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Improving Ergometrine Production by *easO* and *easP* Knockout in *Claviceps paspali*

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Abstract: Ergometrine is widely used for the treatment of excessive postpartum uterine bleeding. *Claviceps paspali* is a common species for industrial production of ergometrine, which is often accompanied by lysergic acid α-hydroxyethylamide (LAH) and lysergic acid amide (LAA). Currently, direct evidence on the biosynthetic mechanism of LAH and LAA from lysergic acid in *C. paspali* is absent, except that LAH and LAA share the common precursor with ergometrine and LAA is spontaneously transformed from LAH. A comparison of the gene clusters between *C. purpurea* and *C. paspali* showed that the latter harbored the additional *easO* and *easP* genes. Thus, the knockout of *easO* and *easP* in the species should not only improve the ergometrine production but also elucidate the function. In this study, gene knockout of *C. paspali* by homologous recombination yielded two mutants Δ$easO_{hetero}$-1 and Δ$easP_{hetero}$-34 with ergometrine titers of 1559.36 mg·L^{-1} and 837.57 mg·L^{-1}, which were four and two times higher than that of the wild-type control, respectively. While the total titer of LAH and LAA of Δ$easO_{hetero}$-1 was lower than that of the wild-type control. The *Aspergillus nidulans* expression system was adopted to verify the function of *easO* and *easP*. Heterologous expression in *A. nidulans* further demonstrated that *easO*, but not *easP*, determines the formation of LAA.

Keywords: *Claviceps paspali*; ergometrine production; gene knockout; function of *easO* and *easP*; lysergic acid α-hydroxyethylamide

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1. Introduction

Claviceps species produce a lot of bioactive ergot alkaloids, some of which have been developed into clinically important drugs [1,2]. For example, ergometrine is used to cure postpartum uterus bleeding [3]; ergotamine is used for the treatment of migraine [4,5]; bromocriptine can be used for treating hyperprolactinemia and Parkinson's disease [6,7]. On the other hand, ergot alkaloids are responsible for the toxicity of ergotism, a common cereal epidemics [8]. These alkaloids are generally biosynthesized from the common precursor *D*-lysergic acid. This step is catalyzed by the non-ribosomal peptide synthetases (NRPSs). From this reaction, a class of ergopeptides (also named ergopeptines), including ergotamine, ergocristine, ergocornine, α-ergocriptine and β-ergocriptine are synthesized [9,10]. Alternatively, *D*-lysergic acid can also be converted into simple lysergic acid amides, including ergometrine, lysergic acid α-hydroxyethylamide (LAH) and lysergic acid amide (LAA) [1,4,11,12].

C. purpurea produces not only ergopeptides but also ergometrine [13]. The ergot alkaloid synthesis (EAS) pathway of ergometrine in *C. purpurea* has been deciphered [4,9,14–22]. In comparison, *C. paspali* does not produce ergopeptides but produces ergometrine and other lysergic acid amides, including LAH and LAA [23]. A comparison of the EAS gene clusters of *C. purpurea* and *C. paspali* shows that both *C. purpurea* and *C. paspali* harbor

lpsB (encoding NRPS) and *lpsC* (encoding NRPS), which guarantee the biosynthesis of ergometrine. However, *lpsA1* and *lpsA2* (encoding NRPSs) are missing from *C. paspali*, which results in the inability of *C. paspali* to produce ergopeptines (Figure 1a). Surprisingly, the EAS gene cluster of *C. paspali* additionally harbors *easO* and *easP*, which are also present in other non-*Claviceps* fungal species, such as *Periglandula ipomoeae*, *Metarhizium robertsii* and *Metarhizium*, which can produce the simpler lysergic acid amides [10]. The additional *easO* and *easP* are likely involved in the biosynthesis of LAH and LAA, in which LAH can spontaneously convert to LAA by a non-enzymatic process [10,24]. Recently, *easO* has been shown to control the biosynthesis of LAH in *M. brunneum* [25], suggesting the role of the enzyme in the biosynthesis of LAH in *C. paspali*.

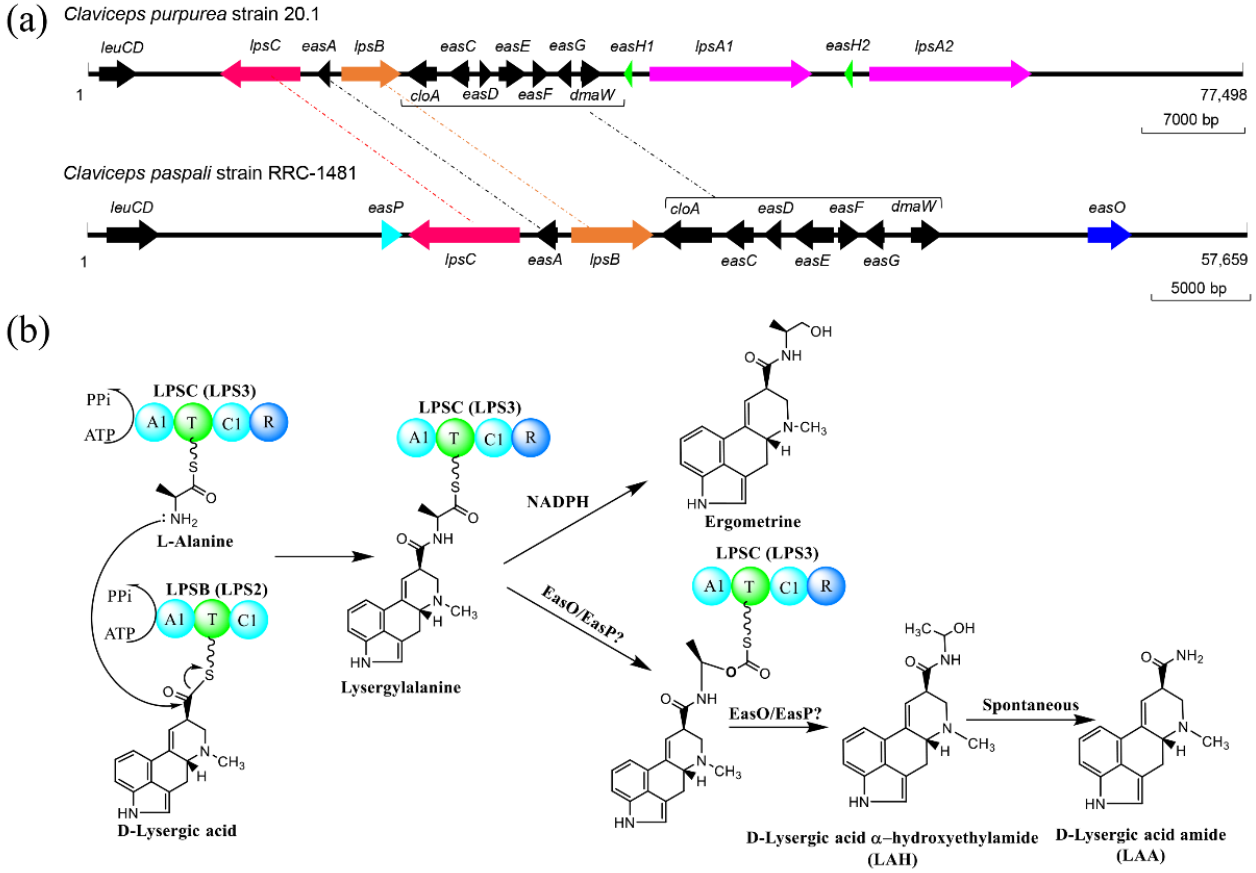

Figure 1. (**a**) Comparison of the two EAS gene clusters from *C. purpurea* and *C. paspali*. (**b**) The proposed biosynthetic pathway of lysergic acid amides in *C. paspali*.

Multiple sequence alignment displayed that the enzyme EasO of *C. paspali* harbors two 'fingerprint' motifs which are characteristic of type I Baeyer-Villiger monooxygenase (BVMO): (FXGXXXHXXXW[P/D]) and ([A/G]GXWXXXX[F/Y]P[G/M]XXXD), and the latter is located between the two Rossmann folds and contains the critically conserved active site asparagine (Figure S1). This type of BVMO acts on intermediates linked to an acyl carrier protein (ACP) and forms thiocarbonates through BV oxidation of the carbonyl group [26,27]. The proposed biosynthetic pathway of lysergic acid amides produced in *C. paspali* is shown in Figure 1b [9,24,28,29].

In addition to validating the function of the additional genes in the EAS gene cluster, down-regulating of *easO* and *easP* in *C. paspali* should be a good strategy for improving the ergometrine production by diverting the metabolic flux away from LAH and into ergometrine. In this study, a protoplast-mediated genetic transformation system for the *C. paspali* WL721 strain was established to disrupt *easO* and *easP* in the *C. paspali* genome, resulting in the mutants $\Delta easO_{hetero}$ and $\Delta easP_{hetero}$. Fermentation products of the mutants

were subsequently analyzed. Compared with the control, the titer of ergometrine in the mutant Δ*easO*$_{hetero}$ was significantly increased along with a sharp decline in the titers of LAH and LAA, demonstrating that *easO* plays a key role in the biosynthesis of LAH and LAA. The titer of ergometrine was also increased in the mutant Δ*easP*$_{hetero}$, however, the titers of LAH and LAA did not decline compared with those of the wild-type strain. The role of *easO* in the biosynthesis of LAA was further demonstrated in the heterologous expression system of *A. nidulans*, by co-expressing *easO* with *lpsB* and *lpsC* from *C. paspali*.

2. Materials and Methods

2.1. Strains

C. paspali WL721 (GenBank No. OK326692) was preserved at 4 °C in our laboratory and was used throughout this study. *A. nidulans* A1145 was used for heterologous expression. *Saccharomyces cerevisiae* BJ5464 was used for in vivo yeast DNA recombination cloning. The nucleotide sequences of *C. paspali* RRC-1481(GenBank No. AFRC01000001.1) and *C. purpurea* (GenBank No. CAGA01000001.1) were used.

2.2. Establishment of the Protoplast-Mediated Transformation System

Fungal genomic DNA isolation was performed according to the previous method [30,31]. Preparation and transformation of protoplast were performed according to a modified version of the method previously used in other filamentous fungi [32]. Protoplasts were successively diluted to three concentration gradients: 5×10^7, 5×10^6 and 5×10^5 protoplasts/mL with STC buffer. Two different protoplast regeneration methods were used and tested, namely the monolayer medium culture method and the layered medium culture method (Figure S2). Follow-up experiments for protoplast regeneration were carried out on the basis of the most suitable protoplast concentration and regeneration mode. The minimal inhibitory concentrations (MICs) of 5 antibiotics were tested on *C. paspali* WL721 for screening the proper antibiotic as a dominant selection marker. Specific operation methods and details are provided in the supporting information.

2.3. Construction of Deletion Cassettes and Characterization of Δ*easO* and Δ*easP* Mutants

The nucleotide sequence of the EAS gene cluster of *C. paspali* RRC-1481 was obtained from the National Center for Biotechnology Information (NCBI), with the GenBank accession number JN186800.1, and was used to design primers for the PCR amplification of gene sequences from the genome of *C. paspali* WL721. The deletion cassettes for *easO* and *easP* knockout were constructed as described by Szewczyk et al. [33] DNA fragments comprising the 5′ and 3′ DNA sequences flanking the target gene and the hygromycin B phosphotransferase gene (*hph*) cassette was assembled by the fusion PCR method to construct the fusion fragments. The size of 5′- and 3′-flanking fragments were 1152 bp and 1657 bp for *easO*, and 2055 bp and 2077 bp for *easP*, respectively. The *hph* cassette was 2.6 kb. The fusion fragments and *pEasy*-Blunt vectors were combined to form the gene disruption plasmids, designated as *pEasy*-Blunt-fusion-*easO*-*hph* and *pEasy*-Blunt-fusion-*easP*-*hph*, respectively. Then, the deletion cassettes were generated by PCR based on the above disruption plasmids. These two deletion cassettes were separately transformed into protoplast of *C. paspali* WL721. The genes of *easO* and *easP* were knocked out by homologous recombination. The primers used for the amplification of DNA fragments and PCR verification of transformants are shown in Table S1.

2.4. Detection and Analysis of Ergot Alkaloid Production for C. paspali Mutants

The culture method and fermentation conditions of the mutants are described in the supporting information. For ergot alkaloid extraction, the fermentation broth was adjusted to pH 8–9 by using NH_4OH, and 5 mL of cultures were extracted with 20 mL of ethyl acetate and concentrated with a rotary evaporator followed by dissolving with 1 mL of methanol. The solution was filtered through a 0.22 μm MultiScreen filter plate (Merck Millipore, Burlington, MA, USA). Then, the Agilent 1200 high-performance liquid chromatography

(HPLC) system was used, and the separation was carried out on a C18 column (Capcel pak C18 MG II, 4.6 mm I.D. × 150 mm, 5 μm) at the flow rate of 1 mL·min^{-1}, operated at the temperature of 28 °C. The detection UV wavelength was 314 nm. Solvents were: (A) HPLC grade H_2O containing 20 mM of ammonium formate; (B) HPLC grade CH_3CN. Gradients were as follows: 0–15 min, 10–18% B; 15–20 min, 18–42% B; 20–25 min, 42–100% B; 25–26 min, 100–10% B; 26–30 min, 10% B. LC-MS analysis was conducted on an LCMS-2020 system (Shimadzu Corporation, Japan, Kyoto) with LC-20AT pumps, a PDA detector, an electrospray ionization (ESI) source interface, and an SQ mass detector (Shimadzu, 5 μm, 2.1 × 100 mm, C18 column) using positive and negative mode electrospray ionization. Water (solvent A) and acetonitrile (solvent B) were used at a flow rate of 0.3 mL·min^{-1}. Samples were analyzed with the following gradient: 0–20 min, 10–42% B; 20–25 min, 42% B; 25–30 min, 42–100% B; 30–31 min, 100–10% B; 31–36 min, 10% B. Ergometrine, LAH, LAA and *D*-lysergic acid prepared by our lab were used as standards.

The titer of ergometrine was calculated by the standard curve of ergometrine, which was plotted based on the different concentrations of standard sample ergometrine (0.1625, 0.325, 0.65, 0.8125 and 0.975 mg·L^{-1}). The regression equation was y = 19304x + 385.55 (R^2 = 0.9953). In addition, this regression equation was utilized to roughly estimate the titers of LAH, LAA and the isomers.

2.5. Heterologous Expression in A. nidulans A1145

The plasmids pYTU-*lpsB*, pYTP-*lpsC*, pYTR-*easO*, pYTR-*easP* and pYTR-*easO*-*glaA*-*easP* for *A. nidulans* expression were assembled by yeast homologous recombination [34]. The genes of *lpsB*, *lpsC*, *easO* and *easP* carrying 200 bp terminators were amplified from the genomic DNA of *C. paspali* WL721 by Q5 high fidelity DNA polymerase (NEB, Cat# M0491L) using primers containing 30 bp overlapping regions with the *A. nidulans* vectors. The PCR products were co-transformed into *S. cerevisiae* BJ5464 with Pac I digested pYTU and pYTP and BamH I digested pYTR, respectively. The plasmid was extracted from yeast using the Zymoprep Yeast Miniprep Kit (Zymo Research, Cat# D2001) and transformed into *E. coli* T1 for sequencing. The combinations of pYTU-*lpsB*/pYTP-*lpsC*, pYTU-*lpsB*/pYTP-*lpsC*/pYTR-*easO*, pYTU-*lpsB*/pYTP-*lpsC*/pYTR-*easP* and pYTU-*lpsB*/pYTP-*lpsC*/pYTR-*easO*-*glaA*-*easP* were, respectively, co-transformed into the protoplast of *A. nidulans* A1145. Preparation of the protoplast and transformation of *A. nidulans* A1145 were performed as previously described [35]. Spores of each transformant were collected separately and suspended in sterile distilled water before use.

2.6. Product Analysis for Transformants of A. nidulans

For product analysis, the transformants were cultured on solid and in liquid CD-ST medium (GMM liquid medium containing 20 g·L^{-1} starch without glucose), respectively. For solid cultivation, 2 mg of *D*-lysergic acid was dissolved in 100 μL of DMSO and mixed with 20 mL of CD-ST for each plate. The spore solution of each transformant of *A. nidulans* was spread on the plate containing the substrate *D*-lysergic acid and cultivated for 5 days at 30 °C, then, the culture was soaked in methanol overnight and extracted with methanol. For liquid cultivation, each transformant was incubated in 50 mL of liquid CD-ST medium at 25 °C for 2 days, and 5 mg of *D*-lysergic acid dissolved in 250 μL of DMSO was added to the medium and cultivation was continued for another 3 days. The culture broth was extracted with ethyl acetate. The extracts were concentrated by rotary evaporator and subjected to the liquid chromatograph-mass spectrometer (LC-MS) analysis. The equipment and conditions used in LC-MS analysis were the same as those in the previous steps.

2.7. Statistical Analysis

Data are means ± standard deviation (SD) of three biological repeats. One-way ANOVA was used to analyze the statistical significance of the differences between means of the yield of ergometrine, total ergot alkaloids, the total of LAA, LAH and their isomers

for wild-type and Δ*easO*$_{hetero}$-1, wild-type and Δ*easP*$_{hetero}$-34. * $p < 0.05$ and ** $p < 0.01$ are relative to the wild-type. Differences with * $p < 0.05$ were considered statically significant.

3. Results

3.1. The Protoplast-Mediated Transformation of C. paspali

Since *C. paspali* could not generate spores under laboratory conditions, protoplasts were used for genetic manipulation. The first step of our study was to investigate the conditions for the protoplast preparation and regeneration system for *C. paspali* WL721.

The commercial lywallzyme worked well in releasing the protoplast from the mycelia of *C. paspali* WL721. The number of protoplasts released from mycelium increased obviously with the prolonged incubation and reached its maximum value at 90 min. Beyond 90 min, the number of protoplasts remained basically unchanged (Figure 2a). Based on the regenerated colony dispersion, the layered medium culture method was chosen for further study and the optimal protoplast concentration was 10^5 protoplasts/mL (Figure S2). After preliminary screening, hygromycin B resistance was chosen as the dominant selectable marker. Hygromycin B could inhibit hyphal growth at the suitable concentration of 0.3 mg·L^{-1} after cultivation for 7 days (Figure 2b). The PEG-mediated transformation method was used and the protoplasts could be successfully transformed via pAN7-1 [36] which harbored the *hph* cassette containing the hygromycin B-resistance gene (hygromycin phosphotransferase gene, *hph*) (Figure 2c). The selected transformants could still grow normally on the solid medium containing 0.6 mg·L^{-1} of hygromycin B (Figure 2d). The *hph* cassette (2.6 kb in length) could be amplified from 14 of the 19 transformants (transformation frequency: ~74%), indicating that the genetic transformation system was successfully established (Figure 2e).

3.2. Knockout of easO and easP and Characterization of ΔeasO and ΔeasP Mutants

The established genetic transformation system was used to knock out the genes *easO* and *easP* from the genome of *C. paspali* WL721. The gene knockout efficiency (per regenerated colony) was ~17%. The diagnostic lengths and sequences of the PCR products for Δ*easO* and Δ*easP* are summarized in Figure 3 and Tables S2–S8, respectively.

For analyzing the *easO* deletion, the two overlapped fragments with 3.5 kb and 3.3 kb in lengths were yielded when the primer pairs P1/P8 and P7/P6 were used, respectively, which harbored the 5′ flank and 3′ flank regions of the *easO* gene and the whole *hph* expression cassette (Figure 3b). This result, together with the 2.6 kb-*hph* amplified fragment, demonstrated that the deletion cassette has replaced the corresponding part of *easO* and led to the knockout of *easO* from the genome of *C. paspali* WL721 (Figure 3b). However, the 1.9 kb-*easO* gene could still be amplified from both the wild-type control and each of the six recombinants (Figure 3b), indicating that all the recombinants were heterokaryons. In other words, the *easO*-deleted nucleus (Δ*easO*) and the *easO*-preserved nucleus were simultaneously present in each cell. These recombinants are designated as Δ*easO*$_{hetero}$ mutants.

Similarly, the Δ*easP*$_{hetero}$ mutants were obtained in which the fragments of 3.9 kb (5′ flank regions of *easP* gene), 3.8 kb (3′ flank regions of *easP* gene), the *hph* cassette (2.6 kb), and the 1.1 kb-*easP* gene could be simultaneously amplified (Figure 3c).

3.3. Analysis of Ergot Alkaloid Production

The alkaloid-producing capabilities of these partial gene knockout mutants were analyzed in which the mutants Δ*easO*$_{hetero}$-1 and Δ*easP*$_{hetero}$-34 were chosen as the representatives for further investigation. The HPLC profiles of the product yields were shown in Figure 4, which also included the MS spectra data of LAA, LAH and ergometrine, as well as their isomers.

Compared with the wild-type strain, Δ*easO*$_{hetero}$-1 accumulated more ergometrine, however, the production of LAA and LAH decreased significantly, indicating that the partial deletion of *easO* has redirected the lysergic acid flux away from LAH/LAA to ergometrine. More ergometrine was also accumulated in Δ*easP*$_{hetero}$-34, although the yields

of LAH and LAA seemed unchanged compared with those of the wild-type strain. This result implied that *easP* may play a different role in the production of ergometrine.

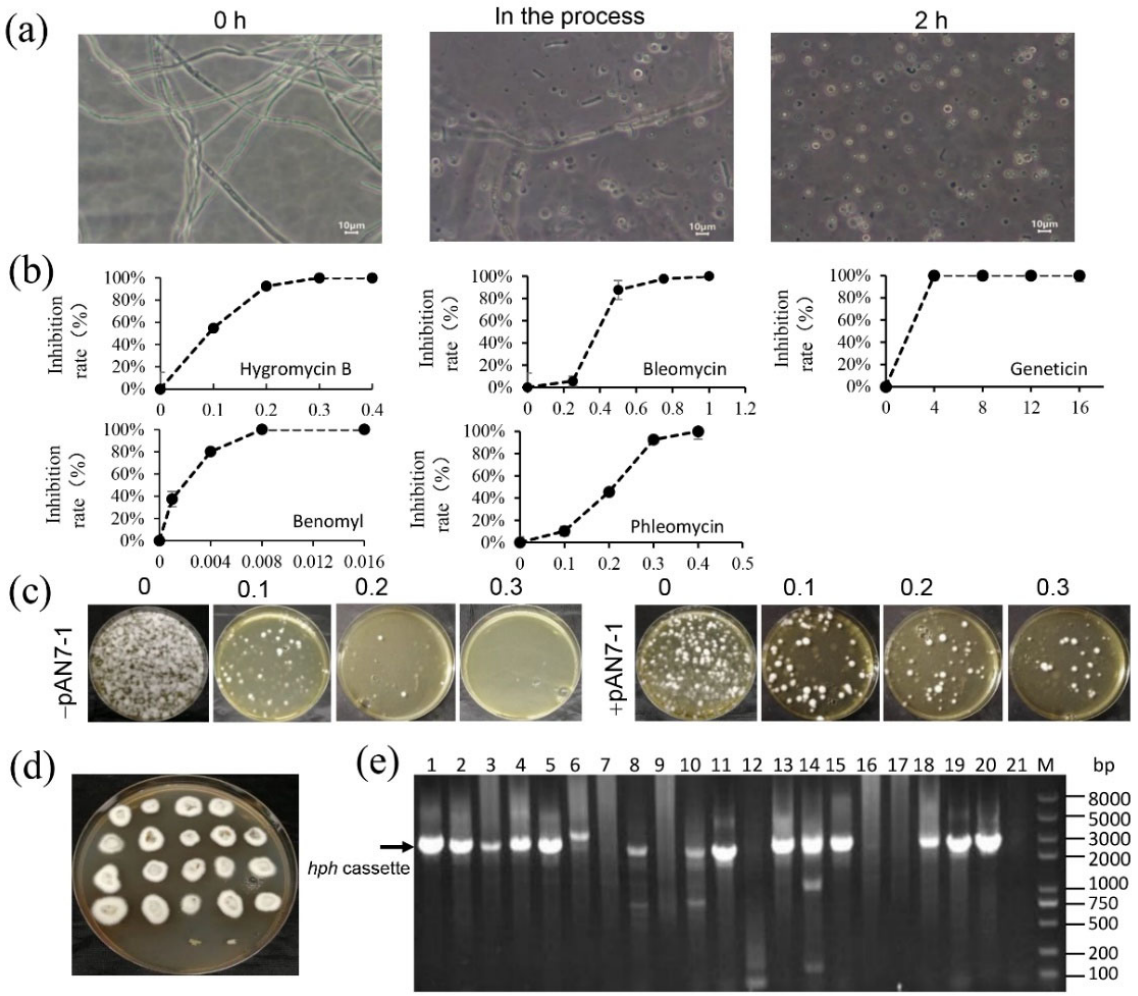

Figure 2. Establishment of the protoplast-mediated transformation system for *C. paspali*. (**a**) Protoplast formation at different time of enzymatic hydrolysis. The bar is 10 μm. (**b**) Determination of minimal inhibitory concentrations (MICs) of 5 antibiotics. The concentrations of antibiotics are measured in mg·mL^{-1}. (**c**) Protoplast regeneration on the regeneration plates with different concentrations of hygromycin B. −pAN7-1: protoplasts transformed with mock plasmid (negative control). +pAN7-1: protoplasts transformed with dominant pAN7-1 harboring *hph* gene. The layered medium culture method was used and the upper layer medium (5 mL) contained 0, 0.1, 0.2, and 0.3 mg·mL^{-1} hygromycin B, respectively. (**d**) Growth of the transformants on fresh PGA medium containing 0.6 mg·mL^{-1} hygromycin B. (**e**) Identification of positive transformants via PCR. Lanes 1–19, transformants; Lane 20, pAN7-1 (positive control); Lane 21, the wild-type strain (negative control). M: DNA marker.

The titer of ergometrine in Δ*easO*$_{hetero}$-1 reached 1559.36 mg·L^{-1} after 14 days of fermentation, which is nearly 4 times higher than that of the wild-type (400.84 mg·L^{-1}) (Figure 5). The proportion of ergometrine in the total ergot alkaloid fermentation products of the Δ*easO*$_{hetero}$-1 mutant was more than 80%. On the other hand, the total titer of LAH, LAA and their isomers of the Δ*easO*$_{hetero}$-1 mutant was lower than that of the wild-type control (459.13 mg·L^{-1} vs. 521.03 mg·L^{-1}), and the significant decreases in the LAH and LAA titers suggested that *easO* is indispensable for the biosynthesis of these two alkaloids. The mutant could maintain the productivity and quantity of the alkaloids for at least three generations. The ergometrine titer of the Δ*easP*$_{hetero}$-34 reached 837.57 mg·L^{-1} after 14 days

of fermentation, which was 2.1 times that of the wild-type. Additionally, the ergometrine content was more than 50% of the total alkaloids produced by the mutant. However, the total titer of LAH, LAA and their isomers of Δ*easP*$_{hetero}$-34 mutant was even higher than that of the wild-type control (641.12 mg·L^{-1} vs. 521.03 mg·L^{-1}) (Figure 5), suggesting that *easP* may not be involved in the biosynthesis of LAH and LAA.

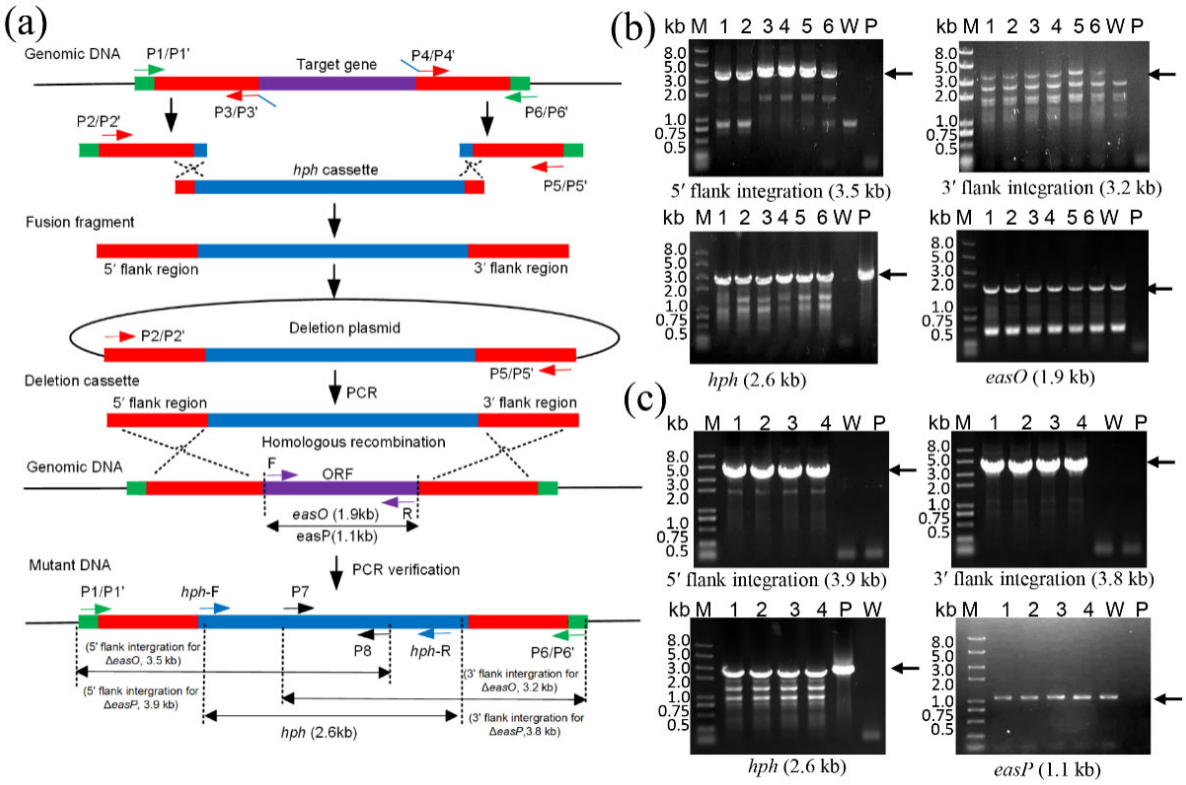

Figure 3. Schematic diagram of the progress of constructing mutant strains and screening of gene knockout mutants. (**a**) Knockout of *easO* and *easP* in *C. paspali* WL721. 5′ and 3′ flank fragments are amplified separately from genomic DNA with primers P1/P3 and P4/P6 for *easO* or P1′/P3′ and P4′/P6′ for *easP*, respectively. Primers P3 (or P3′) and P4 (or P4′) have 5′ tails homologous to the *hph* cassette. The two flanks and the *hph* cassette are assembled by overlapping PCR method and the fusion fragment containing the deletion cassette is obtained by amplification with primers P2 (or P2′ for *easP*) and P5 (or P5′ for *easP*). Homologous recombination creates the circular construct. The deletion cassette harboring the 5′ and 3′ flank regions of *easO* or *easP* was obtained by PCR amplification with primers P2 and P5 for *easO* or primers P2′ and P5′ for *easP*. The fragment harboring the *easO* or *easP* in the genomic DNA of *C. paspali* W721 can be replaced by the deletion cassette containing the *hph* gene through the double crossover between the deletion cassette and the genomic DNA. The *hph* is transcribed in the antisense direction relative to the target gene. Transformants carrying the homologous integration of the replacement construct were identified via PCR with primers P1 (or P1′) and P8, P7 and P6 (or P6′), respectively. Other pairs of primers were used to conduct PCR verification at the same time: *easO*-F and *easO*-R (1900 bp of amplified fragment), *hph*-F and *hph*-R (2660 bp of amplified fragment), *easP*-F and *easP*-R (1100 bp of amplified fragment). The *easP* gene was knocked out in the same way as the *easO* gene. Transformants carrying the homologous integration of the replacement construct were identified via PCR with two different pairs of primers P1′ and P8, P6′ and P7, respectively. Two additional pairs of primers, *easP*-F and *easP*-R (1100 bp of amplified fragment), *hph*-F and *hph*-R (2660 bp of amplified fragment), were used to conduct PCR verification. (**b**) Screening of Δ*easO* transformants via PCR. M: DNA marker. 1–6: Δ*easO* transformants. (**c**) Screening of Δ*easP* transformants via PCR. M: DNA marker. 1–4: Δ*easP* transformants. W: *C. paspali* WL721 wild strain. P: pAN7-1 plasmid.

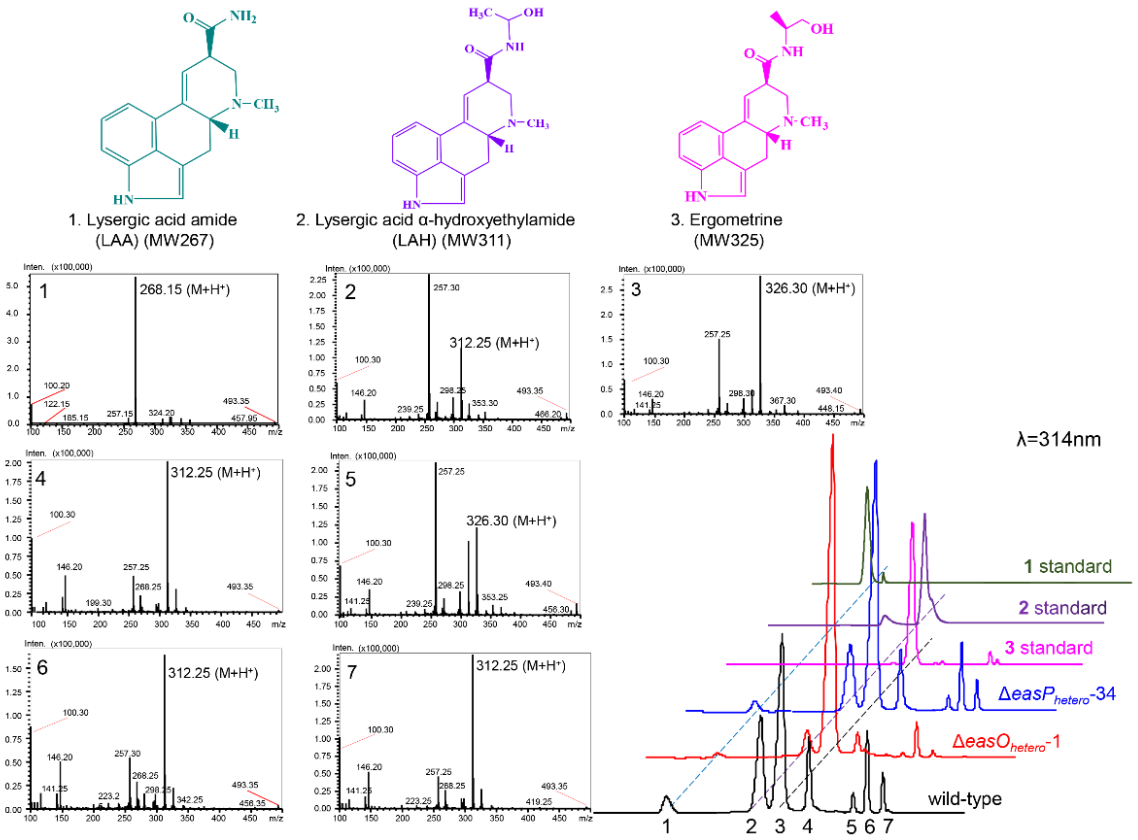

Figure 4. LC-MS analysis of fermentation products of Δ*easO*$_{hetero}$-1, Δ*easP*$_{hetero}$-34 and the wild-type strain. Notes: Ergot alkaloids might convert into their isomers. 1, LAA; 2, LAH; 3, ergometrine; 4, 6 and 7, isomers of LAH; 5, isomer of ergometrine. The standards of ergometrine, LAH and LAA were used, respectively.

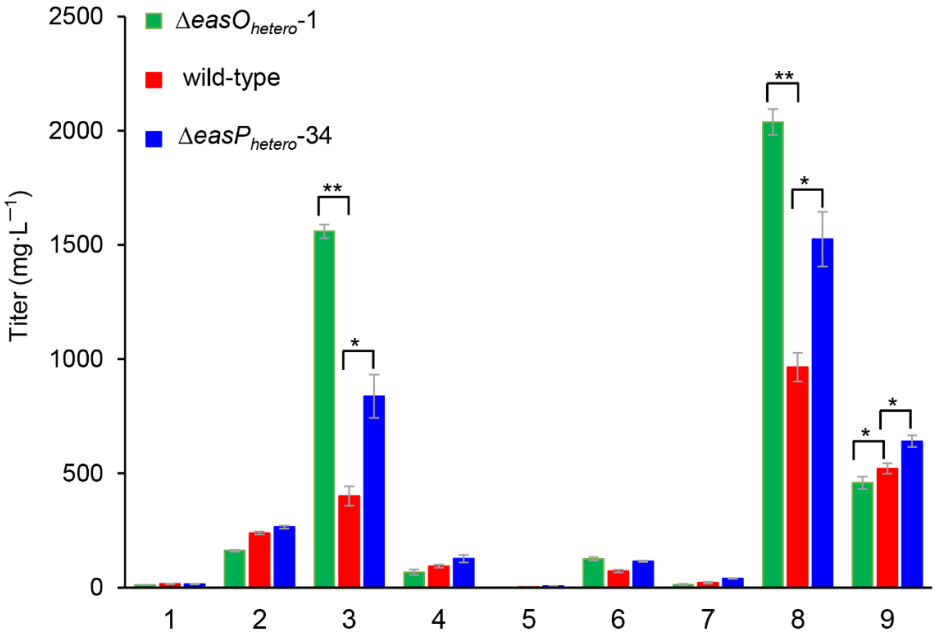

Figure 5. The quantitative analysis of ergot alkaloids production (mg·L^{-1}) from different strains after 14 days of fermentation. One-way ANOVA was used to analyze the significance between means. * $p < 0.05$, ** $p < 0.01$ relative to the wild-type. Notes: 1, LAA; 2, LAH; 3, ergometrine; 4, 6 and 7, isomers of LAH; 5, isomer of ergometrine; 8, total ergot alkaloids; 9, total of LAA, LAH & isomers.

3.4. Heterologous Expression of the Ergot Alkaloid Biosynthetic Genes in A. nidulans

To examine the function of *easO* and *easP* in the biosynthesis of LAA based on the proposed biosynthetic pathway, different combinations of *lpsB*, *lpsC*, *easO* and *easP* were expressed in the heterologous host *A. nidulans*, and the recombinants were cultured on the solid medium fed with the substrate lysergic acid during cultivation. The metabolic products from *A. nidulans* were analyzed by LC-MS.

As shown in Figure 6, co-expression of *lpsB* and *lpsC* in *A. nidulans* led to the production of ergometrine, confirming the roles of the two genes in the biosynthesis of ergometrine. The product LAA, along with the major products ergometrine, were produced when *lpsB*, *lpsC* and *easO* were heterologously co-expressed in *A. nidulans*, demonstrating the key function of *easO* in the formation of LAA. LAH was not detected in the *lpsB*/*lpsC*/*easO* recombinant, and the most possible reason is that an unknown factor in *A. nidulans* triggered the rapid conversion from LAH to LAA; and/or after a long time soaking in methanol, nearly all the remaining LAH was spontaneously converted into LAA [10,24]. However, when *lpsB*, *lpsC* and *easP* were co-expressed in *A. nidulans*, only ergometrine could be detected, confirming that *easP* is not responsible for the biosynthesis of LAH/LAA. Specifically, when *easO* was co-expressed with *lpsB*, *lpsC* and *easP* genes, the production of additional minor LAA was restored, which again demonstrated that *easO* catalyzed the formation of LAA. Interestingly, the recombinant *A. nidulans* with the *lpsB*/*lpsC*/*easO* combination grew very poorly in the medium while the addition of *easP* in the combination (*lpsB*/*lpsC*/*easO*/*easP*) could restore growth (Figure 6), which deserves further analysis. In fact, the recombinant with the *lpsB*/*lpsC*/*easO* combination almost stopped growth in the liquid medium, and the experiment could not be completed for this mutant.

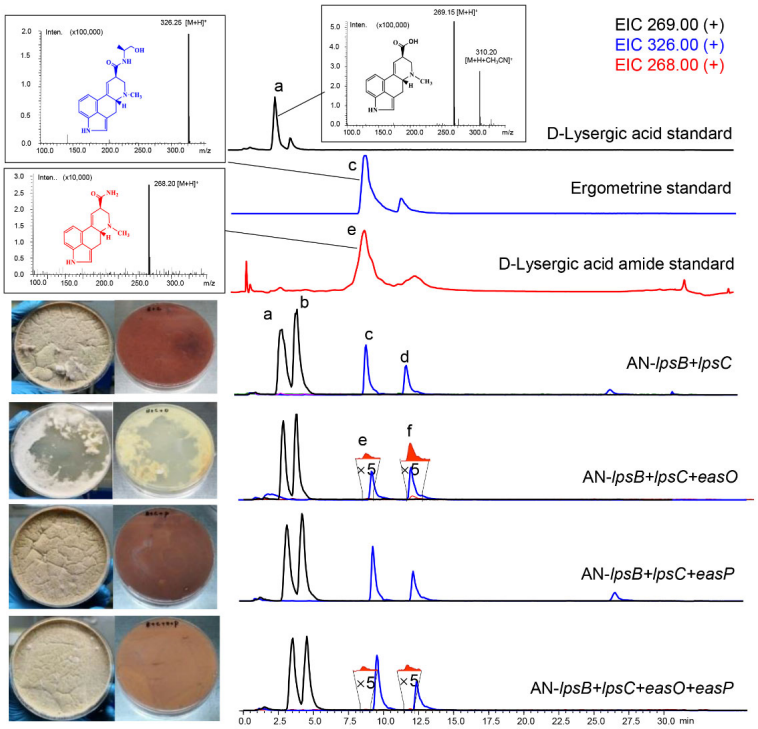

Figure 6. LC-MS analysis and morphologies of the corresponding strains. Note: Ergot alkaloids might convert into their isomers. a and b, *D*-lysergic acid and isomer; c and d, ergometrine and isomer; e and f, LAA and isomer. a, c and e were the standards of *D*-lysergic acid, ergometrine and LAA, respectively. AN-*lpsB* + *lpsC*: co-expression of *lpsB* and *lpsC* in *A. nidulans* host. AN-*lpsB* + *lpsC* + *easO*: co-expression of *lpsB*, *lpsC* and *easO* in *A. nidulans* host. AN-*lpsB* + *lpsC* + *easP*: co-expression of *lpsB*, *lpsC* and *easP* in *A. nidulans* host. AN-*lpsB* + *lpsC* + *easO* + *easP*: co-expression of *lpsB*, *lpsC*, *easO* and *easP* in *A. nidulans* host. The morphologies of different recombinants on the solid medium show the sporulation (**left**) and the color patterns (**right**) after incubation at 30 °C for 5 days.

4. Discussion

Ergometrine is clinically used for the treatment of postpartum uterus bleeding. The valuable commodity is mainly produced by *C. paspali*, which also produces other simple lysergic acid amides, including LAH and LAA. Both ergometrine and LAH/LAA are derived from D-lysergic acid, given that LAA is spontaneously converted from LAH. The presence of additional genes (*easO* and *easP*) in the EAS gene cluster of *C. paspali* suggests their functions in the biosynthesis of LAH/LAA. Thus, the proposed biosynthetic pathway suggested that *easO* and *easP* may be involved in the biosynthesis of LAH/LAA. Moreover, down-regulation of *easO* and *easP* should be a good strategy to improve the productivity of the ergometrine of *C. paspali*. On the other hand, although the function of *easO* in *M. brunneum* has recently been illustrated to control the biosynthesis of LAH by the gene knockout test, additional evidence is required to demonstrate the roles of *easO* and *easP* in the biosynthesis of LAH/LAA in *C. paspali*.

Currently, there is no universal genetic transformation method that can be applied to every fungal species. Moreover, the current protoplast-mediated transformation protocols of *C. paspali* are undermined by its inefficiencies in protoplast regeneration, low frequency of DNA integration and low mitotic stability of the nascent transformants [31]. Therefore, in the present study, a protoplast-mediated genetic transformation system for the *C. paspali* WL721 strain was first established to enable subsequent gene manipulation.

C. paspali mutants were produced using homologous fragment recombination, and they were screened with a high concentration of hygromycin B. Figure 3 showed that the event of gene replacement between the deletion cassette and *easO* (Figure 3b) or *easP* (Figure 3c) has taken place in every tested gene knockout mutant, indicating the high efficacy of the homologous recombination approach. On the other hand, due to the multinucleate property of the strain, the nucleus harboring the *easO* or *easP* gene was hardly eliminated from the mutants. However, ergometrine production of the heterokaryon Δ$easO_{hetero}$-1 was significantly increased compared with that of the wild-type control (1559.36 mg·L^{-1} vs. 400.84 mg·L^{-1}), accompanied by a significant decrease in the production of LAH and LAA (Figure 5), which supported the key role of the enzyme in the formation of LAH/LAA (Figure 1b). Furthermore, the down-expression of the gene could attenuate the lysergic acid (the common precursor of ergometrine and LAH/LAA) flux towards LAH/LAA but enhance it towards ergometrine biosynthesis. Typically, the function of *easO* has been demonstrated in this study by heterologous expression of it in the *A. nidulans* system (Figure 6). To the best of our knowledge, this study is the first to demonstrate the role of the *easO* in the biosynthesis of LAA of *C. paspali*. Although the mutant of *C. paspali* was a heterokaryon to the gene *easO*, the increase in ergometrine productivity was maintained for at least four generations, suggesting that the mutant can be utilized for industrial production if the strain improvement is accompanied during the industrial process. On the other hand, the great increase in the titer of ergometrine and the significant decrease in the impurities of the fermentation products may further benefit the pharmaceutical industry.

The productivity of LAH and LAA was slightly enhanced in the Δ$easP_{hetero}$-34 strain compared with that in the wild-type control (641.12 mg·L^{-1} vs. 521.03 mg·L^{-1} in total), suggesting that *easP* was not involved in the biosynthesis of LAH/LAA. The non-involvement of *easP* in the biosynthesis of LAH/LAA was further supported by the heterologous expression analysis in the *A. nidulans* system (Figure 6). However, the titer of ergometrine in the mutant was surely increased. Taken together, this enzyme does not participate in the formation of LAH/LAA, but knocking it out indirectly improved the biosynthesis of ergometrine.

In summary, a protoplast-mediated genetic transformation system for the *C. paspali* WL721 strain was established to delete *easO* and *easP* in the *C. paspali* genome. *A. nidulans* system was used to further investigate the function of the additional genes of *easO* and *easP* in the EAS gene cluster of *C. paspali*. The two ergot mutants, Δ$easO_{hetero}$-1 and Δ$easP_{hetero}$-34, were obtained. The ergometrine yields of Δ$easO_{hetero}$-1 and Δ$easP_{hetero}$-34 at the flask fermentation level reached 1559.36 mg·L^{-1} and 837.57 mg·L^{-1}, which were 4 and 2 times

higher than that of the wild-type control, respectively. Meanwhile, the yields of LAH and LAA in Δ*easO*$_{hetero}$-1 were significantly decreased, which strongly supported that the gene of *easO* was involved in the branch pathway of the biosynthesis of ergot alkaloids. The function of *easO* was further demonstrated by heterologous expression of it in the *A. nidulans* system. In addition, the increase in the ergometrine yield in Δ*easP*$_{hetero}$-34 suggests that although *easP* is not involved in the biosynthetic pathway of LAH/LAA, knocking it out can indirectly improve the formation of ergometrine.

5. Conclusions

By means of protoplast-mediated genetic transformation and homologous recombination, we obtained two mutants, Δ*easO*$_{hetero}$-1 and Δ*easP*$_{hetero}$-34, from the *C. paspali* WL721 strain, with ergometrine titers of 1559.36 mg·L^{-1} and 837.57 mg·L^{-1}, which were 4 and 2 times higher than that of the wild-type control, respectively, and have practical implication for improving ergometrine production of *C. paspali*. Meanwhile, the yields of LAH and LAA in Δ*easO*$_{hetero}$-1 were significantly decreased, supporting that at least the gene of *easO* was involved in the branch pathway of the biosynthesis of ergot alkaloids. Heterologous expression of *easO* or *easP*, together with *lpsB* and *lpsC* from *C. paspali* in the *A. nidulans* system, further demonstrated that *easO*, but not *easP*, determines the formation of LAA. To the best of our knowledge, this is the first evidence of the biosynthetic mechanism of LAH and LAA from lysergic acid in *C. paspali* and paves the way for the improvement of ergometrine production of *C. paspali*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8060263/s1. Table S1. Primers used in this study. Table S2. The sequence of *hph* cassette. Table S3. The sequence of 5′ homologous integration of the replacement construct in the location. Table S4. The sequence of 3′ homologous integration of the replacement construct in the location of target gene in Δ*easO* strain when primers P6 and P7 were used. Table S5. The sequence of 5′ homologous integration of the replacement construct in the location of target gene in Δ*easP* strain when primers P1′ and P8 were used. Table S6. The sequence of 3′ homologous integration of the replacement construct in the location of target gene in Δ*easP* strain when primers P6′ and P7 were used. Table S7. The sequence of *easO*. Table S8. The sequence of *easP*. Table S9. ITS sequence of *Claviceps paspali* WL721 strain. Figure S1. Sequence alignment of representative Baeyer-Villiger monooxygenase and EasO. Figure S2. (a) Protoplast regeneration by two different methods. (1) Layered medium culture method. (2) Monolayer medium culture method. (b) Determination of minimal inhibitory concentrations (MICs) of 5 antibiotics. The concentrations of antibiotics are measured in mg·mL^{-1}.

Author Contributions: Y.-M.Q. designed and conducted the experiments, collected data, created the graphs and wrote the manuscript; Y.-H.W. performed parts of the experiments; T.G. analyzed the data of mass spectra and helped in taking the photographs; J.-J.C., T.-J.C. and J.-L.Y. helped to analyze the data; P.Z. conceived the experiments and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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