Homologous Overexpression of Genes in Cordyceps militaris Improves the Production of Polysaccharide

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Research

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Abstract

Background *Cordyceps* polysaccharides have been used around the globe for its bioactivity for millennia. However, the study and medicinal of *Cordyceps militaris* polysaccharides has been hampered by the low in natural abundances. Recently, the genetic engineered *C. militaris* developed for production of exopolysaccharides (EPS) had received extensive attention.

Results In this study, based on the biosynthetic pathway and metabolization mechanism of exopolysaccharides, the crucial biosynthetic genes of *Cordyceps* polysaccharides were introduced by *Agrobacterium* transformation to provide a high flux of EPS. 21 mutants of *C. militaris* were identified through antibiotic screening and DNA sequencing. The maximum yield of EPS produced by mutant CM-*pgm*-H was 4.63 ± 0.23 g/L, while the yield of wild-type strain was 3.43 ± 0.26 g/L. And the data obtained in the present study indicated that the yield of EPS produced by the engineered strain treated with co-overexpression of phosphoglucomutase and UDP-glucose 6-dehydrogenase genes achieved 6.11 ± 0.21 g/L, which was increased by 78.13% compared with the wild-type strain.

Conclusions CM-*pgm*-H obtained the highest EPS content than that of mutants glucokinase, UDP-glucose pyrophosphorylase, UDP-glucose 6-dehydrogenase. It indicated that the content of protein phosphoglucomutase was the most critical influencing factor on the CP production in *C.militaris*. Furthermore, the EPS production of CM-*ugdh*-*pgm*-M was significantly improved 1.78-fold by co-overexpression. It anticipated that our engineering strategies will play an important role in the development of *C. militaris* for sustainable production of *Cordyceps* polysaccharides.

Background

Cordyceps militaris (L.:Fr.) Fr. is an entomogenous fungus belonging to *Ascomycetes*, *Hypocreales*, *Cordycipitaceae*, *Cordyceps* (L.:Fr.) Link [1], and has received extensive research and attention because of its potential medicinal properties. The production of natural *Cordyceps* decreased year by year, while its market demand is still great, so more and more research groups are focusing on submerge fermentation of *Cordyceps* mycelia. Nowadays, many biologically active substances have been isolated from *C. militaris*, such as *Cordyceps* polysaccharides (CP), *Cordycepic* acid, cordycepin, mannitol, alkaloids, vitamins B1, B2, and minerals. It was reported that CP has a good protection of the kidneys and liver, lowering blood sugar, antibacterial, anti-inflammatory, anti-tumor, anti-arrhythmia, improving immunity and reducing body fatigue [2–4]. There are two ways to enhance the value of CP. One is to improve the activity of polysaccharides, such as the selenium-enrich fermentation of CP, which has been reported by our group before [5]. Another way is increasing the production of CP without decreasing the activity [6]. However, there are many factors that can affect the production of CP, including nutritional content, cultivation environment, cultivation time and fermentation method [7, 8]. Zhu [9] found that these factors also play an important role on the expression of gene and the activity of enzymes in the biosynthesis of CP. Therefore, it is a potential effective way to improve polysaccharides production by regulating the biosynthesis process of CP.

The biosynthetic pathway of fungal polysaccharides has been gradually revealed, mainly including three steps about the biosynthetic of the nucleotide sugar precursors, the assembly of nucleotide sugar precursors to form repeating units, and the polymerization of repeating units [10]. The generation of nucleotide sugar precursor is an important and basic part in the process of CP biosynthesis. As researchers have discovered in their work, the overexpression of genes involved in the polysaccharide biosynthetic pathway can increase the production of *Ganoderma lucidum* polysaccharides [11, 12]. Knocking out genes in the branch pathway and controlling the metabolic pathways direction of products can also affect the yield of polysaccharides too. Overexpression of *S. thermophilus* polysaccharide biosynthetic gene alone may not have much effect on polysaccharides production, but the polysaccharides yield was significantly improved after two of genes were combined [13].

The whole genome sequencing work of *C. militaris* 01 was completed by Zheng et al [14]. Gene annotation work has been continuously improved in subsequent *C. militaris* 01 research. Among the polysaccharide biosynthetic enzymes, glucokinase (*gk*), phosphoglucomutase (*pgm*), UDP-glucose pyrophosphorylase (*ugp*), and UDP-glucose 6-dehydrogenase (*ugdh*) are important enzymes in the biosynthetic pathway of nucleotide sugar precursors. Fungal genetic transformation methods mainly include PEG/$CaCl_2$-Mediated transformation; electro-transformation; biolistic transformation; *Agrobacterium*-mediated transformation and restriction enzyme-mediated integration [15, 16]. The most widely used method of fungal genetic modification was based on protoplast transformation, but it's very difficult to introduce foreign genes into *C. militaris* cells due to its thick cell wall [17]. *Agrobacterium tumefaciens*-mediated transformation (ATMT) is an alternative method to protoplast transformation and a simple way to generate stable transformants [18]. The ATMT system has two different selectable markers that can be used for bacterial resistance screening and fungal resistance screening, respectively. At present, ATMT has realized the transformation of a variety of fungi, including *Penicillium chrysogenum* [19], *Malassezia* [20], *Aspergillus flavus* [21], and *C. militaris* [22].

In this study, the cell growth and EPS production of *C. militaris* affected by four over-expressed genes *gk*, *pgm*, *ugp*, *ugdh* and three combinations of *ugdh-pgm*, *ugdh-ugp*, *pgm-ugp* were investigated. These are helpful to further study on the regulation of polysaccharide biosynthesis and develop high-yield strains of *C. militaris* EPS.

Results

Target gene sequences and Recombinant vector maps

C.militaris 01 genomic DNA has been sequenced and reported by Zheng et al [14], and the datebase was submitted to DDBJ/EMBL/GenBank under accession number AEVU00000000. In this work, the genes *gk*, *pgm*, and *ugp* involved in the synthesis of nucleotide-activated glucose were successfully amplified from *C. militaris* 01 genomic DNA (Table 1), while *ugdh* was synthesized (TsingKe, Beijing). Genes involved in the glucose activation process will be overexpressed to increase carbon flux, and increasing EPS production. The same 15–20 bp DNA as the vector was introduced at both ends of the target gene by

PCR, and then four inserts were got, respectively. The vector was linearized at the 175th bp after the promoter gpdA of the T-DNA and followed by the terminator cbh1. The inserts were linked to the linearized vector by seamless cloning homologous recombination (TransGen, Beijing), and four recombinant vectors were obtained, respectively (Fig. 1b). The target gene have replaced the position of the enhanced green fluorescent protein (EGFP) and was controlled by the promoter gpdA, and then the transcription ended by the terminator cbh1.

Table 1
Target gene data amplified from *C. militaris* 01 genomic DNA in this experiment.

Fragment	Genome Length(bp)	Amino acid	Molecular mass(kDA)	product
CCM_03320	1822	549	61.62	glucokinase
CCM_02278	1887	553	60.95	phosphoglucomutase
CCM_06235	2708	552	63.09	UTP-glucose-1-phosphate uridylyltransferase
CCM_01686	1875*	624	69.08	UDP glucose 6-dehydrogenase
**ugdh* full-length was identified to 2037 bp, and orf 1875 bp				

The recombinant vector carries two selection markers, namely the hygromycin B resistance gene *hph* and the kanamycin resistance gene *kan*. They can enable the host to survive under the antibiotic screening after the introduction of the recombinant vector, thereby obtaining potentially positive clones through resistance screening. The *hph* gene on the recombinant vector was controlled by another promoter TrpC, but it shares the same terminator with the target gene. After inserting the target gene into the vector to obtain a recombinant vector, the primer F9 and R9 were used to linearize the recombinant vector. And the primers F10 and R10 were used to amplify the target gene that has been inserted into the recombinant vector together with the promoter gpdA and the terminator cbh1 to obtain a new insert. The two ends of the amplification piece were respectively introduced with homologous fragment to the linearization gap of the recombinant vector through corresponding primers to obtain an insert. The insert and the linearized vector were re-ligated to obtain a recombinant vector, and then two genes were combined (Fig. 1c). In this experiment, three combination types were selected, including *ugdh-pgm*, *ugdh-ugp*, and *pgm-ugp* [21].

Recombinant vector enters A. tumefaciens

The structure of the recombinant vector obtained by seamless cloning is unstable in vitro and would be affected by external factors to cause the loop to break again. Therefore, the recombinant vector needs to be introduced into *E. coli* DH5α immediately after completing the looping reaction. After 24 h of growth of *E. coli* DH5α on the selection medium, potential *E. coli* clones were selected for further testing. The kanamycin-resistant strains inserted with the target-gene was verified by PCR with F6, R6. The PCR

products showed a very clear band for the *gk*, *pgm*, *ugp*, *ugdh* gene fragment (1.8 kb, 1.8 kb, 2.7 kb, 1.8 kb, respectively) in the transformant (Fig. 2a). On the other hand, all three types of two-gene recombinant vectors were also introduced into *E. coli* DH5α, respectively, and potential transformants were obtained through kanamycin resistance. Every kanamycin-resistant strain was picked, and confirmed by PCR for both two target-genes, and the PCR products showed a very clear band for *ugdh*, *pgm*, *ugp* fragment (Fig. 2b).

After the recombinant vector successfully introduced in *E. coli* DH5α, the loop structure of the vector became more stable. Therefore, many *E. coli* clones can be obtained through fermentation, and then a high-concentration recombinant vector of *gk*, *pgm*, *ugp*, *ugdh ugdh-pgm*, *ugdh-ugp*, *pgm-ugp* can be extracted from it, for the preparation of *A. tumefaciens* transformation. In order to reduce the interference of other bacteria to *A. tumefaciens* transformants results, two antibiotics were added to the LB screening medium, namely kanamycin (50 μg/mL) and rifampicin (25 μg/mL). *A. tumefaciens* clones were picked from the screening medium, and the potential-transformants were verified by PCR..

Agrobacterium transformation of Cordyceps militaris

The *C. militaris* WT stored in our laboratory was fermented for 7 days and filtered to obtain a spore suspension. The spore concentration was diluted to 10^6/mL with sterile water, and then cultured with *A. tumefaciens* transformants. Hygromycin-resistant *C. militaris* strains were selected and transferred to a selective CO-IM. Stable inherited potential-transformants were obtained through five subcultures, each generation lasted 4 days (Fig. 3c). Mycelium of each *C. militaris* potential-transformants was obtained by fermentation, and genomic DNA was extracted, and the *hph* gene (1.0 kb) was used as an identification marker for PCR verification by prime F7, R7. The PCR results showed that each transformant had clear bands and the size was close to the expected result, while the *C. militaris* WT strains did not have corresponding bands (Fig. 3a).

The EPS production, biomass of *C. militaris* transformed strains and *C. militaris* WT were measured and compared. All strains were transferred to SFM after three days of seed culture, the EPS and biomass were measured at 9th day. The biomass dry weight results of all *C. militaris* transformants were shown (Table 2), and *C. militaris* WT was analyzed as control group. The EPS production of transformants CM-*ugdh*, *gk*, *pgm*, *ugp*-A ~ L and *C. militaris* WT were assayed (Fig. 4a). There is no significant difference in biomass dry weight between *C. militaris* WT and transformants. However, the EPS production has improved significantly compared with that of the *C. militaris* WT strain. It shows that the highest EPS production is 4.63 ± 0.23 g/L, which was about 1.35 times the EPS production of the wild strain. Transformant CM-*pgm*-H with the highest EPS yield was chosen with the *C. militaris* WT strain to study the kinetic profiles of glucose consumption and EPS production (Fig. 4b), and the EPS production stopped increasing after 7 days of fermentation. The EPS test results showed that the EPS of *gk*-transformants and *pgm*-transformants were significantly improved, while *ugdh*-transformants and *ugp*-transformants had no significant difference, compared with *C. militaris* WT. The results indicated that the

overexpression of *gk* or *pgm* gene can increase the carbon flux to a higher level and achieve the purpose of increasing EPS production, and the overexpression of *pgm* gene has a more significant effect on the EPS production of *C. militaris* than that of *gk*-transformants.

Table 2
Biomass dry weight of *C. militaris* WT and *C. militaris* trantsformants mycelium after 7 days of fermentation

Strains	Biomass DW(g/L)	Strains	Biomass DW(g/L)
CM-*ugdh*-A	2.85 ± 0.46	CM-*ugp*-L	3.70 ± 0.57
CM-*ugdh*-B	2.78 ± 0.37	CM-*ugdh-pgm*-M	2.81 ± 0.55
CM-*ugdh*-C	2.54 ± 0.80	CM-*ugdh-pgm*-N	2.75 ± 0.43
CM-*gk*-D	3.15 ± 0.78	CM-*ugdh-pgm*-O	2.69 ± 0.60
CM-*gk*-E	3.25 ± 0.52	CM-*ugdh-ugp*-P	2.75 ± 0.23
CM-*gk*-F	2.92 ± 0.34	CM-*ugdh-ugp*-Q	2.56 ± 0.43
CM-*pgm*-G	3.28 ± 0.18	CM-*ugdh-ugp*-R	2.82 ± 0.39
CM-*pgm*-H	2.80 ± 0.39	CM-*pgm-ugp*-S	2.51 ± 0.34
CM-*pgm*-I	3.06 ± 0.44	CM-*pgm-ugp*-T	2.84 ± 0.56
CM-*ugp*-J	3.39 ± 0.56	CM-*pgm-ugp*-U	3.04 ± 0.45
CM-*ugp*-K	3.36 ± 0.20	CM-WT	3.12 ± 0.56

Levander's [13] research on increasing the EPS production of *Streptococcus thermophilus* found that although *pgm* gene and *ugp* gene overexpressed alone had no increase effect on EPS production, but the production increased to 1.82 times when *pgm* and *ugp* were combined. In order to study the effect of two-gene overexpression on *C. militaris* EPS production, the genes *ugdh* and *pgm*, *ugdh* and *ugp*, *pgm* and *ugp* were combined in this work. Three potential transformants were selected for each combination, namely CM-*ugdh-pgm*-M ~ O, CM-*ugdh-ugp*-P ~ R, CM-*pgm-ugp*-S ~ U, and hygromycin-resistance gene (1.0 kb) were verified by PCR (Fig. 3b). Although the DNA fragment introduced into the genomic DNA of *C. militaris* WT became longer, the morphology of the 9 mutants did not differ from that of the *C. militaris* WT strain (Fig. 3d). Biomass DW of all 9 mutants was measured, and results were showed in Table 2. EPS production of transformants and *C. militaris* WT were assayed (Fig. 4a). The results showed that EPS production of the transformants of the co-overexpression of *pgm* and *ugdh*, *ugdh* and *ugp* were increased, and the most significant improvement (6.11 ± 0.21 g/L) was the transformant CM-*ugdh-pgm*-M. The highest EPS production strain CM-*ugdh-pgm*-M was choosed with CM-*pgm*-H, CM-WT to study the kinetic profiles (Fig. 4c). *C. militaris* EPS production reached its maximum after 7 days of fermentation, and stopped accumulating.

Discussion

The homologous overexpression system constructed by using the characteristics of *A. tumefaciens* to transfer its own T-DNA into the host fungal genomic DNA under the induction of AS, has potential application value for the regulation and design of *C. militaris* biological metabolic pathway. Since the glycosyltransferase gene of polysaccharide synthesis has not been revealed, the subsequent process of polysaccharide synthesis is still unknown. Therefore, overexpression of genes on the polysaccharide synthesis pathway to achieve a accumulation of polysaccharide synthesis precursors is currently the main research direction. In the biosynthesis of fungal polysaccharides, significant results were found that the polysaccharide yield through the accumulation of nucleotide sugar precursors. Phosphoglucomutase (E.C.5.4.2.2, PGM) catalyzes the reversible conversion of glucose-6-phosphate (Glc6P) to glucose-1-phosphate (Glc1P) in the polysaccharide biosynthesis and glycolysis pathways. And many studies have shown that overexpression of *pgm* gene can effectively increase the yield of polysaccharides, and the combination of *pgm*, *gk*, *ugp*, and *ugdh* genes also affects the production of polysaccharides [12, 23, 24]. In this experiment, the EPS production of the transformant CM-*pgm*-H increased most significantly, which was 1.34 times that of the *C. militaris* WT. The results indicated that the content of protein PGM was the most critical influencing factor and had the most direct effect on the CP production in the experiments of overexpressing *gk*, *pgm*, *ugp*, and *ugdh* to investigate the key enzymes of CP synthesis.

Glucose is catalyzed to Glc6P by glucokinase (E.C.2.7.1.1, GK). High content of GK is closely related to glucose phosphorylation and can stimulate glycolytic reactions. Glc6P is an important synthetic substrate for CP, and its concentration also affects the synthesis of polysaccharides. The control coefficient (1.4–1.7) of GK on Glc6P synthesis was not affected by glucose concentration, while the flux control coefficient (3.7–1.8) of GK on glycogen synthesis was positively correlated with glucose concentration [25]. Overexpression of *gk* increased the concentration of Glc6P and further affected glycogen synthesis. However, GK is the basic enzyme for the biological utilization of glucose, and the flow of Glc6P is complex, that's why only transformant CM-*gk*-F showed a significant increase in EPS synthesis of *C. militaris*. Uridine diphosphate glucose (UDPG) is a donor of glycogen synthesis, which is obtained by UDP glucose pyrophosphorylase (E.C.2.7.7.9, UGP) catalyzing Glc1P [26]. The genetically engineered strain of *Ganoderma lucidum* constructed by Xu et al [12] increased the transcription level to 1.51 times in fermentation of the overexpression polysaccharide biosynthetic gene *ugp*, but the EPS production did not significantly increase. In the process of synthesizing polysaccharides by different strains, the effects of protein UGP are also different. In experiments to study the function of UGP in *Populus deltoides*, it indicated that UGP is involved in the regulation of primary and secondary metabolism, and even led to a reduction in sugar and starch levels [26]. However, during the growth process of transgenic *Arabidopsis thaliana*, overexpression of the *upg* gene cloned from *Larix gmelinii* enhanced the vegetative growth of *Arabidopsis thaliana*, and the content of soluble sugar and cellulose was also increased [27]. Overexpression of *ugp* also increased cellulose content in Jute [28]. In this work, overexpression of the *ugp* gene did not result in a significant increase in EPS, and indicated that *ugp* is not a key gene for CP synthesis. UDPG and uridine 5'-bisphosphate glucuronic acid (UDP-GlcA) are both glycosyl donor units of glycogen and glycan compounds. UDP-glucose 6-dehydrogenase (E.C.1.1.1.22, UGDH) can convert UDPG to UDP-GlcA, which plays an important role in the uronic acid pathway and

hyaluronic acid synthesis [29, 30]. The expression level of UGDH also plays an important role in the monitoring of lung adenocarcinoma conditions [31]. In the research of Wang et al [32], it was found that the content of biosynthetic *Polygonatum sibiricum* polysaccharides was positively correlated with the expression of *ugdh*, and negatively correlated with the expression of *gk*. UDP-GlcA plays an important role in the biosynthesis of hemicellulose in plants and it's a key nucleotide sugar involved in the formation of plant cell walls [33]. Combined with the EPS content level of transformants CM-*ugdh*-A ~ C obtained in this experiment, it was shown that overexpression of *ugdh* did not have a significantly increase in the EPS production of *C. militaris*, and only a slightly increase here. It may be due to the increase in the concentration of UDP-GlcA which promoted the formation of *C. militaris* cell walls and the production of polysaccharide polymer on the membrane.

In order to explore the effect of gene expression on CP production, the T-DNA fragment in Fig. 1c was constructed, and three combinations of *pgm-ugp*, *ugdh-pgm*, and *ugdh-ugp* were obtained. Comparing the EPS production of all transformants CM- *ugdh-pgm*, *ugdh-ugp*, *pgm-ugp*-M ~ U, it showed that the transformants CM-*ugdh-pgm*-M, CM-*ugdh-pgm*-O had a significant increase on EPS production than *C. militaris* WT, 1.78-fold and 1.49-fold, respectively. Although the EPS output of CM-*pgm-ugp*-T increased by 1.30 times, it is still lower than that of CM-*pgm*-H. Therefore, the results cannot be ruled out that the impact of *pgm* on CP synthesis is even greater, nor can it be affirmed that the increase in EPS production comes from the combination of *pgm* and *ugp*.

Conclusion

In this study, a total of 21 transformants was successfully constructed, and explored the relationship between genes including *gk*, *pgm*, *ugp*, *ugdh* and CP biosynthesis by the overexpression of these target genes. The results showed that *pgm* is an important regulatory gene that affects EPS biosynthesis of *C. militaris*. *Pgm* overexpression increased carbon flux and EPS production. In addition, the EPS production of the transformants CM-*ugdh-pgm*-M (combination of the gene *pgm* and *ugdh*) was significantly improved compared with *C. militaris* WT. The constructed engineering strain created a suitable basis for the further development of industrial production of *C. militaris* EPS.

Material And Methods

Cordyceps militaris and culture conditions

Cordyceps militaris 01(CGMCC 3.14242) was obtained from the Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences. And the *C. militaris* 01 was maintained on potato dextrose agar slants and cultured at 25 °C for 7 days [14]. The *C. militaris* wild-type (WT) strains for transformation experiments was preserved by our group and cultured on sabouraud medium. A 0.5-cm^2 mycelial block of *C. militaris* was transferred to shake flasks (250 mL) containing 50 mL of submerge fermentation medium (SFM: 2%glucose, 0.70% $(NH_4)_2SO_4$, 0.05% $K_2HPO_4{\cdot}3H_2O$, 0.05% KH_2PO_4, 0.05%

$MgSO_4 \cdot 7H_2O$, 0.10% L-Glycine). Inoculated submerge fermentation flasks were shaken at 180 rpm at 25 °C for 9 days.

Recombinant Vector Construction

Escherichia coli DH5α with plasmid pCAMBIA-PgpdA-Tcbh1-*hph*-PtrpC (11852 base pair) was used in this work [34]. PCAMBIA-PgpdA-Tcbh1-*hph*-PtrpC was used as a T-DNA donor for maintenance of constructs and for *C. militaris* WT transformation. The plasmid also had *hph* (hygromycin B phosphotransferase) gene for transformants selection with Trpc promoter and cbh1 terminator (Fig. 5). The *C. militaris* 01 *gk*, *pgm*, *ugp*, and *ugdh* gene were amplified by using primers (F1 ~ 4, R1 ~ 4) respectively (Table 3). Plasmid pCAMBIA-PgpdA-Tcbh1-*hph*-PtrpC was linearized and amplified using primers (F5, R5) (Table 3). Seamless cloning enzyme pEASY-Uni Seamless Cloning and Assembly Kit used throughout this study was purchased from TransGen Biotech (Beijing City, China).

Table 3
Primers used in this experiment.

primers	Sequences (5′-3′)	notes
gk-F1	GCAGACATCACAATGGGCCTGCAAGAAGAAACCAAAA	Cloning
gk-R1	TTTCGCCACGGAGCCTAATGACGCCTCCAGTACCACAAC	Cloning
pgm-F2	GCAGACATCACAATGGACGTCAAGACTGTTGAGTTTA	Cloning
pgm-R2	TTTCGCCACGGAGCTCAAGTGCGGACATTAGGCTCGTCG	Cloning
ugp-F3	GCAGACATCACAATGGTTGTGGCAGGGGGGAGGAGGG	Cloning
ugp-R3	TTTCGCCACGGAGCTTAATGCTCAAGCAGGCGTAGCGAG	Cloning
ugdh-F4	GCAGACATCACAATGTCTTCATCCATCGTCGACACCG	Cloning
ugdh-R4	TTTCGCCACGGAGCTTAGAAGCGGTGTTGACGACCAACG	Cloning
Plasmid-F5	AGCTCCGTGGCGAAAGCCTGACGCA	Cloning
Plasmid-R5	CATTGTGATGTCTGCTCAAGCGGGGT	Cloning
F6	CCCCGCTTGAGCAGACATCACAATG	Identification
R6	TGCGTCAGGCTTTCGCCACGGAGCT	Identification
F7	CTATTCCTTTGCCCTCGGACGA	Identification
R7	ATGCCTGAACTCACCGCGACGT	Identification
F8	GTATATTCATCTTCCCATCCAAGAACCT	Sequencing
R8	ATATTTGAAAAGGGTCAGAAGTAGATAC	Sequencing
F9	ACCTATTCCTTTGCCCTCGGACGA	Cloning
R9	CAAGTTGGTCTCCAACAGTGCTTT	Cloning
F10	TTGGAGACCAACTTGGCTTGTATCTCTACACACAGG	Cloning
R10	GGCAAAGGAATAGGTAAGTTGGTCTCCAACAGTGCTT	Cloning

The Ti mini-recombinant vector with the gene was transformed into *E. coli* DH5α, which were grown on Luria–Bertani (LB) agar plates containing 100 μg/mL kanamycin. Then it was identified by PCR and further confirmed by DNA sequencing (TsingKe, Beijing). *E. coli* DH5α transformants are stored in -80 °C and ready for use.

Agrobacterium transformation

Agrobacterium tumefaciens strain AGL-1 (provided by Dr. Linian Cai of Zhejiang University, China) was cultured on LB medium containing 25 μg/mL rifampin (28 ℃). The recombinant vector carrying the target gene were extracted from *E. coli* DH5α and transformed into *A. tumefaciens* [35]. The transformed clones are then screened from LB agar plates containing 25 μg/mL rifampicin and 50 μg/mL kanamycin. *A. tumefaciens* clones was transferred in induction liquid medium (AIM: 0.145% KH_2PO_4, 0.205% K_2HPO_4, 0.06% $MgSO_4{\cdot}7H_2O$, 0.03% NaCl, 0.01‰ $CaCl_2$, 0.001‰ $FeSO_4$, 0.05% NH_4NO_3, 5 mL/L glycerol, 0.2% glucose, 5 mL/L trace element stock solution, 40 mL/L MES buffer (1 mol/L, pH = 5.5)) containing 200 μM acetyl syringone (AS) and shaken at 180 rpm for activation (28 °C). Induction solid medium (CO-AIM: add 1.5% agar to IM medium and reduces glucose by half) was prepared for cocultivation of pre-cultured *A. tumefaciens* clones and *C. militaris* WT. Selective CO-IM (CO-AIM without AS) containing 200 μg/mL hygromycin B, 200 μg/mL ceftriaxone sodium, and 200 μg/mL kanamycin was used for inhibiting *A. tumefaciens* growth and screening for *C. militaris* transformants.

Fungal transformation and identification

A. tumefaciens, carrying the binary vector pCAMBIA-PgpdA-Tcbh1-*hph*-PtrpC with *hph* gene under the *Aspergillus nidulans* Trpc promoter, was used to introduce *C. militaris* 01 gene (*gk*, *pgm*, *ugp*, *ugdh*) to *C. militaris* WT with the already described method. In order to test the inhibition ability of hygromycin B to the growth of *C. militaris* WT, different concentrations of hygromycin B (0, 50, 100, 150, 200 and 250 μg/mL) were added to the sabouraud medium plates, and then conidiospores were inoculated at a same concentration. The hygromycin B concentration at which *C. militaris* WT cannot grow was selected as the hygromycin B screening concentration for *C. militaris* transformantion.

A. tumefaciens clones was grown for 36 h on LB with kanamycin (50 μg/mL), rifampin (25 μg/mL) in a shaker (180 rpm, 28 °C). Then 1% (v/v) *A. tumefaciens* were transferred to shake flasks (250 mL) which containing 50 mL AIM, and re-incubated at 28 °C until the OD600 was 0.80. *C. militaris* WT conidia were obtained by cultivating the fungi on PDA plates for 7 day and the plates was washed gently with sterile water. Hemocytometer was used to calculated the conidiospores density of conidial suspension that has been filtered by four layers of sterile gauze to remove large particles. The conidial suspension was diluted to 10^6/mL with sterile water and mixed with the equal volume (150 μL) of *A. tumefaciens* clones that have been induced to obtain a mixed solution.

The mixed solution was inoculated onto a sterile cellophane sheet (diameter 90 mm), and placed on CO-AIM medium (24 °C, 48 h) with three replicates. After co-cultivation, the cellophane was transferred to the selective CO-IM while being covered by another layer of selective CO-IM. *C. militaris* transformants were picked by sterile toothpick and transferred to sabouraud medium containing 200 μg/mL hygromycin B. To determine the genetic stability of the transformants, all *C. militaris* transformants were cultured on sabouraud medium (25 °C, 4 d) containing 200 μg/mL hygromycin B. At the end of each generation of genetic stability cultivation, a spot of mycelia at the edge of each transformant was picked with a sterile

toothpick and transferred to a new sabouraud medium plate (hygromycin B 200 μg/mL), which was repeated additional four times.

Potential transformants resistant to hygromycin B were verified by PCR, and confirmed by DNA sequencing. *C. militaris* transformants mycelia were harvested, frozen in liquid nitrogen, and ground to powder. The genomic DNA was extracted using the urea method. Standard PCR and primers (F6, R6; F7, R7) were used to detect whether Target-gene and *hph* were successfully recombined into the genomic DNA of potential transformants.

Sampling, analysis of residual sugar in medium and EPS production

A 0.5-cm^2 mycelial block of *C. militaris* WT and all transformants were inoculated to shake flasks (250 mL) containing 50 mL PDB (PDA without ager, 200 μg/mL ceftriaxone sodium), and the flasks were shaken at 180 rpm at 25 °C for 3 days to obtain a seed liquid. A 1% (v/w) seed liquid was transferred to 50 mL of SFM (200 μg/mL ceftriaxone sodium) in a 250 mL flask and cultivated for 9 days. The sample was vacuum filtered to obtain mycelium, washed three times with deionized water and dried to constant weight at 50 ℃.

The fermentation sample was centrifuged at 10,000 × *g* (model: 5810R, Eppendorf, Hamburg Germany) for 10 minutes at 4 °C, and the supernatant was taken to measure the glucose and EPS concentrations, respectively. As the carbon source in the medium is derived from glucose, the residual amount of glucose in the fermentation supernatant was determined by the 3,5-dinitrosalicylic acid method described by Miller [36]. The crude EPS was precipitated by addition of four volumes of absolute ethanol and stored at 4 °C overnight. After centrifugation at 13000 g for 15 min, the precipitate was suspended in 1 M NaOH at 60 °C for 1 h. Phenol-sulfuric acid method was used for the determination of EPS in the supernatant [37].

Statistics Processing

All dates are the average of three independent sample measurements and expressed as the mean ± standard error. The error bars indicate the standard deviations from the means of triplicates. Statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, USA).

Declarations

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Authors’ contributions

YSL and ZH conceived and designed research. YX and CP conducted experiments. WYF and FLL analyzed data, YX wrote the manuscript. All authors read and approved the manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests

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Figures

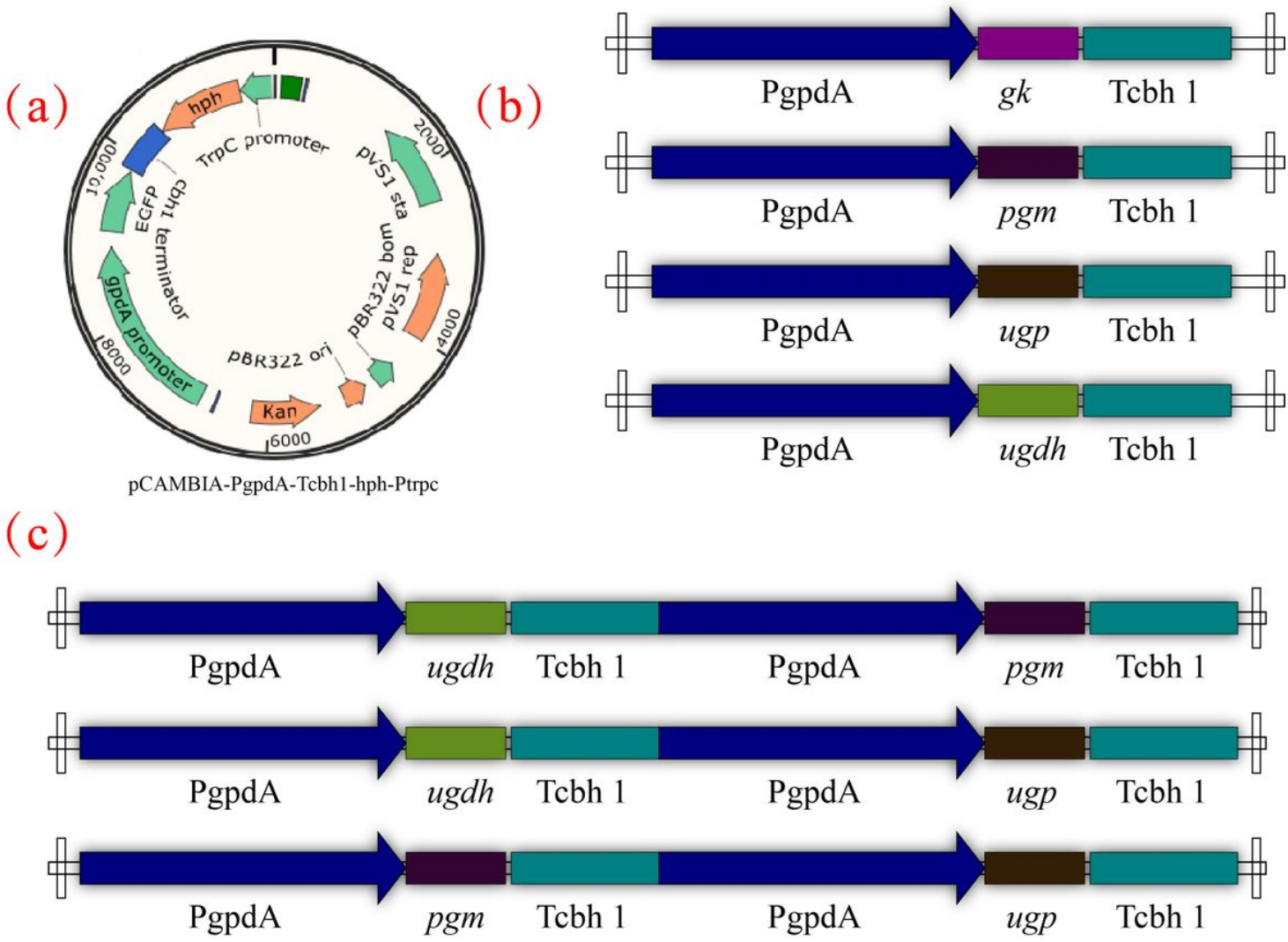

Figure 1

(a) A. tumefaciens binary vector pCAMBIA-PgpdA-Tcbh1-hph-PtrpC was choosed to construct recombinant vector for C.militaris;(b) All the target genes gk, pgm, ugp, and ugdh were inserted into the vector through homologous recombination, as well as T-DNA partial fragments;(c) Target gene of the

recombination vector constructed in (b) was recombined with the promoter and the terminator all three fragments behind the terminator cbh 1 of another recombination vector to achieve the combination of the two target genes.

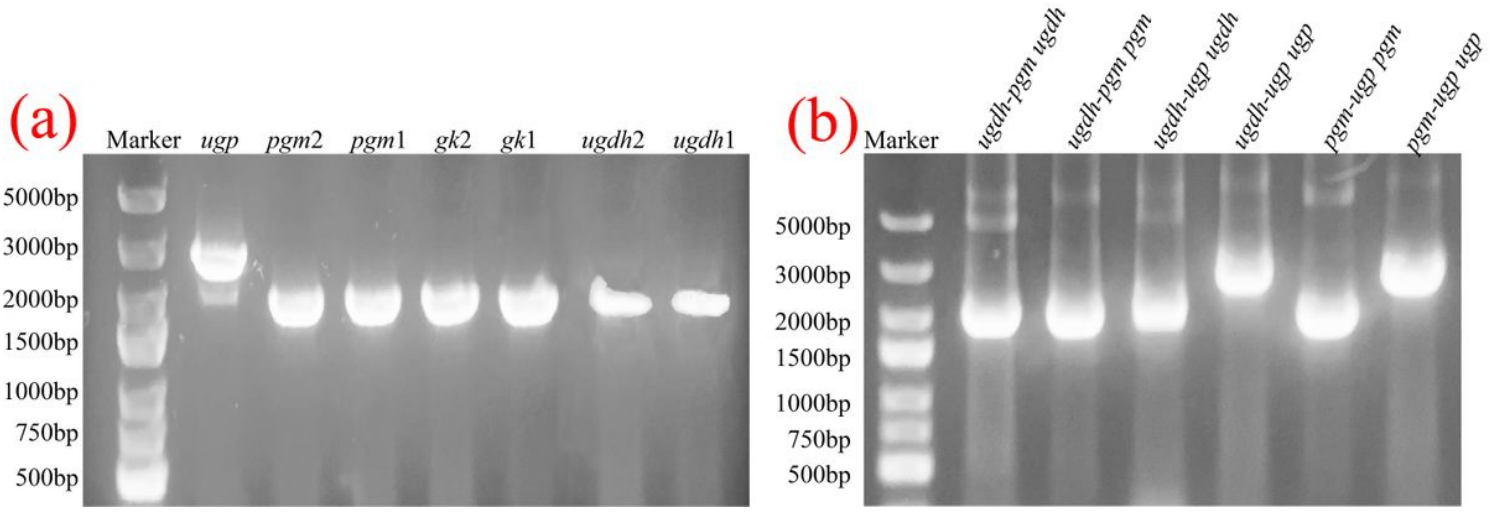

Figure 2

(a) Recombinant vectors were introduced into E. coli DH5α transformed strains for PCR identification, including gk, pgm, ugp, ugdh. Two E.coli DH5α transformants were selected for each, except for ugp; (b) PCR identification results of potential E. coli DH5α transformed strain, verifyed successful transformation of the two-gene recombinant vector.

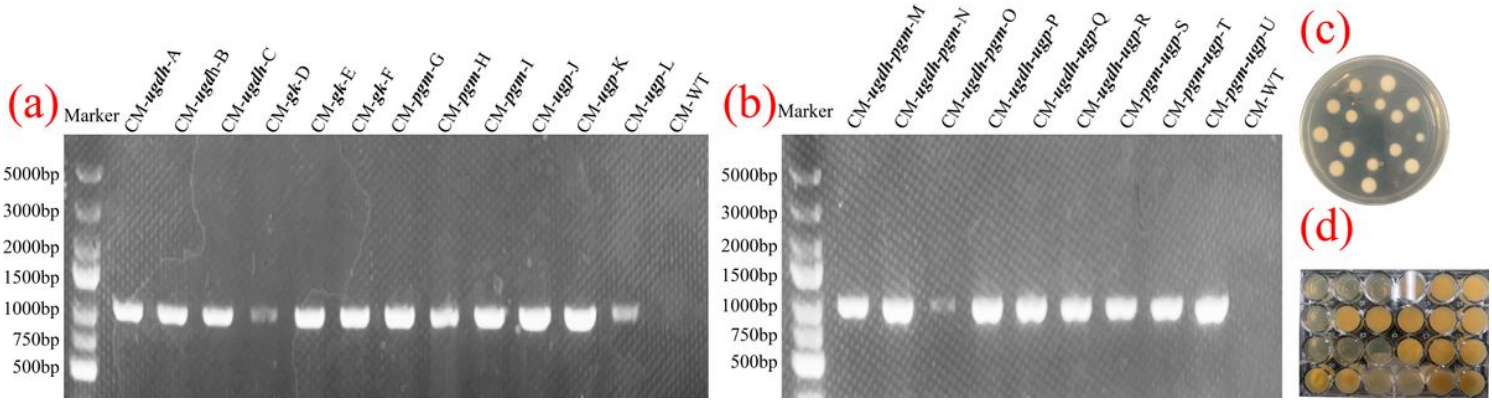

Figure 3

(a) PCR results of C. militaris potential-transformants obtained by introducing the T-DNA carrying the single-gene into C. militaris WT, and three transformants were selected for ugdh (CM-ugdh-A~C), gk (CM-gk-D~F), pgm (CM-pgm-G~I), ugp (CM-ugp-J~L); (b) T-DNA carrying two target-genes were introduced into C. militaris WT, and transformants (CM-ugdh-pgm-M~O, CM-ugdh-ugp-P~R, CM-pgm-ugp-S~U) were selected to verify the hygromycin-resistance gene; (c) Morphological characteristics of all ugdh potential-transformants; (d) Morphology of transformants growing on 24-well plates. The first row is the ugdh-pgm transformant, the second row is the ugdh-ugp transformant, the third row is the pgm-ugp transformant, and the last is the ugp transformant.

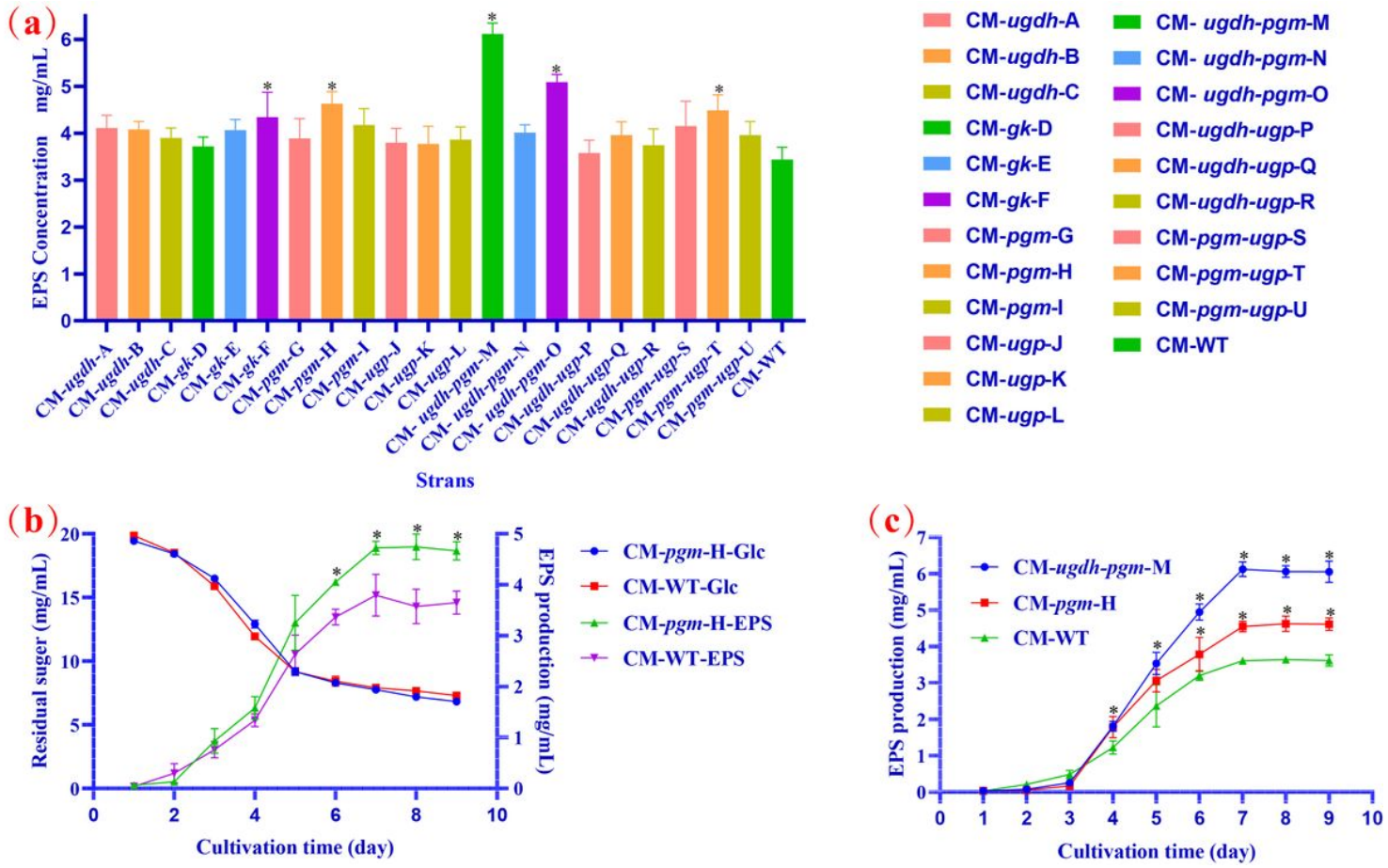

Figure 4

(a) EPS production of C. militaris WT and transformed strains CM-ugdh, gk, pgm, ugp, ugdh-pgm, ugdh-ugp, pgm-ugp-A~U. EPS production of C.militaris WT is 3.43±0.26 g/L; (b) Kinetic profiles of EPS production (CM-pgm-H green ▲, CM-WT purple▼) and residual sugar (CM-pgm-H blue●, CM-WT red ■) in the C. militaris WT strain and the pgm transformant CM-pgm-H; (c) Kinetic profiles of EPS production (CM-ugdh-pgm-M blue ●, CM-pgm-H red ■, CM-WT green ▲) in the C. militaris WT strain, transformants CM-pgm-H and CM-ugdh-pgm-M. The error bars indicate the standard deviations from three independent samples. * Significantly different from value for C. militaris WT (P<0.05)

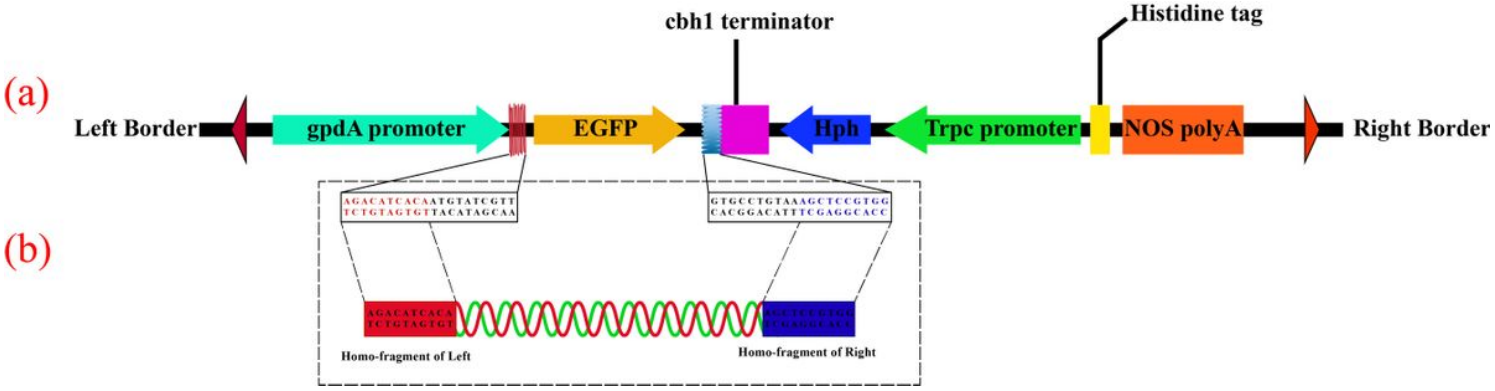

Figure 5

(a) The T-DNA backbone of the vector is integrated into the C. militaris genome from LB to RB; (b) indicates the homologous recombination of the target gene and the vector; EGFP on the linearized vector was replaced by gk, pgm, ugp, and ugdh, respectively, to form a recombinant vector. Vector pCAMBIA-PgpdA-Tcbh1-hph-PtrpC and inserts are ligated to construct a recombinant vector.

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