Constitutive hyperproduction of sorbicillinoids in *Trichoderma reesei* ZC121

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**Abstract**

**Background:** In addition to its outstanding cellulase production ability, *Trichoderma reesei* produces a wide variety of valuable secondary metabolites, the production of which has not received much attention to date. Among them, sorbicillinoids, a large group of hexaketide secondary metabolites derived from polyketides, are drawing a growing interest from researchers because they exhibit a variety of important biological functions, including anticancer, antioxidant, antiviral, and antimicrobial properties. The development of fungi strains with constitutive, hyperproduction of sorbicillinoids is thus desired for future industry application but is not well-studied. Moreover, although *T. reesei* has been demonstrated to produce sorbicillinoids with the corresponding gene cluster and biosynthesis pathway proposed, the underlying molecular mechanism governing sorbicillinoid biosynthesis remains unknown.

**Results:** Recombinant *T. reesei* ZC121 was constructed from strain RUT-C30 by the insertion of the gene 121121-knockout cassette at the telomere of *T. reesei* chromosome IV in consideration of the off-target mutagenesis encountered during the unsuccessful deletion of gene 121121. Strain ZC121, when grown on cellulose, showed a sharp reduction of cellulase production, but yet a remarkable enhancement of sorbicillinoids production as compared to strain RUT-C30. The hyperproduction of sorbicillinoids is a constitutive process, independent of culture conditions such as carbon source, light, pH, and temperature. To the best of our knowledge, strain ZC121 displays record sorbicillinoid production levels when grown on both glucose and cellulose. Sorbicillinol and bisvertinolone are the two major sorbicillinoid compounds produced. ZC121 displayed a different morphology and markedly reduced sporulation compared to RUT-C30 but had a similar growth rate and biomass. Transcriptome analysis showed that most genes involved in cellulase production were downregulated significantly in ZC121 grown on cellulose, whereas remarkably all genes in the sorbicillinoid gene cluster were upregulated on both cellulose and glucose.

**Conclusion:** A constitutive sorbicillinoid-hyperproduction strain *T. reesei* ZC121 was obtained by off-target mutagenesis, displaying an overwhelming shift from cellulase production to sorbicillinoid production on cellulose, leading to a record for sorbicillinoid production. For the first time, *T. reesei* degraded cellulose to produce platform chemical compounds other than protein in high yield. We propose that the off-target mutagenesis occurring at the telomere region might cause chromosome remodeling and subsequently alter the cell structure and the global gene expression pattern of strain ZC121, as shown by phenotype profiling and comparative transcriptome analysis of ZC121. Overall, *T. reesei* ZC121 holds great promise for the industrial production of sorbicillinoids and serves as a good model to explore the regulation mechanism of sorbicillinoids’ biosynthesis.

**Keywords:** Biosynthetic gene cluster, Secondary metabolites, Natural product, Yellow pigment, Sorbicillinoids, *Trichoderma reesei*
Background
Secondary metabolites are structurally heterogeneous, highly bioactive, and low-molecular weight compounds synthesized by bacteria, fungi, algae, plants, and animals [1–5]. Unlike primary metabolites, they are not directly essential for the growth of the corresponding production organism but can broaden the inhabitable environments or beat other competitive organisms in a given ecological niche [6]. Most secondary metabolites are derived from either nonribosomal peptides (NRPs) or polyketides (PKSs) or both mixed together, whereas others are derived from terpenes or fatty acids [7]. These molecules include important pharmaceuticals (e.g., penicillin, cyclosporin and statins), potent toxins (e.g., aflatoxins and trichotheccenes) and Janus-faced compounds (e.g., ergot alkaloids), holding both economical and health implications for humans [8].

Sorbicillinoids, also termed “yellow pigment,” are derived from PKS and a large group of hexaketide secondary metabolites and include the cyclization on the carboxylate terminus. Sorbicillinoids are produced and secreted by both marine and terrestrial ascomycetes, including Trichoderma [9], Aspergillus [10], Penicillium [11], Streptomyces [1], Acremonium [12], Paecilomyces [13], and Eurotiomycete [14]. Most of these compounds possess the characteristic C1–C6 sorbyl sidechain and bi- or tri-cyclic frameworks that are extremely complex and highly oxygenated. Based on their structure, sorbicillinoids fall into four classes: monomeric sorbicillinoids, bisorbicillinoids, trisorbicillinoids and hybrid sorbicillinoids. They have a variety of biological activities, including anticancer [15], antioxidant [16], antiviral [17] and antimicrobial [18], showing promising applications in the agriculture, pharmaceutical, and food industries. Therefore, these yellow pigments have attracted considerable interest. Historically, however, most work was aimed at eliminating yellow pigments from fungal fermentation cultures for the production of products like β-lactams [19] and cellulase [9].

Studies of sorbicillinoids have been performed primarily with Trichoderma [9] and Penicillium species [11]. As a well-known industrial strain for the production of cellulases, hemicellulases, and recombinant proteins [20], T. reesei is also a rich source of secondary metabolites [21, 22], a point which often is overlooked. It has been observed that T. reesei forms yellow pigments during growth [23, 24], which have been identified as a mixture of sorbicillin, sorbicillinol and sorbicillinoids [9, 25, 26]. More recently, the biosynthesis pathway of sorbicillinoids in P. chrysogenum [27] and T. reesei [25] has been proposed, wherein all the related genes are clustered on the genome the same way as most genes involved generally in secondary metabolites production [9, 19, 25]. This cluster also contains two transcriptional factors, YPR1 and YRP2, and a transporter [9]. Still, the biosynthesis mechanism of sorbicillinoids remains obscure and strain improvement for sorbicillinoids’ hyperproduction has not been reported.

In this study, the recombinant T. reesei strain ZC121 was obtained by off-target mutagenesis resulting from the unsuccessful deletion of gene 121121. Strain ZC121 was found to produce only a small amount of cellulase and hemicellulase, but a record yield of sorbicillinoids. The effect of culture conditions on this sorbicillinoids’ hyperproduction was determined, including carbon source, light, pH and temperature. Furthermore, identification of the off-target mutagenesis, phenotype profiling and comparative transcriptional profiling were carried out to reveal the molecular mechanism(s) underlying sorbicillinoid hyperproduction of ZC121.

Materials and methods
Materials
Construction and propagation of plasmids were performed in Escherichia coli DH5α. Agrobacterium tumefaciens AGL-1 served as a T-DNA donor for transformation of T. reesei RUT-C30 (CICC 13052) [28]. E. coli DH5α and A. tumefaciens AGL-1 were cultured in Luria–Bertani (LB) with 220 rpm at 37 °C and 28 °C, respectively. T. reesei RUT-C30 and its derivatives were cultured on potato dextrose agar (PDA) plates at 28 °C with mixing at 200 rpm for conidia production and in Trichoderma minimal media (TMM) [29] with 2% (w/t) cellulose or other carbon sources (as indicated) for cellulase and sorbicillinoid production. Plasmid pXBthg was employed to construct plasmid pXBthg-121121 (Additional file 1: Fig. S1) [30]. The primers used in this study can be found in Additional file 1: Table S1. Fifty μg/mL of hygromycin B was utilized as the selection marker. All chemicals used in this study were purchased from Sigma-Aldrich, USA.

Strain construction
Genomic DNA was extracted from T. reesei cells grown in sabouraud dextrose broth (SDB) medium for 48 h at 28 °C using the E-Z 96 Fungal DNA Kit (Omega Bio-tek, Germany). The 1500-bp upstream or downstream region abutting gene 121121 (Additional file 1: Fig. S2) were amplified from the prepared DNA template, respectively. The 1500-bp downstream fragment was cloned into plasmid pXBthg at BamHI using ClonExpress™ II One Step Cloning Kit (Vazyme, China), which was followed by the cloning of the 1500-bp upstream one at XhoI, resulting in the plasmid pXBthg-121121 (Additional file 1: Fig. S1). pXBthg-121121 was then introduced into T. reesei RUT-C30 by the Agrobacterium tumefaciens-mediated transformation (AMT) method.
Four transformants ZC121-1, ZC121-2, ZC121-3, and ZC121-4 were obtained after selection on PDA plates containing 50 μg/mL hygromycin B and 200 μM cefotaxime.

Shake flask cultivation
Five percent (v/v) 10^7/mL conidia from T. reesei grown on PDA plates at 28 °C for 7 days were inoculated into 10 mL SDB and incubated at 28 °C with mixing at 200 rpm for 2 days. Ten percent (v/v) pre-grown mycelia were inoculated into 50 mL TMM media (pH 6) with 2% cellulose, lactose, glucose, galactose, or glycerol, and then incubated at 28 °C with mixing at 200 rpm for 5 days. A 0.5 mL culture sample was taken every 12 h. The samples were centrifuged at 14,000×g for 10 min at 4 °C and the supernatants were filtrated with 0.22 μm filter membranes and stored at −80 °C for sorbicillinoid analysis and the cellulase activity assay.

The absorbance at 370 nm of the prepared supernatant as mentioned above was recorded using a UV spectrophotometer to determine the amount of sorbicillinoids present [31]. To determine the exact yield of sorbicillinoids, the prepared supernatant was dried at 40 °C overnight. Methanol was used to extract the sorbicillinoids from the dried supernatant powder several times until the powder became white. The supernatant containing sorbicillinoids in methanol was collected after centrifuging and methanol was removed by rotary evaporation. The crude sorbicillinoids product was then obtained and utilized to determine the standard curve (Additional file 1: Fig. S3) for the yield calculation of sorbicillinoids.

The composition of the crude sorbicillinoids product from T. reesei ZC121 and RUT-C30 were analyzed using LC–MS system (G2-XS QTof, Waters) coupled with a UPLC column (2.1×100 mm ACQUITY UPLC BEH C18 column containing 1.7 μm particles). Reserpine was used as the internal standard [32]. Two microlitre samples dissolved in methanol were injected onto the C18 column at a flow rate of 0.4 mL/min. The gradient for elution was 2% buffer B (0.1% formic acid in water) for 0.5 min, 2–20% buffer B for 5 min, 20–95% buffer B for 6 min, and finally 95% buffer B for 2 min. Mass spectrometry was operated using the electrospray source in positive ion mode with MSe acquisition according to the following settings: the selected mass ranged from 50 to 1200 m/z and leucine enkephalin (m/z 556.2771) was used as the “lock mass option” for recalibration. The ionization parameters were set as follows: capillary voltage was 2.5 kV, collision energy was 40 eV, source temperature was 120 °C, and the desolvation gas temperature was 400 °C. Data acquisition and processing were performed using Masslynx 4.1.
Results

*Trichoderma reesei* recombinant strains with off-target mutagenesis displayed markedly reduced cellulase production, but significantly enhanced sorbicillinoids production

Gene 121121 encodes a candidate fungal regulatory protein that contains a Zn(2)Cys(6) fungal-type DNA binding domain. Gene 121121 is located next to β-glucosidase gene, *cel3d* [46]. Both genes belong to a tightly co-expressed genome region as identified by analysis of transcriptional data of *T. reesei* producing cellulases [46]. Moreover, gene *cel3d* was considered to be significantly involved in cellulase production by *T. reesei* [33]. Based on these previous findings, we presumed that gene 121121 might play a role in *T. reesei* cellulase production. Gene 121121 did not affect cellulase production when cells were grown on lactose [46], although no study was found which explored the effect of deletion of gene 121121 on cellulase synthesis. Therefore, we attempted to knockout gene 121121 in *T. reesei* RUT-C30 using homologous recombination mediated by AMT (Fig. 1a and Additional file 1: Fig. S1).

Four transformants, ZC121-1, -2, -3, -4, were obtained after selection using the marker, hygromycin. Unfortunately, the deletion of 121121 was not successful as shown by PCR result that gene 121121 was successfully cloned from all of four transformants, individually (Fig. 1b). This unsuccessful deletion was further confirmed by whole genome resequencing using NGS sequencing (Additional file 1: Fig. S4). However, we have successfully cloned the gene, hyg, in the four transformants mentioned above, demonstrating that the knockout cassette was randomly inserted into the chromosome of these strains by off-target mutagenesis [36].

Cellulase production in the four recombinant strains induced by cellulose on day 5 was assayed as described (Fig. 1c). The FPase, pNPCase, CMCase, pNPase, and pNPXase activities of all four mutants were in the range of 0–1.9 IU/mL, 0.007–0.21 IU/mL, 0–0.37 IU/mL, 0.03–1.2 IU/mL, and 0.1–2.9 IU/mL, respectively, showing noticeably reduced cellulase activities when compared to that of RUT-C30, which was 6.1 IU/mL FPase, 0.5 IU/mL pNPCase, 6.8 IU/mL CMCase, 2.1 IU/...
mL pNPGase, and 4.6 IU/mL pNPXase. Obviously, the off-target mutagenesis caused serious inhibition of cellulase and hemicellulase production in *T. reesei*.

Surprisingly, we observed that these mutants displayed a much greater yellow color production than RUT-C30 both in liquid culture (Additional file 1: Fig. S4) and on the PDA plates (Additional file 1: Fig. S5), indicating far more sorbicillinoids were produced. For quantitative comparison, the absorbance of liquid cultures of *T. reesei* at 370 nm was used to measure the sorbicillinoids’ production (Fig. 1d) on cellulose [31]. The sorbicillinoids’ production in strains ZC121-1, ZC121-2, ZC121-3, and ZC121-4 was increased by 6, 3.3, 4.5, 4.6-fold, respectively, when compared to RUT-C30 production. In contrast to the dramatic decrease of cellulase and hemicellulase activities on cellulose, a significantly enhancement of yellow pigment production was observed by these recombinant strains. This finding demonstrated that off-target mutagenesis in strain ZC121 has switched cellulase production to sorbicillinoids’ production on cellulose. The recombinant strain ZC121-1 was selected for further study and was referred to as ZC121 because it displayed the highest yellow pigment production found in this study.

Hyperproduction of sorbicillinoids in strain ZC121 is a constitutive process

In previous studies, the ability of *T. reesei* to produce sorbicillinoids was reported to vary with different carbon sources [19, 37–39]. To see whether the superior sorbicillinoids’ production of strain ZC121 compared to strain RUT-C30 is dependent on carbon source, both the sorbicillinoids and cellulase production of strains ZC121 were measured during the time course of growth on TMM containing cellulose, lactose, glucose, galactose or glycerol as the individual carbon source (Fig. 2 and Additional file 1: Fig. S6). The maximal absorbance at 370 nm of the culture supernatant of strain ZC121 was 6.6, 8.4, 17.3, 13.3 and 10.5 for cellulose, lactose, glucose, galactose and glycerol, respectively. Note that these values are 5.1, 8.4, 4.3, 5.1 and 4.5-fold that of RUT-C30 (Fig. 2). Obviously, strain ZC121 produced remarkably increased sorbicillinoids on all tested carbon sources. The highest sorbicillinoids’ production was found with glucose as the carbon source at 120 h of growth, followed by galactose, glycerol, lactose, and cellulose—in descending order. An absorbance of $\text{OD}_{370} = 17.3$ for the culture supernatant from growth on glucose corresponds to a concentration of 627 μg/mL yellow pigments (see “Materials and methods” section). Except for growth on cellulose, both
ZC121 and RUT-C30 display very low cellulase production on lactose, glucose, galactose, and glycerol (Additional file 1: Fig. S5), which is reasonable because these four carbon sources have been shown to be inefficient inducers cellulasles.

Using TMM + glucose as the culture medium, we also tested the effects of other culture conditions on the sorbicillinoids’ production ability of ZC121, including light, pH and temperature. Strain ZC121 was grown under normal lab light condition, constant light, constant darkness, or cycles of 12 h light–12 h dark (light–dark cycles) for 120 h. The highest absorbance (OD$_{370}$=24.7) was observed for cell growth under constant darkness, whereas the absorbance under the other three light conditions were comparable internally (Fig. 3). It seems that the lighting condition only affects the production of sorbicillinoids in strain ZC121 to some limited extent, with the highest sorbicillinoids’ production observed for growth under constant darkness (Fig. 3a). However, strain ZC121 displayed excellent sorbicillinoids’ production ability under all tested light conditions. Strain ZC121 exhibited the hyperproduction of yellow pigment at pH 4, 6 and 7, but displayed sharply reduced pigment production at extreme pH values (pH 2, 10 and 12), which was probably related to the poor growth of strain ZC121 under these extreme pH conditions (Fig. 3b). The sorbicillinoids’ production declined somewhat when the culture temperature was decreased to 18 °C, but growth at 18 °C and 24 °C still maintained 74% and 87% of growth at 28 °C, respectively (Fig. 3c). In contrast, yellow pigment production was enhanced along with the increased temperature up to 42 °C (Fig. 3c). Beyond 42 °C, the sorbicillinoids’ production was reduced sharply, because strain ZC121 did not grow at all (Fig. 3c). Obviously, the high production performance of ZC121 was maintained over a limited range as the culture temperature was varied from the optimum temperature of 28 °C. In summary, the hyperproduction of sorbicillinoids in T. reesei ZC121 was a constitutive process, independent of carbon source, light, pH, and temperature.

**Identification of the off-target mutagenesis in recombinant strain ZC121**

The random insertion of the knockout cassette of pxBthg-121121 (Additional file 1: Fig. S2) into the chromosome of T. reesei ZC121 could cause collateral mutations, which might contribute to the outstanding yellow pigment production shown by ZC121. Therefore, the identification of the insertional site might help us further understand the regulatory mechanism of sorbicillinoids’ biosynthesis. To this end, the whole genome resequencing using NGS sequencing was employed to find the random insertional sites. The whole genome resequencing of T. reesei ZC121 resulted in a total of 16,078,934 150-bp paired-end reads with mean depth coverage of 89.78% and Q30 percentage of 92.42%. These clean reads covered 89.78% of the reference genome of RUT-C30. The NGS sequencing result (NCBI Accession Number: SRR6906202) show that one copy of the cassette was probably inserted at 1–60 bp of T. reesei RUT-C30 genome KI911238.1 (https://www.ncbi.nlm.nih.gov/nuccore/572281258/). The insertion site was at the telomere of chromosome IV of T. reesei. No coding genes were found in the neighboring sequence of 3 kb.

**Identification of sorbicillinoids in the culture supernatant of strain ZC121**

To determine which types of sorbicillinoids were produced by strain ZC121, LC–MS analysis of the culture supernatant of strain ZC121 cultivated on glucose for 5 days was performed (Table 1). A total of seven known sorbicillinoid-related compounds were identified, including sorbicillin, sorbicillinol, bisorbicillinol, dihydrosorbicillinol, oxosorbicillinol, bisvertinolone, and dihydrobisvertinolone. Furthermore, five structure-unknown compounds were detected. These compounds

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**Fig. 3** The effect of light (a), pH (b) and temperature (c) on the sorbicillinoids’ production ability of T. reesei ZC121. Unless otherwise indicated, T. reesei was cultivated on TMM + glucose (pH 6) at 28 °C with 200 rpm for 5 days. Error bars indicate SDs from three independently grown cultures.
have been reported to be sorbicillinoid-related but are not named [19]. Peak area was utilized to roughly assess the abundance of these compounds (Table 1). Sorbicillinol was the most abundant product; bivertinolone was second. Sorbicillinol has been found to be the major sorbicillinoid-related product in several studies [13, 19, 25] and considered to be the building block for the other sorbicillinoids [19]. Bivertinolone can inhibit the biosynthesis of β-1,6-glucan and is an effective 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenger [40]. Its direct precursor, oxosorbicillinol, was also abundantly accumulated in the supernatant of strain ZC121. Only a trace amount of sorbicillin was detected, which agreed with the early findings [41].

Characterization of T. reesei ZC121

Growth of strains ZC121 and RUT-C30 was studied by measuring colony diameters on PDA plates and TMM plates with different carbon sources (i.e., cellulose, lactose, glucose, galactose or glycerol) (Fig. 4a and Additional file 1: Fig. S6). The colony diameters of these two strains were nearly the same regardless the carbon sources (Fig. 4a). Furthermore, the growth of strain ZC121 and RUT-C30 in TMM + 2% cellulose was assayed by DNA content measurement (Fig. 4b), as we were unable to accurately measure the dry T. reesei biomass due to the interference from insoluble cellulose. No significant difference of growth was observed between strain ZC121 and RUT-C30 (Fig. 4b). These results suggest that off-target mutagenesis did not affect the growth of T. reesei on different carbon sources.

The morphology of ZC121 was different from that of RUT-C30. The number of the branched hyphae of ZC121 decreased and its hyphae became thinner and longer when compared to RUT-C30 (Fig. 4c). Also, the sorbicillinoids were not only secreted out of the cells, but also filled the fungal cell resulting in blue fluorescence emission at 405 nm excitation—as observed under fluorescence confocal microscopy (Fig. 4c). Furthermore, the spore amount of strain ZC121 was 1 × 10⁶/mL, only 4.8% of that of RUT-C30 (2.1 × 10⁷/mL) (Fig. 4d), demonstrating that the off-target mutagenesis leads to noticeable reduction of sporulation in T. reesei ZC121. This negative link between secondary metabolite production and conidiation in fungi was frequently found in early studies [20]. For example, when deleting the velvet protein VeA in A. nidulans, the abolishment of penicillin and aflatoxin secondary metabolite production was accompanied with the enhancement in asexual conidiation. The low level expression of sorbicillinoid-related genes during asexual growth was also reported [12]. Overall, with the off-target mutagenesis, the growth rate and biomass of T. reesei ZC121 was not affected significantly, but its morphology changed with marked sporulation reduction.

Transcription patterns of strain ZC121

RNA-seq analysis was performed to understand how the off-target mutagenesis influences the transcriptional level of strain ZC121. The sequences of the total reads were mapped to the reference genome of T. reesei RUT-C30 (https://www.ncbi.nlm.nih.gov/genome/323?assembly_id=49799) with coverage of 93.24–95.63%. A total of 9544 unique transcripts were detected. Genes were differentially expressed between the two strains when the average reads of the corresponding transcripts differed with |log2Ratio| ≥ 1 and adjusted p values ≤ 0.05. By comparing strain ZC121 to RUT-C30, we obtained 638 and 1006 differentially expressed genes (DEGs) under cellulose and glucose growth conditions, respectively (Additional file 1: Table S4). Among these, 341 DEGs

Table 1  Metabolite profiling for sorbicillinoids in the culture broth of strain ZC121 grown on glucose for 5 days

| Compound | Name | Formula | Acquired [M+H]+ | RT (min) | Peak area |
|----------|------|---------|-----------------|---------|-----------|
| 1        | Sorbicillinol | C₁₄H₁₆O₄ | 249.1135 | 6.94 | 333,191 |
| 2        | Bivertinolone | C₂₈H₃₂O₉ | 513.2125 | 12.08 | 300,654 |
| 3        | Oxosorbicillinol | C₁₄H₁₆O₅ | 265.1080 | 9.03 | 111,253 |
| 4        | Bisorbicillinol | C₂₈H₃₂O₈ | 497.2175 | 11.89 | 99,103 |
| 5        | Dihydrobivertinolone | C₂₈H₃₄O₉ | 515.2280 | 12.57 | 40,780 |
| 6        | Dihydrosorbicillinol | C₁₄H₁₆O₄ | 251.13 | 7.89 | 22,754 |
| 7        | Sorbicillin | C₁₄H₁₆O₃ | 233.1178 | 5.63 | 7002 |
| 8        | Unknown | C₁₂H₁₇ON | 192.1388 | 5.26 | 36,046 |
| 9        | Unknown | C₁₂H₁₇O₃ | 193.0865 | 6.77 | 35,210 |
| 10       | Unknown | C₁₂H₂₀O₄N₂ | 291.1501 | 4.56 | 9430 |
| 11       | Unknown | C₁₂H₂₀O₅N₂ | 207.1021 | 6.94 | 5678 |
| 12       | Unknown | C₁₂H₂₀O₅N₂ | 309.145 | 4.58 | 1021 |
were found under both glucose and cellulose growth conditions, of which 327 DEGs expression changing trends were identical between these two carbon sources, whereas 14 DEGs expression changing trends were opposite (Additional file 1: Table S4).

KEGG pathway enrichment analysis showed that 14 out of the top 20 enriched pathways were shared by growth on both glucose and cellulose conditions (Fig. 5c). This result implies that the impact of the off-target mutagenesis in ZC121 at the transcription level share a lot in common between growth on different carbon sources. The “other glycan degradation” was enriched only on cellulose (Fig. 5a). Most genes in this pathway were related to cellulase production induced by cellulose and were downregulated, notably. This result is in line with reports of the inhibited cellulase production in strain ZC121 for growth on cellulose in comparison with RUT-C30.

For the enriched cellular components, both “membrane” and “extracellular region” were enriched in the presence of glucose or cellulose (Fig. 6b), which is reasonable given that both cellulases and sorbicoloids are secreted outside of the fungal cells. Under the “membrane” category, DEGs are mainly involved in the intrinsic component of membrane (Fig. 6b), including the plasma membrane, vacuolar membrane, nuclear membrane, and mitochondrial membrane. Deletion of gene 121121 might pose a significant change on cell structure of T. reesei, which might be responsible for the morphology change mentioned above (Fig. 4c); as well as the condition defect (Fig. 4d).

In the presence of cellulose, the most enriched DEGs in the category of biological process belong to “carbohydrate metabolic process” which mainly included “xylan catabolic process” and “cellulose catabolic process” (Fig. 6c). Most of these DEGs were downregulated, which is consistent with the markedly reduced cellulase production and the result of the KEGG pathway enrichment analysis. Under glucose growth conditions, the most enriched biological process changed to “transmembrane transport” and “oxidation–reduction process” (Fig. 6c).
Fig. 5 Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs in strain ZC121 in comparison with the parental strain RUT-C30 on cellulose or glucose. The y axis represents the name of the top 20 enriched pathways, and the x axis shows the number of DEGs in the enriched pathways. The enriched pathways were categorized into pathways enriched only under glucose condition (a), only under cellulose condition (b), and under both glucose and cellulose conditions (c).

Fig. 6 Gene ontology (GO) functional enrichment analysis of DEGs of strain ZC121 compared to the parental strain RUT-C30 grown on cellulose and glucose as the sole carbon source, respectively. The y axis represents the name of the most enriched GOs that belong to different ontologies: (a) the molecular function, (b) the cellular component and (c) the biological process, while the x axis represents the number of DEGs in each enriched GO.
Most DEGs involved in the cellulase production were downregulated in *T. reesei* ZC121 on cellulose

There are 74 genes known or predicted to be related to cellulase/hemicellulase production in *T. reesei*, of which 43 were DEGs with notably downregulated mRNA levels, except gene *xdh1* (Additional file 1: Table S5). Specifically, the transcriptional levels of cellulase/hemicellulase genes, such as cellobiohydrolase cel6a (CBH II), endoglucanases egII, egII2, egIII, egIII4, and egIII5, β-glucosidases cel3a (bgII1), cel3d, cel3e, celIIa, and celIIb, β-xyllosidase bxII, and xylanases xyn1, xyn2 and xyn3 were all reduced markedly in strain ZC121 (Table 2), matching well with the nearly abolished cellulase/hemicellulase production in *T. reesei* of strain ZC121 on cellulose, whereas cellulase transcription activators xyR1 and XyR2 [45], a glycoside hydrolyase Family 61 (GH61 s) GH61b and GH61a [43] and the cellulose-induced proteins CIP-1 and CIP-2 [44], which have been shown to improve the hydrolysis of cellulose, were also downregulated significantly in strain ZC121 grown on cellulose. In addition, three well-known cellulase transcription activators *xyn1* [45], *ace3* [46], and *crt1* [47] were significantly downregulated in strain ZC121 grown on cellulose, whereas cellulase transcription repressor, *cre1* [48], was downregulated under both cellulose and glucose growth conditions.

**Table 2** The main DEGs related to (hemi) cellulase in strain ZC121 (C121)

| Gene ID | Gene name | Description | log2 (C121/CC30) | p value | Category |
|---------|-----------|-------------|-----------------|---------|----------|
| 122470  | cel6a     | Exoglucanase 2 | −6.43           | 5.00E−05 | Cellulase |
| 5304    | egII      | Endoglucanase I precursor | −5.75           | 5.00E−05 | Cellulase |
| 124931  | egII2     | Endoxylanase II  | −2.63           | 0.0001  | Cellulase |
| 72489   | egIII     | Endoglucanase III | −6.74           | 5.00E−05 | Cellulase |
| 139633  | egIII4    | Endoglucanase-4  | −5.70           | 5.00E−05 | Cellulase |
| 25940   | egIII5    | Endo-1,4-beta-glucanase V | −2.50           | 5.00E−05 | Cellulase |
| 136547  | cel3a     | Beta-1,4-glucosidase glucohydrolase | −4.35           | 5.00E−05 | Cellulase |
| 122639  | cel3d     | Hypothetical protein | −1.94           | 5.00E−05 | Cellulase |
| 74305   | cel3e     | Hypothetical protein | −1.88           | 5.00E−05 | Cellulase |
| 127115  | celIIa    | Beta-glucosidase | −3.13           | 5.00E−05 | Cellulase |
| 77989   | celIIb    | Glycoside hydrolase | −2.90           | 5.00E−05 | Cellulase |
| 122518  | celIIb1   | Endoglucanase VII | −6.84           | 5.00E−05 | Cellulase |
| 77521   | bxIII     | Family 43 glycoside hydrolase | −1.32           | 0.0017  | TF       |
| 38418   | xyn1      | Endo-1,4-β-xylanase 1 | −1.45           | 0.0009  | Hemicellulases |
| 124931  | xyn2      | Endo-1,4-β-xylanase 2 | −2.63           | 0.0001  | Hemicellulases |
| 23616   | xyn3      | Xylanase III | −6.17           | 5.00E−05 | Hemicellulases |
| 104220  | swoI      | Swollenin | −3.52           | 5.00E−05 | Nonenzymatic cellulose attacking enzymes |
| 121449  | cip1      | Hypothetical protein | −6.30           | 5.00E−05 | Nonenzymatic cellulose attacking enzymes |
| 125575  | cip2      | Hypothetical protein | −5.19           | 5.00E−05 | Nonenzymatic cellulose attacking enzymes |
| 98788   | xyr1      | Xylanase regulator 1 | −2.28           | 5.00E−05 | TF       |
| 98455   | ace3      | Hypothetical protein | −2.29           | 5.00E−05 | TF       |
| 109243  | cre1      | General substrate transporter | −3.04           | 5.00E−05 | TF       |
| 23706   | cre1      | Hypothetical protein | −1.63           | 0.0001  | TF       |

* Gene ID was assigned based on the *T. reesei* RUT-C30 genome database (https://www.ncbi.nlm.nih.gov/genome/3237?genome_assembly_id=49799)
ZC121, but not in RUT-C30 (Fig. 7b and Additional file 1: Table S2). Gene sor5 in this study, which is designated as sor7 in the literature, encodes a short-chain dehydrogenase/reductase [14]. Its ortholog from P. rubens was not located in the sorbicillinoid gene cluster. Moreover, gene sor5 was not found in most other fungi. It seems that the off-target mutagenesis activates the expression of gene sor5, which might play a role in the notable overexpression of genes in the sorbicillinoid cluster. However, how the activation of gene Sor5 functions in T. reesei ZC121 is unclear, because studies performed on gene sor5 are rare.

In the previous study, an extensive search for PKS-, NRPS- and hybrid synthetase genes using basic local alignment search tool (BLAST) revealed the 11 PKS-, 11 NRPS- and 4 hybrid synthetase genes in the T. reesei genome (http://genom e.jgips f.org/pages/blast .jsf?db=Trire2), which could be appointed to 23 distinct gene clusters [21]. Also, a total of 31 predicted transcription factor (TF) genes were found in a 50 kb radius of these synthase genes. Among them, five synthases and four TFs were significantly upregulated in strain ZC121 under both tested conditions (Additional file 1: Table S6), of which two synthases and two TFs (Additional file 1: Table S2) are involved in the sorbicillin biosynthesis pathway, as we discussed above. The transcription level of pks8 (90904) were increased under both conditions (Table 3). Its homologues, adaA from A. niger [10] or vrtA from Penicillium aethiopicum [11], were involved in anthracenone and naphthacenedione biosynthesis, respectively. A hybrid PKS-NRPS synthase (128011) and its neighboring TF (74475) was significantly upregulated (Table 3), whose closest annotated hit was fusaproliferin synthase [21]. Fusaproliferin, a mycotoxin from Fusarium spp. PKS 77957, is a nonribosomal peptide synthetase, whose homologue in Aspergillus fumigatus is essential for fumigaclavine C production. Fusaproliferin has been studied extensively [49]. This outcome shows that other secondary metabolites, other than sorbicillinoids, might also be produced in strain ZC121 and thus it is worth studying them in the future.

**Discussion**

Sorbicillinoids have potential pharmaceutical value as antimicrobial, antiviral, and anticancer agents [50]. Moreover, they could be utilized as pigments and food colorants (yellow pigments) as well. Since they were first discovered in 1948 from Penicillium notatum [51], studies related to sorbicillinoids have focused on finding...
new compounds with similar structure [52], elucidating chemical structures and biological activities [16, 50], establishing the complex biosynthetic pathway [41] and developing chemical synthesis methods [53]. However, both strain engineering to increase sorbicillinoids’ production and the relevant regulatory mechanism is less studied. *T. reesei* is well-known for its prominent enzyme-secreted ability using cellulose as the efficient inducer and widely utilized in industry as a work horse for the production of both cellulase and heterologous recombinant proteins [54]. For the first time, an overwhelming switch from cellulase production to sorbicillinoids’ production using cellulose as carbon source was reported in recombinant *T. reesei* strain ZC121. Coincidentally, an expression shift from cellulase-related genes to genes in the sorbicillinoid gene cluster was found by transcriptional profiling with steep downregulation of 42 genes involved in cellulase production and significant upregulation of all genes in the sorbicillinoid cluster. This switch would enable *T. reesei* to contribute more energy and metabolites to sorbicillinoids’ production, considering that cellulase production is a heightened energy-efficient process. This shift, at the same time, would allow the easier separation of sorbicillinoids from a culture supernatant that contains only low titers of cellulases/hemicellulases to support the host cells in the production of valuable products in high yield.

In most studies related to sorbicillinoids, glucose is usually used as the carbon source with the highest sorbicillinoids’ production of OD_{370} = 13 reported in the literature [31], which is lower than OD_{370} = 17.3 of strain ZC121 in this study. Upon growth on cellulose, *T. reesei* cultures with the deletion of gene XPP1 can produce a small amount of yellow pigment with OD_{370} ≈ 0.32 [31], much less than the OD_{370} = 6.6 we observed in strain ZC121. Strain ZC121 generated the highest reported amounts of sorbicillinoids when grown on cellulose or glucose, as far as we know. Other carbon sources, such as lactose, glycerol, and galactose have been reported to be utilized by *T. reesei* to produce remarkable amounts of sorbicillinoids. Furthermore, the sorbicillinoids’ production in fungi has been reported to be generally impacted by culture conditions, such as carbon source [9, 37], light exposure time [39], temperature, and pH. By contrast, the recombinant strain ZC121 displayed hyperproduction of sorbicillinoids regardless the culture conditions, so long as *T. reesei* can grow well. This constitutive hyperproduction would benefit the future industry application of strain ZC121 for sorbicillinoids’ production with great flexibility and easy fermentation operation.

Currently, sorbicillinoids are not produced by industry. Various efforts have been made to access these compounds by chemical synthesis in the laboratory and only for research purposes [41]. However, most of the chemical synthesis routes are cumbersome, as the structure of sorbicillinoids is complex [53]. Whether or not these chemical methods are applicable in the industry is still unknown. Moreover, the chemical method is less environment-friendly than the biosynthesis. Therefore, it is highly desired to have recombinant microorganisms that can produce constantly sorbicillinoids with high yield.

The global regulator *laeA* is well-known for being involved in the secondary metabolism by regulating some
polyketides [57]. Protein LaeA directly interacts in the nucleus with transcription factors of the trimeric Velvet complex, consisting of the Velvet domain proteins VeA and VelB [57]. The heterotrimeric velvet complex VelB/ VeA/LaeA correlates fungal development and secondary metabolism in response to light. Moreover, it is found that both the knockout and overexpression of gene laeA affected the expression of some sor genes [14]. Nevertheless, the mRNA level of all these three genes remained unchanged in strain ZC121 in comparison with RUT-C30. Moreover, VosA, a recently identified regulator of fungal sporogenesis [58], another binding partner of VelB [57], was also not affected in strain ZC121. This finding is in line with the observation that T. reesei ZC121 exhibited high sorbicillinoids’ production as a function of light exposure time as we showed above.

The major types of sorbicillinoids generated in strain ZC121 from growth on glucose were sorbicillinol and bisvertinolone as analyzed by LC–MS (Table 1). It is worth noting that bisvertinolone is a potential antican-

cer agent (acts by inhibiting β-1,6-glucan biosynthesis or serving as an effective 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenger [40]. Currently, it is only available by intricate chemical methods [53]. Bisvertinolone has never been reported to be the major product in T. reesei. Furthermore, the precursor of bisvertinolone, oxosorbicillinol, was the third most abundant compound in the culture medium (Table 1). Thus, it seems that T. reesei ZC121 might serve as a good starting strain to obtain mutants that primarily produce bisvertinolone by further strain optimization.

Interestingly, four genes related to cellulase produc-
tion are in close vicinity to the sorbicillinoid gene cluster, including gene 102500, axel1, cip1 and cel61a, forming a “sorbicillinoid-cellulase” supercluster (Fig. 7a). In con-
trast to the remarkably upregulated expression of genes in the sorbicillinoid gene cluster, these four genes were all DEGs steeply downregulated in ZC121 on cellulose growth, while only two genes were DEGs markedly reduced on glucose growth (Fig. 7b). Genes encoding candidate carbohydrate-active enzymes (CAZy) for polysaccharide degradation are prone to clustering in the T. reesei genome, leading to 25 genomic regions of high CAZy gene density [59]. These regions usually possess genes participating in secondary metabolism as well, forming “superclusters”. These kinds of superclusters have also been observed for other secondary metabolites in other fungi, playing a key role in the correspond-
ing secondary metabolite production [60–62]. However, whether and how the “sorbicillinoid–cellulase” supercluster impacts the secondary metabolism for sorbicillinoids and cellulase is still unknown and is worth exploring in future studies.

We have initially aimed to specifically knockout gene 121121 in T. reesei RUT-C30 to study its function on cellu-

lase production by AMT utilizing homologous recom-

bination; however, this approach unfortunately failed. Continued work gave rise to the unexpected off-target mutagenesis at the telomere region of chromosome IV. The insertion of T-DNA into the genome of the recipient cell is random [63]. This randomness of T-DNA integration leads to inefficient targeting, resulting in off-target mutagenesis. It has been shown that T-DNA can insert into the telomeric region by non-homologous recom-

bination (NHR) [64]. Therefore, although the similarity between the flanking 1500-bp regions of gene 121121 and the integration site at the telomere of chromosome IV of T. reesei is not high (40.4% and 39.0% for the upstream and downstream flanking regions, respectively), off-target integration was observed at the telomere of chro-

mosome IV of T. reesei. The inefficient gene targeting by AMT has been a long-standing issue in plant and fungi [36]. Strategies like generation of double strand breaks at genomic positions of interest, downregulation of enzymes involved in NHEJ pathway, and concomitant translocation of the homing endonuclease I-SceI, have been explored to improve targeted integration of AMT [36]. Nevertheless, target mutagenesis can be embraced as a potent mutagenesis strategy for strain engineering as we showed here, together with the traditional meth-

ods using nitrosoguanidine (NTG) or UV irradiation. The insertion of T-DNA into the telomeric region induced gross chromosome rearrangement [64]. Chromosome remodeling plays an important role in cellulase produc-
tion [33, 34, 65]. It is tempting to speculate that the off-
target mutagenesis probably gave rise to the excellent sorbicillinoids’ production ability of strain ZC121, which came about by chromosome rearrangement and subse-
quent affected gene expression as we found using tran-
scriptome analyses.

Conclusion

We constructed recombinant T. reesei strain ZC121 from T. reesei RUT-C30 by off-target mutagenesis dur-

ing the failed knockout of gene 121121. The knockout cassette for gene 121121 was found to insert at the telo-
mere of chromosome IV of T. reesei. Strain ZC121 exhibited constitutive hyperproduction of sorbicillinoids under all tested culture conditions, including varied carbon source, light, pH, and temperature. Particularly, an overwhelming switch from cellulase production to sor-

bicillinoids production was observed in strain ZC121 on cellulose. Coincidently, a similar shift was also observed at the transcriptional level in ZC121 cultivated on cellulose, with steep downregulation of 42 genes involved in cellulase production and significant upregulation of all
genes in the sorbicillinoid cluster. For the first time, T. reesei alone can degrade cellulose to directly produce valuable compounds (sorbicillinoids in this case) other than proteins, paving the way for the industrial production of cellulase-based chemical compounds.

Additional file

Additional file 1: Fig. S1. Schematic illustration of the plasmid pXBthg‑121121. hyg: homocystine resistance; LB, left border of binary vector; RB, right border of binary vector; Kan: kanamycin resistance; 121121-up: the 1500 bp upstream of gene 121121; 121121-down: the 1500 bp downstream of gene 121121. Fig. S2. The 1500-bp upstream and downstream regions of gene 121121. Fig. S3. The standard curve of sorbicillinoids. Fig. S4. The whole genome resequencing of strain ZC121 shows the unsuccessful deletion of gene 121121. Fig. S5. The color of the cell supernatant of T. reesei RUT‑C30 (a) and ZC121 (b) grown on glucose. Fig. S6. Cellulolytic enzyme activities in the culture supernatant of T. reesei ZC121 and RUT‑C30 grown on 2% cellulose, lactose, glucose, galactose and glycerol were assayed on day 5, including the activities of FPhase (the filter paper activity) (a), pNPGase (the BGL activity) (b), pNPCase (the CBH activity) (c), CMCase (the CMC activity) (d) and pNPXase (the β‑xylosidase activity) (e). The error bars indicate the standard deviations of three biological replicates. Table S1. Primers used in this study. Table S2. Comparative transcription levels of the sorbicillinoid gene cluster and its neighboring cellulase-related genes in T. reesei ZC121 grown on cellulose and glucose.

Abbreviations

pNPase: the β‑glucosidase activity; pNPCase: the CBH activity; CMCase: the CMC activity; FPhase: the filter paper activity; pNPXase: the β‑xylosidase activity; AMT: agrobacterium tumefaciens-mediated transformation; PDA: potato dextrose agar; SDB: sabouraud dextrose broth; TMM: Trichoderma minimal medium; MFS: major facilitator superfamily sugar transporter; PKSs: polyketides; NRP: non‑ribosomal peptides; DEGs: differentially expressed genes; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; NGS: next-generation sequencing; FPKMs: fragments per kilobase of transcript sequence per million bases pairs sequenced; RT‑qPCR: quantitative real-time PCR.

Authors’ contributions

CL and FL conceived and designed the study. CL carried out the majority of the experiments. WS conducted part of enzyme activity measurement experiments and the extraction of sorbicillinoids. ZZ helped us to design and construct the plasmid pXBthg‑121121 used in this study. SY helped us to analyze part of the RNA sequencing results. CL, FL and ZC analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

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Acknowledgements

Authors thank the National Natural Science Foundation of China (31700040), the Fundamental Research Funds for the Central Universities, and a project funded by the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions. ZC thanks the University of Michigan for supporting his sabbatical leave.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethical approval and consent to participate

Not applicable.

Funding

This work was supported by the National Natural Science Foundation of China (31700040), the Fundamental Research Funds for the Central Universities, and a project funded by the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions. ZC acknowledges the support from University of Michigan.

Publisher’s Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 7 June 2018 Accepted: 16 October 2018

Published online: 25 October 2018

References

1. Omura S, Ikeda H, Ishikawa J, Hanamoto A, Takahashi C, Shinose M, Kikuchi H. Genome sequence of an intestinal microorganism Streptomyces avermillos: deducing the ability of producing secondary metabolites. Proc Natl Acad Sci USA. 2001;98:12215–20.
2. Schulz B, Boyl C, Dreger S, Römert AK, Krohn K. Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol Res. 2002;106:996–1004.
3. Leffaive J, Ten‑Hage L. Algcal and cyanobacterial secondary metabolites in freshwaters: a comparison of allelopathic compounds and toxins. Freshw Biol. 2007;52:199–214.
4. Pichersky E, Gang DR. Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. Trends Plant Sci. 2000;5:439–45.
5. Forbey JS, Harvey AL, Huffman MA, Provenza FD, Sullivan R, Tasdemir D. Exploitation of secondary metabolites by animals: a response to homeostatic challenges. Integr Comp Biol. 2009;49:314–28.
6. Wallace RJ. Antimicrobial properties of plant secondary metabolites. Proc Nutr Soc. 2004;63:621–9.
7. Walsh CT. Polyketide and nonribosomal peptide antibiotics: modularity and versatility. Science. 2004;303:1805–10.
8. Gershenzon J, Dudareva N. The function of terpene natural products in the natural world. Nat Chem Biol. 2007;3:3408.
9. Deiml C, Rassinger A, Srebrotik E, Mach RL, Mach‑Aigner AR. Identification of the main regulator responsible for synthesis of the typical yellow pigment produced by Trichoderma reesei. Appl Environ Microbiol. 2016;82:6247–57.
10. Aerts D, Hauer EE, Ohm RA, Arenshorst M, Teertstra WR, Phippen C, Wösten HA. The FIBA-regulated predicted transcription factor Fum21 of Aspergillus niger is involved in fumonisin production. Antonie Van Leeuwenhoek. 2018;111:311–22.
11. Chooi YH, Cacho R, Tang Y. Identification of the vindicatumtoxin and griseofulvin gene clusters from Penicillium aethiopicum. Chem Biol. 2010;17:483–94.
12. Kontani M, Sakagami Y, Marumo S. First β-1,6-glucan biosynthesis inhibitor, bisvertinolide isolated from fungus, Acromonium striatum and its absolute stereochemistry. Tetrahedron Lett. 1994;35:2577–80.
13. Sugaya K, Kosihono H, Hongo Y, Yasunaga K, Ohse JJ, Yoshikawa K, Abe N. The biosynthesis of sorbicillinoids in Trichoderma sp. USF:2690: prospect for the existence of a common precursor to sorbicillinol and 5-epihydroxyvertinolide, a new sorbicillinoid member. Tetrahedron Lett. 2008;49:654–7.
14. Druzzhina IS, Kubicek EMI, Kubicek CP. Several steps of lateral gene transfer followed by events of birth and death evolution shaped a fungal sorbicillin biosynthetic gene cluster. BMC Evol Biol. 2016;16:269.
15. Balde EHS, Andolfi A, Bruyère C, Cimmino A, Lamoral‑Theys D, Vurro M, Damme MV, Almarea C, Mathieu V, Kiss R, Evidente A. Investigations of
fungal secondary metabolites with potential anticancer activity. J Nat Prod. 2010;73:969–71.
16. Abe N, Hirota A. Chemical studies of the radical scavenging mechanism of bisorbicillinol using the 1,1-diphenyl-2-picrylhydrazyl radical. Chem Commun. 2002;2002:662–3.
17. Peng J, Zhang X, Du L, Wang W, Zhu T, Gu Q, Li D. Sorbitin biosynthesis from the marine-derived fungus Penicillium chrysogenum PJX-17. J Nat Prod. 2014;77:424–8.
18. Maskey RP, Grun-Wollny I, Laatsch H. Sorbicillin analogues and related dimeric compounds from Penicillium notatum. J Nat Prod. 2005;68:865–70.
19. Salo O, Guzmán-Chávez F, Ries MI, Lankhorst PP, Bovenberg RA, Vreeken PJX. J Nat Prod. 2018;81:1499–508.
20. Gupta VK, Steindorff AS, de Paula RG, Silva-Rocha R, Mach-Aigner AR, Nakari-Setälä T, Aro N, Kalkkinen N, Alatalo E, Penttilä M. Genetic and biochemical characterization of the Trichoderma reesei hydrophobin HFB1. Eur J Biochem. 1996;235:248–55.
21. Seiboth B, Karimi RA, Phatale PA, Link R, Hartl L, Sauer DG, Kristina M, Guzmán-Chávez F, Mello-de-Sousa TM, Busse HJ, Driessen AJ. Identification of a polyketide synthase involved in sorbicillin biosynthesis by Penicillium chrysogenum. Appl Environ Microbiol. 2016;82:3971–8.
22. Jørgensen MS. Unraveling the secondary metabolism of the biotechno-technos. Microb Biotechnol. 2017;10:958–68.
23. Nakari-Setälä T, Aro N, Kalkkinen N, Alatalo E, Penttila M. Genetic analysis and finding significantly enriched Gene Ontology terms and sequence similarity to the plant expansins, exhibits disruption activity on cellulose materials. Eur J Biochem. 2002;269:402–7.
24. Portnoy T, Margeot A, Fekete E, Sándor E, Hartl L, Karaffa L, Druzhinina IS, Baker SE, The putative protein methyltransferase LAET controls cellulase gene expression in Trichoderma reesei. Mol Microbiol. 2012;84:1150–60.
25. Derntl C, Guzmán-Chávez F, Mello-de-Sousa TM, Busse HJ, Driessen AJ, Mach RL, Mach-Aigner AR. In vivo study of the sorbicillinogen gene cluster in Trichoderma reesei. Front Microbiol. 2017;8:2037.
26. Jørgensen MS. Unraveling the secondary metabolism of the biotechnological important filamentous fungus Trichoderma reesei (Teleomorph Hypoecia jeconina). Technical University of Denmark. 2013. p. 164.
27. Guzmán-Chávez F, Salo O, Nygård Y, Lankhorst PP, Bovenberg RA, Driessen AJ. Mechanism and regulation of sorbicillin biosynthesis by Penicillium chrysogenum. Microb Biotechnol. 2017;10:958–68.
28. Zhong YH, Wang XL, Wang TH, Jiang Q. Agrobacterium-mediated transformation (AMT) of Trichoderma reesei as an efficient tool for random insertional mutagenesis. Appl Microbiol Biotechnol. 2007;73:1348–54.
29. Minty JJ, Singer ME, Scholz SA, Bae CH, Ahn JH, Foster CE, Liao JC, Lin XN. Design and characterization of synthetic fungal–bacterial consortia for direct production of iso-butyral from cellulose biomass. Proc Natl Acad Sci USA. 2013;110:14592–7.
30. Ma L, Zhang J, Zou G, Wang C, Zhou Z. Improvement of cellulase activity in Trichoderma reesei by heterologous expression of a beta-glucosidase gene from Penicillium decumbens. Enzyme Microb Technol. 2011;49:366–71.
31. Derntl C, Kluger B, Bueschl C, Schuhmacher R, Mach RL, Mach-Aigner AR. Transcription factor Xpp1 is a switch between primary and secondary fungal metabolism. Proc Natl Acad Sci USA. 2017;114:5606–9.
32. Ali H, Ries M, Nijland JG, Lankhorst PP, Hankemeier T, Bovenberg RA, Vreeken RJ, Driessen AJ. A branched biosynthetic pathway is involved in production of roquefortine and related compounds in Penicillium chrysogenum. PLoS ONE. 2013;8:e65328.
33. Li C, Lin F, Li Y, Wei W, Wang H, Qin L, Zhou ZH, Li BZ, Wu F-G, Chen Z. A beta-glucosidase hyper-production in Trichoderma reesei mutant reveals a potential role of cel3D in cellulase production. Microb Cell Fact. 2016;15:151.
34. Li C, Lin F, Zhou L, Qin L, Li B, Zhou ZH, Jin MJ, Chen Z. Cellulase hyper-production by Trichoderma reesei mutant SEU7 on lactose. Biotechnol Biofuels. 2017;10:2288.
35. Boyle EI, Weng S, Gollub J, Jin H, Botstein D, Cherry JM, Sherlock G. GO-TermFinder—open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics. 2004;20:3710–5.
36. Rolloos M, Hooykaas M, Biehl J, Van Der Zaal BJ. Enhanced targeted integration mediated by translocated I-SceI during the agrobacterium mediated transformation of yeast. Sci Rep. 2015;5:8345.
60. Wiemann P, Sieber CM, von Bargen KW, Studt L, Niehaus EM, Espino JJ, et al. Deciphering the cryptic genome: genome-wide analyses of the rice pathogen Fusarium fujikuroi reveal complex regulation of secondary metabolism and novel metabolites. PLoS Pathog. 2013;9:e1003475.

61. Santamarta I, López-García MT, Kurt A, Nárdiz N, Álvarez-Álvarez R, Pérez-Redondo R, Martín JF, Liras P. Characterization of DNA-binding sequences for CcaR in the cephamycin–clavulanic acid supercluster of Streptomyces clavuligerus. Mol Microbiol. 2011;81:968–81.

62. Mast Y, Weber T, Götz M, Ort-Winklbauer R, Gondran A, Wohlleben W, Schinko E. Characterization of the pristinamycin supercluster of Streptomyces pristinaespiralis. Microb Biotechnol. 2011;4:192–206.

63. Kim SI, Veena, Gelvin SB. Genome-wide analysis of Agrobacterium T-DNA integration sites in the Arabidopsis genome generated under non-selective conditions. Plant J. 2007;51:779–91.

64. van Hattikum H, Bunduck P, Hooykaas PJJ. Non-homologous end-joining proteins are required for Agrobacterium T-DNA integration. EMBO J. 2001;22:6550–8.

65. Lin H, Wang Q, Shen Q, Ma J, Fu J, Zhao Y. Engineering Aspergillus oryzae A-4 through the chromosomal insertion of foreign cellulase expression cassette to improve conversion of cellulosic biomass into lipids. PLoS ONE. 2014;9:e108442.