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RESEARCH ARTICLE

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New insight into the production improvement and resource generation of chaetoglobosin A in *Chaetomium globosum*

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Abstract

Chaetoglobosin A is a complex macrocyclic alkaloid with potent antimycotic, antiparasitic and antitumor properties. However, the low output and high cost of chaetoglobosin A biosynthesis have hampered the application and commercialization of chaetoglobosin A in agriculture and biomedicine. Here, the *CgMfs1* gene, which encodes the major facilitator superfamily secondary transporter, was identified based on bioinformatics analysis, and an intensive study of its effects on chaetoglobosin A biosynthesis and secretion was performed using *CgMfs1*-silencing and *CgMfs1*-overexpression strategies. Inactivation of *CgMfs1* caused a notable decrease in chaetoglobosin A yield from 58.66 mg/L to 19.95 mg/L (MFS1–3) and 17.13 mg/L (MFS1–4). The use of an efficient expression plasmid in *Chaetomium globosum* W7 to generate the overexpression mutant OEX13 resulted in the highest chaetoglobosin A increase to 298.77 mg/L. Interestingly, the transcription level of the polyketide synthase gene significantly fluctuated with the change in *CgMfs1*, confirming that the predicted efflux gene *CgMfs1* could play a crucial role in chaetoglobosin A transportation. Effective efflux of chaetoglobosin A could possibly alleviate feedback inhibition, resulting in notable increase in the expression of the polyketide synthase gene. Furthermore, we utilized cornstalk as the fermentation substrate to produce chaetoglobosin A, and scanning electron microscopy and Fourier transform-infrared spectroscopy revealed that the strain OEX13 could well degrade cornstalk, presenting significant increases in the chaetoglobosin A yield, when compared with that produced by the wild-type strain (from 40.32 to 191.90 mg/L). Thus, this research provides a novel analogous engineering strategy for the construction of high-yielding strain and offers new insight into large-scale chaetoglobosin A production.

INTRODUCTION

Cytochalasans, a large group of secondary metabolites catalysed and synthesized by polyketide-non-ribosomal peptide synthetase (PKS-NRPS) (Skellam, 2017), exhibit a broad range of biological activities such as phytotoxic (cytochalasin B and chaetoglobosin K) (Cutler et al., 1980), antitumor (periconiasins A–C) (Zhang et al., 2013a, 2013b, 2013c), antifouling (Zheng et al., 2013) and immunomodulatory (Chaetoglobosin Fex) activities (Dou et al., 2011). Although members of this family share a similar feature in structure, they can be divided into five subunits based on variations in their size and substitution pattern connected to the perhydro-isoindolone scaffold as follows: cytochalasins, pyrichalasins, chaetoglobosins, aspochalasins and alachalasins (Scherlach et al., 2010). Chaetoglobosin A, the most abundant member of this family, was first reported in 1973 (Seklta et al., 1973) and is predominantly biosynthesized by the biological control species *Chaetomium globosum*. Its major, and most well-known, distinctive inhibitory activity is against the phytopathogenic fungi *Fusarium sporotrichioides*, *Setosphaeria turcica and Rhizopus stolonifer* (Jiang et al., 2017; Zhang et al., 2013a, 2013b, 2013c); and plant parasitic nematodes *Meloidogyne incognita* (Hu et al., 2013). Besides, chaetoglobosin A has also been noted to exhibit potent cytotoxicity in tumour cells A549 and HepG2 (Zhang et al., 2021). Owing to its significant research and commercial importance, many investigators have successively analysed chaetoglobosin A. For instance, by using RNA-mediated gene-silencing technology, the gene cluster responsible for chaetoglobosin A biosynthesis has been predicted and identified in *Penicillium expansum* (Schümann & Hertweck, 2007). Besides, it has been reported that acetate and malonate are the starting compounds for chaetoglobosin A biosynthesis, and that PKS-NRPS hybrid synthase is responsible for polymerization of the monomeric substance to form the carbon backbone. Furthermore, a stand-alone enoyl reductase (ER) located downstream of the gene cluster, which functions as a chaperone protein, has been noted to have an extremely crucial effect on carbon-chain elongation (Ishiuchi et al., 2013). Furthermore, intramolecular Diels-Alder cyclization of an earlier precursor prochaetoglobosin I has been identified to be an important step in chaetoglobosin A biosynthesis (Cox & Simpson, 2009). Moreover, three redox enzymes, including one FAD-dependent monooxygenase and two cytochrome P450 oxygenases, have been found to transform the nascent substrate into terminal product chaetoglobosin A (Ishiuchi et al., 2013). In the past 5 years, our research group conducted stepwise investigation of the factors influencing chaetoglobosin A production. A global regulator CgLaeA was identified, which positively regulated the expression of pathway-specific transcription and negatively adjusted another regulator CgVelB, and increased the chaetoglobosin A yield from 52.0 up to 79.6 mg/L (Cheng et al., 2021a). Furthermore, a GAL4-like $Zn(II)_2Cys_6$ transcription factor CgCheR was detected in *C. globosum*, which was found to play a critical role in chaetoglobosin A production, increasing the target product titre by five-fold, when compared with that noted in the parent cell (Cheng et al., 2021b). Jiang et al., (2017) attempted to utilize cornstalk as the fermentation matrix instead of the traditional medium to produce it and obtained 0.34 mg/g chaetoglobosin A. Although chaetoglobosin A has shown diversiform application potential in plant protection, the low output and high cost of fermentation still limit its widespread applications in agriculture. To overcome this problem, improvement of chaetoglobosin A producing capacity of *C. globosum* and selection of an economical substrate are crucial.

Major facilitator superfamily (MFS) represents the largest group of secondary active transporters that are ubiquitously present across all living organisms, and mediates antiportation, uniportation and symportation of sugars, peptides, vitamins, deleterious substances, inorganic ions, chromophores, neurotransmitters and many other small molecules (Chen et al., 2008; Chittrakanwong et al., 2021; Lee et al., 2021, 2016; Liu et al., 2019; Yen et al., 2010). Owing to their characteristics such as low substrate specificity and high drugs sensitivity, MFS transporters exert an important effect on various physiological processes and are progressively being identified as potential drug targets. In fungi, transporters can be regarded as the 'first-line defence barrier' to protect the fungal cells against the toxic effects of metabolites in natural environments (Rahman et al., 2017). Ma et al. (2020) identified an MFS transporter gene cluster by comparative transcriptional analysis with or without propionic acid (PA) pressure and found that overexpression of this cluster effectively enhanced the tolerance of the host cells to PA. It has been reported that overexpression of such efflux pump genes not only enhanced multidrug resistance of microorganisms to achieve self-protection (Monod et al., 2019; Samaras et al., 2020), but also effectively improved the secretion of target compounds (Yamada et al., 2021). In *Aspergillus niger*, the gene encoding CexA, which belongs to the MFS subclass DHA1, was found to function as the main transporter for citric acid, and the strong-constitutive or inducible expression system of the gene effectively increased the target product (Steiger et al., 2019). Chu et al. (2021) utilized a TuPPE module with replaceable promoters and ribosome-binding sites to optimize the expression levels of three ATP-binding cassette transporters for milbemycin titre optimization, which improved the titres of milbemycin A3/A4, avermectin $B_{1a\ and}$ nemadectin α by 24.2%, 53.0% and 41.0%, respectively. Furthermore, Jin et al. (2020) altered the sugar uptake to improve milbemycins titre and yield by employing appropriate native

temporal promoters, which significantly regulated the expression of two ATP-binding cassette transporters, TP2 and TP5, in *Streptomyces* spp., resulting in a dramatic increase in the production of milbemycin A3/A4.

Therefore, improvement in the export efficiency of chaetoglobosin A could be a promising approach to elevate its yield; however, knowledge about the efflux gene or transport mechanisms of chaetoglobosin A in *C. globosum* is still limited. In the present study, the *CgMfs1* gene in *C. globosum*, which encodes an MFS protein proved to be involved in chaetoglobosin A transport, was characterized, and its effects on chaetoglobosin A biosynthesis and secretion were verified by *CgMfs1*-silencing and *CgMfs1*-overexpression strategies. As development of an effective and economically feasible chaetoglobosin A production method is crucially important to reduce the cost of production, an inexpensive substrate, namely, cornstalk, was employed to produce chaetoglobosin A. Scanning electron microscopy (SEM) and Fourier transform-infrared spectroscopy (FT-IR) were utilized to evaluate the digestion of cornstalk samples by wild-type *C. globosum* and optimal *CgMfs1*-overexpressing mutants. The findings of the present study provide novel insights for improving secondary metabolites production and resource generation in filamentous fungi.

EXPERIMENTAL PROCEDURES

Strains, media and culture conditions

Chaetomium globosum W7 (preservation number CGMCC 3.14974), the chaetoglobosin A production species, was obtained from the Microbial Genetic Engineering Lab of Harbin Institute of Technology. *Escherichia coli* DH5α was used to propagate all plasmids. Other strains employed in this study are summarized in Table S1. The *E. coli* strains DH5α, pSilent1 and pBARGPE1-EGFP carrier were grown in Luria–Bertani medium (LB culture comprised of 10 g tryptone, 10 g NaCl and 5 g yeast extraction per litre, with a pH of 7.2). Where appropriate, experiments in petri dishes were made with the same media supplemented with ampicillin (100 μg/ml) and agar (1.5% w/v). Cultures (20 ml) were routinely grown in 100 ml flasks at 180 rpm and 37°C. *C. globosum* W7 and its mutants were incubated at 28°C statically or shaken at 180 rpm. For spore preparation and morphological comparison, *C. globosum* W7 (the parent isolate) and its mutants were cultivated at 28°C on PDA, which containing potato 200 g, dextrose 20 g, agar 15 g and 1000 ml water. For transformation of protoplasts, protoplasts-genetic modification carrier and mediated PEG4000 mixture was added to the first layer of the double screening plate, protoplast regeneration medium, which was composed of yeast extract and peptone 0.1 g, sucrose 3.42 g, agar 0.15 g and 10 ml water. Twelve hours later, covered with a selection medium PDA supplemented with hygromycin (200 μg/ml) and incubated at 28°C. For chaetoglobosin A production, the spore suspensions of species *C. globosum* and its mutants were inoculated into 50 ml PDA broth in 150 ml flasks at 180 rpm and fermented for various time points. For cornstalk fermentation, the dried cornstalk was shattered by a muller and passed through a 40-mesh sieve, fermentation medium contained cornstalk 5 g, $(NH_4)_2SO_4$ 0.06 g, NaCl 0.2 g, $MgSO_4{\cdot}7H_2O$ 0.02 g, K_2HPO_4 0.5 g, yeast extract 0.25 g and 100 ml water. *C. globosum* and its mutants were inoculated into 100 ml broth in 250 ml flasks and fermented for 15 days.

Plasmid constructions

All plasmids used in this work are listed in Table S2. All primers designed for *C. globosum* W7 in this paper are shown in Table S3. To construct the *CgMfs1* interference plasmid, plasmid pSilent1 was employed to silence the *CgMfs1* gene, promoting the target destruction of *CgMfs1*-derived transcripts in *C. globosum* W7, was constructed as follows. The amino acid sequence of the MFS transporter of *C. globosum* W7 (GenBank: XP_001220482.1) was evaluated by the online Simple Modular Architecture Research Tool (SMART) (available online: http://smart.embl-heidelberg.de/) to identify the conserved domains of the MFS protein. The essential function area of gene *CgMfs1* has been determined, a 461 bp long sequence named *CgMfs1*-R, and amplified by PCR from *C. globosum* W7 genomic DNA using as primers oligonucleotides SRF and SRR (Table S3). The amplification product was digested with BglII and SphI, and ligated with BglII/SphI double digested pSilent1 (Table S2) skeleton plasmid to generate *CgMfs1*-pSR7 (Table S2). Subsequently, the primers SLF and SLR were used to produce *CgMfs1*-L and cloned to the HindIII and XhoI sites of *CgMfs1*-pSR7 by the similarity method mentioned above to obtain *CgMfs1*-pSL2 (Table S2). The vector *CgMfs1*-pSL2 contains *CgMfs1*-R and *CgMfs1*-L that can form the hairpin structure to disrupt the express of the target gene.

The *CgMfs1* scramble control plasmid pSilent-SCL1 was constructed using an approach similar to that of *CgMfs1*-pSL2. Two fragments *CgMfs1*-SCR and *CgMfs1*-SCL were obtained by PCR from the pUC57 vector using as primers oligonucleotides SCRF and SCRR, SCLF and SCLR (Table S3). The enzyme sites were BglII and SphI for *CgMfs1*-SCR and HindIII and XhoI for *CgMfs1*-SCL on the pSilent1 plasmid to produce pSilent-SCL1. The detailed information about the selectable area and its scramble sequence of the *CgMfs1* were listed in the Table S4.

For regulator overexpression, vector pBARGPE-*CgMfs1*-OE6 was constructed to increase the

expression level of *CgMfs1* in *C. globosum* W7 following next guide. The *CgMfs1* CDS was amplified by PCR from *C. globosum* W7 using primers listed in Table S3. The 1716 bp product was digested with EcoRV and XhoI. The digested *CgMFS1* fragment was ligated into the pBARGPE1-EGFP, which was prepared by digesting with the same set of restriction enzymes to generate pBARGPE-*CgMfs1*-OE6.

Transformation of *C. globosum* W7 protoplasts

After constructing the plasmids *CgMfs1*-pSL2, pSilent-SCL1 and pBARGPE-*CgMfs1*-OE6, PEG4000 mediated plastid transformation was performed. The obtainment and transformation of protoplasts were carried out according to the steps described by Anthony et al. (1994) and Liu et al. (2019). Transformational strains were selected by their resistance to hygromycin (200 μg/ml). Following purification, all genetic modification products were maintained on PDA medium for spore collection and preserved as glycerol suspensions (50%, v/v) at −80°C.

Scanning electron microscopy (SEM) observation

Morphology properties of the *C. globosum* W7 and its mutants were observed by light microscopy (Nikon ECLIPSE E200) and electron microscopy (JSM-6700F) using cultures grown on PDA medium at 28°C for 2 weeks. Samples for scanning electron microscopy were prepared as follows. All the test specimens were prepared by cutting a square from an agar plate and fixing in 2.5% glutaraldehyde at 4°C for approximately 1.5 h. Subsequently, samples were washed twice with the phosphate buffer and further treated with a graded series of ethanol (the processing gradient of ethanol were 50%, 70%, 90%, 100%). Passing all samples through tert-butanol and critical-point drying by the lyophilizer. The dried samples were placed onto a stub bearing adhesive, sputter coated with gold layer and viewed in scanning electron microscope operating. The morphology of cornstalk samples that digestion by *C. globosum* W7 and its mutants were captured by the similarity method mentioned above.

Extraction of RNA and quantitative real-time PCR

Transcription levels of *CgMfs1* and biosynthetic gene clusters relative genes were compared between *C. globosum* W7 and its derivatives by quantitative real-time PCR (qRT-PCR) analysis. For this purpose, total RNA from wild-type isolate and different transformants were isolated from mycelia grown at various time points (6, 9, 12 and 15 days) in PDA medium following a protocol previously described by Long et al. (2012). UV spectroscopy and agarose gel electrophoresis were used to examine the RNA quality and quantity. The cDNA was synthesized using the EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China). RT-qPCR was performed using the Go Taq qPCR Master Mix (Promega) in a Real-time PCR System (Thermo Fisher Scientific). The sequences of the gene-specific primers were designed using Primer 5.0 and listed in Table S3. The β-actin gene was used as an internal control, which of the accession number is CH408033.1 from the genomics of *C. globosum* W7. The relative gene expression levels ($2^{-\Delta\Delta CT}$) were measured automatically (Livak & Schmittgen, 2001). All assays were conducted in triplicates and each experiment was also repeated three times.

Cellulose, hemicellulose and lignin content analysis

The content of cellulose, hemicellulose and lignin in cornstalk samples, that were decomposed by *C. globosum* W7 and its mutants, were determined by the method described by Van Soest et al. (1963, 1991). The collected residual cornstalk were centrifuged at 5000 rpm for 10 min, the supernatant were discarded, and then dried at 60°C overnight. After this, the dried samples were pulverized and weighed. Samples (1.0 g) mixed with moderate amount of neutral detergent were placed into the heating jacket and boiled for 1 h. Filtered insoluble matter by using the bachner funnel and washed with acetone. Harvested the residues, dried at 105°C overnight and weighed. The quality of the dry matter lost was calculated. Subsamples from the residue were mixed with acid detergent and then treated by using the similar method. After heating, collected the mixture and filtered, washed with hot water until free of detergent and subsequently flushed with acetone until the solvent did not appear to remove any more coloured material. Harvested the residues, dried and weighed. Finally, samples were hydrolysed with H_2SO_4 (72%) and ashed in the muffle furnace for 2.5 h. After this, the dried residues were measured and analysed.

Fourier transform-infrared spectroscopy (FT-IR) analysis

Fourier transform-infrared spectroscopy (SPECTRUM-ONE) was used to detect the changes of cornstalk samples specimens before and after digestion by different transformants. Briefly, samples for FT-IR were prepared by freeze-dried, grinded with KBr pellets and

analysed by PerkinElmer 100 operating at a resolution of 4 cm^{-1} over 4000–450 cm^{-1}.

Mycelial biomass and chaetoglobosin A determination

Approximately 1×10^7 spores of the detective species were cultivated into Fernbach flasks, each flask containing 50 ml PDA broth, fermentation was carried out at 28°C for 9 days. When needed, the culture broth was taken at an interval time of 3 days to track changes in the yield of *C. globosum* W7 and its mutants. After centrifuging at 12,000 rpm for 10 min, the fermentation broths were extracted with equal volumes of ethyl acetate (EtOAc). Mycelial pellets were subjected to crush with an ultrasonic disruptor and extracted with an equal volume of EtOAc. Absorbing the supernatant with the pipettor and dehydrating with anhydrous sodium sulphate. After that, the extractive was filtered and enriched in a rotary evaporator at 35°C. The crude extract was dissolved in 1.5 ml of MeOH and filtered using 0.22 mm polyethylene filter before injection. The concentration of chaetoglobosin A was quantitated by Waters 2695–2489 HPLC with a TC-C18 column (Agilent, 4.6 mm × 250 mm, 5 μm) on an isocratic elution system of 45% CH_3CN (v/v) in H_2O at a flow rate of 1.0 ml/min. The chaetoglobosin A (C32H36N2O5) standard used as control was purchased from Sigma-Aldrich (Germany). For detection the yield of the chaetoglobosin A produced by fermentation cornstalk, the same concentration of spores was incubated into fermentation medium at 28°C for various days. And then centrifuged at 12,000 rpm for 15 min to separate culture broth from the mycelia and residues. The pretreatment and examination method of samples are as described above.

For determination of mycelial biomass, 50-millilitre cell cultures that grown in the PDA liquid medium were collected by vacuum filtration and dried at 60°C to a constant weight. Using cornstalk as substrate to produce chaetoglobosin A, 100 ml fermentation broth was thoroughly washed three times with distilled water through a 40-mesh sifter to make sure the mycelia were free from cornstalk residues. Collected the mycelium, which was larger than 40-mesh aperture and dried at 60°C to constant weight for mycelial biomass determination.

RESULTS

Identification of chaetoglobosin a transporter gene *CgMfs1* in *C. globosum* W7

BlastP algorithm analysis from the online platform NCBI (National Center for Biotechnology Information) suggested that CgMfs1 (GenBank: XP_001220482.1), which was adjacent to the chaetoglobosin A biosynthetic gene cluster, was a member of the MFS efflux pump. Sequence research revealed that the *CgMfs1* gene has a 1716-bp open reading frame that encodes a polypeptide of 571 amino acids with an estimated molecular weight of 61.40 kDa and a theoretical isoelectric point of 5.77. The results of conservative region analysis by using NCBI database revealed that CgMfs1 contains a highly conserved MFS multi-domain between amino acids 100 and 552. TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) program showed that CgMfs1 comprises 12 putative transmembrane spanning domains (TMS) uniformly distributed at positions 98–273 and 338–545 (Figure S1A). The results of hydropathy profile predicted by SACS HMMTOP Transmembrane Prediction Page (http://www.sacs.ucsf.edu/cgi-bin/hmmtop.py) are presented in Figure S1B. These findings indicated that the transmembrane structure of CgMfs1 *w*as similar to the typical configuration of H^+ antiporter (DHA) 12 involved in toxin export. Besides, CgMfs1 could have also evolved from a single 2-TMS hairpin structure that triplicated to produce a 6-TMS unit and then replicated to form a 6TMS/6TMS arrangement, which is the most frequent topological type of the MFS secondary transporters (Paulsen et al., 1996; Reddy et al., 2012). Moreover, three-dimensional modelling of CgMfs1 predicted by using SwissProt (http://swissmodel.expasy.org/) proposed that the multi-MFS protein systematically arranged around a central channel may be associated with substrate binding and translocation (Figure S1C) and that it contains four highly conserved motifs (motifs B, C, D2 and G) in the sequence (Figure S1D) that belong to MFS signature structures (Paulsen et al., 1996). The results of in silico analyses of the hypothetic transporter CgMfs1 unconditionally support that the hydrophobic protein with 12 putative TMS could act as a transporter of the MFS superfamily.

Silencing strategy leads to decrease in chaetoglobosin A production

The DHA12/DHA14 subfamily proteins from bacteria and fungi have been reported to be involved in secondary metabolite transport and multidrug resistance (Fernandez-Aguado et al., 2014; Liu et al., 2012; Roohparvar et al., 2007). To investigate the functions of CgMfs1 transporter in *C. globosum*, the expression of the target gene was silenced, and the resulting effects were analysed. The vector pSilent1 was employed to interrupt the objective gene in the wild-type *C. globosum*, and the flow diagram is shown in Figure 1. *CgMfs1-R* (Figure 1A) and *CgMfs1-L* (Figure 1B) were connected to carrier pSilent1 through the enzyme sites HindIII/XhoI and BglII/SphI. Each step of the construction was chemically transformed into *Escherichia coli* DH5α

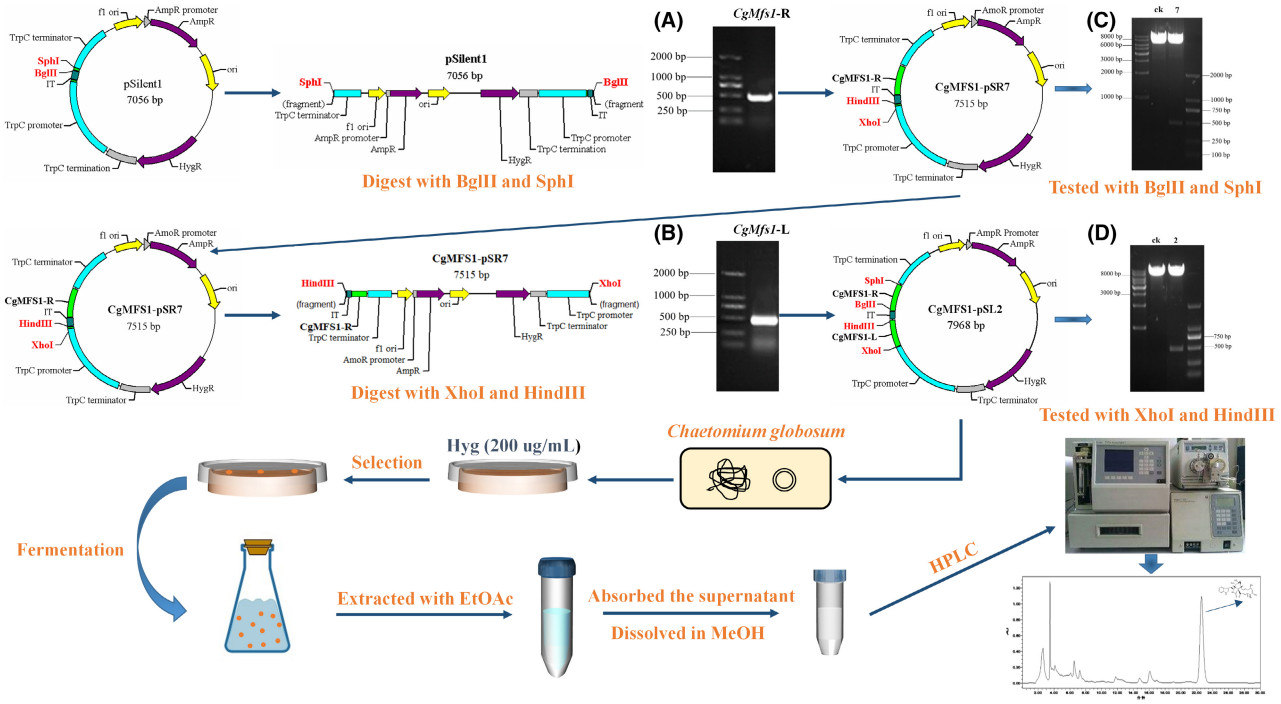

FIGURE 1 Schematic for silencing strategy of the *CgMfs1* gene in *C. globosum* and chaetoglobosin A yield detection by HPLC. (A) the amplified result of *CgMfs1*-R by using as primers oligonucleotides SRF and SRR; (B) the amplified result of reverse fragment *CgMfs1*-L by using as primers oligonucleotides SLF and SLR; (C) CK: Empty carrier pSilent1 digested with BglII and SphI used as the negative control. 7: *CgMfs1*-pSR7, contains pSilent1 plasmid (7056 bp) and interference sequence (461 bp) after BglII and SphI digestion. (D) CK: Empty carrier pSilent1 digested with HindIII and XhoI used as the negative control. 2: *CgMfs1*-pSL2, contains pSilent1 plasmid (7056 bp) and interference sequence (461 bp) after HindIII and XhoI digestion.

competent cells, and the transformants were digested by an appropriate enzyme(s) and verified by agarose gel electrophoresis (Figure 1C, D). Only *CgMfs1*-pSL2 isolate carried a successful integration of *CgMfs1-L* and *CgMfs1-R* within the original plasmid. The scramble control plasmid was created by utilizing a method similar to that of *CgMfs1*-pSL2. First, we tested the mutants that had an expected infusion of the target gene as evidenced by diagnostic PCR with the primers pSF and pSR (Table S3) designed according to the pSilent1 skeleton sequence. When using the primers, a 1242-bp DNA fragment was amplified from the transformants, except No. 4 isolate, whereas a 320-bp fragment was amplified from the empty pSilent1 carrier (Figure S2A). During the establishment process, restriction assays were used to verify if the fragment had accurately fused with the vector, and the results are presented in Figure S2B, C. All the three transformants could be cut and formed two bright bands, demonstrating that the empty vector had accurately connected to the sequence. The plasmids *CgMfs1*-pSL2 and pSilent-SCL1, which were used for further investigation, were sequenced after double-enzyme digestion and were found to be 100% identical to the template.

CgMfs1-pSL2 was transformed by utilizing polyethylene glycol (PEG) mediated protoplasts transformation. A total of eight independent hygromycin-resistant colonies were separated, and SC1–1 (derived from pSilent-SCL1 transformation) and wild-type *C. globosum* W7 were used as control. First, the integrated hairpin structure was identified by diagnostic PCR, and a 1242-bp silencing cassette was amplified with the flank primers (pSF and pSR) of the seven mutants and reference strain SC1–1, whereas no fragment of the wild-type strain was amplified (Figure S3), confirming that the mutants has a complete hairpin structure that can be used to achieve gene silencing. Furthermore, to test the influence of *CgMfs1* interference on chaetoglobosin A production, the seven mutants and the parental strain were cultivated in potato dextrose agar (PDA) medium for 9 days at 28°C. The fermentation broth was treated as described in the experimental procedures and the samples were analysed by high-performance liquid chromatography (HPLC). Two of the seven pure separated mutants, namely, MFS1–3 and MFS1–4, presented a significant decrease in the yield of chaetoglobosin A, with the reduction percentages reaching 73.54% and 80.40%, respectively (Figure S4A). Subsequently, morphological characteristics of the optimal mutants were further examined by cultivating the mutants in PDA medium for 14 days at 28°C and observing them under optical microscope and SEM. The aerial hyphae and substrate mycelium of all the examined strains were well developed and distinctly verrucose with

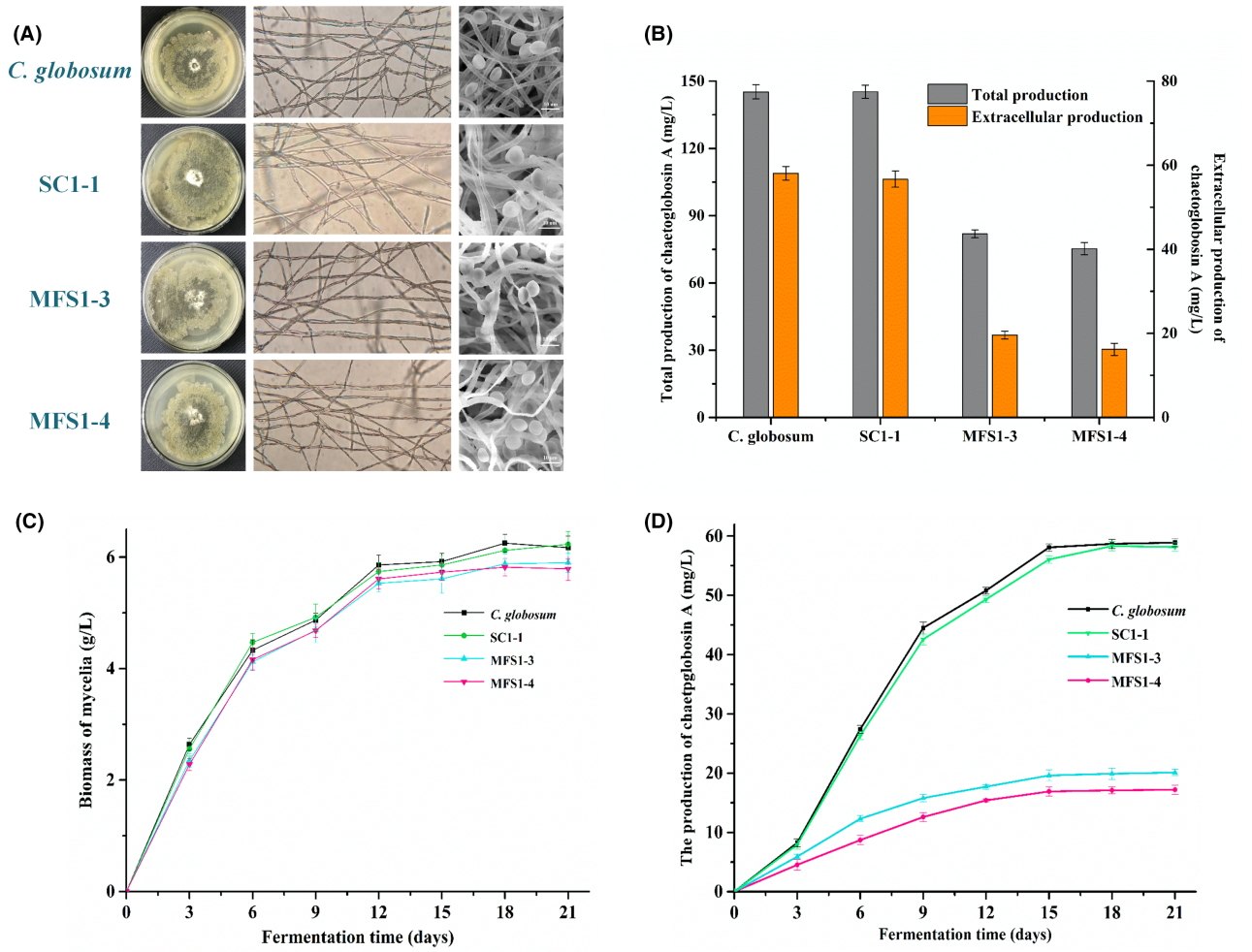

FIGURE 2 Effects of *CgMfs1* disruption on phenotypic development, cell growth and chaetoglobosin A production. (A) Morphological profile of detected isolates on PDA medium and photographs of cultures observed by optical microscope and scanning electron microscopy (SEM). Bar, 10 μm. (B) the extracellular and total production of chaetoglobosin a in different *CgMfs1*-silencing transformants after 21 days cultivation in the PDA liquid medium. (C) Growth curves of *C. globosum*, SC1–1, MFS1–3 and MFS1–4 cultured in fermentation medium. Biomass is expressed as dry cell weight. (D) Quantitative chaetoglobosin a production of detective isolates cultured in fermentation medium. Error bars show standard deviations.

2.6–3.3 μm diameter. Olivaceous-brown, irregularly arranged, non-motile, ovoid and one-celled spores (8.8–9.5 × 7.0–7.6 μm) with a smooth surface on aerial mycelium were noted (Figure 2A), which revealed that the mutant strains exhibited representative traits of the genus *Chaetomium* (Lodha, 1964), with no differences between the reference strain and *CgMfs1*-silenced mutants. These results suggested that the *CgMfs1* gene is not involved in the regulation of morphological development. In addition, to clarify the changes in the chaetoglobosin A biosynthesis in *CgMfs1*-inactive mutants, the key enzymes genes such as polyketide synthase (*CgPKS*, Protein Accession No. CAO91861), enoyl reductase (*CgER*, Protein Accession No. CAO91860), cytochrome P450 oxygenase (*CgP450*, Protein Accession No. CAO91862) and FAD-dependent monooxygenase (*CgFMO*, Protein Accession No. CAO91863) involved in chaetoglobosin A biosynthetic pathway (Figure S5) were examined by quantitative real-time PCR (qRT-PCR). During the fermentation process, samples were collected every 3 days, and the relative expression levels of each transcript were calculated using $2^{-\Delta\Delta CT}$ formula, considering the expression level of each gene of the parental strain *C. globosum* W7 (incubated in PDA medium for 6 days) as 1.0 (Figure 3). As expected, transcription of *CgMfs1* was markedly decreased in the mutants at all the tested time points, whereas that in SC1–1 and wild-type strain was similar. Moreover, *CgPKS*, which is responsible for the carbon skeleton synthesis of chaetoglobosin A (Ishiuchi et al., 2013), was also found to be significantly reduced in the mutants. At 12 days of fermentation, the transcription of *CgMfs1*, *CgPKS*, *CgP450*, *CgFMO and CgER* in MFS1–3 was reduced to 0.36, 0.65, 0.90, 0.88 and 0.79, and to 0.29, 0.52, 0.86, 0.76 and 0.75 in MFS1–4, respectively, when compared with that observed in the wild-type

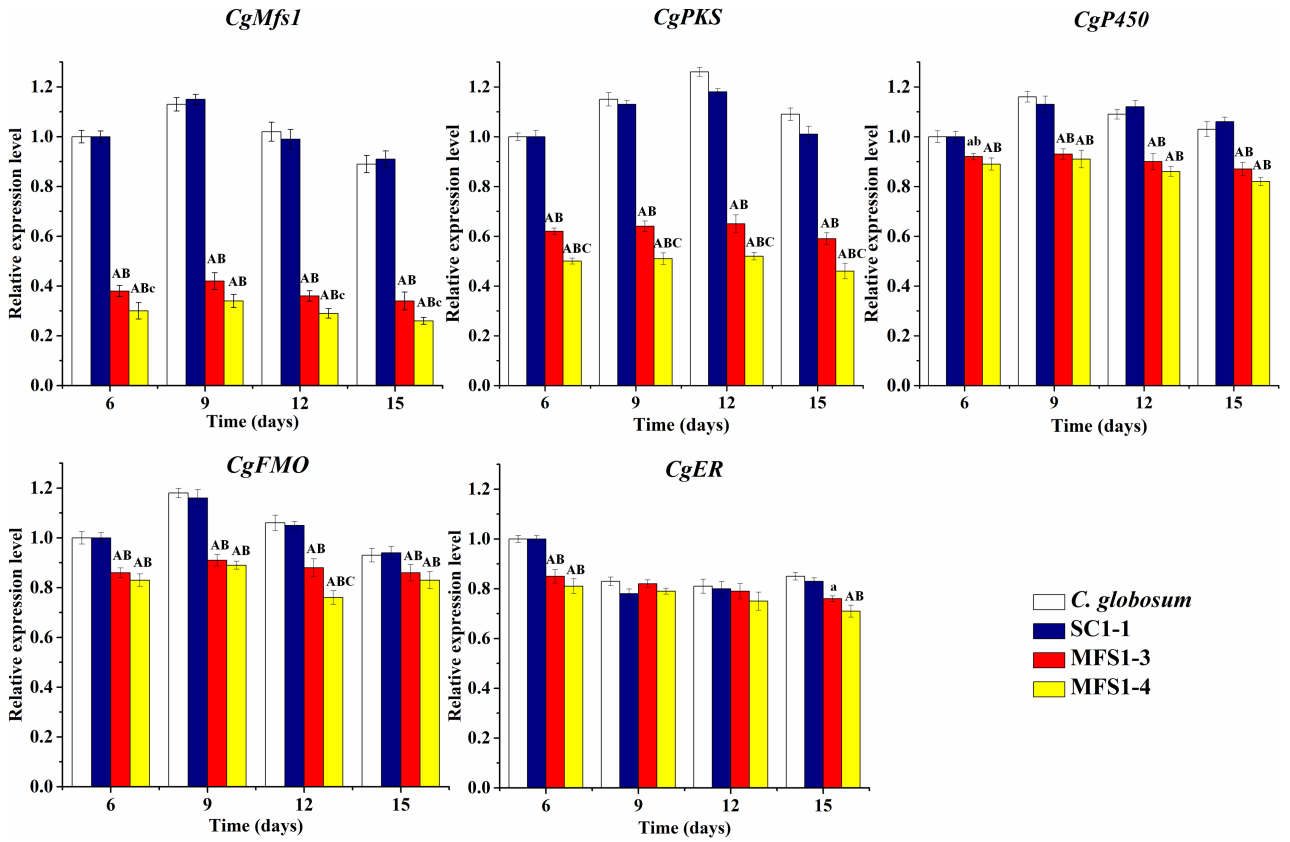

FIGURE 3 Relative expression levels of *CgMfs1*, *CgPKS*, *CgP450*, *CgFMO* and *CgER* in *C. globosum* W7 (wild type), SC1–1 (scramble control), MFS1–3 and MFS1–4 (*CgMfs1*-silent mutants) grown in PDA medium. All RNA samples were isolated from 6, 9, 12 and 15 days cultures. The primers employed for qRT-PCR are listed in Table S3 and β-Actin gene from the *C. globosum* W7 genomics was used as an internal control. The transcriptional levels of biosynthetic related genes are presented relative to that of wild-type species collected after fermentation for 6 days, which was arbitrarily assigned a value of 1.0. The error bars represent standard deviations from three independent experiments. *p*-values were determined by Student's *t*-test. Different lowercase letters indicate values that are significantly different ($p < 0.05$), different majuscule letters indicate values that are significantly different ($p < 0.01$).

strain. These findings implied that efficient silencing of *CgMfs1* in the engineered strains possibly influenced the transcription of chaetoglobosin A biosynthesis related genes.

Subsequently, the yield of chaetoglobosin A and biomass of mycelia extracted from the fungal strains were evaluated at the same time points. HPLC quantification revealed a significant decline in the chaetoglobosin A yield of two *CgMfs1* silent mutants throughout the time course, when compared with the parental and control strains (Figure 2D), and all the tested strains reached equilibrium after 15 days of fermentation. At equilibrium, the chaetoglobosin A yield of MFS1–3 and MFS1–4 declined from 58.66 to 19.95 and 17.13 mg/L (calculated by using a chaetoglobosin A standard curve), respectively. In addition, as shown in Figure 2B, the extracellular and total production of target compound in the *CgMfs1*-inactivated species was evidently lower than that in the reference strains. Nevertheless, the results of biomass showed that the two mutants presented similar biomass with no apparent decrease, when compared with those of the reference strains (Figure 2C). Combined with the findings of qRT-PCR, these data indicated that disruption of *CgMfs1* reduced chaetoglobosin A production, presumably by decreasing chaetoglobosin A export and thus increasing feedback inhibition, which might possibly affect the expression levels of key genes involved in chaetoglobosin A biosynthesis.

CgMfs1-overexpressing mutants showed enhanced chaetoglobosin A production

To confirm the assumption that *CgMfs1* is involved in chaetoglobosin A transport, the expression of *CgMfs1* was enhanced by using a strong promoter *gpd*A in *C. globosum* W7. Figure S6 shows the design and construction of the integrative plasmid pBARGPE1-*CgMfs1*-OE6 that was used to improve *CgMfs1* expression. The *CgMfs1*-overexpressing carrier pBARGPE1-*CgMfs1*-OE6 and empty vector pBARGPE1-EGFP were introduced into the protoplasts of *C. globosum* W7, and various transformants were selected based on their ability to grow in hygromycin-supplemented medium. Diagnostic PCR assay of all the

isolates, using the primers OEXEG-F and OEXEG-R to confirm accurate integration of *CgMfs1* overexpression cassette, detected a 1851-bp sequence in 10 independent mutants (Figure S6D). Subsequently, these 10 candidates were incubated in PDA medium for 9 days at 28°C and the fermentation broth was preprocessed and investigated for chaetoglobosin A production. As expected, chaetoglobosin A production of most of the isolates was increased at varying degrees; in particular, OEX1, OEX13 and OEX19 showed 226.06%, 444.97% and 228.38% increases in chaetoglobosin A production, respectively (Figure S4B). Subsequently, the phenotypic properties of the optimal *CgMfs1*-overexpressing strains were further examined by incubating the strains in PDA medium for 14 days at 28°C and observing the colonies under a light microscope, standard electron microscope and fluorescence microscope. As shown in Figure 4A, the colony diameter, aerial hyphae diameter, shape and size of the spores of the *CgMfs1*-overexpressing strains remained unchanged, when compared with those of the parental strain and the control isolate pB-CK1. These results suggested that unlike the structural gene of the biosynthetic cluster and global regulator, the transporter gene *CgMfs1* has no significant effect on cellular development (Cheng et al., 2021a, 2021b; Hu et al., 2012). In addition, green fluorescence signals (Figure 4A) corresponding to EGFP-*CgMfs1* were coincident as inferred, confirming that the fluorescent protein gene had been integrated into the genome of the target cells and correctly expressed. Moreover, to further examine the impact of *CgMfs1* overexpression on the transcription of its targets, the expression of four genes involved in chaetoglobosin A biosynthetic cluster, namely, *CgPKS*,

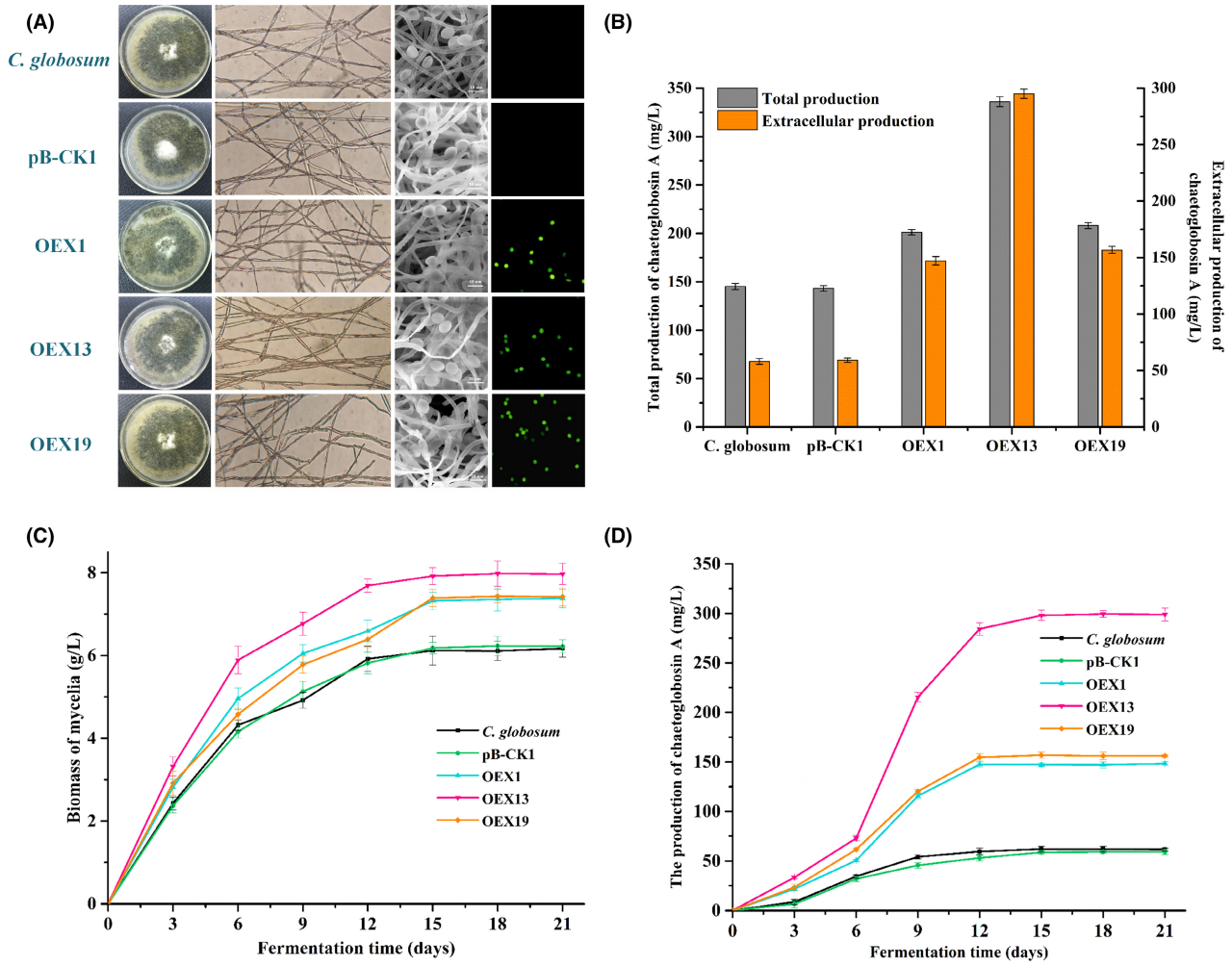

FIGURE 4 Phenotypic characteristics, cell growth and chaetoglobosin A production of the *CgMfs1* overexpress mutants OEX1, OEX13 and OEX19, in addition, *C. globosum* W7 and pB-CK1 (containing empty vector) were employed as the control. (A) Morphological profile of detected species on PDA medium and photographs of cultures observed by optical microscope, fluorescence microscope and scanning electron microscopy. Bar, 10 μm. (B) the extracellular and total production of chaetoglobosin a in different *CgMfs1*-overexpression derivatives after 21 days incubation. (C) Influence of the *CgMfs1* mutants in *C. globosum* W7 on cell growth. *C. globosum* W7, pB-CK1 (vector control), OEX1, OEX13 and OEX19 (containing *CgMfs1* overexpression cassette) incubated in fermentation broths and sampled interval of 3 days. (D) Yield curve of chaetoglobosin a of overexpression mutants at different culture time. Error bars show standard deviations.

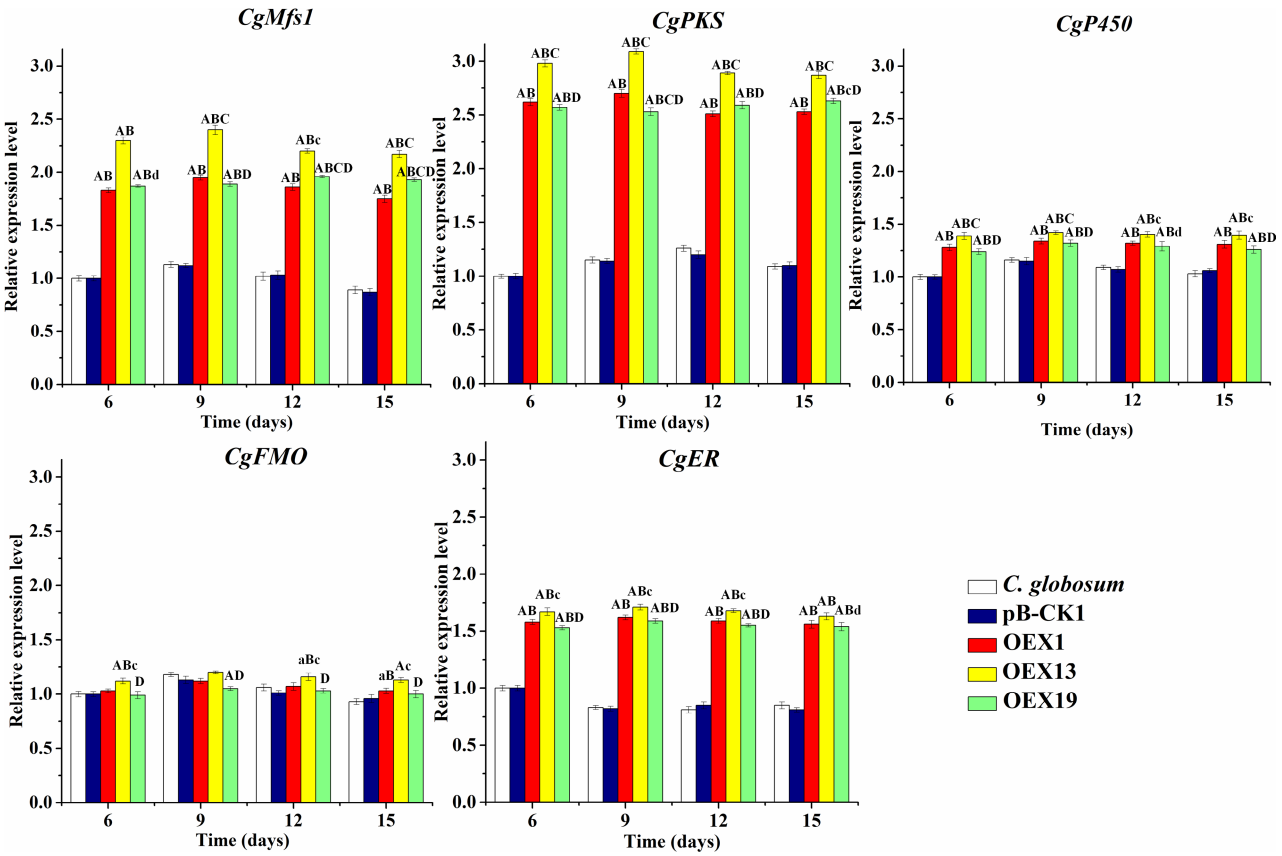

FIGURE 5 Transcriptional activity in wild-type *C. globosum* W7 in response to multiple copies of *CgMfs1*. Relative transcription levels of *CgPKS* (PKS-NRPS hybrid), *CgP450* (cytochrome P450 oxygenases), *CgFMO* (FAD-dependent oxidoreductase), *CgER* (enoyl reductase) and *CgMfs1* (MFS transporter) in OEX1, OEX13 and OEX19 (containing *CgMfs1* overexpression cassette) were compared to the vector control (pB-CK1) and parent cell. Samples were collected from PDA medium with a various fermentation time (6, 9, 12 and 15 days). The β-Actin gene was used as an internal control. Each sample was tested in triplicate by quantitative RT-PCR, using gene-specific primers (Table S3). The error bars represent standard deviations from three independent experiments. *P*-values were determined by Student's *t*-test. Different lowercase letters indicate values that are significantly different ($p < 0.05$), different majuscule letters indicate values that are significantly different ($p < 0.01$).

CgER, *CgP450* and *CgFMO*, was examined at different time points. For this purpose, the RNA samples were extracted from the mycelia of the candidate strains cultivated for various days (6, 9, 12 and 15 days), and the relative expression levels were tested by qRT-PCR. The results showed that transcription of *CgMfs1* was obviously improved in the mutants at all the tested time points, whereas that in the vector control was similar to the *CgMfs1* transcription in the parent strain (Figure 5). Interestingly, the transcription of *CgPKS* was up to 2.51-, 2.89- and 2.59-fold higher in the mutants at 12 days, when compared with that in the wild-type and vector control strains, respectively. As the *CgPKS* gene is most likely to catalyse the first crucial step of chaetoglobosin A production (Ishiuchi et al., 2013), this transcription pattern of *CgPKS* might possibly be another reason for the higher levels of chaetoglobosin A in the mutants, when compared with those in the reference strains. Meanwhile, the transcription levels of *CgER* and *CgP450* were also observed to be higher in the *CgMfs1*-overexpressing mutants, when compared with those in the control strains, which could be owing to the fact that *CgER* and *CgP450* are necessary for extending the carbon framework and modifying the precursors of chaetoglobosin A (Ishiuchi et al., 2013).

As can be seen from Figure 4C, the mycelial biomass rapidly increased from 3 to 12 days and reached an equilibrium from 15 to 21 days, and the maximum mycelial biomass of OEX1, OEX13 and OEX19 increased from 6.13 g/L to 7.35, 7.41 and 7.96 g/L, respectively. The chaetoglobosin A accumulation curve of the control strains and OEX13 stabilized in 15 days of fermentation, whereas that of the two mutants, OEX1 and OEX19, reached an equilibrium in 12 days. HPLC analysis revealed that OEX13 produced the highest titre of chaetoglobosin A, which was increased from 61.96 to 298.77 mg/L, whereas OEX1 and OEX19 generated 147.56 and 155.92 mg/L chaetoglobosin A, respectively (Figure 4D). Meanwhile, effective enhancement of *CgMfs1* gene expression could significantly increase the excretion and production of chaetoglobosin A (Figure 4B). These results clearly demonstrated that

the hypothetical gene *CgMfs1* is not only advantageous to chaetoglobosin A secretion, but also affects the expression of critical genes in the chaetoglobosin A biosynthetic cluster, and that the efflux pump located downstream of the gene cluster could have a crucial role in chaetoglobosin A production.

Morphology of cornstalk after digestion with C. globosum W7 and its mutants

Estimation of the economic value of biomass supply from crop residues in China revealed an annual production of about 174.4–248.6 million metric tons of agricultural residues, with corn stalk accounting for 28% of the agricultural residues (Chen, 2016). Thus, rational utilization of straw resources is an important task, which not only involves sustainable development of agriculture, but is also related to environmental safety. The biocontrol species, *C. globosum* W7, can decompose cornstalk by producing numerous hydrolytic enzymes such as endoglucanase, exoglucanase, cellobiose dehydrogenase and xylanase (Jiang et al., 2017). Therefore, exploitation of *C. globosum* W7 to generate bioactive secondary metabolites such as chaetoglobosin A from cornstalk could be a promising strategy for recycling agricultural residues. In the present study, we employed optimal *CgMfs1*-overexpressing mutant OEX13 to produce chaetoglobosin A using cornstalk as the substrate, with wild-type *C. globosum* W7 as control. As shown in Figure S7, the untreated straw samples were intact and well-structured with a smooth surface (Figure S7A), whereas those digested with *C. globosum* W7 (Figure S7B) and OEX13 (Figure S7C) showed irregular superficies and lamellar morphology with numerous apertures. These results suggested that the saprophytic fungus *C. globosum* and its *CgMfs1*-overexpressing mutant can effectively destroy the recalcitrant structure of straw. The structural characteristics of cornstalk observed in this study are in well accordance with those of specimens pretreated with alkaline solution of ionic liquids (Liu et al., 2018).

Structural analysis of cornstalk by FT-IR

The FT-IR spectra of cornstalk samples (Figure 6) revealed that the peak located at 3400 cm^{-1} represented O-H vibrations and is caused by the stretching of O-H in cellulose. The strong absorption peaks appeared at 2946 cm^{-1}, which corresponded to C-H stretching vibration in lignin. When compared with the peak of untreated cornstalk sample, that of cornstalk samples treated with *C. globosum* and OEX13 decreased at 3400 and 2946 cm^{-1}, suggesting that the two *C. globosum* strains destroyed the structure of cellulose and

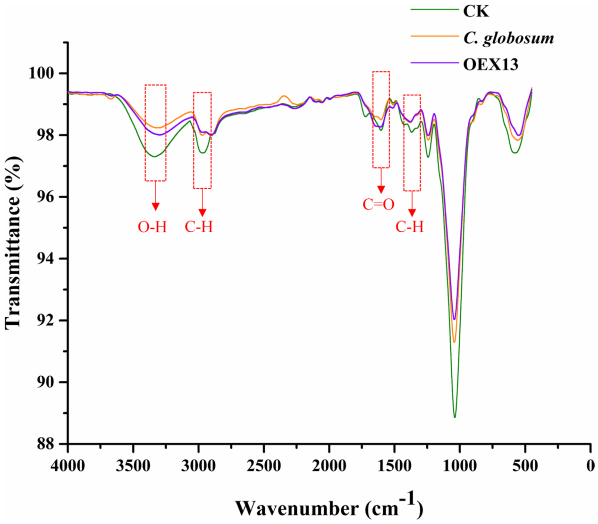

FIGURE 6 FT-IR results of different cornstalk samples.

lignin of cornstalk, at least to some extent. In addition, absorption bands at 1690 and 1425 cm^{-1} corresponding to C=O and C-H bonds, respectively, were detected in the control group, but were not observed in the other test groups. These results revealed that *C. globosum* and OEX13 can destroy the connections between lignin and aromatic rings or aliphatic compounds in cornstalk.

Mycelial growth, chaetoglobosin a production and cornstalk degradation

The biomass variation curves of *C. globosum* W7 and OEX13 using cornstalk as the single substrate were determined by adopting the dry weight approach. As can be seen from Figure 7A, the growth of hyphae linearly varied from 3 to 9 days and became steady in 12–18 days, reaching the peak in 15 days. The maximum mycelial biomass of *C. globosum* W7 and OEX13 was 5.04 and 5.39 g/L, respectively. Furthermore, a time course detection of chaetoglobosin A production revealed the generation of only a small amount of chaetoglobosin A by *C. globosum* W7 and OEX13 in the preliminary stage of fermentation, indicating that the fungal cells were still at the primary metabolic level. However, the chaetoglobosin A yield rapidly increased from 6 to 15 days and stabilized in 18 days. When compared with the parental strain, OEX13 presented a dramatic increase in chaetoglobosin A production by 475.94% (from 40.32 to 191.90 mg/L) (Figure 7B). In spite of OEX13 decreased the levels of chaetoglobosin A 35.77% (from 298.77 mg/L to 191.90 mg/L), which was lower than that cultivated in the PDA medium. Nonetheless, the chaetoglobosin A productivities were enhanced by 258.06% (from 0.30 mg/g to 0.81 mg/g) and 257.72% (from 1.49 mg/g to 3.84 mg/g) of *C. globosum* W7 and OEX13, respectively (Figure 7D), when incubated in the cornstalk fermentation medium.

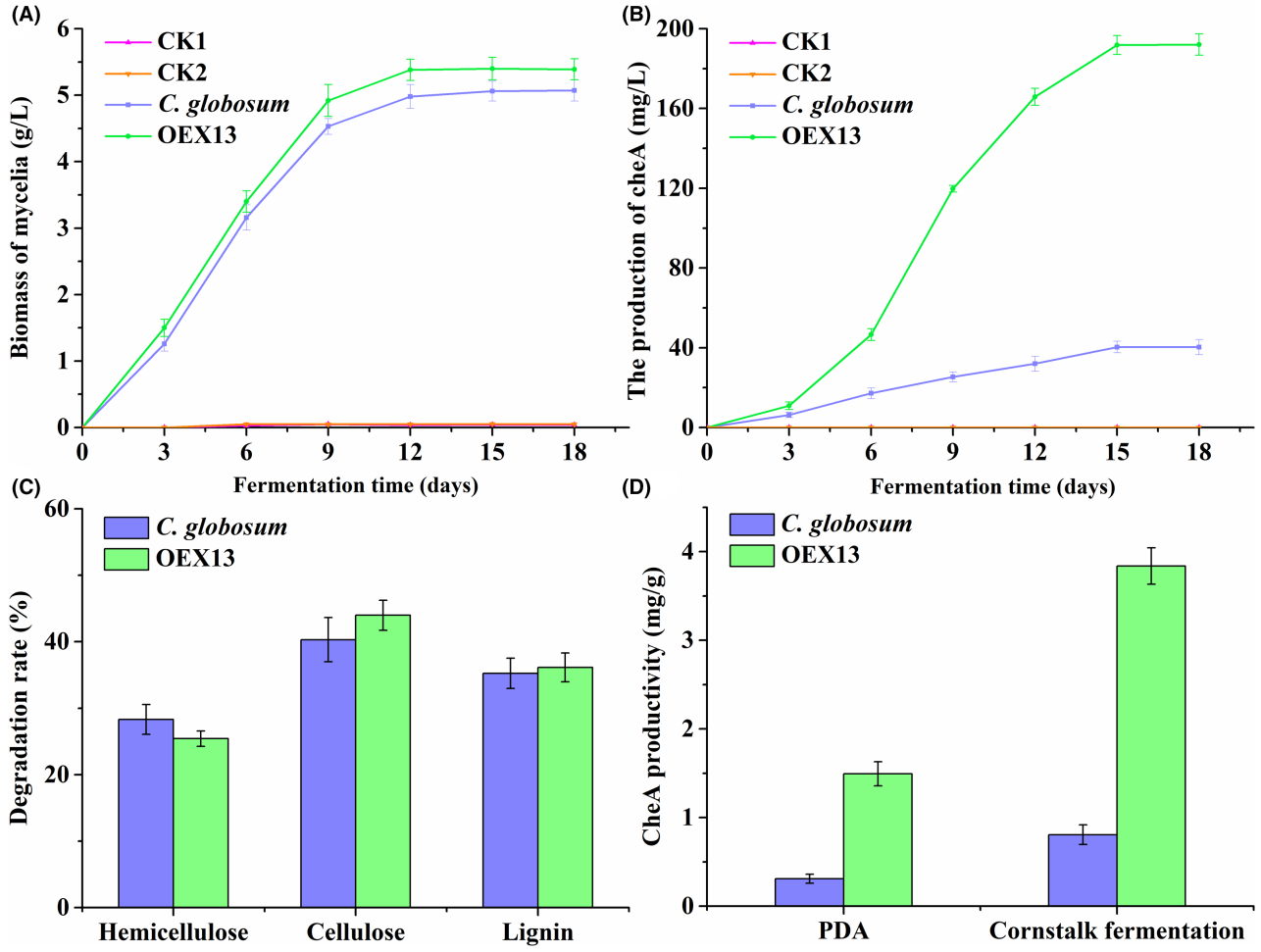

FIGURE 7 Mycelial growth, chaetoglobosin a production, cornstalk degradation and chaetoglobosin A productivity of species *C. globosum* W7 and OEX13. (A) the mycelial biomass of *C. globosum* and OEX13 under submerged fermentation and sampled interval of 3 days. CK1: Incubation of wild-type species in the fermentation medium without addition of cornstalk; CK2: Cultivation of the overexpression mutant OEX13 in the fermentation medium without addition of cornstalk. (B) Yield curve of chaetoglobosin A of two detected species at different culture time. CK1 and CK2 are the same as above mentioned. (C) Degradation rates of various cornstalk samples that digested by *C. globosum* W7 and OEX13. (D) Chaetoglobosin A productivities of species *C. globosum* W7 and OEX13 in different fermentation medium. Error bars show standard deviations.

These findings indicated that recycling of agricultural wastes biomass to produce chaetoglobosin A displayed higher productivity could result in substantial cost reduction. Figure 7C shows the degradation rates of different cornstalk samples digested by *C. globosum* W7 and OEX13. The degradation rates of hemicellulose, cellulose and lignin in cornstalk by *C. globosum* W7 were 28.31%, 40.31% and 35.26%, respectively, while those by OEX13 were 25.42%, 43.98% and 36.13%, respectively, with no significant differences between the two strains in their cornstalk degradation capacity.

Taken together, these results demonstrated that both *C. globosum* W7 and OEX13 could decompose the cornstalk structure and transform it to produce chaetoglobosin A. Moreover, the chaetoglobosin A yield and productivity generated by OEX13 was higher than that produced by the parental strain, indicating that overexpression of the efflux pump genes could effectively enhance the transport of the target products.

DISCUSSION

Cytochalasins, which were discovered by Rothweiler and Tamm in 1966, are a well-known class of alkaloids characterized by a polyketide backbone and an amino acid (such as leucine, tryptophan, tyrosine, valine, or phenylalanine). Owing to their complex polycyclic structure and broad spectrum of bioactivity, cytochalasins have attracted the attention of numerous chemists and physicists. Increasing numbers of bioactive cytochalasins and its analogues have been isolated from filamentous fungi, such as *Aspergillus flavipes* (Zhu et al., 2017), *C. globosum* (Chen et al., 2015), *Periconia* sp. (Zhang et al., 2016) and *Trichoderma gamsii* (Chen et al., 2014), and the current estimates suggest that over 300 cytochalasins have been described (Wei et al., 2017). Chaetoglobosin A, a member of the cytochalasins family with diverse antibacterial and antitumor activities, was first identified by Sekita

et al. in 1973. Owing to its numerous beneficial properties, development of an effective channel to enhance chaetoglobosin A production is particularly important. In the present study, we first identified and predicted the function of an MFS secondary transporter gene in *C. globosum* W7, which was adjacent to the chaetoglobosin A biosynthesis cluster.

The vital functions of the cytoplasmic membrane transport systems, which are ubiquitous in bacterial and eukaryotic cells, include uptake of essential nutrients and excretion of toxic compounds to maintain cellular homeostasis. By using the *C. globosum* genome, the present study analysed the function of a putative MFS efflux pump adjacent to the chaetoglobosin A cluster, which was annotated as CgMfs1. The results of transmembrane and hydropathy profile demonstrated that CgMfs1 comprised 12 TMS, similar to the typical configurations of DHA12 involved in secondary metabolites transport (Paulsen et al., 1996; Reddy et al., 2012). Target disruption of *pfmfr3*, a novel mitochondrial transporter in *Plasmodium falciparum* has been reported to decrease the sensitivity of the parasite to antimalarial compounds (Rocamora et al., 2021), and mutants overexpressing an MFS transporter *PdMfs1* in *Penicillium digitatum* have been noted to be more resistant to imazalil (Wang et al., 2012). Furthermore, interruption of the expression level of the novel MFS transporter gene *pen*M via gene-silencing strategy led to a marked decrease in benzylpenicillin production; by contrast, overexpression of the target gene by the efficiently expressed promoter increased the yield and promoted the biosynthetic of benzylpenicillin (Fernandez-Aguado et al., 2014). These results are consistent with the findings of the present study, which revealed that inactivation of the efflux pump gene *CgMfs1* directly caused a significant decline in chaetoglobosin A output from 58.66 to 19.95 mg/L (MFS1–3) and 17.13 mg/L (MFS1–4) during the equilibrium period of fermentation. On the contrary, chaetoglobosin A production by *CgMfs1*-overexpressing strains evidently improved by 4.8-fold (OEX13), when compared with that by the wild-type strain, which proved that the predicted efflux gene *CgMfs1* may play a crucial role in chaetoglobosin A transportation. Interestingly, the transcription level of the *CgPKS* gene dramatically varied in the *CgMfs1*-silenced and *CgMfs1*-overexpressing mutants; however, the diameters of the mycelia and spores were not affected by the expression of *CgMfs1*, suggesting that excessive accumulation of compounds in vivo could result in feedback inhibition. In *CgMfs1*-silenced mutants, the expression level of *CgPKS* in the biosynthesis cluster, which is responsible for the synthesis of precursor skeleton of chaetoglobosin A, was significantly reduced. In contrast, in *CgMfs1*-overexpressing mutants, the timely expulsion of the generated products outside of the cells could effectively reduce antibiotic accumulation and alleviate feedback inhibition, thus significantly increasing the expression level of *CgPKS*. Qiu et al. (2011) used a multi-copy plasmid to improve the expression of *avtAB* gene, which encoded the multidrug efflux pump AvtAB in *Streptomyces avermitilis*, and the additional copies of *avtAB* resulted in increased avermectin production both in wild-type and high-yield producer strains, which could be owing to the reduction in feedback inhibition and improvement in the ability to export avermectin by the overexpression mutants. Zhao et al. (2021) identified a novel efflux pump designated as LexABC in *Lysobacter antibioticus*, which could be sufficiently induced by myxin and potentially regulated by the LysR-type transcriptional regulator LexR. Inactivation of the *lexABC or lexR* genes resulted in a dramatic increase in the susceptibility of the mutants to myxin and loss of capacity to produce this compound, which could possibly be owing to the important role of the efflux system (LexABC) in exporting the intolerable myxin to preserve *L. antibioticus* from the attack of continually synthetized product. Rahman et al. (2017) speculated that, at the single cell level, the effectively expressed metabolite efflux transporter gene could decrease the intracellular level of antibiotic and maintain the internal concentration of antibiotic at a sufficiently low level, which may be considered as the first line of defence to protect antibiotic-producing microorganisms from autotoxicity.

In China, about 174.4–248.6 million metric tons of agricultural residues are produced annually, and cornstalk is a common and abundant resource (Chen, 2016). Nevertheless, because of the lack of effective application, cornstalk is improperly disposed or directly burned, which not only results in wastage of resources, but also causes serious environmental pollution. Therefore, rational utilization of straw resources is an essential task for alleviating energy and environmental pressure. As an important underutilized and rich biomass energy, straw resources have been widely researched. For instance, Zhao et al. (2020) accomplished liquefaction of cornstalk in a high-temperature and high-pressure reactor and obtained water-soluble products that exhibited excellent growth inhibitory effect on the soilborne plant pathogen, *Fusarium oxysporum*. Furthermore, lignocellulosic materials such as wheat straw, sugarcane bagasse and rice straw have been employed to produce antioxidative phenolic compounds (Zhu & Xu, 2013). Similarly, in the present study, we attempted to use cornstalk as the fermentation substrate for chaetoglobosin A production using *C. globosum*, considering the requirements of large-scale production and rational application of straw resources. SEM and FT-IR analyses revealed that both *C. globosum* and OEX13 could degrade the cornstalk substrate and produce chaetoglobosin A. The chaetoglobosin A production by OEX13 was significantly improved by 475.94% (from 40.32 to 191.90 mg/L), and a further enhancement in the chaetoglobosin A yield could possibly be achieved

by straw pretreatment and humidity adjustment (Wang et al., 2020).

In summary, the present work ascertained a novel unidentified MFS efflux gene, which had a crucial effect on chaetoglobosin A transportation and provided new insights for studying secondary metabolites of the genus of *Chaetomium*. In future, production of bioactive substances with high economic value from agricultural residues will be an important waste management strategy as well as a novel research direction for reducing fermentation costs and accomplishing commercial production.

AUTHOR CONTRIBUTIONS

Shanshan Zhao and Qian Yang conceived the idea of the study; Shanshan Zhao performed the main experiments. Shanshan Zhao, Congyu Lin and Ming Cheng analysed the data; Kai Zhang, Zhengran Wang and Tong Zhao participated in the fermentation experiments. Shanshan Zhao wrote the paper; all authors discussed the results and revised the manuscript.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest.

DATA AVAILABILITY STATEMENT

All study data are included in the article and supplementary materials.

ETHICAL APPROVAL

I certify that this manuscript is original and has not been published and will not be submitted elsewhere for publication while being considered by Qian Yang. And the study is not split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time. No data have been fabricated or manipulated (including images) to support our conclusions. The submission has been received explicitly from all co-authors. And authors whose names appear on the submission have contributed sufficiently to the scientific work and, therefore, share collective responsibility and accountability for the results.

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REFERENCES

Anthony, P., Davey, M.R., Power, J.B., Washington, C. & Lowe, K.C. (1994) Synergistic enhancement of protoplast growth by oxygenated perfluorocarbon and Pluronic F-68. *Plant Cell Reports*, 13, 251–255.

Chen, C.M., Zhu, H.C., Li, X.N., Yang, J., Wang, J.P., Li, G.T. et al. (2015) Armochaeglobines a and B, two new indole-based alkaloids from the arthropod-derived fungus *Chaetomium globosum*. *Organic Letters*, 17, 644–647.

Chen, D.E., Podell, S., Sauer, J.D., Swanson, M.S. & Saier, M.H. (2008) The phagosomal nutrient transporter (Pht) family. *Microbiology*, 154, 42–53.

Chen, L., Liu, Y.T., Song, B., Zhang, H.W., Ding, G., Liu, X.Z. et al. (2014) Stereochemical determination of new cytochalasans from the plant endophytic fungus *Trichoderma gamsii*. *Fitoterapia*, 96, 115–122.

Chen, X.G. (2016) Economic potential of biomass supply from crop residues in China. *Applied Energy*, 166, 141–149.

Cheng, M., Zhao, S.S., Lin, C.Y., Song, J.Z. & Yang, Q. (2021a) Requirement of LaeA for sporulation, pigmentation and secondary metabolism in *Chaetomium globosum*. *Fungal Biology-UK*, 125, 305–315.

Cheng, M., Zhao, S.S., Liu, H., Liu, Y.T., Lin, C.Y., Song, J.Z. et al. (2021b) Functional analysis of a chaetoglobosin a biosynthetic regulator in *Chaetomium globosum*. *Fungal Biology-UK*, 125, 201–210.

Chittrakanwong, J., Charoenlap, N., Mongkolsuk, S. & Vattanaviboon, P. (2021) mfsQ encoding an MFS efflux pump mediates adaptive protection of *Stenotrophomonas maltophilia* against benzalkonium chloride. *Canadian Journal of Microbiology*, 67, 491–495.

Chu, L.Y., Li, S.S., Dong, Z.X., Zhang, Y.Y., Jin, P.J., Ye, L. et al. (2021) Mining and engineering exporters for titer improvement of macrolide biopesticides in *Streptomyces*. *Microbial Biotechnology*, 15, 1120–1132.

Cox, R.J. & Simpson, T.J. (2009) Fungal type I polyketide synthases. *Methods in Enzymology*, 459, 49–78.

Cutler, H.G., Crumley, F.G., Cox, R.H., Cole, R.J., Dorner, J.W., Springer, J.P. et al. (1980) Chaetoglobosin K: a new plant growth inhibitor and toxin from *Diplodia macrospora*. *Journal of Agricultural and Food Chemistry*, 28, 139–142.

Dou, H., Song, Y.X., Liu, X.Q., Gong, W., Li, E.G., Tan, R.X. et al. (2011) Chaetoglobosin Fex from the marine-derived endophytic fungus inhibits induction of inflammatory mediators via toll-like receptor 4 signaling in macrophages. *Biological & Pharmaceutical Bulletin*, 34, 1864–1873.

Fernandez-Aguado, M., Martin, J.F., Rodriguez-Castro, R., Garcia-Estrada, C., Albillos, S.M., Teijeira, F. et al. (2014) New insights into the isopenicillin N transport in *penicillium chrysogenum*. *Metabolic Engineering*, 22, 89–103.

Hu, Y., Hao, X.R., Lou, J., Zhang, P., Pan, J. & Zhu, X.D. (2012) A PKS gene, *pks-1*, is involved in chaetoglobosin biosynthesis, pigmentation and sporulation in *Chaetomium globosum*. *Science China Life Sciences*, 55, 1100–1108.

Hu, Y., Zhang, W.P., Zhang, P., Ruan, W.B. & Zhu, X.D. (2013) Nematicidal activity of chaetoglobosin a poduced by *Chaetomium globosum* NK102 against *Meloidogyne incognita*. *Journal of Agricultural and Food Chemistry*, 61, 41–46.

Ishiuchi, K., Nakazawa, T., Yagishita, F., Mino, T., Noguchi, H., Hotta, K. et al. (2013) Combinatorial generation of complexity by redox enzymes in the Chaetoglobosin a biosynthesis. *Journal of the American Chemical Society*, 135, 7371–7377.

Jiang, C., Song, J.Z., Zhang, J.Z. & Yang, Q. (2017) Identification and characterization of the major antifungal substance against *fusarium sporotrichioides* from *Chaetomium globosum*. *World Journal of Microbiology and Biotechnology*, 33, 108.

Jin, P.J., Li, S.S., Zhang, Y.Y., Chu, L.Y., He, H.R., Dong, Z.X. et al. (2020) Mining and fine-tuning sugar uptake system for titer improvement of milbemycins in *Streptomyces bingchenggensis*. *Synthetic and Systems Biotechnology*, 5, 214–221.

Lee, J., Sands, Z.A. & Biggin, P.C. (2016) A numbering system for MFS transporter proteins. *Frontiers in Molecular Biosciences*, 3, 1–13.

Lee, S., Ricachenevsky, F.K. & Punshon, T. (2021) Functional overlap of two major facilitator superfamily transporter, ZIF1, and ZIFL1 in zinc and iron homeostasis. *Biochemical and Biophysical Research Communications*, 560, 7–13.

Liu, H., Cheng, M., Zhao, S.S., Lin, C.Y., Song, J.Z. & Yang, Q. (2019) ATP-binding cassette transporter regulates N, N'-diacetylchitobiose transportation and chitinase production in *Trichoderma asperellum* T4. *International Journal of Molecular Sciences*, 20, 2412.

Liu, Z., Li, L.F., Liu, C. & Xu, A.R. (2018) Pretreatment of corn straw using the alkaline solution of ionic liquids. *Bioresource Technology*, 260, 417–420.

Liu, M., Liu, J. & Wang, W.M. (2012) Isolation and functional analysis of Thmfs1, the first major facilitator superfamily transporter from the biocontrol fungus *Trichoderma harzianum*. *Biotechnology Letters*, 34, 1857–1862.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C [T]) method. *Methods*, 25, 402–408.

Lodha, B.C. (1964) Studies on coprophilous fungi. Ii. *Chaetomium*. *Antonie Van Leeuwenhoek International Journal of General and Molecular*, 30, 163–167.

Long, L.K., Yang, J., An, Y. & Liu, G. (2012) Disruption of a glutathione reductase encoding gene in *acremonium chrysogenum* leads to reduction of its growth, cephalosporin production and antioxidative ability which is recovered by exogenous methionine. *Fungal Genetics and Biology*, 49, 114–122.

Ma, C., Mu, Q.X., Xue, Y.B., Xue, Y.F., Yu, B. & Ma, Y.H. (2020) One major facilitator superfamily transporter is responsible for propionic acid tolerance in *pseudomonas putida* KT2440. *Microbial Biotechnology*, 14, 386–391.

Monod, M., Feuermann, M., Salamin, K., Fratti, M., Makino, M., Alshahni, M.M. et al. (2019) *Trichophyton rubrum* azole resistance mediated by a new ABC transporter, TruMDR3. *Antimicrobial Agents and Chemotherapy*, 63, 00863–00819.

Qiu, J.F., Zhuo, Y., Zhu, D.Q., Zhou, X.F., Zhang, L.X., Bai, L.Q. et al. (2011) Overexpression of the ABC transporter AvtAB increases avermectin production in *Streptomyces avermitilis*. *Applied Microbiology and Biotechnology*, 92, 337–345.

Paulsen, I.T., Brown, M.H. & Skurray, R.A. (1996) Proton-dependent multidrug efflux systems. *Microbiological Reviews*, 60, 575–608.

Rahman, T., Yarnall, B. & Doyle, D.A. (2017) Efflux drug transporters at the forefront of antimicrobial resistance. *European Biophysics Journal*, 46, 647–653.

Reddy, V.S., Shlykov, M.A., Castillo, R., Sun, E.I. & Saier, M.H. (2012) The major facilitator superfamily (MFS) revisited. *The FEBS Journal*, 279, 2022–2035.

Rocamora, F., Gupta, P., Istvan, E.S., Luth, M.R., Carpenter, E.F., Kümpornsin, K. et al. (2021) PfMFR3: A multidrug-resistant modulator in *Plasmodium falciparum*. *ACS Infectious Diseases*, 7, 811–825.

Roohparvar, R., De Waard, M.A., Kema, G.H.J. & Zwiers, L.H. (2007) MgMfs1, a major facilitator superfamily transporter from the fungal wheat pathogen *Mycosphaerella graminicola*, is a strong protectant against natural toxic compounds and fungicides. *Fungal Genetics and Biology*, 44, 378–388.

Rothweiler, W. & Tamm, C. (1966) Isolation and structure of phomin. *Experientia*, 22, 750–752.

Samaras, A., Ntasiou, P., Myresiotis, C. & Karaoglanidis, G. (2020) Multidrug resistance of *penicillium expansum* to fungicides: whole transcriptome analysis of MDR strains reveals overexpression of efflux transporter genes. *International Journal of Food Microbiology*, 335, 108896.

Scherlach, K., Boettger, D., Remme, N. & Hertweck, C. (2010) The chemistry and biology of cytochalasans. *Natural Product Reports*, 27, 869–886.

Schümann, J. & Hertweck, C. (2007) Molecular basis of cytochalasan biosynthesis in fungi: gene cluster analysis and evidence for the involvement of a PKS-NRPS hybrid synthase by RNA silencing. *Journal of the American Chemical Society*, 129, 9564–9565.

Sekita, S., Yoshihira, K. & Natori, S. (1973) Structures of chaetoglobosin a and B, cytotoxic metabolites of *Chaetomium globosum*. *Tetrahedron Letters*, 23, 2109–2112.

Skellam, E. (2017) The biosynthesis of cytochalasans. *Natural Product Reports*, 34, 1252–1263.

Steiger, M.G., Rassinger, A., Mattanovich, D. & Sauer, M. (2019) Engineering of the citrate exporter protein enables high citric acid production in *Aspergillus Niger*. *Metabolic Engineering*, 52, 224–231.

Van Soest, P.J. (1963) Use of detergents in the analysis of fibrous feeds. I. Preparation of fiber residues of low nitrogen content. *Journal of AOAC International*, 46, 825–829.

Van Soest, P.J., Robertson, J.B. & Lewis, B.A. (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, 74, 3583–3597.

Wang, J.Y., Sun, X.P., Lin, L.Y., Zhang, T.Y., Ma, Z.H., Li, H.Y. et al. (2012) *PdMfs1*, a major facilitator superfamily transporter from *penicillium digitatum*, is partially involved in the imazalil-resistance and pathogenicity. *African Journal of Biomedical Research*, 6, 95–105.

Wang, Z.C., Cui, J.W., Gao, W.S., Yang, Q., Chen, L.Z., Yang, L.B. et al. (2020) Effects of rice straw structure on chaetoglobosin a production by *Chaetomium globosum* CGMCC 6882. *International Journal of Biological Macromolecules*, 150, 1223–1228.

Wei, G., Tan, D., Chen, C., Tong, Q., Li, X.N., Huang, J. et al. (2017) Flavichalasines A-M, cytochalasan alkaloids from *Aspergillus flavipes*. *Scientific Reports*, 7, 42434.

Yamada, Y., Urui, M., Oki, H., Inoue, K., Matsui, H., Ikeda, Y. et al. (2021) Transport engineering for improving production and secretion of valuable alkaloids in *Escherichia coli*. *Metabolic Engineering Communications*, 13, e00184.

Yen, M.R., Chen, J.S., Marquez, J.L., Sun, E.I. & Saier, M.H. (2010) Multidrug resistance: phylogenetic characterization of superfamilies of secondary carriers that include drug exporters. *Methods in Molecular Biology*, 637, 47–64.

Zhang, D., Ge, H.L., Xie, D., Chen, R.D., Zou, J.H., Tao, X.Y. et al. (2013a) Periconiasins A-C, new cytotoxic cytochalasans with an unprecedented 9/6/5 tricyclic ring system from endophytic fungus *Periconia* sp. *Organic Letters*, 15, 1674–1677.

Zhang, D.W., Tao, X.Y., Liu, J.M., Chen, R.D., Zhang, M., Li, L. et al. (2016) Periconiasin G, a new cytochalasan with unprecedented 7/6/5 tricyclic ring system from the endophytic fungus *Periconia* sp. *Tetrahedron Letters*, 57, 796–799.

Zhang, G.Z., Wang, F.T., Qin, J.C., Wang, D., Zhang, J.Y., Zhang, Y.H. et al. (2013b) Efficacy assessment of antifungal metabolites from *Chaetomium globosum* no.05, a new biocontrol agent, against *Setosphaeria turcica*. *Biological Control*, 64, 90–98.

Zhang, G.Z., Zhang, Y.H., Qin, J.C., Qu, X.Y., Liu, J.L., Li, X. et al. (2013c) Antifungal metabolites produced by *Chaetomium globosum* no.04, an endophytic fungus isolated from *Ginkgo biloba*. *Indian Journal of Microbiology*, 53, 175–180.

Zhang, X.Y., Tan, X.M., Yu, M., Yang, J., Sun, B.D., Qin, J.C. et al. (2021) Bioactive metabolites from the desert plant-associated endophytic fungus *Chaetomium globosum* (*Chaetomiaceae*). *Phytochemistry*, 185, 112701.

Zhao, S.G., Li, Z.Y., Zhou, Z.X., Xu, L.F., He, S.H., Dou, Y.M. et al. (2020) Antifungal activity of water-soluble products obtained following the liquefaction of cornstalk with subcritical water. *Pesticide Biochemistry and Physiology*, 163, 263–270.

Zheng, C.J., Shao, C.L., Wu, L.Y., Chen, M., Wang, K.L., Zhao, D.L. et al. (2013) Bioactive phenylalanine derivatives and cytochalasins from the soft coral-derived fungus, *Aspergillus elegans*. *Marine Drugs*, 11, 2054–2068.

Zhu, H.C., Chen, C.M., Tong, Q.Y., Yang, J., Wei, G.Z., Xue, Y.B. et al. (2017) Asperflavipine a: a cytochalasan heterotetramer uniquely defined by a highly complex tetradecacyclic ring system from *Aspergillus flavipes* QCS12. *Angewandte Chemie, International Edition*, 56, 5242–5246.

Zhu, L.H. & Xu, X.Q. (2013) Stimulatory effect of different lignocellulosic materials for phenolic compound production and antioxidant activity from *Inonotus obliquus* in submerged fermentation. *Applied Biochemistry and Biotechnology*, 169, 2138–2152.

Zhao, Y.Y., Liu, J.Y., Jiang, T.P., Hou, R.X., Xu, G.G., Liu, F. et al. (2021) Resistance-nodulation division efflux pump, LexABC, contributes to self-resistance of the phenazine Di-N-oxide natural product myxin in *Lysobacter antibioticus*. *Frontiers in Microbiology*, 12, 618513.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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