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(54) **VISUALIZED SCREENING METHOD FOR ASPERGILLUS RECOMBINANT STRAINS WITH MULTIGENE EDITING**

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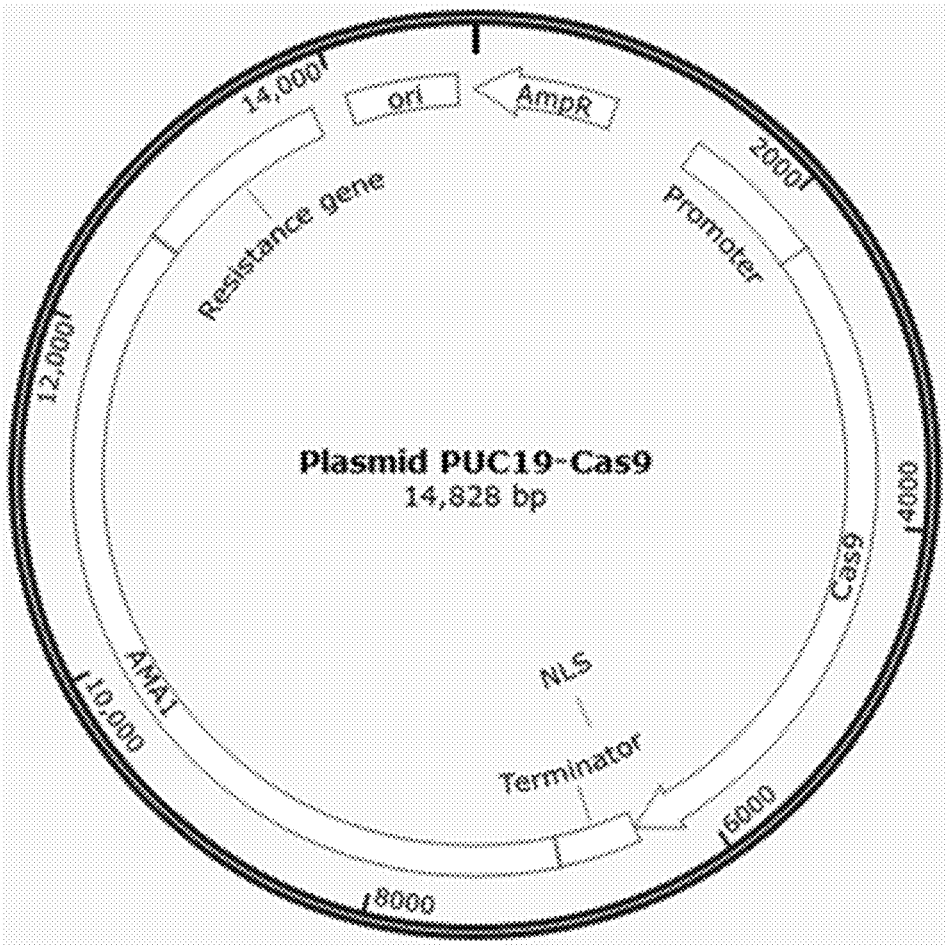
(57) **ABSTRACT**

The present disclosure discloses a visualized screening method for an *Aspergillus* recombinant strain with multigene editing and belongs to the technical field of gene engineering. CRISPR-Cas9 is used in the disclosure to cleave spore color change-related genes and a target gene in *Aspergillus* at the same time, such that editing of the target gene is visualized and an *Aspergillus niger* strain with multigene editing can be rapidly and efficiently screened out through spore phenotypes. Through different combinations of visualized genes and non-phenotypic change genes, rapid screening of the strain with multigene editing and simultaneous screening of multiple visualized genes are realized, and use of resistance genes in industrial strains is reduced.

Specification includes a Sequence Listing.



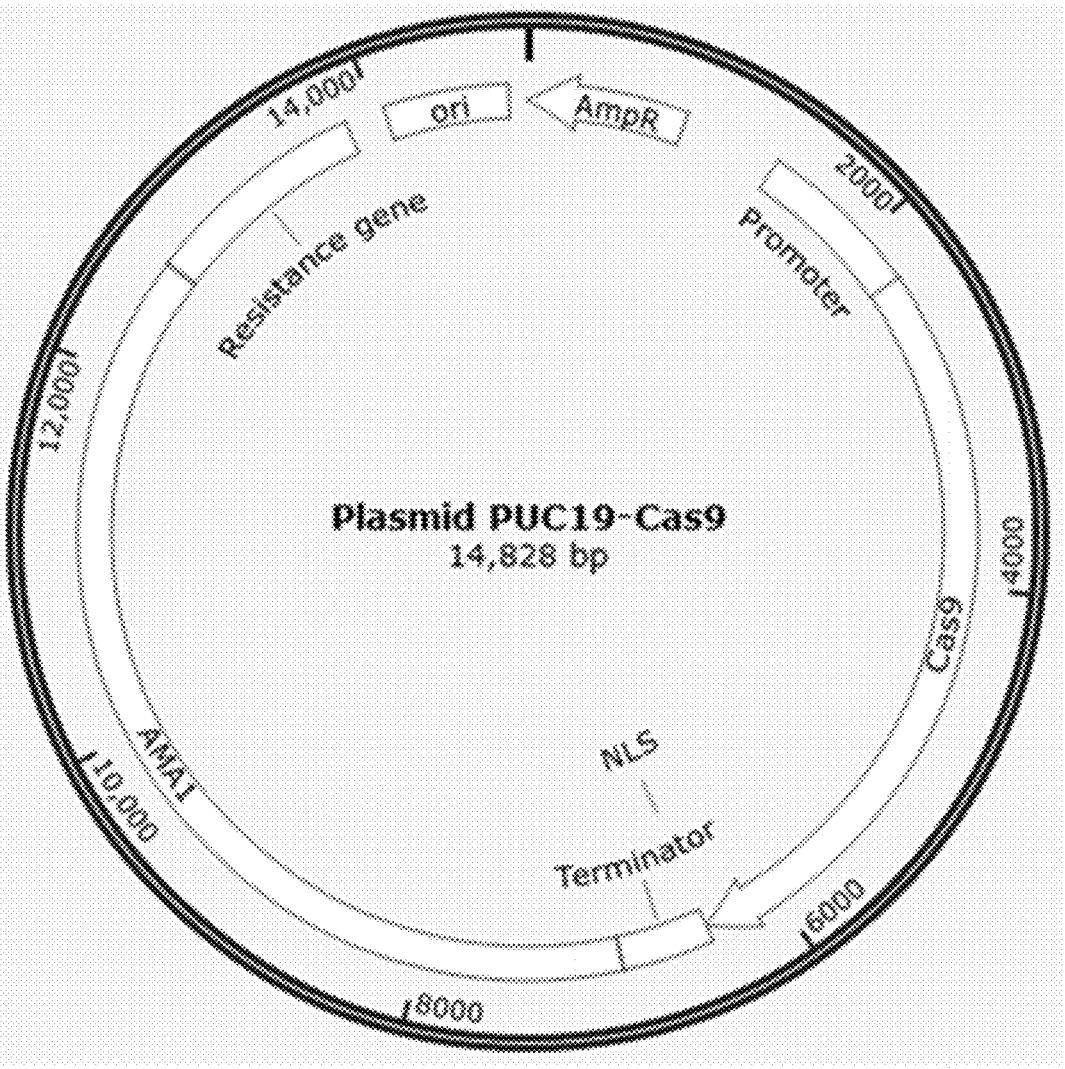

Plasmid PUC19-Cas9
14,828 bp
ori
AmpR
Promoter
Cas9
NLS
Terminator
AMA1
Resistance gene
2000
4000
6000
8000
10,000
12,000
14,000

FIG. 1

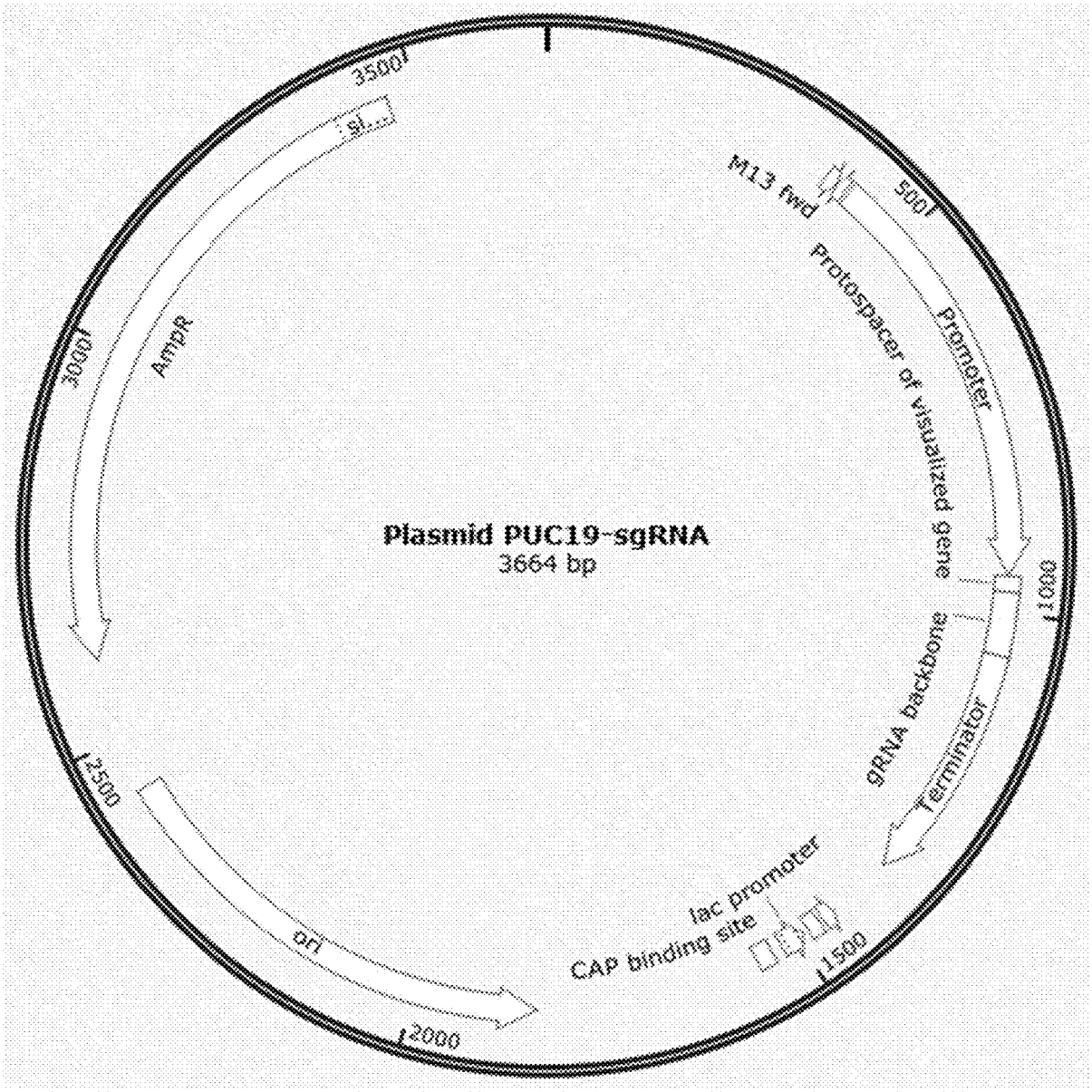

Plasmid PUC19-sgRNA
3664 bp
500
1000
1500
2000
2500
3000
3500
M13 fwd
Protospacer of visualized gene
Promoter
gRNA backbone
Terminator
lac promoter
CAP binding site
ori
AmpR

FIG. 2

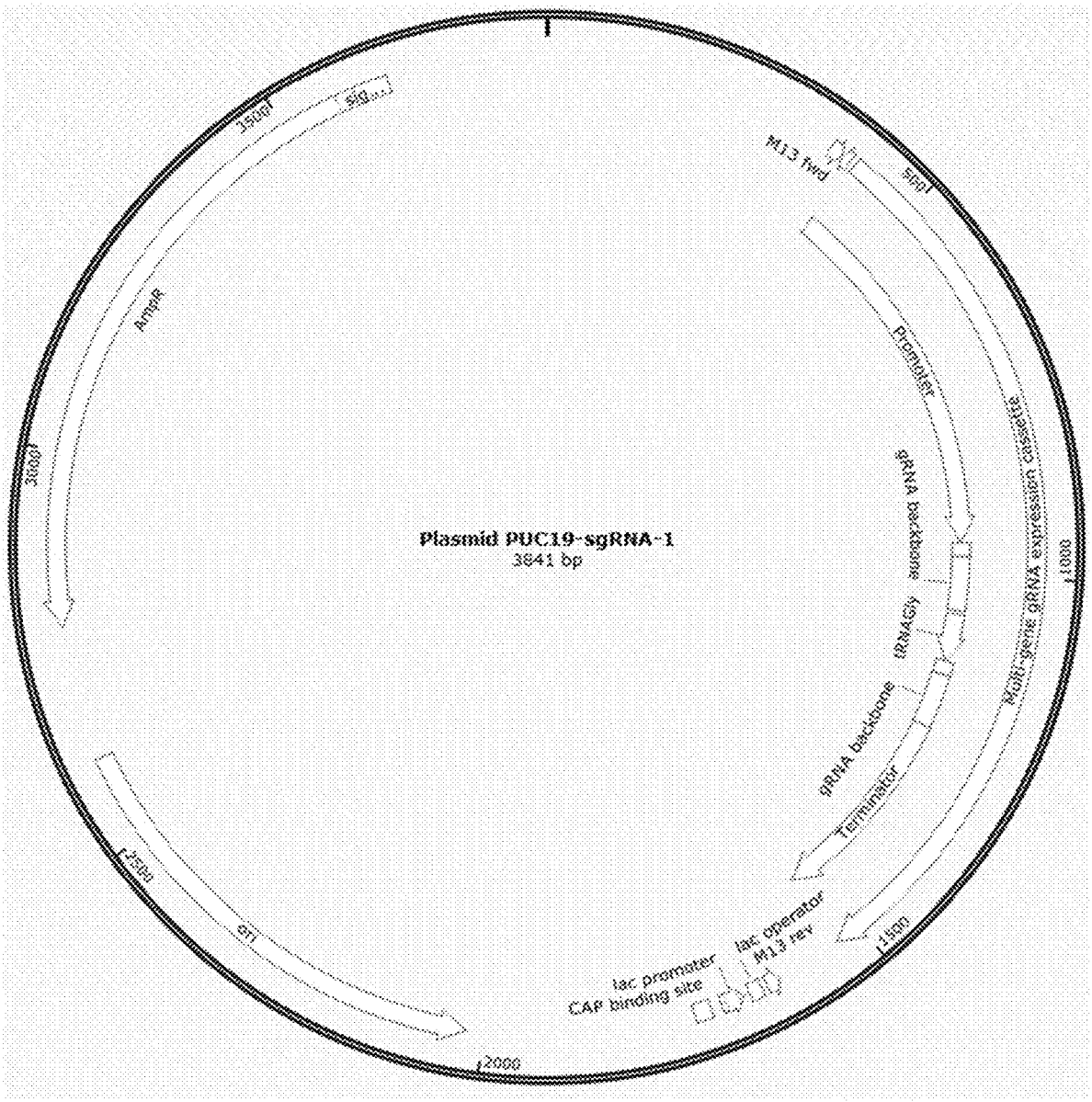

Plasmid PUC19-sgRNA-1
3841 bp
1
500
1000
1500
2000
2500
3000
3500
M13 fwd
Promoter
gRNA backbone
tRNA(Gly)
gRNA backbone
Terminator
lac operator
M13 rev
lac promoter
CAP binding site
ori
AmpR
MCS-gene gRNA expression cassette

FIG. 3A

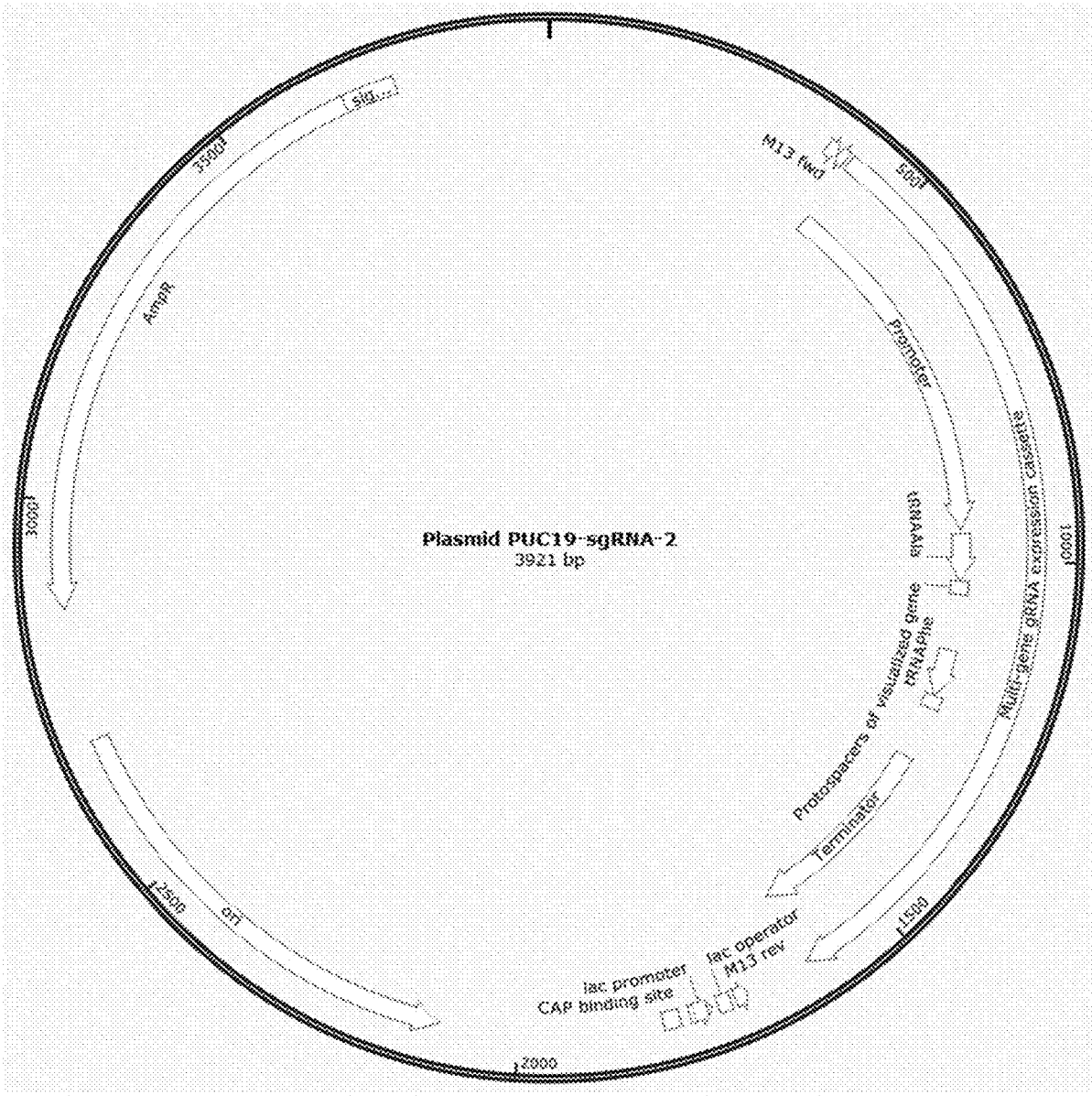

Plasmid PUC19-sgRNA-2
3921 bp
Sig...
M13 fwd
Promoter
tRNAAla
tRNAPhe
Protospacers of visualized gene
Terminator
Multi-gene gRNA expression cassette
M13 rev
lac operator
lac promoter
CAP binding site
ori
AmpR
500
1000
1500
2000
2500
3000
3500

FIG. 3B

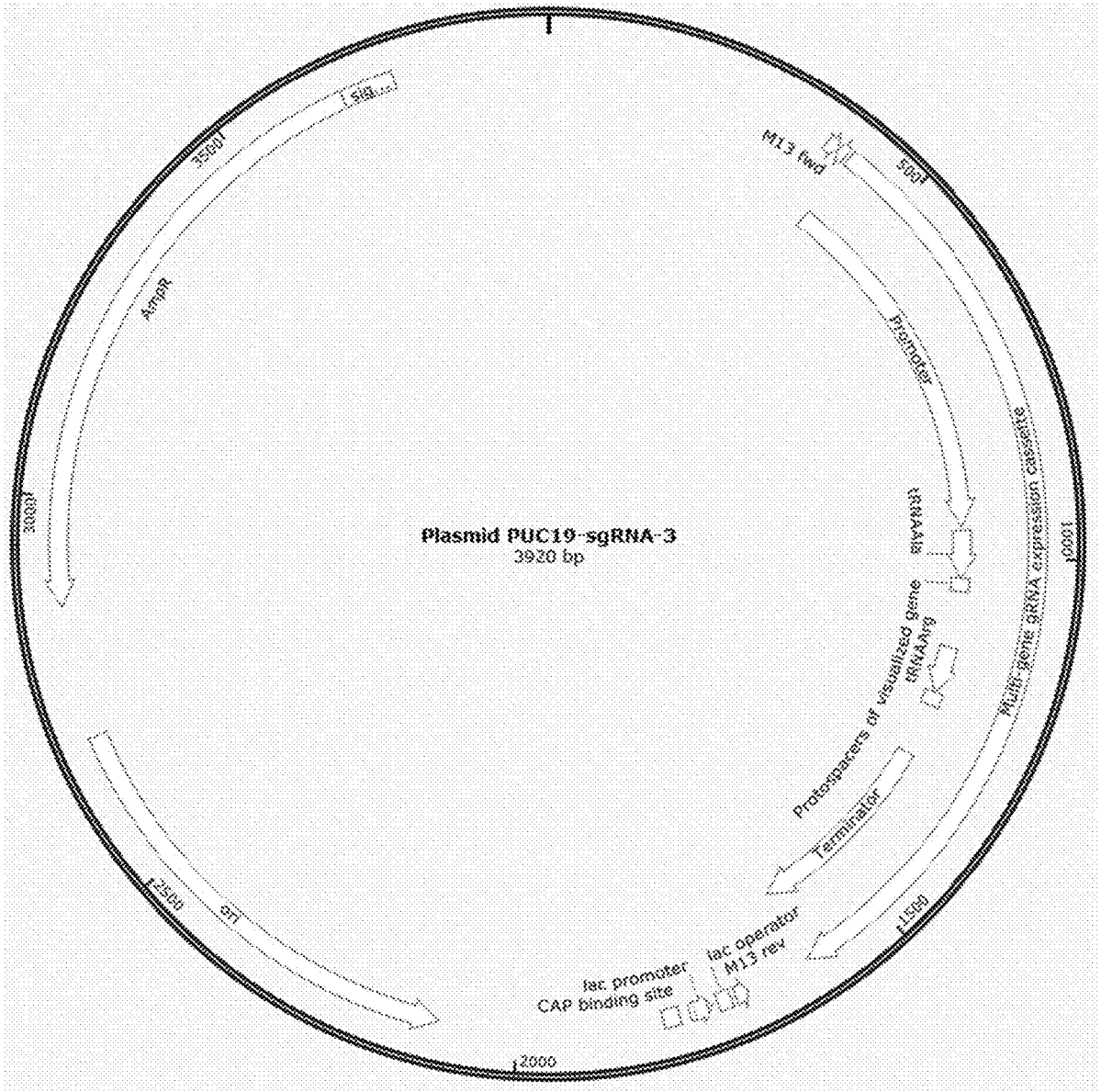

Plasmid PUC19-sgRNA-3
3920 bp
M13 fwd
500
1000
1500
2000
2500
3000
3500
AmpR
Promoter
tRNAArg
Protospacers of visualized gene
tRNAArg
Terminator
Multi-gene gRNA expression cassette
lac promoter
CAP binding site
lac operator
M13 rev

FIG. 3C

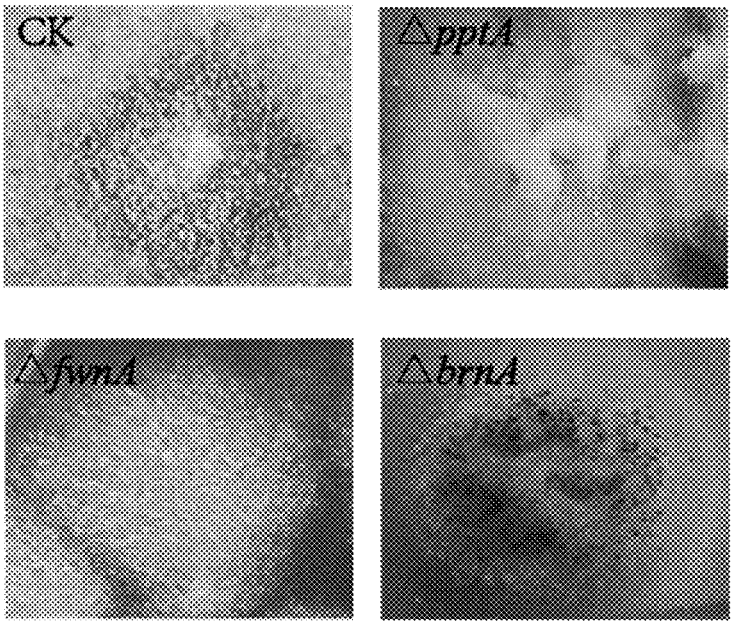


FIG. 4

VISUALIZED SCREENING METHOD FOR ASPERGILLUS RECOMBINANT STRAINS WITH MULTIGENE EDITING

TECHNICAL FIELD

[0001] The present disclosure relates to a visualized screening method for *Aspergillus* recombinant strains with multigene editing and belongs to the technical field of genetic engineering.

BACKGROUND

[0002] Filamentous fungi have a long history of development in the traditional fermentation industry. *Aspergillus* is used for food fermentation and production of enzymes and organic acids. Commercially valuable *Aspergillus* species include *Aspergillus niger, Aspergillus oryzae*, etc. However, a molecular modification process of industrial *Aspergillus* strain is hindered by lack of an efficient gene editing method and a method for rapidly screening recombinant strains.

[0003] *Aspergillus* cells are wrapped by hyphae, have a cell wall structure more complex compared with prokaryotic cells such as *Escherichia coli* and *Bacillus subtilis*. Therefore, visualized screening of single *Aspergillus* colony is difficult to realize. In the common visualized screening method, green fluorescent protein (GFP) was fused to N-terminal target sequence, and screening by fluorescence. In prokaryotic cells such as *Escherichia coli* and *Bacillus subtilis*, single recombinant colony can be picked by green fluorescence deposition of the microorganisms, or single cell is sorted by flow cytometer. However, when the GFP was expressed in *Aspergillus* for sorting, a fluorescence microscope is needed to observe fluorescence of the microorganisms, which is difficult to realize sorting. Screening *Aspergillus* recombinant strain by color reaction of chromogenic culture medium not only has use limitation, but also has complex procedure and inaccurate effect. Therefore, a more rational "visualized" procedure is needed for screening *Aspergillus* recombinant strains.

[0004] Different types of *Aspergillus* have different color of spores. The different colors of the spores can be used as visualized features to distinguish *Aspergillus*. The color of host spores can be changed by editing spore pigmentation related genes, which are used as a "visualized" screening marker for *Aspergillus* recombinant strain. Generally, mature spores of *Aspergillus niger* wild type strains are black. When the genes related to spore color change and a target gene are simultaneously mutated in *Aspergillus* using CRISPR-Cas9, the target gene editing can be visualized.

SUMMARY

[0005] A first object of the present disclosure is to disclose a visualized screening method for *Aspergillus* strains with gene editing, in which a CRISPR-Cas9 gene editing technology is used to simultaneously knock out genes (a) and (b) in the *Aspergillus*, where (a) are genes affecting a spore color change and (b) are genes with unchanged phenotypes of *Aspergillus* before and after knockout; and

[0006] the genes affecting a spore color change are fwnA, pptA and/or brnA.

[0007] Preferably, the gene affecting a spore color change is fwnA.

[0008] In one implementation, the genes with unchanged phenotypes of *Aspergillus* before and after knockout include, but are not limited to, amyA, ammA, pepA and kusA.

[0009] Preferably, the genes with unchanged phenotypes of *Aspergillus* before and after knockout are amyA and/or ammA.

[0010] In one embodiment, the fwnA has a nucleotide sequence as set forth in SEQ ID NO:24.

[0011] In one embodiment, the pptA has a Gene ID of 4985743 (a nucleotide sequence as set forth in SEQ ID NO:50) and the brnA has a Gene ID of 4987395 (a nucleotide sequence as set forth in SEQ ID NO:51).

[0012] In one embodiment, the amyA has a Gene ID of 4980947 (a nucleotide sequence as set forth in SEQ ID NO:52), the ammA has a Gene ID of 4984565 (a nucleotide sequence as set forth in SEQ ID NO:53), the pepA has a Gene ID of 4987328 (a nucleotide sequence as set forth in SEQ ID NO:54), and the kusA has a Gene ID of 4987871 (a nucleotide sequence as set forth in SEQ ID NO:55).

[0013] In one embodiment, *Aspergillus* includes *Aspergillus flavus, Aspergillus* niger, *Aspergillus* fumigatus, *Aspergillus versicolor* and *Aspergillus* nidulans.

[0014] Preferably, the host is *Aspergillus niger*; more preferably, the host is *Aspergillus niger* CCTCC M 2018881, which has been disclosed in Chinese Patent Application No. CN110438018A.

[0015] In one embodiment, the method is to construct a Cas9 expression plasmid and an sgRNA expression cassette and transfer the Cas9 expression plasmid and the sgRNA expression cassette into *Aspergillus niger* for co-expression.

[0016] In one embodiment, a codon-optimized Cas9 gene sequence of *Aspergillus niger* is as set forth in SEQ ID NO:4.

[0017] In one embodiment, the method is to construct an sgRNA expression cassette by using a Pu3 or Pu6 promoter and a corresponding Tu3 or Tu6 terminator.

[0018] In one embodiment, the Pu6 promoter has a nucleotide sequence as set forth in SEQ ID NO:3; and the Tu6 terminator has a nucleotide sequence as set forth in SEQ ID NO:5.

[0019] In one embodiment, the Pu3 promoter has a nucleotide sequence as set forth in SEQ ID NO:2; and the Tu3 terminator has a nucleotide sequence as set forth in SEQ ID NO:4.

[0020] In one embodiment, sgRNA is released by using *Aspergillus* endogenous tRNAs.

[0021] In one embodiment, the endogenous tRNAs include $tRNA^{Ala}$, $tRNA^{Arg}$, $tRNA^{Cys}$, $tRNA^{Ile}$, $tRNA^{Leu}$, $tRNA^{Lys}$, $tRNA^{Met}$, $tRNA^{Phe}$, $tRNA^{Ser}$, $tRNA^{Thr}$, $tRNA^{Val}$, $tRNA^{Glu}$, $tRNA^{Pro}$, $tRNA^{Glu}$, $tRNA^{Gln}$ and $tRNA^{Gly}$.

[0022] In one embodiment, when a single gene is edited, a first tRNA after a promoter is preferably $tRNA^{Ala}$.

[0023] In one embodiment, when two genes are edited, a first tRNA after a promoter is preferably $tRNA^{Ala}$ and a second tRNA is preferably $tRNA^{Arg}$ or $tRNA^{Phe}$.

[0024] In one embodiment, when multiple genes are edited, a first tRNA after a promoter is preferably $tRNA^{Ala}$, a second tRNA is preferably $tRNA^{Phe}$, and a tRNA in a last sgRNA is preferably $tRNA^{Arg}$ or $tRNA^{Ile}$.

[0025] In one embodiment, the $tRNA^{Ala}$ has a nucleotide sequence as set forth in SEQ ID NO:10; the $tRNA^{Arg}$ and the $tRNA^{Phe}$ respectively have a nucleotide sequence as set

forth in SEQ ID NO:20 and SEQ ID NO:16; and the $tRNA^{Ile}$ has a nucleotide sequence as set forth in SEQ ID NO:19.

[0026] A second object of the present disclosure is to disclose a visualized system for gene knockout in *Aspergillus*, the system includes a gene encoding a Cas9 protein, an sgRNA expression cassette and a screening marker; the sgRNA expression cassette contains target sequences of genes affecting a spore color change and target sequences of genes not affecting phenotypes of *Aspergillus*.

[0027] In one embodiment, sgRNA is released by using *Aspergillus* endogenous tRNAs.

[0028] In one embodiment, the endogenous tRNAs include $tRNA^{Ala}$, $tRNA^{Arg}$, $tRNA^{Cys}$, $tRNA^{Ile}$, $tRNA^{Leu}$, $tRNA^{Lys}$, $tRNA^{Met}$, $tRNA^{Phe}$, $tRNA^{Ser}$, $tRNA^{Thr}$, $tRNA^{Val}$, $tRNA^{Glu}$, $tRNA^{Pro}$, $tRNA^{Glu}$, $tRNA^{Gln}$ and $tRNA^{Gly}$.

[0029] In one embodiment,

[0030] when a single gene is edited, a first tRNA after a promoter is $tRNA^{Ala}$;

[0031] when two genes are edited, a first tRNA after a promoter is $tRNA^{Ala}$ and a second tRNA is $tRNA^{Arg}$;

[0032] when multiple genes are edited, a first tRNA after a promoter is $tRNA^{Ala}$, a tRNA in a last sgRNA is $tRNA^{Arg}$ or $tRNA^{Ile}$; or a first tRNA after a promoter is $tRNA^{Ala}$, a tRNA in a last sgRNA is $tRNA^{Arg}$ or $tRNA^{Ile}$ and a Pu6 promoter and a Tu6 terminator are used to separately promote and terminate an expression of the last sgRNA;

[0033] the Pu6 promoter has a nucleotide sequence as set forth in SEQ ID NO:3; the Tu6 terminator has a nucleotide sequence as set forth in SEQ ID NO:5; and

[0034] the $tRNA^{Ala}$ has a nucleotide sequence as set forth in SEQ ID NO:10; the $tRNA^{Arg}$ and the $tRNA^{Phe}$ respectively have a nucleotide sequence as set forth in SEQ ID NO:20 and SEQ ID NO:16; and the $tRNA^{Ile}$ has a nucleotide sequence as set forth in SEQ ID NO:19.

[0035] A third object of the present disclosure is to disclose a method for improving screen efficiency of gene editing in *Aspergillus*, in which a CRISPR-Cas9 gene editing technology is used to simultaneously knock out genes (a) and (b) in the *Aspergillus*, where (a) are genes affecting a spore color change; (b) are genes with unchanged phenotypes of *Aspergillus* before and after knockout; the genes affecting a spore color change include fwnA, pptA and brnA; and the genes with unchanged phenotypes of *Aspergillus* before and after knockout include, but are not limited to, amyA, ammA, pepA and kusA.

[0036] The present disclosure also discloses use of a visualized screening method for an *Aspergillus* strain with gene editing in gene editing of *Aspergillus*.

[0037] The present disclosure also discloses use of a gene knockout visualized system for *Aspergillus* in gene editing of *Aspergillus*.

[0038] The present disclosure also discloses use of a method for improving screen efficiency in gene editing of *Aspergillus* in gene editing of *Aspergillus*.

[0039] In the present disclosure, CRISPR-Cas9 is used to cleave spore color change-related genes and a target gene in *Aspergillus* at the same time, such that editing of the target gene is visualized and an *Aspergillus niger* strain with multigene editing can be rapidly and efficiently screened out through spore phenotypes. Through different combinations of visualized genes and non-phenotypic change genes, rapid screening of the strain with multigene editing and simultaneous screening of multiple visualized genes are realized, and use of resistance genes in industrial strains is reduced. The method of the present disclosure can also be generally used in other *Aspergillus* species and a target gene can be quickly and accurately knocked out by combining visualized genes with a color change and the target gene to be knocked out.

BRIEF DESCRIPTION OF FIGURES

[0040] FIG. **1** is a map of an *Aspergillus niger* Cas9 expression plasmid pUC19-Cas9.

[0041] FIG. **2** is a map of an *Aspergillus niger* sgRNA co-expression plasmid pUC19-sgRNA.

[0042] FIG. **3**A is a map of an *Aspergillus niger* non-phenotypic gene sgRNA and a visualized phenotypic gene sgRNA co-expression plasmid pUC19-sgRNA-1.

[0043] FIG. **3**B is a map of an *Aspergillus niger* non-phenotypic gene sgRNA and a visualized phenotypic gene sgRNA co-expression plasmid pUC19-sgRNA-2.

[0044] FIG. **3**C is a map of an *Aspergillus niger* non-phenotypic gene sgRNA and a visualized phenotypic gene sgRNA co-expression plasmid pUC19-sgRNA-3.

[0045] FIG. **4** shows comparison of a spore color of an *Aspergillus niger* visualized transformant with gene editing.

DETAILED DESCRIPTION

[0046] (I) Culture Medium

[0047] PDA culture medium: 200 g of potatoes, 20 g of glucose, 15-20 g of agar and the balanced water to a constant volume of 1 L.

[0048] LB culture medium: 10 g of peptone, 5 g of yeast powder, 10 g of NaCl and the balanced water to a constant volume of 1 L.

[0049] (II) Reagent Formula

[0050] STC buffer solution: 1.2 M sorbitol, 50 mM $CaCl_2$ and 10 mM Tris, and pH of 7.5-8.

[0051] PEG buffer solution: 25% PEG 6000, 50 mM $CaCl_2$ and 10 mM Tris, and pH of 7.5-8.

TABLE 1

PCR primers for verification of targeted sites

Name of primers	Sequence	
pptA-F	TAACCCAACCCCTCACTTCACCT	SEQ ID NO: 25
pptA-R	TGGAGACGTATTCCAGGAAGGCT	SEQ ID NO: 26
brnA-F	TGTTTGGATCTGATGCCGAGGC	SEQ ID NO: 27
brnA-R	GGCTTGACGCTGATCTTGGT	SEQ ID NO: 28
fwnA-F	GACCAATGACAAGACTCTGTGGGT	SEQ ID NO: 29
fwnA-R	TCTTCTTCCCCTCCGCAGTGAC	SEQ ID NO: 30
kusA-F	TCAAATGCGCCTATCACTTCATGC	SEQ ID NO: 31
kusA-R	CCGCCGGTTAATACGATGTCATAT	SEQ ID NO: 32
amyA-F	GCAGGGCATCATCGACAAGGT	SEQ ID NO: 33
amyA-R	GGTGGTATCGAGATCAGGCAAGG	SEQ ID NO: 34
pepA-F	TCCATCATGACGGCTGCCA	SEQ ID NO: 35
pepA-R	CGAACTCGGAGCTGATCTTGC	SEQ ID NO: 36

TABLE 1-continued

PCR primers for verification of targeted sites

Name of primers	Sequence	
ammA-F	GACGCTGTTCTGTCGCTTTGT	SEQ ID NO: 37
ammA-R	GATCAGGCAGTATGGGTGGAAGT	SEQ ID NO: 38

TABLE 2

Sequence of tRNA

Name	Sequence	
$tRNA^{Pro}$	GCCCGGGTGGTCTAGTGGTATGATTCTCGCTTAGGGATAC AAACCCAAGCATATCTGCGAGAGGTCCCGCGTTCGATCCG CGGCTCGGGCC	SEQ ID NO: 9
$tRNA^{Ala}$	GGGGCTGTGGTTTAGTGGTATAATATTCCCTTAGCATGGG AGAGGtCCGGGGTTCGATTCCCCGCAGCTCCA	SEQ ID NO: 10
$tRNA^{Gly}$	ACAACCATACGTTAATTGGTAAACTAGTCGTCTTCCAAAC GATAAtTGTGAGTTCGAACCTCACTGGTTGTA	SEQ ID NO: 11
$tRNA^{Thr}$	GCTTCTATGGCTCAGTTGGTAGAGCGCATGACTAGTAATC ATGAGGtCCGCGGTTCGAATCCGCGTGGAAGCA	SEQ ID NO: 12
$tRNA^{Val}$	GGCCGGATGGTGTAGTTGGTtATCACGTATCGTTAACACC GATAAGGtCCTGGGATCGAGCCCCAGTCTGGTCA	SEQ ID NO: 13
$tRNA^{Ser}$	GTCAGTGTGGCCGAGTGGTtAAGGCGATAGACTAGAAATCT ATTGGGTTCGCCCGCACAGGTTCGAGTCCTGTCGCTGACG	SEQ ID NO: 14
$tRNA^{Leu}$	GGCAAGATGGCCGAGTTGGTCCAAGGCGTCAGGTTAAGGT CCACCTTAATACCCAGCTTCACAGCTTCCTGATCATCGTA AGATGGCGTGGGTTCGAATCCCACTCTTGTCA	SEQ ID NO: 15
$tRNA^{Phe}$	GGGGCAATGGCGCATCTGGGAGCGCGTCAGACTGAAGATC TGGAGGtGGCCGGTTCAAGCCCGGCTTGCCCCA	SEQ ID NO: 16
$tRNA^{Lys}$	GCCCGGCTAGCTCAATCGGTAGAGCGTGAGACTCTTAATC TCAAGGcTGCGGGTTCGAGCCCCGCGTTGGGCT	SEQ ID NO: 17
$tRNA^{Glu}$	TCCGATATGGTGTAGTGGCtAACATCGCCGTCTCTCACAC GGCAGCCGGGGGTTCGATTCCCCCTATCGGAG	SEQ ID NO: 18
$tRNA^{Ile}$	GGTCCCATAGCTCAGTTGGTtAGAGCGTGACGCTAATAAC GTCAAAGtCGAGGGTTCGAGCCCCTCTGGGACCA	SEQ ID NO: 19
$tRNA^{Arg}$	GGCCTGCTGGCCCAATGGTAAGGCGCTTGACTACGGATCA AGAGAtTGCAGGTTCGAGTCCTGCGTAGGTCA	SEQ ID NO: 20
$tRNA^{Met}$	AGCATGTTAGCTCAGGGGAAGAGCGCCGGGCTCATAACCC GGAGGtCCCTGGATCGAAACCAGGACATGCTA	SEQ ID NO: 21
$tRNA^{Gln}$	GGTTGTGTAGTGTAATGGTcATCACTCTGGATTCTGATTC CAGCAATCCCGGTTCGATCCCGGGCACGACCT	SEQ ID NO: 22
$tRNA^{Cys}$	GGGCCGGTAGCTCAGGGGTAGAGCGTGGGACTGCAGATCT TAAGGtCACGCGTTCAAATCGCGTTCGGCCCT	SEQ ID NO: 23

Example 1: Construction of Visualized Recombinant *Aspergillus niger* Strain with Single Gene Editing by Using CRISPR-Cas9

[0052] A CRISPR-Cas9 system included a gene encoding a Cas9 protein (a Cas9 gene), sgRNA and a screening marker.

[0053] (1) Construction of Cas9 Expression Vector

[0054] An *Aspergillus* promoter PglaA (with a nucleotide sequence as set forth in SEQ ID NO:7) or Ptef1 (with a nucleotide sequence as set forth in SEQ ID NO:6) and other *Aspergillus* strong promoters were used to express a Cas9 protein (with a nucleotide sequence as set forth in SEQ ID NO:1).

[0055] A ClonExpress® II One Step Cloning Kit (Vazyme) was used, pUC19 was used as a vector framework, an *Aspergillus* promoter sequence, a gene sequence encoding a Cas9 protein, a resistance gene and an AMA1 (GenBank: X78051.1) sequence were subjected to homologous recombination twice to obtain a Cas9 expression plasmid pUC19-Cas9 (a plasmid map was shown in FIG. **1**). A nuclear localization signal (NLS) sequence (CCCAAGAAGAAGCGCAAGGTC, SEQ ID NO:56) was added at a or a C-terminal of a gene encoding a Cas9 protein (as set forth in SEQ ID NO:1).

[0056] (2) Construction of sgRNA Expression Cassette

[0057] A promoter Pu3, a protospacers sequence of a target gene (See Table 3), a gRNA backbone sequence (a

nucleotide sequence as set forth in SEQ ID NO:8) and a terminator Tu3 are used to construct an sgRNA expression cassette.

TABLE 3

Sequence listing of target gene protospacers

Target gene	Target gene sequence	
fwnA	AGTGGGATCTCAAGAACTAC	SEQ ID NO: 39
pptA	GGCGGGTGTCGATGTACCAC	SEQ ID NO: 40
brnA	ACCATGCCAATGGATTCCGG	SEQ ID NO: 41
kusA	CGAGCACTGGTAGATGATGA	SEQ ID NO: 42

[0058] Screening markers included filamentous fungal markers of similar effects as hygromycin B (hygB), orotidine-5'-phosphate dehydrogenase, acetamidase and the like commonly used in *Aspergillus*. A hygromycin resistance gene in a recombinant plasmid was obtained from a plasmid PAN7-1, expression cassette primers Hyg-F/R were shown in Table 4, and hyg in other resistance replaceable expression cassettes were selected for construction.

TABLE 4

Listing of primers

Name of primers	Primer sequence	
Hyg-F	GAATTCCCTTGTATCTCTAC ACACAG	SEQ ID NO: 43
Hyg-R	TGAAGAACGAATACCGCGAC ATCCAACCCATC	SEQ ID NO: 44

[0059] A ClonExpress® II One Step Cloning Kit (Vazyme) was used and pUC19 was used as a vector framework for recombination with the sgRNA expression cassette to construct an sgRNA expression plasmid (a plasmid map was shown in FIG. **2**).

[0060] (3) Transformation of Cas9 Expression Plasmid and sgRNA Expression Cassette

[0061] The Cas9 expression plasmid and the sgRNA expression cassette were transferred into a host by using a protoplast transformation method.

[0062] *Aspergillus niger* hyphae were cultured in a PDA culture medium overnight and mycelia were collected and washed three times with normal saline; the washed mycelia were enzyme-digested with a Lysozyme for 3 h and filtered with four layers of lens paper to prepare a protoplast; the protoplast was collected by centrifugation at 4° C., and 1,000 rpm and washed 2-3 times with pre-cooled STC; and 100 μL of the prepared protoplast was taken, into which 10 μL of the Cas9 expression plasmid and 10 μL of the sgRNA expression cassette were added and mixed evenly, 2 mL of PEG 6000 was added, and the corresponding resistance was added to the culture medium for screening. The culture was conducted at 30° C. for 5-7 d, a genome of a transformant was extracted, and editing of a targeting site of a target gene was verified by PCR.

[0063] A positive single colony was picked and transferred to a plate, and each single colony was transferred three times (that is, a single colony was picked to a new culture medium for culture).

[0064] A spore color of a starting strain of *Aspergillus niger* CCTCC M 2018881 (the strain has been disclosed in the patent application document with the publication number of CN110438018A) was used as a control, changes in a color phenotype of *Aspergillus niger* spores after transformation were observed, after fwnA or pptA were destroyed, a spore color was changed from brown to white, and after brnA was destroyed, the spore color was changed from brown to olive (See FIG. **4**). When a non-phenotypic change gene kusA was destroyed, phenotypes of microorganisms did not change. A spore color change transformant had significantly improved gene editing efficiency, where, a color mutant strain accounted for 14%, the color mutant strain has gene editing efficiency of brnA for 30% and editing efficiency of fwnA or pptA for 100% and editing efficiency of kusA for 4.16%.

Example 2: Visualized Screening for Recombinant *Aspergillus niger* Strain with Double Gene Editing

[0065] (1) Construction of Cas9 Expression Vector

[0066] The specific implementation referred to step (1) in Example 1.

[0067] (2) Construction of sgRNA Expression Cassette

[0068] A Pu3 mutant promoter (to facilitate assembly of multiple sgRNAs, a BsaI site related with the sgRNAs was mutated to facilitate subsequent assembly, specifically a BsaI site mutation of a nucleotide sequence of the Pu3 promoter sequence as set forth in SEQ ID NO:2 was mutated from GAGACC to ACCCAC), target gene protospacers sequences pptA, fwnA and brnA (See Table 1), an amyA sequence (See Table 5), an sgRNA expression cassette realized by using $tRNA^{Gly}$, a gRNA backbone sequence (with a nucleotide sequence as set forth in SEQ ID NO:8), a terminator Tu3 (with a nucleotide sequence as set forth in SEQ ID NO:4) were used to construct the sgRNA expression cassette.

TABLE 5

Sequence listing of target gene protospacers

Target gene	Target gene sequence	
amyA	TCTCTTCGGCCCTTCATGAG	SEQ ID NO: 45

[0069] Screening markers included filamentous fungal markers of similar effects as hygromycin B (hygB), orotidine-5'-phosphate dehydrogenase, acetamidase and the like commonly used in *Aspergillus*. A hygromycin resistance gene in a recombinant plasmid was obtained from a plasmid PAN7-1, expression cassette primers Hyg-F/R were shown in Table 3, and hygB in other resistance replaceable expression cassettes were selected for construction.

[0070] A ClonExpress® II One Step Cloning Kit (Vazyme) was used, pUC19 was used as a vector framework, an *Aspergillus* promoter sequence, a gene sequence encoding a Cas9 protein, a resistance gene and an AMA1 sequence were subjected to homologous recombination twice to pUC19 to obtain a Cas9 expression plasmid pUC19-Cas9 (a plasmid map was shown in FIG. **1**). A nuclear localization signal (NLS) sequence (CC-

CAAGAAGAAGCGCAAGGTC, SEQ ID NO:56) was added to a N-terminal or a C-terminal of a gene encoding a Cas9 protein (as set forth in SEQ ID NO:1).

[0071] A ClonExpress® II One Step Cloning Kit (Vazyme) was used, pUC19 was used as a vector framework, an sgRNA expression cassette was recombined to the pUC19 to construct a double sgRNA expression plasmid PUC19-sgRNA-1 plasmid containing the sgRNA expression cassette with two protospacers sequences, and $tRNA^{Gly}$ was used to release different sgRNAs. The sgRNA expression cassette included a visualized gene sgRNA and a non-phenotypic sgRNA, and the visualized gene sgRNA was pptA-sgRNA, fwnA-sgRNA or brnA-sgRNA, and the other non-phenotypic gene sgRNA was amyA-sgRNA (a plasmid map was shown in FIG. **3**).

[0072] (3) Transformation of Cas9 Expression Plasmid and sgRNA Expression Cassette

[0073] The Cas9 expression plasmid and the double sgRNA expression cassette (including any one of pptA-sgRNA and amyA-sgRNA or fwnA-sgRNA and amyA-sgRNA, or brnA-sgRNA and amyA-sgRNA) were transferred into a host by protoplast transformation, and the sgRNA was directly connected with the tRNA to construct a strain containing the double sgRNA expression cassette.

[0074] *Aspergillus niger* hyphae were cultured in a PDA culture medium overnight and mycelia were collected and washed three times with normal saline; the washed mycelia were enzyme-digested with a Lysozyme for 3 h and filtered with four layers of lens paper to prepare a protoplast; the protoplast was collected by centrifugation at 4° C., and 1,000 rpm and washed 2-3 times with pre-cooled STC; and 100 μL of the prepared protoplast was taken, into which 10 μL of the Cas9 expression plasmid and 10 μL of the sgRNA expression cassette were added and mixed evenly, 2 mL of PEG 6000 was added, and the corresponding resistance was added to the culture medium for screening. The culture was conducted at 30° C. for 5-7 d and a single colony transformant of white spores were picked for sequencing verification.

[0075] A spore color of a starting strain of *Aspergillus niger* CCTCC M 2018881 was used as a control and changes of color phenotypes of *Aspergillus niger* spores after transformation were observed. After fwnA was knocked out, the spore color changed from black to white, a total of 4% of single colony on a plate showed white, the white single colony was directly picked, and the results showed that in the white single colony, a homozygous transformant with double-copy destroy of an amyA gene accounted for 25% and a positive selection rate of a transformant with multi-gene editing was improved. Editing of the double copies of the amyA gene did not require new resistance markers, which was beneficial to industrial production.

Comparative Example 1

[0076] A same method was used as Example 2, a single-copy recombinant strain with gene amyA editing was constructed. A same method was used, the recombinant strain was cultured until a single colony was grown, the single colony was picked and gene editing efficiency was calculated. The gene editing efficiency was 5%.

Example 3: Visualized Screening for Recombinant *Aspergillus niger* Strain with Multigene Editing

[0077] The specific implementation referred to Example 2. The difference was that $tRNA^{Gly}$ behind a promoter in Example 2 was replaced by $tRNA^{Ala}$ and a second tRNA was replaced by $tRNA^{Phe}$.

[0078] The $tRNA^{Ala}$ and the $tRNA^{Phe}$ were used to ligate with different sgRNAs. A visualized gene sgRNA expression cassette was a fwnA-sgRNA expression cassette and the other non-phenotypic gene sgRNA expression cassette was an amyA-sgRNA expression cassette (a plasmid map was shown in FIG. **3**).

[0079] A spore color of a starting strain of *Aspergillus niger* CCTCC M 2018881 was used as a control and changes in color phenotypes of *Aspergillus niger* spores after transformation were observed. After fwnA was knocked out, the spore color changed from black to white, 36% of transformants had a white phenotype, the white single colony was directly picked, a positive selection rate of a transformant with multigene editing was improved, time for strain purification was saved, and the amyA gene editing efficiency was 90%. Editing of the amyA gene did not require new resistance markers, which was beneficial to industrial production.

Comparative Example 2

[0080] A same method was used as Example 3, a recombinant strain with double gene fwnA and amyA editing was constructed by using $tRNA^{Gly}$. A same method was used, the recombinant strain was cultured until a single colony was grown, the single colony was picked and gene editing efficiency was calculated. The results showed that 6% was a white mutant and the amyA gene editing efficiency was 36%.

Comparative Example 3

[0081] A same method was used as Example 3, a recombinant strain with fwnA and amyA gene editing was constructed by using $tRNA^{Ala}$. A same method was used, the recombinant strain was cultured until a single colony was grown, the single colony was picked and gene editing efficiency was calculated. The results showed that 37% was a white mutant and the amyA gene editing efficiency was 70%.

Comparative Example 4

[0082] A same method was used as Example 3, a single copy recombinant strain with gene fwnA and amyA editing was constructed by using $tRNA^{Phe}$. A same method was used, the recombinant strain was cultured until a single colony was grown, the single colony was picked and gene editing efficiency was calculated. The results showed that 15% was a white mutant and the amyA gene editing efficiency was 80%.

Example 4: Visualized Multi-Phenotype Screening for Recombinant *Aspergillus* Niger Strain with Gene Editing

[0083] The specific implementation referred to Example 2. The difference was that the amyA gene in Example 2 was replaced by pepA or ammA (sequence listing of protospacers

was shown in Table 6), the $tRNA^{Gly}$ behind a promoter in Example 2 was replaced by $tRNA^{Ala}$ and a second tRNA was replaced by $tRNA^{Arg}$

TABLE 6

Sequence listing of target gene protospacers

Target gene	Target gene sequence	
pepA	CGGTGTCAAAGTCCAGATGG	SEQ ID NO: 46
ammA	**CTGCCCCAGGATACTGCTGA**	SEQ ID NO: 47

[0084] The $tRNA^{Ala}$ and the $tRNA^{Arg}$ were used to release different sgRNAs. A visualized gene sgRNA expression cassette was a fwnA-sgRNA expression cassette and the other non-phenotypic sgRNA expression cassette was a pepA-sgRNA expression cassette or an ammA-sgRNA expression cassette (a plasmid map was shown in FIG. **3**B).

[0085] The Cas9 expression plasmid and the sgRNA expression cassette were transferred into a host and obtained by sequencing.

[0086] A spore color of a starting strain of *Aspergillus niger* CCTCC M 2018881 was used as a control and changes in color phenotypes of *Aspergillus niger* spores after transformation were observed. After fwnA was knocked out, the spore color changed from black to white, a white single colony was picked, the gene editing efficiency was calculated, and the pepA or ammA gene editing efficiency was 75% and 80% respectively. The method was beneficial to perform an in-vivo screening for an active protospacers sequence of a target gene of *Aspergillus niger*, and saved time and cost of molecular operation.

Example 5: Visualized Multi-Phenotype Screening for Recombinant *Aspergillus Niger* Strain with Gene Editing

[0087] The specific implementation referred to Example 2. The difference was that the amyA gene in Example 2 was replaced by ammA, at the same time, a third amyA-sgRNA (sequence listing of protospacers was shown in Table 7) was ligated with $tRNA^{Ala}$, the $tRNA^{Gly}$ behind a promoter in Example 2 was replaced by $tRNA^{Ala}$ and a second tRNA was replaced by $tRNA^{Phe}$.

TABLE 7

Sequence listing of target gene protospacers

Target gene	Target gene sequence	
ammA	CTGCCCCAGGATACTGCTGA	SEQ ID NO: 48
amyA	TCTCTTCGGCCCTTCATGAG	SEQ ID NO: 49

[0088] The $tRNA^{Ala}$, the $tRNA^{Arg}$ and the $tRNA^{Phe}$ were used to release different sgRNAs. A visualized gene sgRNA was fwnA-sgRNA and the other two non-phenotypic gene sgRNAs were pepA-sgRNA and ammA-sgRNA, respectively.

[0089] The Cas9 expression plasmid and the sgRNA expression cassette were transferred into a host and obtained by sequencing.

[0090] A spore color of a starting strain of *Aspergillus niger* CCTCC M 2018881 was used as a control and changes in color phenotypes of *Aspergillus niger* spores after transformation were observed. After fwnA was knocked out, the spore color changed from black to white. In an optimized three-gene knockout white transformant, the ammA gene editing efficiency was 50%, the amyA gene editing efficiency was 100% and the double gene co-editing efficiency was 50%.

[0091] The method was beneficial to perform an in-vivo screening of an active protospacers sequence of multiple target genes of *Aspergillus niger*, and saved time and cost of molecular operation.

Example 6: Visualized Multi-Phenotype Screening for Recombinant *Aspergillus Niger* Strain with Gene Editing

[0092] The specific implementation referred to Example 2. The difference was that the amyA gene in Example 2 was replaced by ammA, the $tRNA^{Gly}$ behind a promoter in Example 2 was replaced by $tRNA^{Ala}$ and a second $tRNA^{Gly}$ was replaced by $tRNA^{Phe}$; and at the same time, an sgRNA expression cassette was added, $tRNA^{Ile}$ was used for release, and a Pu6 promoter (with a nucleotide sequence as set forth in SEQ ID NO:3) and a Tu6 terminator (with a nucleotide sequence as set forth in SEQ ID NO:5) were used to express $tRNA^{Ile}$ and ammA-sgRNA.

[0093] The $tRNA^{Ala}$, the $tRNA^{Arg}$ and the $tRNA^{Phe}$ were used to release different sgRNAs. A visualized gene sgRNA expression cassette was fwnA-sgRNA and the other two non-phenotypic gene sgRNAs were ammA-sgRNA and amyA-sgRNA.

[0094] The Cas9 expression plasmid and the sgRNA expression cassette were transferred into a host and subjected to sequencing verification.

[0095] A spore color of a starting strain of *Aspergillus niger* CCTCC M 2018881 was used as a control and changes in color phenotypes of *Aspergillus niger* spores after transformation were observed. After fwnA was knocked out, the spore color changed from black to white. In an optimized three-gene knockout white transformant, the ammA gene editing efficiency was 69%, the amyA gene editing efficiency was 100% and the two gene co-editing efficiency was 69%. The method was beneficial to perform an in-vivo screening of an active protospacers sequence of multiple target genes of *Aspergillus niger*, and saved time and cost of molecular operation.

[0096] Although the disclosure has been disclosed as above in the preferred examples, it is not intended to limit the disclosure. Any person skilled in the art can make various changes and modifications without departing from the spirit and scope of the disclosure. Therefore, the protection scope of the disclosure should be as defined in the claims.

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tcactggttg ta 72

<210> SEQ ID NO 12

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<211> LENGTH: 73
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<220> FEATURE:
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ccgcgtggaa gca 73

<210> SEQ ID NO 13
<211> LENGTH: 74
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 13

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ccccagtctg gtca 74

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 14

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gttcgagtcc tgtcgctgac g 81

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<211> LENGTH: 112
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<210> SEQ ID NO 16
<211> LENGTH: 73
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic DNA

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ccggcttgcc cca 73

<210> SEQ ID NO 17
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 17

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cccgcgttgg gct 73

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 18

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cccctatcgg ag 72

<210> SEQ ID NO 19
<211> LENGTH: 74
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 19

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cccctctggg acca 74

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<212> TYPE: DNA
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<220> FEATURE:
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ctgcgtaggt ca 72

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<212> TYPE: DNA
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<220> FEATURE:
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caggacatgc ta 72

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

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cgggcacgac ct 72

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<220> FEATURE:
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gcgttcggcc ct 72

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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ttccatgcaa ttcgtcaaga gatcgcgaag ctcccgccgt ctcatcggaa gctcttccca 180

cgcttcacga gcatcgttga tctcctttcc aggagtcgtg aatcaggtcc tagccctgtc 240

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cggctgtaag ttgcgccaga aatgttggag agcttattcc agctgcagtg gaatcggttg 480

taattgcact gcgactggga atctgcgttt ttcgagttcg agaactggtg gactccgccg 540

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<220> FEATURE:
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<400> SEQUENCE: 25

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<210> SEQ ID NO 26
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 26

tggagacgta ttccaggaag gct 23

<210> SEQ ID NO 27
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 27

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<210> SEQ ID NO 28
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 28

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<210> SEQ ID NO 29
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

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<400> SEQUENCE: 29

gaccaatgac aagactctgt gggt 24

<210> SEQ ID NO 30
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 30

tcttcttccc ctccgcagtg ac 22

<210> SEQ ID NO 31
<211> LENGTH: 24
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<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 31

tcaaatgcgc ctatcacttc atgc 24

<210> SEQ ID NO 32
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ccgccggtta atacgatgtc atat 24

<210> SEQ ID NO 33
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 33

gcagggcatc atcgacaagg t 21

<210> SEQ ID NO 34
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<400> SEQUENCE: 34

ggtggtatcg agatcaggca agg 23

<210> SEQ ID NO 35
<211> LENGTH: 19
<212> TYPE: DNA
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<400> SEQUENCE: 35

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<210> SEQ ID NO 36
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<400> SEQUENCE: 36

cgaactcgga gctgatcttg c 21

<210> SEQ ID NO 37
<211> LENGTH: 21
<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 37

gacgctgttc tgtcgctttg t 21

<210> SEQ ID NO 38
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 38

gatcaggcag tatgggtgga agt 23

<210> SEQ ID NO 39
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<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 39

agtgggatct caagaactac 20

<210> SEQ ID NO 40
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 40

ggcgggtgtc gatgtaccac 20

<210> SEQ ID NO 41
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 41

accatgccaa tggattccgg 20

<210> SEQ ID NO 42
<211> LENGTH: 20
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 42

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<210> SEQ ID NO 43
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 43

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<210> SEQ ID NO 44
<211> LENGTH: 32
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 44

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<210> SEQ ID NO 45
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 45

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<210> SEQ ID NO 46
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 46

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<210> SEQ ID NO 47
<211> LENGTH: 20
<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 47

ctgccccagg atactgctga 20

<210> SEQ ID NO 48
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 48

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<210> SEQ ID NO 49
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 49

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<210> SEQ ID NO 50
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 50

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gccatccaga aatactatca cctgaaggac agacacatgt ccctagcctc caacctcctc 180

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gttagggttg aggtgcaggc gtttgagggg gggtatttgg ttgctacggc ggccagaggg 840

ggaggtgttg ggaggataga gggagaaggg aaagatgtgt gggagggatt gaggaggttg 900

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<400> SEQUENCE: 51

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<400> SEQUENCE: 52

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ctactagctt tcagaaagag gaatgtaaac tgacttgata tagaaatact gtggtggaac 240

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agaagaaaaa taaaataaaa agaactctag tcctaaccat cacatagttg gactatatcc 360

agggaatggg cttcacagcc atctggatca cccccgttac agcccagctg ccccagacca 420

ccgcatatgg agatgcctac catggctact ggcagcagga tatgtaagtc gatttcttta 480

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gcgaacgcaa tccggaacta tgccattagc aaagatacag gattcgtgac ctacaaggta 1680

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<220> FEATURE:
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tatttgctgc agctggatta tatccagggc atgggattca cggccatctg gatctcgcct 360

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cagaagatgt atgcgctcct ccttcccata tcgtaggctt actctcaggc ggcgactgac 480

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<400> SEQUENCE: 54

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acccgcacca tcaacctgcc aggcatgtac gcccgttccc tggccaagtt tggcggtacg 180

gtgccccaga gcgtgaagga ggctgccagc aagggtagtg ccgtgaccac gccccagaac 240

aatgacgagg agtacctgac tcccgtcact gtcggaaagt ccaccctcca tctggacttt 300

gacaccggat ctgcagatct gtaagcttcc ctgctcgggt gttcgggcaa atcgtgacta 360

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cagagctaca aaccagtcaa agatgcggtc ctcttcgcaa tcgatgtcag cgattccatg 180

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gttggatgtc gcggtattcg ttcttcagcg aaacgtaatg gacacgtatt ttaggcgatg   2460

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gg                                                                  2522


<210> SEQ ID NO 56
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 56

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What is claimed is:

1. A method for visualized screening of an *Aspergillus* strain with gene editing, wherein using CRISPR-Cas9 gene editing technology to simultaneously knock out genes (a) and (b) in the *Aspergillus;*

wherein (a) are genes affecting a spore color change; (b) are genes with unchanged phenotypes of *Aspergillus* before and after knockout; and

the genes affecting a spore color change are fwnA, pptA and/or brnA.

2. The method according to claim **1**, wherein the genes with unchanged phenotypes of *Aspergillus* before and after knockout are amyA and/or ammA; and the amyA has a nucleotide sequence as set forth in SEQ ID NO:52 and the ammA has a nucleotide sequence as set forth in SEQ ID NO:53.

3. The method according to claim **2**, wherein the fwnA has a nucleotide sequence as set forth in SEQ ID NO:24, the pptA has a nucleotide sequence as set forth in SEQ ID NO:50 and the brnA has a nucleotide sequence as set forth in SEQ ID NO: 51.

4. The method according to claim **3**, wherein sgRNA is released by using *Aspergillus* endogenous tRNAs and the endogenous tRNAs comprise $tRNA^{Ala}$, $tRNA^{Arg}$, $tRNA^{Cys}$, $tRNA^{Ile}$, $tRNA^{Leu}$, $tRNA^{Lys}$, $tRNA^{Met}$, $tRNA^{Phe}$, $tRNA^{Ser}$, $tRNA^{Thr}$, $tRNA^{Val}$, $tRNA^{Glu}$, $tRNA^{Pro}$, $tRNA^{Glu}$, $tRNA^{Gln}$ and $tRNA^{Gly}$.

5. The method according to claim **4**, wherein when a single gene is edited, a first tRNA after a promoter is $tRNA^{Ala}$; and the $tRNA^{Ala}$ has a nucleotide sequence as set forth in SEQ ID NO:10.

6. The method according to claim **4**, wherein when two genes are edited, a first tRNA after a promoter is $tRNA^{Ala}$ and a second tRNA is $tRNA^{Arg}$ or $tRNA^{Phe}$; and the $tRNA^{Arg}$ and the $tRNA^{Phe}$ respectively have a nucleotide sequence as set forth in SEQ ID NO:20 and SEQ ID NO:16.

7. The method according to claim **4**, wherein when multiple genes are edited, a first tRNA after a promoter is $tRNA^{Ala}$, a second tRNA is $tRNA^{Phe}$, and a tRNA in a last sgRNA is $tRNA^{Arg}$ or $tRNA^{Ile}$; and the $tRNA^{Ile}$ has a nucleotide sequence as set forth in SEQ ID NO:19.

8. The method according to claim **7**, wherein a Pu6 promoter and a Tu6 terminator are used to separately promote and terminate an expression of the last sgRNA; the Pu6 promoter has a nucleotide sequence as set forth in SEQ ID NO:3; and the Tu6 terminator has a nucleotide sequence as set forth in SEQ ID NO:5.

9. The method according to claim **3**, wherein the *Aspergillus* comprises *Aspergillus flavus, Aspergillus* niger, *Aspergillus* fumigatus, *Aspergillus versicolor* and *Aspergillus* nidulans.

10. A visualized system for gene knockout in *Aspergillus*, wherein the visualized system comprises a gene encoding a Cas9 protein, an sgRNA expression cassette and a screening marker; the sgRNA expression cassette contains target sequences of genes affecting a spore color change and target sequences of genes not affecting phenotypes of *Aspergillus*; and the genes affecting a spore color change comprise fwnA, pptA or brnA.

11. The visualized system according to claim **10**, wherein the genes with unchanged phenotypes of *Aspergillus* before and after knockout are amyA and/or ammA; and the amyA has a nucleotide sequence as set forth in SEQ ID NO:52 and the ammA has a nucleotide sequence as set forth in SEQ ID NO:53.

12. The visualized system according to claim **11**, wherein sgRNA is released by using *Aspergillus* endogenous tRNAs and the endogenous tRNAs comprise $tRNA^{Ala}$, $tRNA^{Arg}$, $tRNA^{Cys}$, $tRNA^{Ile}$, $tRNA^{Leu}$, $tRNA^{Lys}$, $tRNA^{Met}$, $tRNA^{Phe}$, $tRNA^{Ser}$, $tRNA^{Thr}$, $tRNA^{Val}$, $tRNA^{Glu}$, $tRNA^{Pro}$, $tRNA^{Glu}$, $tRNA^{Gln}$ and $tRNA^{Gly}$.

13. The visualized system according to claim **12**, wherein

when a single gene is edited, a first tRNA after a promoter is $tRNA^{Ala}$;

when two genes are edited, a first tRNA after a promoter is $tRNA^{Ala}$ and a second tRNA is $tRNA^{Arg}$,

when multiple genes are edited, a first tRNA after a promoter is $tRNA^{Ala}$, a tRNA in a last sgRNA is $tRNA^{Arg}$ or $tRNA^{Ile}$; or a first tRNA after a promoter is $tRNA^{Ala}$, a tRNA in a last sgRNA is $tRNA^{Arg}$ or $tRNA^{Ile}$ and a Pu6 promoter and a Tu6 terminator are used to separately promote and terminate an expression of the last sgRNA;

the Pu6 promoter has a nucleotide sequence as set forth in SEQ ID NO:3; the Tu6 terminator has a nucleotide sequence as set forth in SEQ ID NO:5; and

the $tRNA^{Ala}$ has a nucleotide sequence as set forth in SEQ ID NO:10; the $tRNA^{Arg}$ and the $tRNA^{Phe}$ respectively have a nucleotide sequence as set forth in SEQ ID NO:20 and SEQ ID NO:16;

and the $tRNA^{Ile}$ has a nucleotide sequence as set forth in SEQ ID NO:19.

* * * * *