The Genome of Undifilum oxytropis Provides Insights into Swainsonine Biosynthesis and Locoism

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Undifilum oxytropis is a fungal endophyte of locoweeds. It produces swainsonine, which is the principal toxic ingredient of locoweeds. However, the genes, pathways and mechanisms of swainsonine biosynthesis are not known. In this study, the genome of U. oxytropis was firstly sequenced and assembled into a 70.05 megabases (Mb) draft genome, which encoded 11,057 protein-coding genes, and 54% of them were similar to current publicly available sequences. U. oxytropis genes were annotated and 164 putative genes were annotated into enzymes, such as Saccharopine dehydrogenase, Saccharopine oxidase, and Pyrroline-5-carboxylate reductase, hypothesized to be involved in the biosynthesis pathway of swainsonine. The genome sequence and gene annotation of U. oxytropis will provide new insights into functional analyses. The characterization of genes in swainsonine biosynthesis will greatly facilitate locoweed poisoning research and help direct locoism management.

Results

Genome sequencing and analysis. After filtering low quality and adapter contamination reads, the genome of U. oxytropis was sequenced by whole genome shotgun sequencing strategy and produced 3,950 Mb clean data (Table S1). The assembly was performed by SOAP de novo genome assembler39, which generated 9,757 contigs...
predictive to have 11,057 protein-coding genes, which was similar to the coding capacity of other Ascomycetes. The length of CDS (Table S2). The gene density was 157.8 genes per Mbp and the average size of protein coding genes was 1,664.15 bp. Genes contained small exons (average 505.17 bp) and introns (average 149.64 bp). There were an average of 2.77 exons in one gene, which was similar to that found for other Ascomycetes. The gene number was equal to number of coding sequence (CDS). The total length of exons was also equal to total length of CDS (Table S2). The gene density was 157.8 genes per Mbp and the average size of protein coding genes was 1,664.15 bp. Genes contained small exons (average 505.17 bp) and introns (average 149.64 bp). There were an average of 2.77 exons in one gene, which was similar to that found for other Ascomycetes. In addition, in the U. oxytropis genome, genes of <2000 bp account for 81%, gene length distribution 0 ~ 1000 bp account for 41.6% of the gene length, 1000 ~ 2000 bp account for 39.4% of the gene length, and >2000 bp account for 19% (Figure S5).

**Table 1. Description of genome assembly of Undifilum oxytropis.**

| Type                  | Genome              |
|-----------------------|---------------------|
| Scaffold Total number | 6,367               |
| Scaffold Total length (bp) | 70,048,771          |
| Scaffold N50 (bp)     | 33,191              |
| Scaffold N90 (bp)     | 4,183               |
| Scaffold Max length (bp) | 345,265             |
| Scaffold Min length (bp) | 1,000               |
| Contig Total number  | 9,757               |
| Contig Total length (bp) | 69,684,073          |
| Contig N50 (bp)      | 24,851              |
| Contig N90 (bp)      | 3,035               |
| Contig Max length (bp) | 293,689             |
| Contig Min length (bp) | 200                 |
| GC content (%)       | 40.37               |

The analysis of genome component. We identified 11,057 protein-coding genes with a total length of 18,400,467 bp, accounting for 26.27% of the genome by combining several different gene predictors, (Table S2). The gene number was equal to number of coding sequence (CDS). The total length of exons was also equal to total length of CDS (Table S2). The gene density was 157.8 genes per Mbp and the average size of protein coding genes was 1,664.15 bp. Genes contained small exons (average 505.17 bp) and introns (average 149.64 bp). There were an average of 2.77 exons in one gene, which was similar to that found for other Ascomycetes. In addition, in the U. oxytropis genome, genes of <2000 bp account for 81%, gene length distribution 0 ~ 1000 bp account for 41.6% of the gene length, 1000 ~ 2000 bp account for 39.4% of the gene length, and >2000 bp account for 19% (Figure S5).

**Gene function annotation.** We mapped our predicted proteins to Gene Ontology (GO) using homology search, 5,731 (51.8%) of which were assigned to GO terms, including 10,933, 2,354 and 5,470 genes that mapped to the molecular function, cellular component, and biological process categories, respectively. In molecular function, “metabolic process”, “binding” and “catalytic activity” were the top three categories, which matched 3,383, 3,370 and 3,159 genes, respectively. Within cellular component, “cell” and “cell part” were the top 2 categories, which matched 1,173 and 1,173 genes. In biological process, “metabolic process” and “cellular process” were the top 2 categories, which matched 3,098 and 2,453 genes (Fig. 1). Based on this, we screened 164 genes and assigned GO terms; the highest number of genes were found to be involved in metabolic process and catalytic activity (Fig. 2). Meanwhile, we also assigned 5,965 proteins according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The KEGG function classification is shown in Fig. 3, in which “Xenobiotics Biodegradation and Metabolism”, “Translation” and “replication and repair” were the top 3 categories, followed by “Amino Acid Metabolism” and “Carbohydrate Metabolism”, which included up to 1,065, 944, 912, 791 and 780 predicted U. oxytropis genes, respectively. As a result, about 54% of predicted genes were similar to sequences in public databases and only 5,092 genes were not current public sequences, some of which might be Undifilum specific genes.

**The pathway of swainsonine synthesis.** Swainsonine is of great importance in U. oxytropis because of its significant roles in immune regulation and anticancer activity. Swainsonine can induce toxicosis in animals that consume the alkaloid. Therefore, it is very important to understand the biosynthesis of swainsonine and identify the key genes associated its biosynthesis. According to “Tropane, piperidine and pyridine alkaloid biosynthesis” pathway in KEGG (map00960), we speculated the putative swainsonine biosynthesis pathway in U. oxytropis (Fig. 4). We knew that swainsonine is a final product of L-lysine degradation, from L-Lysine to Saccharopine and L-2-aminoadipate 6-semialdehyde, the two intermediates can be obtained from L-lysine degradation (KEGG map00310), from the genome annotation results, and we found that 5 genes was annotated to a key enzyme, Saccharopine dehydrogenase (SDH, EC:1.5.1.-) of this pathway. In L-Lysine degradation process, from L-2-aminoadipate 6-semialdehyde to Δ1-piperideine-6-carboxylic acid (P6C), 1 gene was annotated to Saccharopine oxidase (FAP2, EC 1.5.3.1), furthermore, we found 2 genes annotated to pyrroline-5-carboxylate reductase (P5CR, EC:1.5.1.2) from P6C to L-pipeolate. However, we also found that many more genes that were annotated to two enzymes, polyketide synthase (PKS, EC: 2.3...-) and cytochrome P450 (P450, EC: 1.14...-), from L-pipeolate to swainsonine, the former was annotated to 79 genes, and the latter was 77 genes. To sum up, we...
Figure 1. The GO function annotation of *U. oxytropis*. Distribution of genes in different GO function classification.

Figure 2. The GO function annotation screened 164 genes of *U. oxytropis*. Distribution screened 164 genes in different GO function classification.

Figure 3. The KEGG function annotation in *U. oxytropis*. Distribution of genes in different KEGG categories.
found that some genes can be annotated to key enzymes, which were connected with swainsonine biosynthesis pathway. The important genes information annotated to some enzymes were shown in Table 2.

**Discussion**

Swainsonine, as the active principle in locoweeds, is a water-soluble indole alkaloid and alpha-mannosidase inhibitor which blocks Golgi oligosaccharide processing. It has gained attention widely, because it represents a new class of compounds that could inhibit tumor growth and metastasis. Swainsonine has also aroused interest because of its immunostimulatory properties and possible use in cancer chemotherapy. However, swainsonine may have detrimental effects, especially on animals such as goats and sheep, which can be poisoned after ingesting swainsonine-containing locoweeds. Recent findings have indicated that swainsonine is produced by the slow growing endophytic fungus, *Undifilum oxytropis*. However, little is known about the biosynthesis of swainsonine by *U. oxytropis*.

In order to gain further understanding into the biosynthesis of swainsonine, genome sequencing and annotation of *U. oxytropis* are crucial. Here we sequenced the genome sequence of *U. oxytropis* by Solexa technology. We assembled the sequences into 6,367 scaffolds in 70.05 Mb sequences represented about 87.88% of the whole genome and annotated 11,057 gene models at genome level. We compared the genome sequencing and assemble of *U. oxytropis* with another swainsonine-producing fungus, *Metarhizium anisopliae*, and we found the genome of *U. oxytropis* is different from the genome of *M. anisopliae*, which is a small genome size (39.04 Mb), the higher G + C content (51.49%) and similar genes number (10,582 bp). *U. oxytropis* was also different from the related, but non-swainsonine produce *Altermaria arborescens*. The *Altermaria arborescens* genome had a genome size of 34 Mb, predicted 9,167 genes with an average length of 1.8 Kb and gene density of 3.7 Kb/gene and GC content of 51.34%. Li et al. (2012) analyzed the proteome of *U. oxytropis* and categorized 52 proteins. Our results were similar to their findings. Within molecular function, our top two categories were binding, where Li et al. found 49% of the proteins were related to binding and catalytic activity, where 10% of the...
proteins were given that category by Li et al.38. In our work, within the biological process area, the largest number of genes were metabolic process which corresponds with 34% of the proteins within the biological process.

Currently, studies on swainsonine biosynthesis have mostly been performed on Rhizoctonia leguminicola, renamed as Slafastronia leguminicola39 and Metarhizium anisopliae40. However the detailed biosynthesis pathway is still unclear. The pathway for swainsonine biosynthesis has been partially characterized in R. leguminicola and M. anisopliae26. In the partially characterized pathway, L-pipecolic acid was shown to serve as a precursor for the production of swainsonine. In fungi like Metarhizium and Rhizoctonia, pipecolic acid is formed by the catalysis of L-lysine that in turn allows for the formation of the alkaid, swainsonine26,28.

In the “Tropane, piperidine and pyridine alkaloid biosynthesis” pathway from KEGG map database (map00960), swainsonine is one of the final products in lysine metabolism. In the Undifilum oxytropis genome, we found some putative genes that were annotated to lysine degradation pathway. Thus, at the genome level, we confirmed that some key enzymes that could be involved in swainsonine biosynthesis are present in the fungus. Previous studies have established two basic routes for converting lysine into pipecolic acid, which are P2C pathway and P6C pathway42. The important intermediates are Δ¹-piperideine-2-carboxylic acid (P2C) and Δ¹-piperideine-6-carboxylic acid (P6C)43. The P6C pathway has been intensively studied in R. leguminicola, because the synthesis of pipecolic acid via this route represents the initial steps in the production of two toxic octahydropodolizolene alkaldoids, slaframine, and swainsonine44. Another experimental result implied that Δ¹-piperideine-6-carboxylic acid, not Δ¹-piperideine-2-carboxylic acid, was involved in the conversion of lysine to pipecolic acid, and this conclusion was fully supported by relevant proton NMR studies27. Subsequently, a chain of reactions have been established through which L-lysine was converted to saccharopine, which was in turn converted to P6C through oxidative cleavage. The latter was then readily reduced to pipecolic acid. A previously unrecognized flavin enzyme, Saccharopine oxidase was identified, which oxidatively cleaves saccharopine to yield P6C. Since saccharopine is a major metabolite in lysine degradation in R. leguminicola, the synthesis of pipecolic acid via this route represents the initial steps in the production of two toxic octahydropodolizolene alkaldoids, slaframine, and swainsonine44. Moreover, combining “Tropane, piperidine and pyridine alkaloid biosynthesis” pathway, we knew that there were 3 enzymes that play an important role in this two pathways, that is L-lysine oxidase (EC1.4.3.14), Δ¹-piperideine-2-carboxylic reductase (EC1.5.1.21) and L-lysine-6-dehydrogenase (EC1.4.1.18). However, no clear gene was annotated to these enzymes, which confirmed previous studies27. In P6C pathway, we found L-2-aminoadipate 6-semialdehyde dehydrogenase (encoded by proC gene) acted efficiently with Flavobacterium lutescens LAT to convert L-lysine into L-pipecolic acid45. It is noteworthy that P5C reductase is present in almost all organisms46. It is possible that in the microorganisms that produce L-pipecolic acid via P6C pathway, the universally conserved P5C reductase is actually responsible, at least in part, for the reduction of P6C into L-pipecolic acid47. Although little is known about the biosynthetic pathway of swainsonine in the endophytic fungus Undifilum oxytropis, genome sequencing and function annotation in this fungus will open avenues for future research on control of loco-disease. According to the putative swainsonine biosynthesis pathway in U. oxytropis, we have gained an insight into swainsonine metabolism pathway and some key enzymes involved in this process such as SDH, FAP2, P5CR, PKS and cytochromes P450 enzymes. These enzymes play a very important role in swainsonine biosynthesis. In our results, we found that many genes might be involved in swainsonine biosynthesis in U. oxytropis. After predicting some key enzymes of swainsonine biosynthesis pathway in U. oxytropis, we found 79 genes were annotated to PKS in genome sequencing and function annotation in this fungus. However, some putative genes that were annotated to lysine degradation pathway. Thus, at the genome level, we confirmed that some key enzymes that could be involved in swainsonine biosynthesis are present in the fungus.

Previous studies reported that P2C and P6C pathways are two basic routes for converting lysine into pipecolic acid41. Moreover, combining “Tropane, piperidine and pyridine alkaloid biosynthesis” pathway, we knew that there were 3 enzymes that play an important role in this two pathways, that is L-lysine oxidase (EC1.4.3.14), Δ¹-piperideine-2-carboxylic reductase (EC1.5.1.21) and L-lysine-6-dehydrogenase (EC1.4.1.18). However, no clear gene was annotated to these enzymes, which confirmed previous studies27. In P6C pathway, we found L-2-aminoadipate 6-semialdehyde, is key intermediate compound in this route, according to L-lysine degradation pathway, it is catalyzed as a product of saccharopine by SDH, and 4 genes were annotated to this enzyme, which indicated that SDH played a very important role in swainsonine biosynthesis in U. oxytropis. Meanwhile, Δ¹-piperideine-6-carboxylic acid (P6C) is also the important intermediates in P6C pathway43. Furthermore, 2 genes were annotated to this enzyme, which indicated PSCR is responsible for the reduction of P6C into pipecolic acid45, our experimental results agreed, and we found that 2 genes were annotated to this enzyme, which indicated PSCR could be a key enzyme in swainsonine biosynthesis.

Polyketides, the ubiquitous products of secondary metabolism in microorganisms, are made by a process resembling fatty acid biosynthesis that allows the suppression of reduction or dehydration reactions at specific biosynthetic steps, giving rise to a wide range of often medically useful products47. The polyketide synthases (PKS) are a large class of natural products, which are produced by bacteria, actinomycetes, fungi and plants. These natural products play an important role in anti-infection, anti-fungus, anti-tumor and immunologic suppression. In our studies, we also found 79 genes were annotated to PKS in U. oxytropis genome. Since 1-indolizinone is a ketone compound, we speculated that L-pipecolic acid could be changed into 1-indolizinone through PKS. But, so far we have no evidence to support this speculation, and this needs be confirmed by future research.

Undifilum oxytropis showed rich P450 family (77 genes annotated to cytochromes P450), which is involved in the biotransformation of drugs, the bioconversion of xenobiotics, the metabolism of chemical carcinogens, the biosynthesis of physiologically important compounds such as steroids, fatty acids, eicosanoids, the conversion of alkanes, terpenes, and aromatic compounds as well as the degradation of herbicides and insecticides. There is also a broad versatility of reactions catalysed by cytochromes P450 such as carbon hydroxylation and aromatic hydroxylation48. In biosynthesis of alkaloids derived from ornithine, lysine and nicotinate (KEGG map01064), we found that 1-indolizinone was hydroxylated into swainsonine. As a result, we speculated that cytochromes P450 hydroxylase could also play a very important role in swainsonine biosynthesis pathway.
In conclusion, in *U. oxytropis* genome research, we clarified the sizes and characteristics of this fungus, and screened some genes that were annotated to key enzymes, which are theoretically involved in swainsonine biosynthesis by using whole genome shotgun sequencing strategy. In future, genes knockout of some key enzymes will be carried out and obtain mutant of *U. oxytropis*, which do not produce swainsonine, and on this basis we will gain a new locoweed species that do not contain swainsonine, this will fundamentally resolve locoweeds poisoning of animals and then implement the comprehensive utilization and management for locoweeds of grasslands in western China.

**Materials and Methods**

**Strains and culture conditions.** *Undifilum oxytropis* (OK3UNF) was isolated from *Oxytropis kansuensis*, a locoweed widely distributed in Qianlan County of Qinghai province in western China (38°3′24.9N, 100°13′6.60E), and deposited at the Animal Toxicology Institute of Northwest A&F University (Yangling, Shaanxi, China). The hyphal tipped culture of *U. oxytropis* was stored in a tube at 4 °C prior to transfer to fresh PDA (Potato Dextrose Agar) media and culture at room temperature. After 10-14 days growth, the mycelium was collected and preserved at 4 °C for genomic DNA isolation and extraction.

**DNA isolation, genome sequencing and assembly.** Genomic DNA of *U. oxytropis* was isolated by an improved cetyl trimethylammonium bromide (CTAB) method and sequenced using a whole-genome shotgun strategy. All data were generated by paired-end sequencing of cloned inserts with insert size (500 bp) using an Illumina Hiseq2000 Sequencer at BGI-Shenzhen. After removing the low complexity, low quality, adapter and duplication contamination raw reads, the clean reads were assembled using the whole-genome *de novo* assembler SOAP de novo.

**Genome Annotation.** Protein coding gene models were predicted using *de novo* prediction tools SNAP, GeneMarks and Augustus with the default parameters. The homology-based and *de novo* gene sets were merged to form a comprehensive, non-redundant reference gene set by Glean. The functional annotation of predicted gene models were mainly based on homology to SwissProt, NR, and PHI, P450, CAZy, and mapped them by function with GO, COGs and KEGG pathways. Since each gene mapped to different database sequences, there could be multiple aligned results meeting the cut-off, so the annotations of the sequences with the best score were chosen to be the annotation of the gene in *U. oxytropis*.

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**Author Contributions**

H.L. and B.Y.Z. conceived and designed the experiments; B.Y.Z. contributed reagents/materials; H.L., H.Y.Q., Z.H.R., S.W. and R.X.X. performed the experiments; H.L. analyzed the data and wrote the paper.
