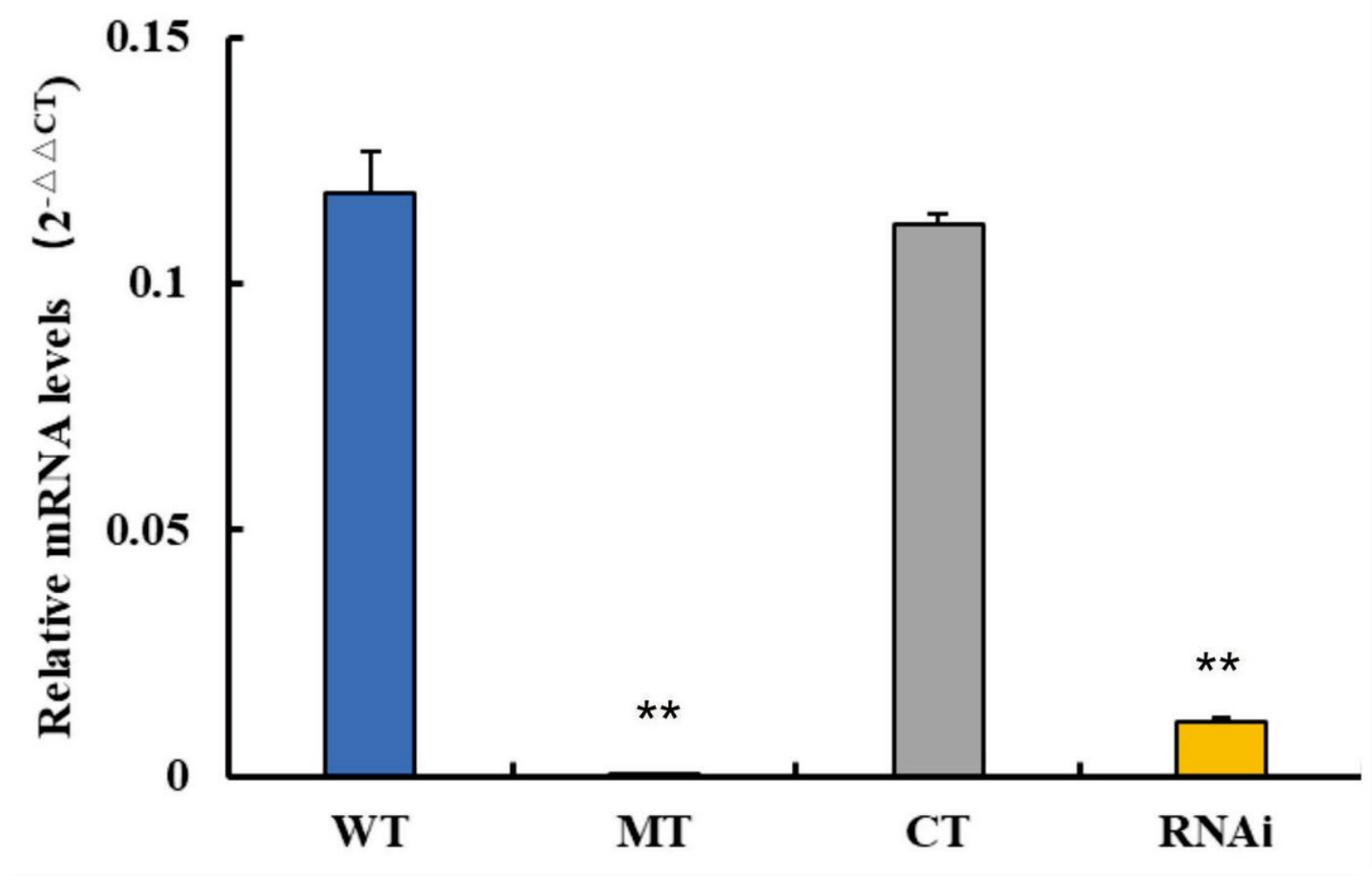

Figure 5

RT-qPCR analysis of *pks* gene. RNA was extracted, converted to cDNA, and the expression in *M. anisopliae* at 5 d was tested. Error bars represent the standard error of the mean (n = 3), *P< 0.05; **P<0.01.

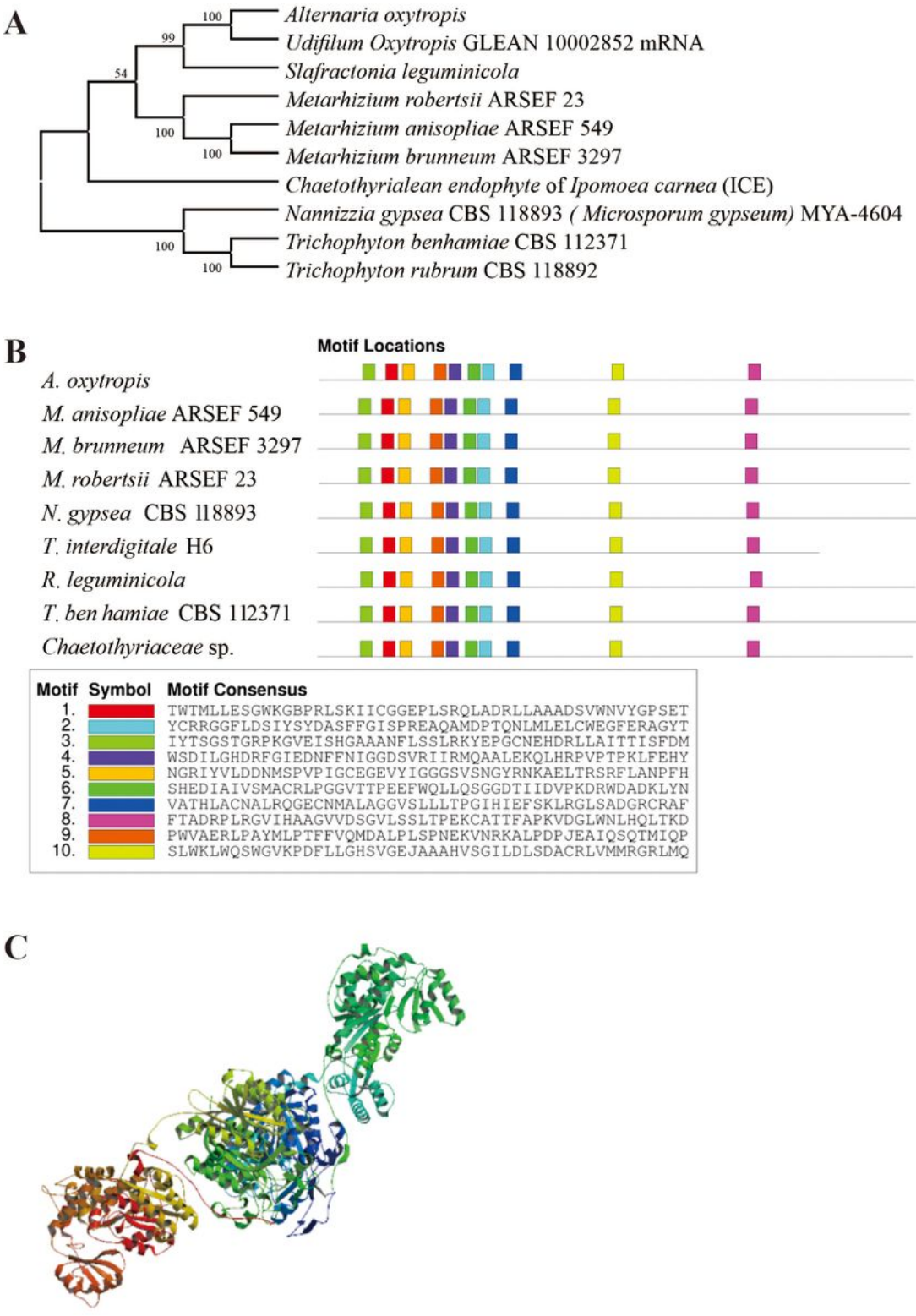

Figure 6

Genetic analysis of *pks* gene of swainsonine-producing fungi. A: Sequence analysis of *pks* gene of swainsonine-producing fungi; B: Analysis and comparison of amino acid motif of *pks* gene of swainsonine producing fungi; C: *Pks* protein model of *M. anisopliae* constructed by homology modeling.

Supplementary Files

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