Synthetic and Systems Biotechnology 9 (2024) 277–284

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology

Original Research Article

The forced activation of asexual conidiation in *Aspergillus niger* simplifies bioproduction

Xingyu Wu [a,1], Tingting Zhang [b,1], Ke Zhang [a], Rui Zhang [a], Man Shi [a], Chenlei Gu [a], Tianqiong Shi [a], Ling Lu [c], Feng Xue [a,***], Qing Xu [a,**], Chi Zhang [a,*]

[a] School of Food Science and Pharmaceutical Engineering, Nanjing Normal University, Nanjing, 210023, China
[b] College of Marine Life Sciences, Ocean University of China, Qingdao, 266003, China
[c] College of Life Sciences, Nanjing Normal University, Nanjing, 210023, China

ARTICLE INFO

Keywords:
Aspergillus niger
L-malic acid
Submerged conidiation
Simplify procedures
Reduce costs

ABSTRACT

Aspergillus niger is an efficient cell factory for organic acids production, particularly L-malic acid, through genetic manipulation. However, the traditional method of collecting *A. niger* spores for inoculation is labor-intensive and resource-consuming. In our study, we used the CRISPR-Cas9 system to replace the promoter of *brlA*, a key gene in *Aspergillus* conidiation, with a xylose-inducible promoter *xylP* in L-malic acid-producing *A. niger* strain RG0095, generating strain $brlA^{xylP}$. When induced with xylose in submerged liquid culture, $brlA^{xylP}$ exhibited significant upregulation of conidiation-related genes. This induction allowed us to easily collect an abundance of $brlA^{xylP}$ spores (>7.1 × 10^6/mL) in liquid xylose medium. Significantly, the submerged conidiation approach preserves the substantial potential of *A. niger* as a foundational cellular platform for the biosynthesis of organic acids, including but not limited to L-malic acid. In summary, our study offers a simplified submerged conidiation strategy to streamline the preparation stage and reduce labor and material costs for industrial organic acid production using *Aspergillus* species.

1. Introduction

Malic acid, a common four-carbon dicarboxylic acid, possesses an asymmetric carbon atom, resulting in three natural forms: D-malic acid, L-malic acid, and the D/L-malic acid mixture. It usually serves as an acidulant and flavor enhancer in the food and beverage industries [1]. Furthermore, malic acid has applications in cosmetics, pharmaceuticals, metal cleaning, textile finishing, and as an animal feed additive. The global malic acid production capacity currently stands at an estimated 80,000 to 100,000 tons per year. However, the annual market demand exceeds 200,000 tons, and this demand continues to rise steadily, indicating significant market potential [2,3]. Across all organisms, the predominant and physiologically relevant form of malic acid is L-malic acid, which plays a fundamental role as an intermediate in cellular metabolism. Currently, L-malic acid production primarily relies on the enzymatic hydration of fumarate, with the substrate fumarate sourced from maleic acid derived from nonrenewable petrochemicals. However, there is a growing interest in the biosynthesis of L-malic acid within microbial cells, using renewable sugars as a substrate. This approach shows promise as a sustainable and environmentally friendly alternative to the traditional enzymatic method [4].

The filamentous fungus *A. niger* ATCC 1015 excels as a cell factory for synthesizing various organic acids, including citric acid and gluconic acid. This is attributed to its heightened expression of elements related to electron transport, carbohydrate transport, and organic acid transport pathways [5]. Recently, genetic engineering has been used to enhance the reductive tricarboxylic acid (rTCA) pathway in ATCC 1015, leading to the accumulation of substantial amounts of L-malic acid. In addition, artificial strengthening of glucose uptake, glycolysis, and L-malic acid secretion pathways has been implemented, while pathways for oxalic acid synthesis and citric acid secretion have been deliberately blocked [6,7]. The final genetically modified strain, S1149, achieved an

Peer review under responsibility of KeAi Communications Co., Ltd.
* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: xuef2020@njnu.edu.cn (F. Xue), xu_qing@njnu.edu.cn (Q. Xu), zhangchi@njnu.edu.cn (C. Zhang).
[1] These authors contributed equally to this work and should be considered co-first authors.

https://doi.org/10.1016/j.synbio.2024.02.007
Received 23 October 2023; Received in revised form 22 February 2024; Accepted 28 February 2024
Available online 4 March 2024

impressive L-malic acid production of up to 201 g/L in an 8-day fed-batch fermentation. Notably, the L-malic acid/glucose ratio reached 1.64 mol/mol, setting a record for the highest yield to date [6]. Optimization of fermentation parameters, including medium composition, pH, inoculum amount, and stirring speed has been conducted. However, for large-scale industrial fermentation, substantial preparatory work is necessary before filling the fermenter. This includes the cultivation of significant quantities of fresh *A. niger* asexual spores on solid media, which consumes considerable time, cost, and labor. Therefore, there's a need to explore methods for controlling asexual development and facilitating the accumulation of spores more efficiently. The life cycle of *Aspergillus* typically involves two main phases: hyphal growth and asexual reproduction. A precise and well-timed transition between these phases is essential for the fungus's long-term survival, successful reproduction, efficient dissemination, and overall biomass increase [8–10].

Aerial hyphae of *Aspergillus* typically give rise to asexual conidiophores and initiate the asexual reproductive process when they encounter conditions of nutrient scarcity and exposure to oxygen [11,12]. However, under submerged liquid culture conditions, *Aspergillus* tends to undergo continuous hyphal growth, with asexual conidiation occurring infrequently [13]. The mechanisms governing asexual conidiation have been extensively studied in several *Aspergillus* species, with particular emphasis on the model fungus *Aspergillus nidulans* [14–19]. In *Aspergillus*, a complex regulatory network controls conidiation. This network consists of central regulators, upstream developmental activators, and negative regulators. The central regulatory pathway involves the C2H2 zinc finger transcription factor BrlA and its downstream targets, AbaA and WetA (BrlA→AbaA→WetA). This pathway plays a pivotal role in controlling the spatial and temporal expression of conidiation-related genes during the process of conidiogenesis [20]. Importantly, in submerged liquid culture, inducing the expression of *brlA* in the hyphae of *Aspergillus fumigatus* can trigger asexual conidiation and halt further growth [21]. This finding suggests that by controlling asexual conidiation in industrial filamentous fungi, there is potential to streamline the preparatory work required before fermentation, leading to potential efficiency gains in industrial processes.

In this study, we introduced a microhomology-mediated CRISPR-Cas9 system that can effectively edit genes of *A. fumigatus*and*A. nidulans* dependent on short homology arm of about 35 bp [22], into the organic acid-producing *A. niger*. This system enabled precise editing of target genes with an impressive 100% efficiency rate. We conditionally expressed *brlA* in the L-malic acid-producing *A. niger* strain, RG0095, by utilizing the xylose-inducible *xylP* promoter [23] and assessed the submerged conidiation and L-malic acid fermentation performance of the conditional *brlA* strain, denoted as $brlA^{xylP}$. In a liquid medium with xylose as the sole carbon source, the $brlA^{xylP}$ strain produced significant amounts of asexual conidia. Crucially, the submerged conidiation strategy did not abolish the high-yield potential of *A. niger* to produce L-malic acid. In summary, our study offers a promising strategy that has the potential to significantly reduce both the time and costs during the preparatory stage before commencing acid-producing fermentation in *A. niger*.

2. Materials and methods

2.1. Strains and media

A high L-malic acid-yielding *A. niger* strain, RG0095 (CCTCC M20221573), was preserved in the China Centre for Type Culture Collection (CCTCC) and served as the parental wild-type strain. The genetically engineered *A. niger* strains used in this study are listed in Table 1. Typically, *A. niger* was cultured on or in the following media, potato-dextrose agar (PDA) medium with or without 5 mM uridine and 10 mM uracil; glucose/xylose medium (GM/XM) containing 1% glucose/D-xylose, 2% agar, 50 mL/L 20 × salt solution and 1 mL/L trace elements [24]; potato dextrose (PD) medium containing 0.6% potato extract and 2% glucose; seed medium containing 4% glucose, 0.6% Bacto peptone, 0.075% K_2HPO_4, 0.075% KH_2PO_4, 0.01% $CaCl_2{\cdot}2H_2O$, 0.01% $MgSO_4{\cdot}7H_2O$, 0.0005% $FeSO_4{\cdot}7H_2O$, and 0.0005% NaCl; fermentation medium containing 10% glucose, 0.6% Bacto peptone, 0.015% K_2HPO_4, 0.015% KH_2PO_4, 0.01% $CaCl_2{\cdot}2H_2O$, 0.01% $MgSO_4{\cdot}7H_2O$, 0.0005% $FeSO_4{\cdot}7H_2O$, and 0.0005% NaCl, and also containing 80 g/L insoluble $CaCO_3$.

Table 1
Strains used in this study.

Strains	Genotype	Source
RG0095	Δ*oahA, pyc, mdhC, Pgpd-C4T318*	CCTCC M20221573 [25]
Δ*pyrG*	Δ*oahA, pyc, mdhC, Pgpd-C4T318*, Δ*pyrG*	This study
$RG0095^{pFC330}$	Δ*oahA, pyc, mdhC, Pgpd-C4T318, Ptef-Cas9::pyrG* (pFC330)	This study
$brlA^{xylP\ pFC330}$	Δ*oahA, pyc, mdhC, Pgpd-C4T318, Ptef-Cas9::pyrG* (pFC330), *xylP-brlA::hph*	This study
$brlA^{xylP}$	Δ*oahA, pyc, mdhC, Pgpd-C4T318, xylP-brlA::hph, pyrG*	This study

2.2. Plasmid preparation

We obtained the two self-replicating Cas9-expressing plasmids, pFC330 (Addgene, #87842) with a *pyrG* (orotidine-5′-decarboxylase gene) selection marker, and pFC332 (Addgene, #87845) with a *hph* (hygromycin-resistance-encoding gene) selection marker, from Addgene. The promoter (*xylP*) of the xylanase-encoding gene (PCH_Pc20g07020) amplified with primer pairs F/R from *Penicillium chrysogenum* genome was integrated into a *hph*-containing plasmid p-zero-*hph* [26], generating p-zero-*hph-xylP*. All primers are listed in Table 2.

2.3. Gene editing for pyrG deletion and brlA promoter substitution

Strains (Δ*pyrG* and $brlA^{xylP}$) were constructed by the CRISPR-Cas9 system as described previously [22,24]. For the deletion of the *pyrG* gene (ASPNIDRAFT_128428), a co-transformation was performed using pFC332 and an in vitro synthesized sgRNA targeting *pyrG*. This was achieved through PEG4000-mediated protoplast transformation [24], with the added selection pressures of 5-Fluoroorotic acid (5-FOA, Sangon Biotech, SA601555, Nanjing, China) and hygromycin B (Sangon Biotech, A600230, Nanjing, China) resulting in the creation of the Δ*pyrG* strain. The final strain, $brlA^{xylP}$, was obtained by removing pFC330 under the stress of 5-FOA and introducing a *pyrG* fragment for complementation. To ensure the accuracy of the genetic modifications, all strains underwent verification through sequencing and/or diagnostic PCR targeting the relevant editing genes.

2.4. Quantitative real-time PCR (qPCR) analysis

The strains were cultured in liquid GM at 28 °C for 24 h and then transferred to new GM or XM for another 24 h. We extracted total RNA from the resulting mycelium pellets using the UNlQ-10 Column TRIzol Total RNA Isolation Kit (Sangon Biotech, B511361, Nanjing, China). Reverse transcription was performed using the HiScript Q RT SuperMix for qPCR Kit (Vazyme, R123-01, Nanjing, China). Subsequently, qPCR was conducted using AceQ qPCR SYBR Green Master Mix (Vazyme, Q111-02, Nanjing, China) and a QuantStudio 3 Real-PCR Instrument (Applied Biosystems, USA). Transcript levels of the target genes were determined through the comparative threshold cycle (ΔCT) method and normalized against the level of *tubA* mRNA. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method. All the qPCR primer pairs are given in Table 2.

Table 2
Primers used in this study.

Name	Sequence	Intention
T7-pyrG-sgRNA-F	TAATACGACTCACTATAGGGACCGAGACCTGCAGTCGTTTTAGAGCTAGAAATAGCA	for the DNA template of *pyrG*-sgRNA (RNA); for mutating *pyrG*
pyrG-seq-F	CAGGGAGCTCTCTGGGGCTTG	diagnostic primer for *pyrG* mutant; for amplifying *pyrG*
pyrG-seq-R	ACTTTCGGAAGTCCCGTATTTCTGCTG	diagnostic primer for *pyrG* mutant; for amplifying *pyrG*
zero-xylP-F	AATTAACCCTCACTAAAGGGCTGCAGTTGGTTCTTCGAGTC	for amplifying the *xylP* promoter
zero-xylP-R	TTAAACCTGCAGGACTAGTCTGCAGGCGGCCGCCGACGG	for amplifying the *xylP* promoter
zero-R	CCCTTTAGTGAGGGTTAATTCTG	for linearizing the p-zero-*hph*
zero-F	ACTAGTCCTGCAGGTTTAAACGAATTG	for linearizing the p-zero-*hph*
T7-brlA-sgRNA-F	TGCTATTTCTAGCTCTAAAACTTGCGCCTTGCCACATTCCCTATAGTGAGTCGTATTA	for the DNA template of *brlA*-sgRNA (RNA); for constructing $brlA^{xylP}$
brlA-xylP-F	TCCACCGTCTCTCACTTGTATATCGCCGCCGTTGGAATTTAGCGGCCGCGAATTGG	for the repair template of constructing $brlA^{xylP}$
brlA-xylP-R	GGTCAGGCGATCGGACATGTTACCCTGGGATCTCATCTGCAGTTGGTTCTTCGAGTC	for the repair template of constructing $brlA^{xylP}$
brlA-seq-F	GCTTGCTTCCCCTCTGCAGAG	diagnostic primer for *brlA* mutant
brlA-seq-R	GTTCCGGTATGCCTTTCATAGC	diagnostic primer for *brlA* mutant
brlA-RT-F	TCCATGGCTTCCAGCTTCTC	qPCR primer
brlA-RT-R	GTTCCGGTATGCCTTTCATAGC	qPCR primer
abaA-RT-F	CGTTTCAACAAGCTCTCGAGG	qPCR primer
abaA-RT-R	GGAGGTGGCTGGAGACTTGC	qPCR primer
wetA-RT-F	CAGTACGTCAATATGGACAGC	qPCR primer
wetA-RT-R	TGGGGTTGCGCTGTTGACGTG	qPCR primer
fwnA-RT-F	TGCAATTCGTCAAGAGATC	qPCR primer
fwnA-RT-R	CCGTAAAAGTGAATGAAACAACC	qPCR primer
rodA-RT-F	ACAACGCCCAGCTCTCTTGC	qPCR primer
rodA-RT-R	AGGTCAAGGGCCTGGAGGTC	qPCR primer
TubA-RT-F	CTCCGAGACTGGTCAGGGCAAG	qPCR primer
TubA-RT-R	GTTCGAGGCATCCTCCTTGC	qPCR primer

2.5. Microscopy observation

To observe submerged conidiation under a microscope, fresh spores or hyphae were initially cultured in liquid GM at 28 °C with continuous shaking at 200 rpm for 20 h. Subsequently, they were subjected to treatment with either new GM or XM for 3 or 5 days. The resulting mycelial pellet was then placed between a microscope slide and coverslip and observed directly using a Zeiss Axio Imager A1 microscope (Zeiss, Jena, Germany).

2.6. Shake flask cultivation

For L-malic acid fermentation, 1×10^8 fresh spores were cultivated in a 50 mL seed medium inside a 250-mL Erlenmeyer flask at 28 °C with continuous shaking at 200 rpm for 20 h. Subsequently, the L-malic acid fermentation process was carried out in 250-mL Erlenmeyer flasks, each containing 50 mL of fermentation medium and 10% (v/v) seed culture. This fermentation was conducted at 28 °C with continuous shaking at 200 rpm for a duration of 6 days.

2.7. Measurement of L-malic acid

For the fermentation process, we set up 18 parallel shake flasks for each strain. Every day, three shake flasks were sampled to measure the L-malic acid content. In this sampling process, 2 mL of the fermentation mixture was transferred into a 50-mL tube, and the same volume of 2 M hydrochloric acid (HCl) was used for sample pretreatment. The mixture was then subjected to centrifugation at 12,000 g for 15 min. After centrifugation, the supernatant was diluted with ultrapure water and filtered through a 0.22-μm filter membrane for subsequent analysis using high-performance liquid chromatography (HPLC). The analysis was performed with a Kromasil 100-5-C8 column and an ultraviolet (UV) detector set at 214 nm. The mobile phase used in the chromatography consisted of 90% phase A (0.1% phosphoric acid) and 10% phase B (methanol) at a flow rate of 0.85 mL/min and a temperature of 40 °C.

3. Results

3.1. Replacing the native promoter of brlA with xylose-induced promoter xylP

In order to control the asexual development of *A. niger*, the MMEJ-CRISPR system established in *A. fumigatus* [22] was used to target the gene *brlA*, encoding a pivotal transcriptional regulator of the central conidiation pathway. To initiate the editing process of the *brlA* gene, we introduced the Cas9-expressing plasmid pFC330, which includes a *pyrG* selection marker and can autonomously replicate in *Aspergillus* species, into a high L-malic acid-yielding strain Δ*pyrG* (RG0095 with uridine auxotroph), generating the Cas9-expressing recipient strain, $RG0095^{pFC330}$. Then, we designed a sgRNA targeting the native *brlA* promoter, termed *brlA*-sgRNA, which was synthesized via in vitro transcription (Fig. 1A). Concurrently, the repair template containing a fused fragment of *hph* (a selective marker) and *xylP* promoter with two short homology arms (34-bp and 37-bp) proximally to the PAM site and initiation codon region, respectively, was amplified by PCR. Then, the *brlA*-sgRNA and repair template were co-transformed into $RG0095^{pFC330}$, generating the $brlA^{xylP}$ ^{pFC330} strain. As illustrated in Fig. 1B and C, the resulting transformants displayed a pigment-less albino phenotype and were incapable of forming asexual conidiogenous structures/conidiophores on solid PDA medium in the absence of the xylose. This observation aligns with findings reported for a conditional mutant of *A. fumigatus brlA* controlled by the Tet-on/off system (Stewart et al., 2020). Further confirmation through diagnostic PCR (Fig. 1D) demonstrated that all isolated albino transformants possessed the expected cleavage sites in the *brlA* locus, along with the insertion of the *hph* gene and *xylP* promoter.

3.2. Xylose can activate the conidiation pathway in $brlA^{xylP}$ mutant

To eliminate any potential interference from Cas9 in subsequent experiments, we removed the plasmid pFC330, which contains the *cas9* and *pyrG* genes, from the $brlA^{xylP}$ ^{pFC330} strain under the stress of 5-FOA. Subsequently, we reintroduced a native ATCC 1015 *pyrG* gene into the *pyrG*-deficient strain, resulting in the creation of the final strain, $brlA^{xylP}$. Under submerged culture conditions, *Aspergillus* typically does not undergo conidiation, and the expression of conidiation-related genes is generally very low [13,27]. To investigate whether forced *brlA*

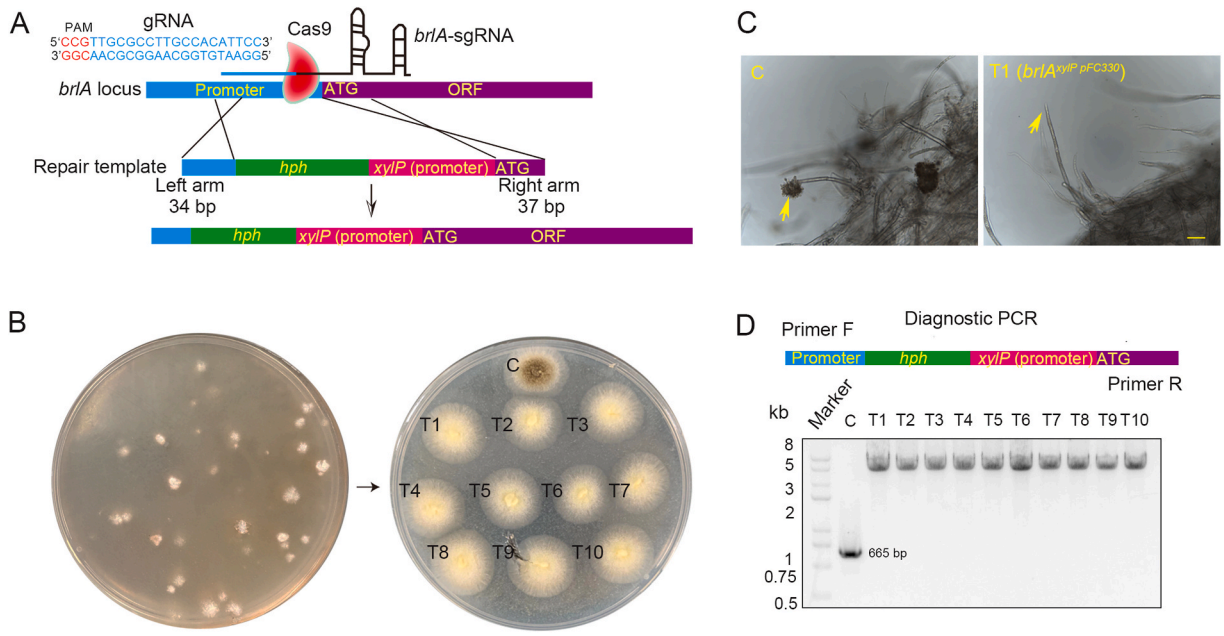

Fig. 1. Replacement of *brlA* promoter with *xylP* promoter. A. This schematic illustrates the replacement of the native *brlA* promoter with the induced *xylP* promoter using the MMEJ-CRISPR system. The Cas9-sgRNA complex targets the start codon region of the *brlA* gene. B. (Left) Transformants were generated on the transformation plates after introducing *brlA*-sgRNA and the corresponding repair template into the Cas9-expressing recipient strain (RG0095^{pFC330}) for 4 days. All resulting transformants displayed the same phenotype. Ten randomly selected transformants (T1-T10) were inoculated onto PDA using hyphae and cultured for 4 days. (Right) RG0095^{pFC330} was used as a control (C). C. The asexual conidiation of the related strains on PDA was observed. Two arrows indicate the conidiogenous structure of RG0095^{pFC330} and the hyphae tip of *brlA*^{xylP} ^{pFC330} (without a conidiogenous structure), respectively. Scale bar represents 10 μm. D. Diagnostic PCR was conducted to verify the integration of the repair template into the *brlA* promoter.

expression under the control of the *xylP* promoter can activate the central regulatory pathway of conidiation, vegetative/pregrown hyphae from both the *brlA*^{xylP} and RG0095 strains were inoculated into liquid GM. After 24 h of cultivation, the resulting mycelium pellets were transferred into either new GM or XM for an additional 24 h (Fig. 2A). The qPCR analysis involved normalizing the mRNA abundance of the target genes to *tubA*, a housekeeping gene encoding α-tubulin that exhibited stable expression across the tested conditions (Fig. 2B). It was observed that under xylose-induced conditions in the *brlA*^{xylP} strain, but not in RG0095, the expression levels of *brlA* and its downstream target genes *abaA* and *wetA* (which encode the other two components of the central conidiation regulatory pathway) exhibited increased levels (Fig. 2C and D). Typically, the asexual conidiation of *Aspergillus* species is characterized by the synthesis and accumulation of melanin and hydrophobin, which are involved in covering the conidial surface [28, 29] (Fig. 2E). The qPCR analysis revealed that the genes encoding hydrophobin (*rodA*) and polyketide synthase (*fwnA*), involved in the synthesis of dihydroxynaphthalene (DHN) melanin, exhibited significant upregulation in *brlA*^{xylP} after xylose treatment (Fig. 2F). As a control, RG0095 exhibited consistently low expression levels of these genes, whether cultured in GM or XM, and these expression levels were comparable to those observed in *brlA*^{xylP} when grown in GM. This upregulation of these genes in *brlA*^{xylP} strongly indicates the activation of the conidiation pathway.

3.3. Xylose can induce the submerged conidiation in *brlA*^{xylP}

The formation of asexual conidia is a highly intricate process that requires a considerable amount of time for the preparation of components and the assembly of conidiophores after receiving the conidiation signaling [30]. To investigate whether forced *brlA* expression induced by xylose could trigger submerged conidiation in *brlA*^{xylP}, we extended the induction time in liquid XM in a shaker for 3 days. As depicted in Fig. 3A, only the xylose-treated *brlA*^{xylP} strain exhibited macroscopically visible black cultures, indicating the occurrence of sporulation. Correspondingly, microscopic observation of these cultures confirmed the presence of numerous spores in the xylose-treated *brlA*^{xylP}, while RG0095 and untreated *brlA*^{xylP} cultures displayed hyphae only (Fig. 3B). In a typical scenario, *Aspergillus* develops a complex conidiophore atop hyphae during asexual sporulation on solid media, including foot cells, stalk, vesicle, phialides, immature conidia, and mature conidia. However, *brlA*^{xylP} didn't form typical conidiophores in liquid XM. Instead, it generated conidia by creating distinct spherical budding structures at the hyphal tips (Fig. 3B). To quantify the conidiation capacity of *brlA*^{xylP}, 1×10^8 *brlA*^{xylP} conidia were initially grown in 50 mL of PD medium (rich medium) for 1 day, and then 5 mL of the resulting culture mixture was transferred into 50 mL of liquid XM with various concentration of xylose (0.5%, 1%, 1.5%, 2% and 3%) for 5 days. After shaking the final culture mixture with an oscillator and counting the separated conidia (Fig. 3C), it was determined that more than 7.1×10^6/mL *brlA*^{xylP} conidia could be obtained in all xylose media tested on the last day, with the highest yield of 7.4×10^6 obtained in 2% xylose media (Fig. 3D). Notably, the xylose dosage has no significant influence on the production of *brlA*^{xylP} conidia. In contrast, the RG0095 strain can generate 7.3×10^6/mL spores on solid PDA medium for 5 days, demonstrating that the liquid-induced sporulation strategy does not sacrifice spore quantity. In summary, the system of xylose-mediated submerged conidiation was successfully established in *A. niger*.

3.4. *brlA*^{xylP} retains the potential for abundant synthesis of L-malic acid

Although gene-modified *brlA*^{xylP} can produce conidia in submerged culture, it was unclear how well these conidia would perform in the fermentation production of L-malic acid. Initially, 1×10^8 *brlA*^{xylP} conidia were cultured in 50 mL of seed medium for 20 h. Subsequently, the seed culture was transferred into fermentation media for acid production (Fig. 4A). For comparison, the conidia of RG0095 grown on solid PDA were cultured using the same two-step system. Residual glucose monitoring indicated that both *brlA*^{xylP} and RG0095 consumed all the glucose by the sixth day (Fig. 4B), suggesting that submerged

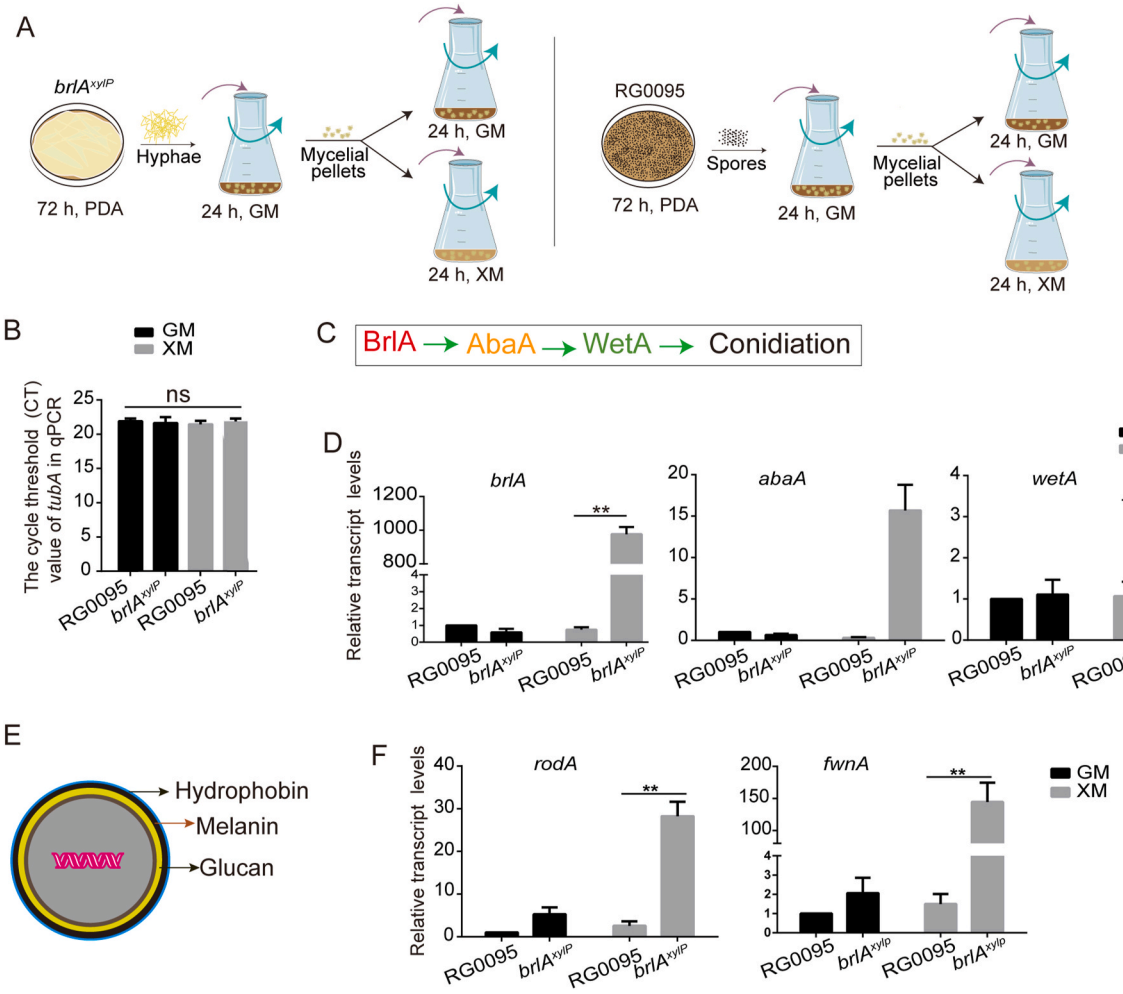

Fig. 2. Expression analysis of conidiation pathway-related genes in RG0095 and *brlA*^{xylP} Strains. A. Schematic diagram illustrating the xylose induction experiment. The related strains were cultured on solid PDA for 72 h. The harvested spores or mycelia were inoculated into liquid GM for 24 h, and then the resulting mycelial pellets were transferred into new liquid GM or XM for an additional 24 h. C. The central genetic model for asexual conidiation in *Aspergillus*. B, D, and F. RG0095 spores/*brlA*^{xylP} hyphae were grown in a liquid GM for 24 h and then transferred into a new GM or XM for another 24 h qPCR was performed to determine the relative transcript levels of target genes. Data represent three biological replicates. All tested samples displayed no significant differences in the expression of the housekeeping gene *tubA*, which was verified using the cycle threshold (CT) values (B). mRNA abundance of target genes was normalized to *tubA* and relative to RG0095 in GM (D and F). Values represent the mean ± SD of three replicates. Statistical significance was determined using Student's *t*-test. ns, not significant. **, $p < 0.01$. E. Schematic diagram illustrating the outer structures of *Aspergillus* conidia.

conidiation did not affect the fermentation progress. In addition, the mycelial pellets of *brlA*^{xylP} were observed to be larger in size than those of RG0095 in the fermentation broth (Fig. 4C–E), indicating that the submerged conidiation strategy influences the morphological development of *A. niger*. In terms of organic acid production, RG0095 produced as much as 67.0 g/L of L-malic acid, along with significant amounts of by-products, including 21.0 g/L of citric acid, 1.7 g/L of fumaric acid, and 6.0 g/L of succinic acid (Fig. 4F–I). In contrast, *brlA*^{xylP} accumulated 57.9 g/L of L-malic acid, along with 27.5 g/L of citric acid, 1.2 g/L of fumaric acid, and 6.7 g/L of succinic acid. The desired product L-malic acid in *brlA*^{xylP} reached 86% of that in RG0095, indicating that *brlA*^{xylP} spores produced in liquid culture retained a high level of yield for L-malic acid. Overall, submerged conidiation of *A. niger* shows promise as a replacement for traditional conidiation on solid media in the preparatory work for L-malic acid biosynthesis.

4. Discussion

L-malic acid, a versatile acidulant and bio-based building block chemical, has traditionally been produced using petroleum-derived substrates through chemical or enzymatic methods [4]. Attention has been directed towards the production of L-malic acid through microbial fermentation, primarily because of its relatively eco-friendly characteristics. In recent years, several microorganisms, including *A. niger*, *Aspergillus oryzae*, *Saccharomyces cerevisiae*, *Myceliophthora thermophila*, and *Ustilago trichophora*, have been genetically modified to yield substantial quantities of L-malic acid in laboratory settings [1]. *A. niger* is an industrial species commonly used as a chassis cell for the commercial production of citric acid. It possesses several key desirable traits, such as high conversion efficiency from sugar to organic acid, a well-defined genetic background, tolerance to low pH, and the ability to utilize inexpensive and renewable carbon sources [31,32]. Additionally, a strain achieving the highest L-malic acid yield to date was developed through multiple rounds of rational genetic engineering in *A. niger* [6]. This underscores the significant potential of *A. niger* for large-scale L-malic acid production. However, despite this potential, industrial microbial fermentation of L-malic acid has not been fully established due to its high input-output ratio. To address this challenge from an industrial standpoint, the focus should be on: 1) optimizing strains, 2) upgrading equipment, and 3) streamlining technological processes.

A. niger produces small hydrophobic asexual conidia/spores as the primary means of reproduction, survival, and propagation. Generally,

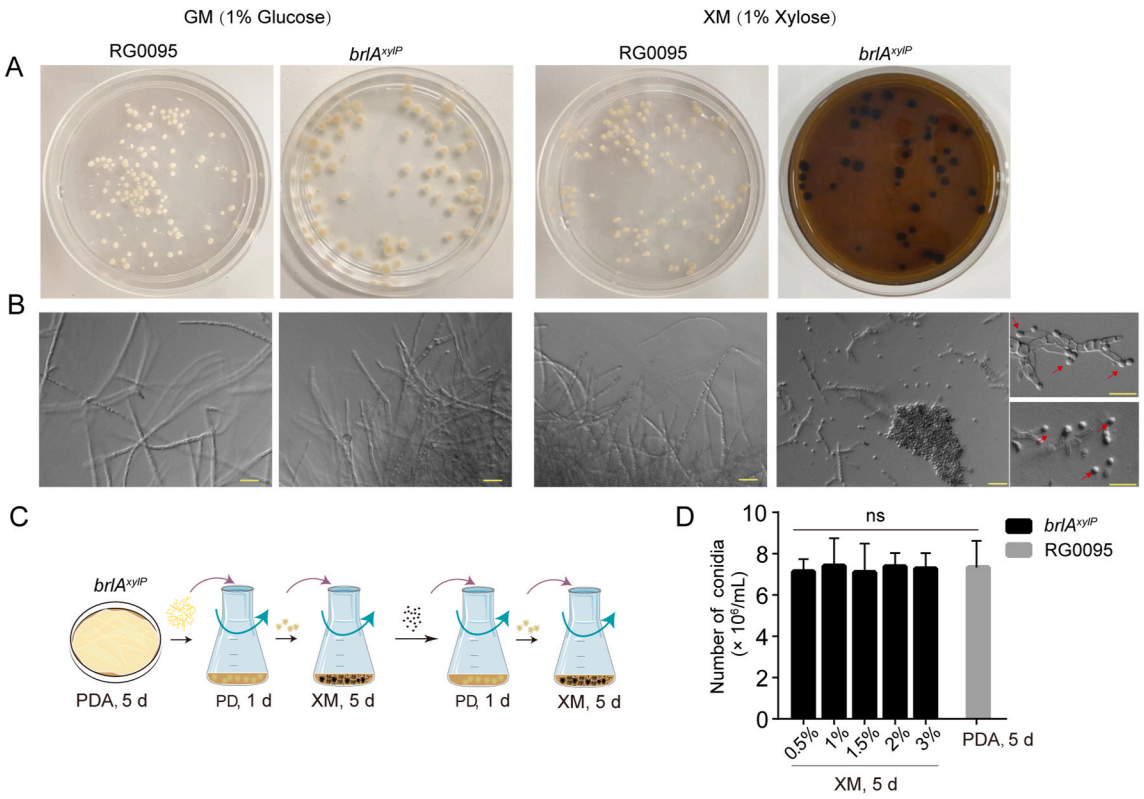

Fig. 3. Asexual conidiation of *brlA*^{xylP} strain in liquid xylose medium. A and B. The harvested spores (RG0095) or mycelia (*brlA*^{xylP}) of the related strains grown on solid PDA were inoculated into liquid GM and cultured in a 250-mL Erlenmeyer flask for 1 day. Subsequently, the resulting mycelial pellets were transferred into new liquid GM or XM for 3 days. The final mycelial pellets were transferred to a Petri dish for macroscopic observation (A). The microscopic phenotypes of mycelial pellets were observed using a microscope (B). Scale bar represents 10 μm. Red arrows indicate spores. C. Schematic diagram illustrating the xylose induction experiment for determining the sporulation ability of *brlA*^{xylP}. D. The number of *brlA*^{xylP} spores produced in 1 mL submerged liquid XM with various xylose dosage and the number of RG0095 conidia produced using on solid PDA with a 5 mm thickness. Statistical significance was determined using Student's t-test. ns, not significant.

the asexual conidiation of *A. niger* only occurs when its vegetative mycelia are exposed to air. Before fermentation, a large number of conidia were required. Hence, it would take a lot of time and money to prepare enough solid media and collect the conidia grown on solid medium, especially for large-scale industrial production. At least 5 days would be spent on medium preparation and plate smearing of parental spores to collect offspring spores for the high L-malic acid-yielding background strain RG0095. In order to simplify this process, with the help of CRISPR-mediated gene editing, we confer the *A. niger* an ability to reproduce asexually in liquid submerged condition by forcibly activating the key central transcriptional regulator of conidiation (*brlA*) under the control of xylose-induced promoter *xylP*. Although specific induction parameters have not been optimized, up to 7.4×10^8 conidia can be produced in 100 mL liquid xylose medium with 2% xylose, which meets the inoculation requirement for 3.7-L fermentation broth. However, the current induction strategy (transfer from non-xylose medium to xylose medium) remains relatively tedious in feasibility operation, and a more direct strategy is needed. A phenomenon of carbon catabolite repression (CCR) is common in most organisms to enable preferential utilization of easily metabolizable carbon sources [33]. Generally, the preferred carbon source glucose would prevent non-preferred carbon sources, such as xylose, from being utilized, which has already been proven in many microorganisms, including *E. coli* [34], *S. cerevisiae* [35], *A. nidulans* [36], etc. Based on CCR, we can use a mixed carbon source medium with different ratios of glucose and xylose to treat the *brlA*^{xylP} strain. In this process, *brlA*^{xylp} primarily utilizes glucose for vegetative growth. Upon glucose depletion, the resulting mycelium is prompted by xylose to undergo asexual conidiation, eliminating the necessity of transferring the mycelium to a xylose medium.

In addition to the target product L-malic acid, the RG0095 background strain synthesizes relatively abundant citric acid, small amounts of succinic acid as well as trace amounts of fumaric acid. Under the condition that only the above four acids are considered, *brlA*^{xylP} and RG0095 have similar total sugar-acid productivity (0.933 vs. 0.957, g acid/g glucose), indicating the submerged conidiation has no effect on the organic acid conversion rate of *A. niger*. Although statistical analysis showed no difference between *brlA*^{xylP} and RG0095 in yields of all the four tested organic acids, the desired product L-malic acid and the main by-product citric acid in *brlA*^{xylP} decreased by 14% and increased by 31%, respectively, compared with that in RG0095, demonstrating that a higher proportion of carbon sources enter mitochondria to synthesize citric acid. Plausible explanations for this alteration could be the nature of submerged conidiation and/or the unnatural expression of *brlA*. However, further genetic engineering is necessary to facilitate the successful bio-manufacture of L-malic acid.

Liu et al., have reported that deleting the citrate transporter-encoding gene *cexA* could reduce citric acid accumulation [6]. Therefore, subsequent knockout of *cexA* in *brlA*^{xylP} could be considered to further improve L-malic acid proportion. It has been reported that *cexA* deletion impedes the glycolysis pathway, causing an inadequate supply of raw material—pyruvate. Hence, overexpressing the key enzymes of glycolysis becomes an essential step. Genetic modifications to the cytoplasmic rTCA pathway enable certain yeasts or filamentous fungi to produce over 200 g/L of L-malic acid, usually in fed-batch fermentation setups [6,37,38]. However, these methods overlook the role of mitochondria in L-malic acid synthesis. Enhancing fumarase expression in the mitochondria, responsible for catalyzing fumaric acid conversion into L-malic acid, may further enhance the efficiency of L-malic acid

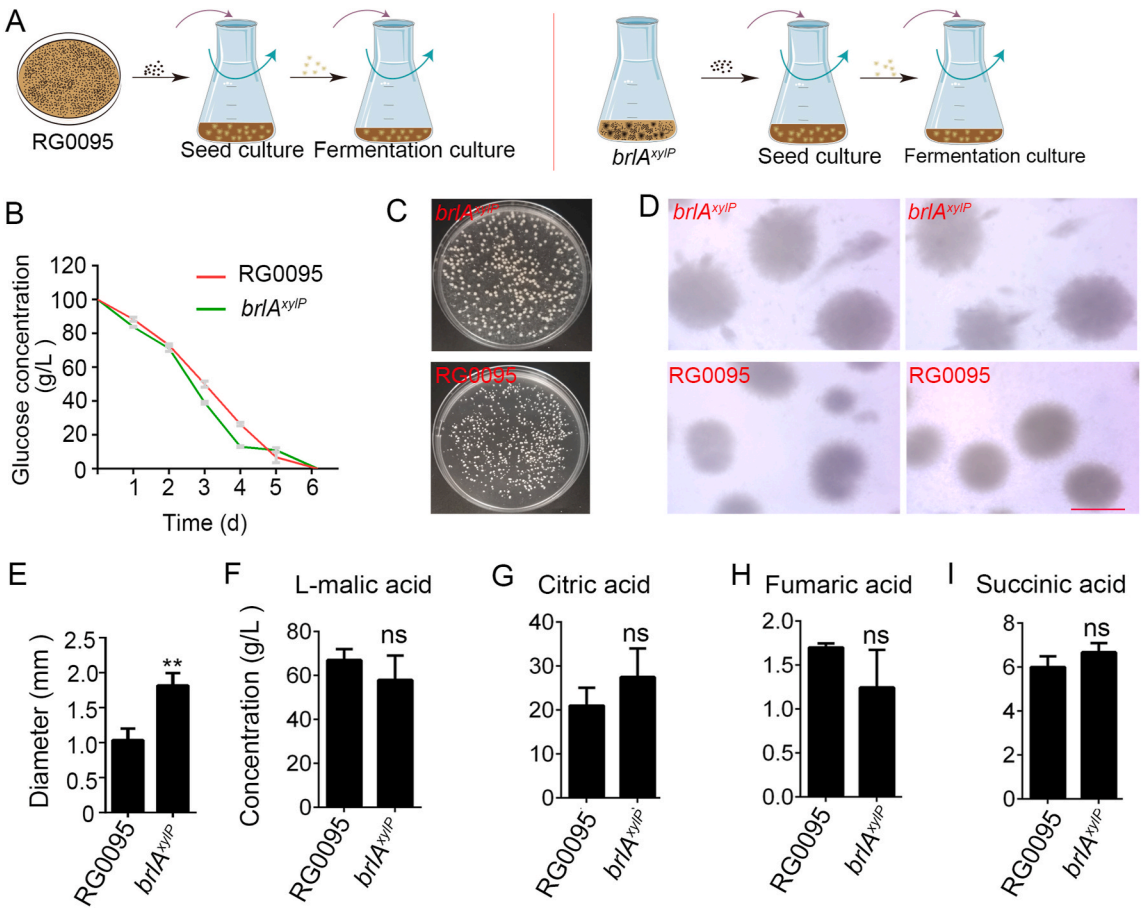

Fig. 4. The titers of various organic acids produced by the indicated strains in shake flasks for 6 days. A. Illustration of *A. niger* fermentation process for L-malic acid synthesis. B. Residual glucose concentrations in *brlA*^{xylP} and RG0095 cultures. C-E. After the completion of fermentation, the resulting mycelial pellets were subjected to three washes with 0.5 M HCl. Subsequently, they were observed and compared visually using the naked eye (C) and a stereomicroscope (D). The bar is 1 mm. The diameters of mycelia pellets were compared between RG0095 and *brlA*^{xylP} (E). F–I. Titers of L-malic acid, citric acid, fumaric acid, and succinic acid on the sixth day. Statistical significance was determined using Student's t-test. "ns" indicates no significance ($p > 0.05$). **, $p < 0.01$.

production. The use of the submerged conidiation strategy results in the volumetric expansion of mycelium pellets in *A. niger*. Nonetheless, the internal regions of larger pellets exhibit limited growth and metabolism, attributed to inadequate oxygen diffusion, potentially constraining product formation [39]. Consequently, editing morphology-related genes is likely to enhance L-malic acid production. On the other hand, the submerged conidiation strategy may achieve better performance when used to specifically synthesize citric acid.

It's important to highlight that the submerged conidiation strategy presents several significant advantages. These include cost savings in culture media, simplified inoculation, convenient spore collection, and reduced contamination risks when compared to the traditional conidiation method using solid media, which necessitates substantial agar usage and substantial human resources. However, this strategy could potentially impact the inherent characteristics of the spores, including their robustness, stress resistance, and hydrophobicity. Consequently, the resulting spores may become ineffective in efficiently synthesizing various target products. In summary, this study offers a novel approach for utilizing *A. niger* in large-scale industrial fermentation processes.

4.1. Ethical statements

This article does not contain any studies with human participants performed by any of the authors.

Declaration of competing interest

The authors declare no conflict of interest.

4.2. Data Availability Statement

All data are publicly available.

CRediT authorship contribution statement

Xingyu Wu: conceived and designed the research, performed the experiments and analyzed the data, wrote the manuscript, All authors read and approved the manuscript. **Tingting Zhang:** conceived and designed the research, performed the experiments and analyzed the data, wrote the manuscript, All authors read and approved the manuscript. **Ke Zhang:** performed the experiments and analyzed the data. **Rui Zhang:** performed the experiments and analyzed the data. **Man Shi:** performed the experiments and analyzed the data. **Chenlei Gu:** performed the experiments and analyzed the data. **Tianqiong Shi:** conceived and designed the research. **Ling Lu:** conceived and designed the research, wrote the manuscript, All authors read and approved the manuscript. **Feng Xue:** conceived and designed the research. **Qing Xu:** conceived and designed the research. **Chi Zhang:** conceived and designed the research, performed the experiments and analyzed the data, wrote the manuscript, All authors read and approved the manuscript.

Acknowledgements

This work was financially supported by the National Key Research and Development Program of China (2021YFC2104300), the National Natural Science Foundation of China (32200055 and 22378210), and the Natural Science Foundation of Jiangsu Province (BK20202002).

References

[1] Wei Z, Xu Y, Xu Q, Cao W, et al. Microbial biosynthesis of L-malic acid and related metabolic engineering strategies: advances and prospects. Front Bioeng Biotechnol 2021;9:765685.
[2] Dai Z, Zhou H, Zhang S, Gu H, et al. Current advance in biological production of malic acid using wild type and metabolic engineered strains. Bioresour Technol 2018;258:345–53.
[3] Kovilein A, Kubisch C, Cai LY, Ochsenreither K. Malic acid production from renewables: a review. J Chem Technol Biot 2020;95:513–26.
[4] Wu N, Zhang J, Chen Y, Xu Q, et al. Recent advances in microbial production of L-malic acid. Appl Microbiol Biotechnol 2022;106:7973–92.
[5] Pel HJ, de Winde JH, Archer DB, Dyer PS, et al. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat Biotechnol 2007;25:221–31.
[6] Xu Y, Zhou Y, Cao W, Liu H. Improved production of malic acid in *Aspergillus niger* by abolishing citric acid accumulation and enhancing glycolyticflux. ACS Synth Biol 2020;9:1418–25.
[7] Xu Y, Shan L, Zhou Y, Xie Z, et al. Development of a Cre-loxP-based genetic system in *Aspergillus niger* ATCC1015 and its application to construction of efficient organic acid-producing cell factories. Appl Microbiol Biotechnol 2019;103:8105–14.
[8] Ren Y, Zhang C, Chen Z, Lu L. The heterotrimeric transcription factor CCAAT-binding complex and Ca(2+)-CrzA signaling reversely regulate the transition between fungal hyphal growth and asexual reproduction. mBio 2021;12:e0300721.
[9] Wu MY, Mead ME, Kim SC, Rokas A, Yu JH. WetA bridges cellular and chemical development in *Aspergillus flavus*. PLoS One 2017;12:e0179571.
[10] Wu MY, Mead ME, Lee MK, Neuhaus GF, et al. Transcriptomic, protein-DNA interaction, and metabolomic studies of VosA, VelB, and WetA in *Aspergillus nidulans* asexual spores. mBio 2021;12.
[11] Soid-Raggi G, Sanchez O, Aguirre J. TmpA, a member of a novel family of putative membrane flavoproteins, regulates asexual development in *Aspergillus nidulans*. Mol Microbiol 2006;59:854–69.
[12] van Munster JM, Dobruchowska JM, Veloo R, Dijkhuizen L, van der Maarel MJ. Characterization of the starvation-induced chitinase CfcA and alpha-1,3-glucanase AgnB of *Aspergillus niger*. Appl Microbiol Biotechnol 2015;99:2209–23.
[13] Lee MK, Kwon NJ, Lee IS, Jung S, et al. Negative regulation and developmental competence in *Aspergillus*. Sci Rep 2016;6:28874.
[14] Oiartzabal-Arano E, Perez-de-Nanclares-Arregi E, Espeso EA, Etxebeste O. Apical control of conidiation in *Aspergillus nidulans*. Curr Genet 2016;62:371–7.
[15] Yu JH. Regulation of development in *Aspergillus nidulans* and *Aspergillus fumigatus*. MYCOBIOLOGY 2010;38:229–37.
[16] Ogawa M, Fukuda R, Iwama R, Koyama Y, Horiuchi H. *srdA* mutations suppress the *rseA/cpsA* deletion mutant conidiation defect in *Aspergillus nidulans*. Sci Rep 2023;13:4285.
[17] Breakspear A, Momany M. *Aspergillus nidulans* conidiation genes *dewA, fluG*, and *stuA* are differentially regulated in early vegetative growth. Eukaryot Cell 2007;6:1697–700.
[18] Son YE, Yu JH, Park HS. Regulators of the asexual life cycle of *Aspergillus nidulans*. Cells 2023:12.
[19] Ruger-Herreros C, Rodriguez-Romero J, Fernandez-Barranco R, Olmedo M, et al. Regulation of conidiation by light in *Aspergillus nidulans*. Genetics 2011;188:809–22.
[20] Mirabito PM, Adams TH, Timberlake WE. Interactions of three sequentially expressed genes control temporal and spatial specificity in *Aspergillus* development. Cell 1989;57:859–68.
[21] Stewart JIP, Fava VM, Kerkaert JD, Subramanian AS, et al. Reducing *Aspergillus fumigatus* virulence through targeted dysregulation of the conidiation pathway. mBio 2020;11:e03202-03219.
[22] Zhang C, Meng X, Wei X, Lu L. Highly efficient CRISPR mutagenesis by microhomology-mediated end joining in *Aspergillus fumigatus*. Fungal Genet Biol 2016;86:47–57.
[23] Zadra I, Abt B, Parson W, Haas H. *xylP* promoter-based expression system and its use for antisense downregulation of the *Penicillium chrysogenum* nitrogen regulator NRE. Appl Environ Microbiol 2000;66:4810–6.
[24] Zhang C, Lu L. Precise and efficient in-frame integration of an exogenous GFP tag in *Aspergillus fumigatus* by a CRISPR System. Methods Mol Biol 2017;1625:249–58.
[25] Wu N, Xing M, Chen Y, Zhang C, et al. Improving the productivity of malic acid by alleviating oxidative stress during *Aspergillus niger* fermentation. Biotechnol Biofuels Bioprod 2022;15:151.
[26] Zhang C, Gu H, Ren Y, Lu L. GlcA-mediated glycerol-3-phosphate synthesis contributes to the oxidation resistance of *Aspergillus fumigatus* via decreasing the cellular ROS. Fungal Genet Biol 2021;149:103531.
[27] Ichinomiya M, Ohta A, Horiuchi H. Expression of asexual developmental regulator gene *abaA* is affected in the double mutants of classes I and II chitin synthase genes, *chsC* and *chsA*, of *Aspergillus nidulans*. Curr Genet 2005;48:171–83.
[28] van de Veerdonk FL, Gresnigt MS, Romani L, Netea MG, Latge JP. *Aspergillus fumigatus* morphology and dynamic host interactions. Nat Rev Microbiol 2017;15:661–74.
[29] Beauvais A, Fontaine T, Aimanianda V, Latge JP. *Aspergillus* cell wall and biofilm. Mycopathologia 2014;178:371–7.
[30] Krijgsheld P, Bleichrodt R, van Veluw GJ, Wang F, et al. Development in *Aspergillus*. Stud Mycol 2013;74:1–29.
[31] Tong Z, Zheng X, Tong Y, Shi YC, Sun J. Systems metabolic engineering for citric acid production by *Aspergillus niger* in the post-genomic era. Microb Cell Fact 2019;18:28.
[32] Amato A, Becci A, Beolchini F. Citric acid bioproduction: the technological innovation change. Crit Rev Biotechnol 2020;40:199–212.
[33] Simpson-Lavy K, Kupiec M. Carbon catabolite repression: not only for glucose. Curr Genet 2019;65:1321–3.
[34] Kim SM, Choi BY, Ryu YS, Jung SH, et al. Simultaneous utilization of glucose and xylose via novel mechanisms in engineered *Escherichia coli*. Metab Eng 2015;30:141–8.
[35] Lane S, Xu HQ, Oh EJ, Kim H, et al. Glucose repression can be alleviated by reducing glucose phosphorylation rate in *Saccharomyces cerevisiae*. Sci Rep-Uk 2018;8.
[36] Kunitake E, Uchida R, Asano K, Kanamaru K, et al. cAMP signaling factors regulate carbon catabolite repression of hemicellulase genes in *Aspergillus nidulans*. Amb Express 2022;12:126.
[37] Sun L, Zhang Q, Kong X, Liu Y, et al. Highly efficient neutralizer-free l-malic acid production using engineered *Saccharomyces cerevisiae*. Bioresour Technol 2023;370:128580.
[38] Chen Y, Wang J, Wang M, Han A, et al. Engineering the metabolism and morphology of the filamentous fungus *Trichoderma reesei* for efficient L-malic acid production. Bioresour Technol 2023;387:129629.
[39] Cairns TC, Zheng X, Zheng P, Sun J, Meyer V. Moulding the mould: understanding and reprogramming filamentous fungal growth and morphogenesis for next generation cell factories. Biotechnol Biofuels 2019;12:77.