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# Saccharopine reductase influences production of Swainsonine in *Alternaria oxytropis*

Ping Lu*, Xin Li, Siyuan Wang, Feng Gao, Bo Yuan, Lingjun Xi, Ling Du, Hu Jiya, & Lina Zhao

College of Life Science and Technology, Inner Mongolia Normal University, Hohhot 010022, China

*e-mail: luping@imnu.edu.cn

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Swainsonine (SW) was first isolated from *Swainsona canescens*, and then identified in several *Astragalus* and *Oxytropis* species, synthesized by the endophytic fungus *Alternaria oxytropis*. It inhibits the activity of alpha-mannosidase in animal cells. Livestock is poisoned when SW accumulates and even dies in serious cases. We isolated an endophytic fungus identified as *A. oxytropis* from *O. glabra* that produced swainsonine. The biosynthetic pathway of SW in endophytic fungus is poorly understood. Partial pathway for SW in *Rhizoctonia solani*, *Metarhizium anisopliae*, and *Alternaria oxytropis* was reported. It was proposed that saccharopine reductase gene (*sac*) played an important role in SW synthesis. In this study, saccharopine reductase gene was cloned, and results showed that it contained 1419 bp of ORF, and encoded a predicted protein with 472 amino acids. The amino acid sequence included 9 key amino acids as the active center of the saccharopine reductase enzyme, and a gene knockout mutant M1 was acquired. The levels of SW in wild-strain OW7.8 were higher than in M1 which suggested that *sac* was correlated with the SW synthesis in endophyte fungus.

Keywords: Saccharopine reductase, *Alternaria oxytropis*, swainsonine, knockout mutant.

*Oxytropis glabra* DC. is a perennial herb of the genus *Oxytropis*. Many plants contain swainsonine (SW), an indolizidine alkaloid poisonous to livestock. The mechanism demonstrated that SW can bind to mannoside competitively inhibiting the activity of alpha-mannosidase in animal cells (Hu et al. 2015a, Lu et al. 2016). *Oxytropis*, *Astragalus* and other poisonous plants containing SW are collectively called Locoweed internationally. Livestock is poisoned when SW accumulates to certain degree and even dies in serious cases resulting in a major loss of grassland animal husbandry in the world. It is very important to study the toxic mechanism of locoweed. In addition, SW is a tool for studying the synthesis of glycoprotein *N*-oligosaccharides in the medicine and chemical industry, and later it was found to have anti-tumor and immunomodulatory effects. Structure of SW possesses an indolizine heterocyclic ring and four chiral centers continuously. Thus chiral isomers could be formed easily in the process of chemical synthesis leading to difficult separation. Though SW can be obtained by purification from plant or fungi, it is very expensive commercially. Therefore, increasing yield of SW through biosynthesis will be a promising way to obtain SW.

Braun et al. (2003) isolated SW-producing endophytic fungi from the three locoweed plants *Oxytropis lambertii*, *Oxytropis sericea* and *Astragalus mollissimus*, and identifed them as *Embellisia oxytropis* Q. Wang, Nagao & Kakish. Pryor et al. (2009) recombined this species in *Undifilum oxytropis*, and Woudenberg et al. (2013) revised it as *Alternaria oxytropis* (Q. Wang, Nagao & Kakish.) Woudenb. & Crous. Our previous studies showed that SW toxicity of *Oxytropis glabra* was caused by its endophytic fungus *Alternaria oxytropis* (Hu et al. 2015b, Huo et al. 2010). The host plant without this endophytic fungus did not contain SW. According to the microbiological characteristics and DNA sequences, this fungus was identified as *A. oxytropis*.

Many details of SW biosynthesis pathways in endophytic fungi are still unknown. It is believed that SW biosynthesis pathways are included in α-aminoadipate pathways of lysine biosynthesis. The pathways of SW biosynthesis in *Rhizotonia leguminicola* and *Metarhizium anisopliae* were partially elucidated (lysine → saccharopine → P6C → Pipecolic acid → SW or lysine → P2C → Pipecolic acid → SW). L-lysine produces saccharopine under the action of saccharopine reductase (Sac). Later SW was synthesized through a series of postmodification reactions (Sim et al. 1995, 1997; Wickwire et al. 1990; Lu et al. 2016; Tan et al. 2018). Lu et al. (2016) predicted that saccharopine reductase (EC:

1.5.1.7, EC: 1.5.1.9 and EC: 1.5.1.10) played an important role in the SW synthesis pathway of *Undifilum oxytropis* after genome sequencing.

In order to study the effects of saccharopine reductase (EC: 1.5.1.9) on the biosynthesis of SW in *A. oxytropis*, we cloned the saccharopine reductase gene (*sac*) and investigated its role in SW biosynthesis. The *sac* deletion mutant M1 was constructed, and the levels of SW in the M1 and the wild-strain OW7.8 were determined and compared.

## Materials and methods

### Strains, media, and culture condition

The endophytes were cultured from leaves and stems in *Oxytropis glabra* collected from Wushenqi, Inner Mongolia, China. The plant samples were cut and dried for subsequent isolation and culture of the endophytes. The tissues were sterilized for 30 sec in 70 % ethanol, followed by 3 min in 20 % bleach, and then in sterile water for 30 sec. Tissues were dried on sterile paper towels and cultured on water agar media. Fungal hyphae were transferred into potato dextrose agar (PDA) plates and grown at room temperature for more than 14 days. Hyphae were transferred onto PDA plates and maintained at about 25 °C. The isolation was preserved as mycelia and stored at 4 °C and –80 °C.

### Degenerate polymerase chain reaction (PCR)

The cDNA and amino acid sequences of Sac from *Magnaporthe grisea*, *Penicillium chrysogenum* and *A. oxytropis* were obtained from the GenBank. Highly conserved regions were identified and degenerate primers were then designed (Tab.1). The *sac* from the fungal DNA was amplified by degenerated PCR (Boerdi, Nanjing, China), and the PCR product was cloned in pGMT vector (Tiangen Biotech, Beijing, China), and sequenced (Sangon Biotech, Shanghai, China).

### Rapid amplification of cDNA ends (RACE)

According to the middle segment of the cloned *sac*, an external forward primer P1 and an internal forward primer P2, an external reverse primer P3 and an internal reverse primer P4 were designed (Tab.1). Cloning was performed using Takara SMARTer RACE5'/3' kit (Takara, China). The conditions for PCR reaction were as follows: denaturation at 94 °C for 45 sec; annealing at 60 °C for 40 sec, extension at 72 °C for 1.5 min for total of 20 and 30 cycles, respectively. PCR products were detected by 1.0 % agar gel electrophoresis, and the products were then sequenced.

DNASTAR software (Laser gene) was used to assembly 3' RACE and 5' RACE sequences to obtain the full-length of cDNA, and the upstream and downstream primers of $P_5$ and $P_6$ were designed (Tab. 1). Total fungal DNA was used as a template to amplify *sac*, and PCR conditions were listed as follows: denaturation at 94 °C for 45 sec, annealing at 57 °C for 40 sec, and extension at 72 °C for 4 min with 35 cycles. The product was purified and inserted into the pGM-T vector, and the vector was then transformed into TOP10 competent cells. The recombinant vector was selected and verified by sequencing as well.

### Correlation analysis between levels of SW and sac expression in *A. oxytropis*

Fungal cultures of the OW7.8 were grown on PDA for 17 to 35 days at 25 °C for correlation analysis between levels of SW and *sac* expression in *A. oxytropis*.

### Extraction and analysis of swainsonine from OW7.8

The extract and detection methods of SW were carried out with reference to Ping Lu (Sarula et al. 2018).

### qRT-PCR of *sac* in OW7.8

Total RNA of OW7.8 was extracted, cDNA was synthesized by reverse transcription, and *actin* was used as an internal reference gene for qRT-PCR reaction. The system and procedure of cDNA synthesis and qRT-PCR reaction were carried out according to the instructions of TAKARA specification (TB Green® Fast qPCR Mix). Primers were listed in Tab.1.

After the qRT-PCR was completed, the specificity of the reaction was determined based on the amplification curve and the dissolution curve. The relative expression level of the gene was then calculated using the $2^{-\Delta\Delta Ct}$ method ($\Delta Ct=Ct_{(sac)}-Ct_{(actin)}$, $\Delta\Delta Ct=\Delta Ct_{(n)}-\Delta Ct_{(1)}$).

### Correlation analysis

Correlation was analyzed between SW and *sac* expression levels in *A. oxytropis* by SPSS.

### Gene knockout

A *sac* deletion vector was constructed with pUC19, with hygromycin phosphotransferase gene (*hph*) as a selection marker gene. A 945 bp fragment

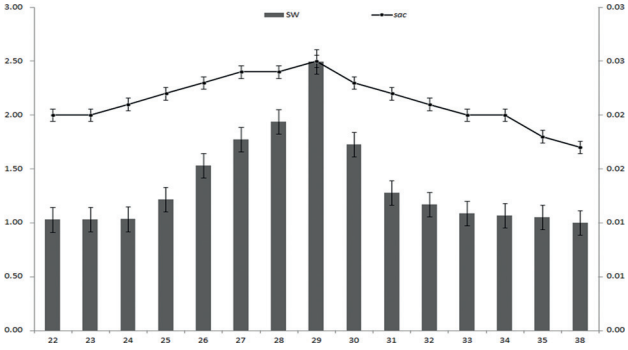


**Fig. 1.** The levels of swainsonine and *sac* expression; The X-axis indicates endophytic fungus culture time; The Y axis (left) show that the level of swainsonine; The Y axis (right) show that expression of *sac*.

**Tab. 1.** Primer sequences in the study

| Names of primer | Sequences of primer |
|---|---|
| P1 | 5'- GTY AACGACGAYGCCGCCCTCGACG -3' |
| P2 | 5'- YCGTCCTTGTTCTCGATCTCGAAC -3' |
| P3 | 5'- TCCTTCAAGTCATACTGCGGTGGT-3' |
| P4 | 5'- GCC ATACTTCATCTACCCTG -3' |
| P5 | 5'- GGCACTTGATG ACTCTGGGAAACCT -3' |
| P6 | 5'- ATGATGGTCTGGCACTCGGGGATGTT -3' |
| SM P1 | 5'- AGAGTAGCCCTTGGGGTCACCGTAGT -3' |
| SMP2 | 5'- TCGCCCTTCCTCCCTTTATTTCAGAT -3' |
| Sac1F | 5'- GGAGCATACAGCGGAAAGAC -3' |
| Sac1R | 5'- CCTTGTAGGGTGTGGAGTCA -3' |
| Sac2F | 5'- GAACCTTATGGGCAACGTC -3' |
| Sac2R | 5'- GAGACCACCGCAGTATGAC -3' |
| Sac3F | 5'- CCTGGAGCATACAGCGGAAAG -3' |
| Sac3R | 5'- GGAATGAGGGAGATTACGACTTG -3' |
| Hph1F | 5'- GGGCGAAGAATCTCGTGCT -3' |
| Hph1R | 5'- TCCAGACAAGCCAACCACGG -3' |
| ACTIN F | 5'- CTTCTATTGTCGGCCGACCGC -3' |
| ACTIN R | 5'- GATGCCAGATCTTCTCCATGCG -3' |
| sac F | 5'- CATGCCGCACTCTTGAGAAG -3' |
| sac R | 5'- CTTCTTGCGGATTGCGGACTTG -3' |

of the gene (nts 1—945) was PCR-amplified using forward and reverse primers carrying *Bam* HI and *Age* I sites for cloning into the upstream end of the *hph* 5' end, the middle part, in which a 1402 bp fragment was *hph* carrying *Age* I and *Sbf*I sites (nts 946—2348), and a 489 bp fragment (nts 2348—2837) was PCR-amplified using forward and reverse primers carrying *Sbf*I and *EcoR*I sites for cloning into the upstream end of the *hph* 3′ end. Therefore, the two ends of the DNA target were the 5′ end and 3′ end sequences of *sac*, respectively. The PCR products were amplified in M1 with primers of SMP1 and SMP2 (Fig.2A).

## Isolation and transformation of fungal protoplasts

Fungal endophytes were inoculated on PDA plates, and the protoplasts were isolated and regenerated according to Hu et al. (2015b). Transformed M1 were subsequently maintained on 20 μg/ml of Hyg B. The transformation plates were incubated at 25 °C for 7 days to observe hyphal growth. Fungal cultures after the protoplast regeneration were then transferred to fresh plates with PDA-Hyg B. Subsequent transfer of fungal cultures was performed after 3 week of growth. The protoplasts were regenerated on PDA plates with Hyg B to test the expression of *hph,* and transformants were also cultured on PDA plates with Hyg B, and the strain was maintained at 4 °C.

## Screening for knockout mutant M1 with PCR

M1 and OW7.8 were screened by PCR to verify the present of *hph* in M1, but not in OW7.8. Primers

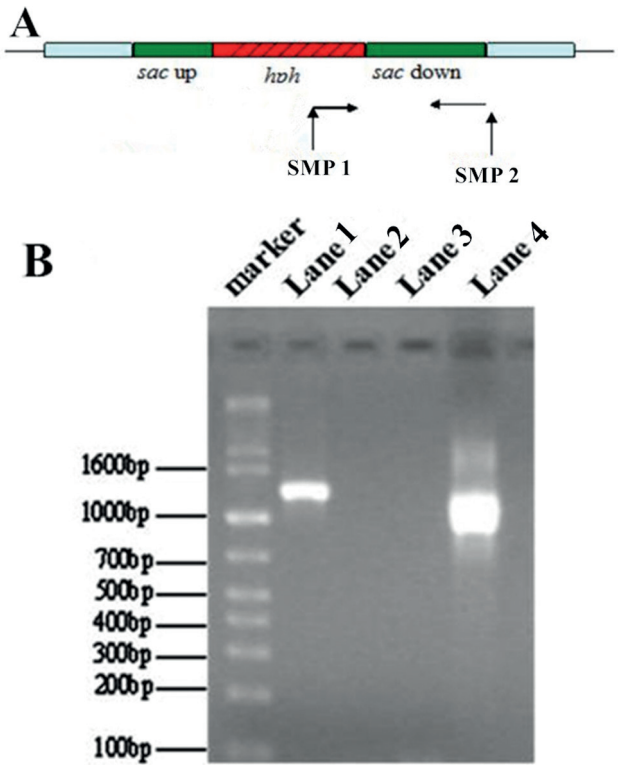


**Fig. 2. A.** Schematic representation of the location of SMP1 and SMP2 on the disruption mutant; **B.** Electrophoresis of PCR products by SMP1 and SMP2 primes; (Marker: 1 kb plus DNA ladder; Lane 1: positive control; Lane 2: negative control; Lane 3: the wild type DNA; Lane 4: the mutant DNA).

were chosen from the sequences of *sac* and *hph* (Fig.2B). M1 and OW7.8 were also screened by reverse transcription PCR (RT-PCR) (Takara, Dalian, China) to detect the expression of *hph* and *sac*. The RT-PCR was carried out using four sets of primers (Tab. 1) to verify the expression of *hph* in M1, but not in OW7.8, and expression of *sac* in OW7.8, but not in M1. Hph1F and Hph 1R primers were also designed according to the sequence of *hph* as well (Tab.1). Sac1F primer was a combination of the sequences from the second and the third exons of *sac*, and Sac1R primer was chosen from the sequence of the fifth exon, and Sac 2F and Sac 3F primers were chosen from the sequence of the second exon, while Sac 2R and Sac 3R primers were selected from the sequence of the fifth exon. The PCR products were tested by gel electrophoresis and then sequenced (Sangon, Shanghai, China). Three *sac* deletion mutants were screened for the latter analysis.

Southern blot analysis of fungal *sac*

The recombinant plasmid pTONO-sac1 (containing *sac* of endophytic fungal) and pCB1003 (containing hygromycin phosphotransferase gene) plasmid were designed according to the endogenous fungal *sac* intermediate sequence and *hph* sequence. The specific primers DSF, DSR, HGF, and YGR were subjected to PCR labeling reaction. At the same time, the same unlabeled PCR reaction was carried out. Electrophoresis was performed, and the probe sequence was recovered by gelation and the concentration was determined.

The total DNA of endophytic fungi was extracted, and digested by the endonucleases *Eco*RV, *Pst*I, *Pci*I and *Pvu*II (Takara) at 37 °C for 12 h, and the enzyme digestion was detected by electrophoresis. The nucleic acid co-precipitating agent was used to purify and concentrate the digested product, and the gel was rinsed, transferred, and fixed. The nylon membrane was taken out and washed twice, and the membrane was transferred to a washing buffer, and 1×blocking solution was shaken for 30 min. The liquid was discarded and the membrane washed again. Finally, 300 µL of NBT/BCIP chromogenic substrate was added and allowed to stand for 20 hr in dark. The membrane was washed with $ddH_2O$ and photographed.

Extraction and analysis of swainsonine from M1 and OW7.8

Fungal endophytes OW7.8 and M1 cultivated for 17, 20, 23, 26, 29, and 32 days were selected as the samples. SW was extracted from mycelia, and SW levels in OW7.8 and M1 were determined by HPLC-MS (Sarula et al. 2018).

## Results

Saccharopine reductase gene cloning

The *sac* cDNA was cloned by RT-PCR and RACE. The size of the cDNA was 1,419 bp (GenBank accession number: KJ944635), predicting to encode a protein with 472 amino acids of 51,551.2 Da molecular mass, and a 1750 bp full length of *sac* was cloned (GenBank accession number: KY052048). There were four introns of 49 bp, 51 bp, 51 bp, and 48 bp, a 5' UTR of 45 bp, and a 3' UTR of 143 bp by comparing the sequences of the gene and the cDNA. The *sac* sequence in the fungus showed 93 % and 90 % homology with those in *Pyrenophora triticirepentis* and *Leptosphaeria maculans*, respectively. The predicted protein was a soluble, non-transmembrane, and intracellular protein.

Correlation analysis between levels of SW and sac expression in *A. oxytropis*

With increase of the expression level of *sac*, the levels of SW increased in OW7.8 (Fig. 1). The correlation between them is significant($P<0.01$).

Saccharopine reductase gene knockout in the *A. oxytropis*

The PCR products were amplified in M1 with primers of SMP1 and SMP2 (Fig. 2A). However, no PCR products were obtained in the OW7.8 and negative control. The RT-PCR products showed about 1000 bp as expected (Fig. 2B). M1 grew on PDA plates with the same characters as white, raised, and regular margin colony, while with more compact structure and faster growth rate compare with OW7.8.

About 1000 bp of *hph* specific RT-PCR products were amplified in the mutant and positive control. However, no such RT-PCR products were detected in the OW7.8 and negative control (Fig. 2B). The results proved that the *sac* deletion mutant M1 was obtained from the *A. oxytropis* fungal endophyte.

Southern blot analysis of fungal *sac*

Southern blot results showed that the intermediate sequence of *sac* was detected in the OW7.8, while the *hph* was detected in M1. However, no intermediate sequence of *sac* was detected in M1, which proved that the *sac* was knocked out in M1 (Fig. 3).

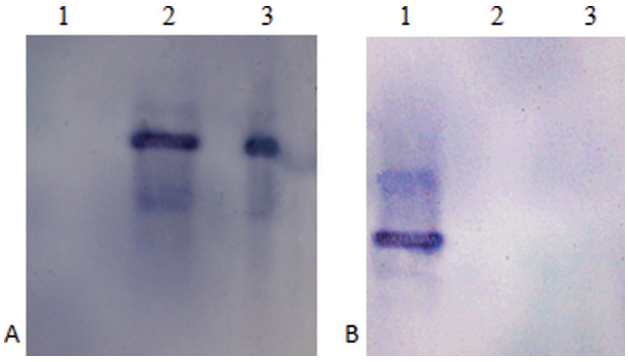


**Fig. 3.** OW7.8 and M1-probe Southern blot; A OW7.8; B M1; Lane 1: The result of hybridization of genomic DNA of M1 cut with *Pvu*II; Lane 2: The result of hybridization of genomic DNA of OW7.8 cut with *Eco*RV; Lane 3: The result of hybridization of genomic DNA of OW7.8 cut with *Pst*I.

**Tab. 2.** Levels of swainsonine in OW7.8 and M1 (μg/mg)

| Day | OW7.8 | M1 |
|---|---|---|
| 14 | 0.036±0.296 | 0.005±0.001 |
| 17 | 0.038±0.018 | 0.008±0.001 |
| 20 | 0.139±0.006 | 0.006±0.001 |
| 23 | 0.194±0.051 | 0.041±0.001 |
| 26 | 0.686±0.006 | 0.005±0.001 |
| 29 | 3.678±0.009 | 0.005±0.001 |
| 32 | 3.569±0.005 | 0.005±0.001 |
| 35 | 2.897±0.141 | 0.005±0.001 |

Swainsonine analysis from OW7.8 and M1

The SW levels in OW7.8 and M1 were determined by HPLC-MS. The SW levels in OW7.8 was higher than that in M1, with the maximum observed in OW7.8 on the day 29 and in M1 on the day 23 of culture (Tab. 2). The variance analysis of SW levels displayed significant difference between OW7.8 and M1 ($P<0.05$).

## Discussion

We identified *sac* in our *A. oxytropis* which contained 4 introns, while the gene identified from *P. chrysogenum* and *Magnaporthe grisea* contained 9 and 6 introns respectively. Homology comparing revealed that the predicted amino acid sequence of the Sac was 93 % and 90 % homologous to that in *Pyrenophora triticirepentis* and *Leptosphaeria maculans*, respectively. However, the homology of Sac amino acid sequences between our *A. oxytropis* and the fungus reported was 57 % (Mukherjee et al. 2012). We carefully examined the cDNA sequences and found, compare with our *A. oxytropis*, that there were several base insertions and replacements at the 5' end of the gene in that fungus, and a C missed at position of 722, which led to a frame-shift mutation from the amino acid site 241, and the original proline (P) became histidine (H). Moreover, a 55 bp fragment was inserted into the place between sites 1,181 and 1,235, which led to several changes in amino acids from positions 241—414. However, of the 9 key amino acids (Y, S, Y, C, G, W, R, T, R) in the enzyme active site, 7 remained unchanged (Y, S, Y, C, G, W, T). The amino acid sequence deduced from the cloned *sac* sequence from OW 7.8 contained all 9 key amino acids which were also found in the crystal structure of the active center of Sac (Johansson et al. 2000), and were located at the same position indicating that the translation product of the sac possessed saccharopine reductase activity.

Lu et al. (2016) speculated that saccharopine reductase (dehydrogenase) played an important role in the biosynthesis pathway of SW based on genome sequencing, which probably catalyzed the saccharopine to produce α-aminoadipate semialdehyde. SW biosynthesis pathways were contained in -aminoadipate pathways of lysine biosynthesis. Few studies reported the relationship between SW and lysine in endophyte fungus. Mukerjee et al. (2012) found that the SW levels increased in the sac deletion mutant, while the levels of saccharopine and lysine decreased in fungi cultured on day 14. We found that the SW levels in M1 were always lower than that in OW7.8 from day 14 to day 45 cultures. We also determined the levels of saccharopine and lysine in OW7.8 and M1, and found that the levels of saccharopine in M1 were lower than that in OW7.8, while the levels of lysine changed little on day 45 cultures (data not published). In addition, we found that the SW levels increased after addition of four precursors (saccharopine, α-aminoadipate, L-lysine and pipecolic acid) to the media both in OW7.8 and M1, and the SW levels in OW7.8 was always higher than that in M1 (Sarula et al. 2018). We speculate that SW levels vary in different growth periods. Moreover, lysine is an essential amino acid, and saccharopine is an intermediate for the lysine synthesis in α-aminoadipate pathway. It was presumed that the synthesis of saccharopine was blocked in the sac knockout mutant. In order to synthesize necessary amount of lysine, a reverse reaction may occur, which was catalyzed by the saccharopine oxidase resulting in a lower level of SW in the sac knockout mutant M1 than that in OW7.8, while there was a similar level of lysine both in M1 and OW7.8, and

6-amino-2-oxohexanoate (P6C) was converted to saccharopine and then lysine in M1.

A complementary strain of *sac*(C1) in *A. oxytropis* was also constructed in our lab. It was found that the levels of SW in C1 were higher than that in OW7.8 and M1 which indicated that the strong promoter increased the expression of *sac* and promoted the SW synthesis in C1 (Wang et al. 2020). We also conducted transcriptome analysis to M1 and OW7.8, in which forty-one Unigenes possibly related to the biosynthesis of SW were identified by data analyzing. The biosynthesis pathways of SW in the fungus were predicted, including two branches of P6C and P2C. Delta1-piperidine-2-carboxylate reductase, lysine 6-dehydrogenase, and saccharopineoxidase/L-pipecolate oxidase were involved in P6C, while 1-piperidine-2-carboxylate/1-pyrroline-2-carboxylate reductase [NAD(P)H] and delta1-piperidine-2-carboxylate reductase were involved in P2C. Saccharopine reductase was involved in both (Li & Lu 2019).

Other SW biosynthesis genes were also investigated. Cook et al. (2017) reported that a gene cluster SWN played an important role in SW biosynthesis in *Metarhizium robertsii*. The SWN contained *swn* A, *swn* H1, *swn* H2, *swn* N, *swn* R, *swn* T and *swn* K (PKS-NRPS multifunctional enzyme gene). The *swn* K included adenylylation (A), acyl transferase (AT), thiolation (T), b-ketoacyl synthase (KS), b-ketoacyl reductase (KR), reductase (SDR) and thioester reductase (SDRe1) domains. It was predicted that these genes encoded different enzymes from pipecolic acid to SW. The T domain deletion mutant of *swn* K did not produce SW, while the complementary strain produced SW. Our group presently identified partial cDNA of *swn* K, and performed genome sequencing of the OW7.8 strain on the Pacbio platform. We will conduct SW biosynthesis gene identification and function analysis to explore more details about the SW biosynthesis in *A. oxytropis* in future.

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**Conflict of interests.** The authors declare that there are no conflicts of interests.

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