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- (71) **Applicant:** BATTELLE MEMORIAL INSTITUTE
[US/US]; P.O. Box 999, M/S K1-53, Richland, WA 99352 (US).
- (72) **Inventors:** DAI, Ziyu; P.O. Box 999, M/S K1-53, Richland, WA 99352 (US). BAKER, Scott, E.; P.O. Box 999, M/S K1-53, Richland, WA 99352 (US).
- (74) **Agent:** CONNOLLY, Jodi, L.; Klarquist Sparkman, LLP, One World Trade Center, Suite 1600, 121 SW Salmon Street, Portland, OR 97204 (US).
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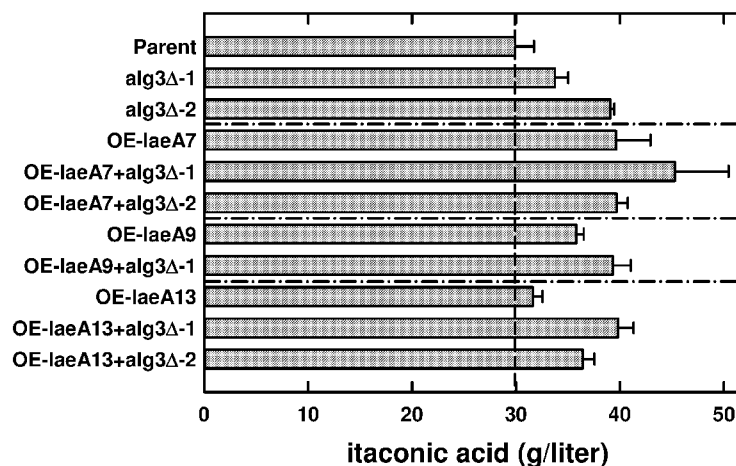
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- (54) **Title:** ENHANCED ITACONIC ACID PRODUCTION IN ASPERGILLUS WITH INCREASED LAEA EXPRESSION

FIG. 28

Itaconic acid production in *A. terreus* after 6 days culture

- (57) **Abstract:** Fungi, such as *Aspergillus niger*, having a dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase (*Alg3*) gene genetic inactivation, increased expression of a loss of *afIR* expression A (*LaeA*), or both, are described. In some examples, such mutants have several phenotypes, including an increased production of citric acid relative to the parental strain. Methods of using the disclosed fungi to make citric acid are also described, as are compositions and kits including the disclosed fungi. Further described are *Aspergillus terreus* fungi overexpressing the *LaeA* gene and the use of such fungi for the production of itaconic acid. In some instances, the *A. terreus* fungi also include a genetic inactivation of the *Alg3* gene.

ENHANCED ITACONIC ACID PRODUCTION IN ASPERGILLUS WITH INCREASED LAEA EXPRESSION

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Patent Application No. 14/928,511, filed October 30, 2015, which is herein incorporated by reference in its entirety. U.S. Patent Application No. 14/928,511 is a continuation-in-part of U.S. Patent Application No. 14/703,499, filed May 4, 2015, issued as U.S. Patent No. 9,206,450 on December 8, 2015, which is a continuation-in-part of U.S. Application No. 13/691,396, filed November 30, 2012, issued as U.S. Patent No. 9,023,637 on May 10 5, 2015, which claims the benefit of U.S. Provisional Application No. 61/565,018, filed November 30, 2011. This application is related to PCT Application No. PCT/US2012/067349, filed November 30, 2012, which claims the benefit of U.S. Provisional Application No. 61/565,018, filed November 30, 2011.

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15 This invention was made with government support under contract number DE-AC05-76RLO1830 awarded by the U.S. Department of Energy. The government has certain rights in the invention.

FIELD

20 This disclosure concerns recombinant *Aspergillus terreus* fungi that are genetically enhanced to increase the expression levels of the loss of *afIR* expression A (*LaeA*) gene, which results in a significant increase in itaconic acid production. In some instances, the *A. terreus* fungi also include a genetic inactivation of the *Alg3* gene. This disclosure further concerns methods of 25 using these fungi to produce itaconic acid.

BACKGROUND

Filamentous fungi, such as *Aspergillus niger*, are well known for their industrial applications in protein and chemical productions. They are used to produce a wide variety of 30 products ranging from human therapeutics, glycosyl hydrolases to specialty chemicals (Punt *et al.*, *Trends Biotechnol* 20(5):200-206, 2002; Schuster *et al.*, *Appl Microbiol Biotechnol* 59(4-5):426-435, 2002; Gerngross, *Nat Biotechnol* 22(11):1409-1414, 2004; Nevalainen *et al.*, *Trends Biotechnol* 23(9):468-474, 2005; Sauer *et al.*, *Trends Biotechnol.* 26(2):100-8, 2008; Magnuson and Lasure (2004). "Organic acid production by filamentous fungi." *Advances in fungal*

biotechnology for industry, agriculture, and medicine, pages 307–340). Some of industrial *A. niger* strains are capable of growing on solutions of glucose or sucrose in excess of 20% (w/v) and converting approximately 90% of the supplied carbohydrate to citric acid. These remarkable properties are the reason that *A. niger* has been used to produce citric acid for more 80 years and is currently the primary source of commercial citric acid production (Magnuson and Lasure (2004). "Organic acid production by filamentous fungi." *Advances in fungal biotechnology for industry, agriculture, and medicine*, pages 307–340).

The maximum product output in fermentation processes is the result of optimal metabolic pathways and cellular formation, which are influenced by endogenous and exogenous factors.

Cellular metabolisms are tightly controlled and highly interconnected, and are regulated spatially and temporally at different levels, such as transcription, post-transcription, translation, and post-translation. Therefore, different approaches have been explored to understand the regulatory mechanisms of metabolic processes and cellular formation for maximizing the product output in filamentous fungi. For example, comparative genomics was used to examine citric-acid-producing versus enzyme-producing *A. niger* strains (Andersen *et al.*, *Genome Res.* 21(6): 885-97, 2011), proteomics was used to examine filamentous fungi related to enzymes or organic acid production (de Oliveira and de Graaff, *Appl. Microbiol. Biotechnol.* 89(2): 225-37, 2011), or combination of both genomics and proteomics were used to examine enzyme production (Jacobs *et al.*, *Fungal Genetics and Biology* 46(1, Supplement):S141-S152, 2009). Although these studies examined the potential involvement of selected genes and proteins in optimizing production of organic acids or proteins in filamentous fungi, methods for altering the complex post-translation modifications (such as N-glycosylation of cellular proteins) for signal transduction, cellular formation and metabolism at different growth and development stages, which may affect product output, have not been examined.

Protein glycosylation is a ubiquitous and structurally diverse form of post translation modification, which occurs at all domains of life. More than two-thirds of eukaryotic proteins are predicted to be glycosylated (Apweiler *et al.*, *Biochim Biophys Acta* 1473(1):4-8, 1999). N- and O-linked protein glycosylation are common types of protein glycosylation, occurring mainly on the asparagine (N) and serine/threonine (S/T) residues, respectively. N-linked glycosylation has been implicated in many biochemical and cellular processes, including protein secretion, stability and translocation, maintenance of cell structure, receptor-ligand interactions and cell signaling, cell-cell recognition, pathogen infection, and host defense in various organisms (Haltiwanger and Lowe, *Ann. Rev. Biochem.* 73(1):491-537, 2004; Dellaporta *et al.*, *Plant Mol. Biol. Reporter* 1(4):19-21, 1983; Nam *et al.*, *Biotech. Bioengineer.* 100(6):1178-1192, 2008; Trombetta and Parodi, *Ann. Rev.*

Cell Dev. Biol. 19(1):649-676, 2003; Tsang *et al.*, *Fungal Genetics and Biology* 46(1): S153-S160, 2009; Pang *et al.*, *Science*, 333(6050):1761-4, 2011).

N-glycosylation is highly complex and has been extensively studied in mammalian systems (Yan and Lennarz, *J. Biol. Chem.* 280(5):3121, 2005; Silberstein and Gilmore, *FASEB J.* 10(8): 849, 1996; Kornfeld and Kornfeld, *Annu. Rev. Biochem.* 54:631-664, 2005; Kim *et al.*, *PLoS ONE* 4(10): e7317, 2009, 2009) and yeast (Kukuruzinska *et al.*, *Annu. Rev. Biochem.* 56(1):915-944, 1987). The protein N-glycosylation pathways in filamentous fungi have also been identified (Deshpande *et al.*, *Glycobiology* 18(8):626-637, 2008; Geysens *et al.*, *Fungal Genetics and Biology* 46(1, Supplement): S121-S140, 2009) on the basis of the known genomic sequences. Several genes involved in N-glycosylation have been studied in filamentous fungi (Kotz *et al.*, *PLoS ONE* 5(12):e15729, 2010; Kainz *et al.*, *Appl Environ Microbiol* 74(4):1076-86, 2008; Maras *et al.*, *J. Biotechnol.* 77(2-3):255-63, 2000; Maddi and Free, *Eukaryot Cell* 9(11):1766-75, 2010; Bowman *et al.*, *Eukaryotic Cell* 5(3):587-600, 2006). In these studies, the effects of gene deletion on N-linked glycan patterns formation, the cell wall formation, overall protein secretion and/or the phenotypic changes were demonstrated.

Alg3 is localized in the ER and catalyzes the initial transfer of a mannose residue from dolichol pyrophosphate-mannose to lipid-linked Man5GlcNAc2-PP-Dol on the ER luminal side. It is involved in the early N-glycan synthesis in eukaryotes for the assembly of a Glc3Man9GlcNAc2 core oligosaccharide that is linked to the lipid carrier dolichol pyrophosphate. The *Alg3* gene and its functions have been identified and studied in *S. cerevisiae*, *P. pastoris*, *T. brucei*, *A. thaliana*, and human (Aebi *et al.*, *Glycobiol.* 6(4):439-444, 1996; Korner *et al.*, *EMBO J.* 18(23): 6816-6822, 1999; Davidson *et al.*, *Glycobiology* 14(5):399-407, 2004; Manthri *et al.*, *Glycobiol.* 18(5):367-83, 2008; Kajiura *et al.*, *Glycobiol.* 20(6):736-51, 2010). In these studies, the *Alg3* mutants exhibited a unique structural profile in the glycoproteins, such as Man3GlcNAc2, Man4GlcNAc2, Man5GlcNAc2, GlcMan5GlcNAc2, and Glc3Man5GlcNAc2, which affected the overall N-glycosylation by incomplete utilization of N-linked glycosites in glycoproteins. No obvious growth phenotype was observed in those *Alg3Δ* mutants of *S. cerevisiae*, *P. pastoris*, *T. brucei*, and plant except that the *Alg3* defect in human caused severe diseases such as profound psychomotor delay, optic atrophy, acquired microcephaly, iris oboles and hypsarrhythmia (Stibler *et al.*, *Neuropediatrics* 26(5): 235-7, 1995; Sun *et al.*, *J. Clin. Endocrinol. Metab.* 90(7):4371-5, 2005; Schollen *et al.*, *Eur. J. Med. Genet.* 48(2):153-158, 2005; Kranz *et al.*, *Am. J. Med. Genet.* 143A(13):1414-20, 2007; Denecke *et al.*, *Pediatr. Res.* 58(2):248-53, 2005).

LaeA, a global regulator gene for the secondary metabolism, was first identified in *A. nidulans* through complementing the *afIR* deficient mutants (Bok and Keller, *Eukaryot Cell* 3:527-

535, 2004). Deletion of *LaeA* gene inhibits the expression of secondary metabolic gene clusters, such as sterigmatocystin, penicillin, and lovastatin, but has no effect on spore production in *A. nidulans*. The *LaeA* that was confirmed as a nuclear protein and a putative methyltransferase does not involve in gene clusters for nutrient utilization (Bok *et al.*, *Mol Microbiol* 61:1636-45, 2006).

5 Furthermore, the role of *LaeA* in secondary metabolism was confirmed in *Aspergillus flavus* and *Aspergillus oryzae* (Kale *et al.*, *Fungal Genet. Biol.* 45:1422-9, 2008; Oda *et al.*, *Biosci Biotechnol Biochem* 75:1832-4, 2011). Evidence indicates that *LaeA* reverses gene repression at the level of the heterochromatin state (Reyes-Dominguez *et al.*, *Molecular Microbiology* 76:1376-86, 2010). *LaeA* is a component of the heterotrimeric VeA/VelB/*LaeA* protein complex (Bayram *et al.*,
 10 *Science Signalling* 320:1504, 2008), which involves in the acetylation signal transduction for secondary metabolite production in *A. nidulans* (Soukup *et al.*, *Mol. Microbiol.*, 86(2):314-30, 2012). The veA/VelB/*LaeA* complex may coordinately respond to environmental cues (Ramamoorthy *et al.*, *Mol. Microbiol.*, 85(4):795-814, 2012) and has a role in fungal morphology (Calvo, *Fungal Genetics and Biology* 45:1053-61, 2008). *LaeA* may direct the formation of the
 15 VelB-VosA and VelB-VelA-*LaeA* complexes, control veA modification and protein levels, and be involved in light regulation of growth and development (Bayram *et al.*, *PLoS genetics* 6: e1001226, 2010).

SUMMARY

20 Provided herein are isolated *Aspergillus terreus* fungi transformed with a heterologous nucleic acid molecule comprising an *Aspergillus* species gene. In some instances, the *A. terreus* fungi also include a genetic inactivation of the *Alg3* gene. Also provided is a method of making itaconic acid by culturing an isolated *A. terreus* fungus transformed with a heterologous nucleic acid molecule comprising an *Aspergillus* species *LaeA* gene (optionally with a genetic inactivation
 25 of the *Alg3* gene) under conditions that permit the fungus to make itaconic acid. In some embodiments, the heterologous nucleic acid construct includes a heterologous *LaeA* gene, a heterologous promoter, a heterologous transcription terminator, and/or a heterologous selective marker gene. In non-limiting examples, the heterologous *Aspergillus* species *LaeA* gene is an *A. fumigatus laeA* gene, an *A. nidulans laeA* gene, an *A. niger LaeA* gene, or an *A. oryzae laeA* gene.

30 The foregoing and other objects and features of the disclosure will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a schematic drawing showing a restriction map of the 10.9 kb fragment containing the *A. niger Alg3* gene.

FIG. 1B is a schematic drawing showing the introduction of the hyg-selective marker, which was flanked by the upstream and downstream DNA sequences of *Alg3*. Integration of the linear molecules by homologous recombination replaces *Alg3* with *hph* in the chromosome.

FIG. 1C is a digital image of a Southern blot showing the genomic DNA hybridization of parent and *Alg3Δ* strains. One parent and two selected *Alg3Δ* strains are shown, which have the correct enzyme restriction pattern.

FIG. 2 is a series of digital images showing the *Alg3Δ* and parent 11414*kusA* strains grown on agar plates of complete medium (CM), potato dextrose (PDA), and minimal medium (MM) at 30°C for 15 hours or 48 hours. Parent strain (panel A), *Alg3Δ* strain (panel B), and both parent and *Alg3Δ* strains (panel C) grown on complete medium plate. Parent strain (panel D), *Alg3Δ* strain (panel E), and both parent and *Alg3Δ* strains (panel F) were grown on PDA agar plates. Parent strain (panel G), *Alg3Δ* strain (panel H), and both parent and *Alg3Δ* strains (panel I) were grown on MM agar plates.

FIG. 3A is a series of digital images showing the spore germination of parent 11414*kusA* and *Alg3Δ* strains in liquid cultures of complete medium (CM), potato dextrose medium (PDA), and minimal medium (MM) at 30°C for 7 hours. Panels A and B are germinated spores in CM liquid culture. Panels C and D are germinated spores in PDA liquid culture. Panels E and F are germinated spores in MM liquid culture. The left panels for the parent 11414*kusA* strain and the right panels for the *Alg3Δ* strain.

FIG. 3B provides graphs showing the time courses of the percentage of spore germination of parent 11414*kusA* and *Alg3Δ* strains grown in the liquid cultures of complete medium (CM; top graph), potato dextrose medium (PDA; middle graph), or minimal medium (MM; bottom graph). The solid filled cycle is the parent 11414*kusA* strain and open cycle is the *Alg3Δ* strain.

FIG. 4 shows a series of digital images (panels A and D) and stereo microscopy digital images (panels B, C, E and F) of parent and *Alg3Δ* strains grown on agar plates of complete medium (CM) and potato dextrose (PDA) at 30°C for 4 days.

FIG. 5 provides stereo microscopy digital images parent 11414*kusA* and *Alg3Δ* strains grown on citric acid production (CAP) medium plates at different pH levels for 27 hours. The left panels for parent strain 11414*kusA* and right panels for the *Alg3Δ* strain.

FIGS. 6A and 6B show spore germination of parent 11414*kusA* and *Alg3Δ* strains in citric acid production (CAP) liquid medium. (FIG. 6A, top left and bottom left panel) Inverted

microscopic images for parent 11414*kusA* strain. (FIG. 6A, top right and bottom right panel)
 Inverted microscopic images for *Alg3Δ* strain. (FIG. 6A, top panels) Strains grown in CAP liquid
 culture at 30°C for 8 hours. (FIG. 6A, bottom panels) Strains grown in CAP liquid culture at 30°C
 for 15 hours. (FIG. 6B) Time course of the spore germination rate (%) of parent and *Alg3Δ* strains
 5 grown in CAP liquid culture. The solid cycle for parent 11414*kusA* strain and open cycle for the
Alg3Δ strain.

FIG. 7 is a graph showing the time course of citric acid production by parent 11414*kusA*
 and *Alg3Δ* strains in the liquid culture of citric acid production at 30°C and 200 rpm.

FIGS. 8A and 8B show (FIG. 8A) a schematic diagram showing the construction of the
 10 transgene used to complement the *Alg3Δ* mutant, and (FIG. 8B) a is a graph showing the citric acid
 production by parent strain 11414*kusA* (*kusA*), *Alg3Δ* mutant (*Alg3*) and *Alg3Δ* mutant
 complemented with *Alg3* gene (*cAlg3*) after growth at 30°C, 200 rpm for 12 days

FIG. 9 shows an alignment of *Alg3* nucleic acid sequences from *A. niger* (top strand,
 nucleotides 1986-2518 of SEQ ID NO: 1) and *A. oryzae* (bottom strand, nucleotides 643-1175 of
 15 SEQ ID NO: 3).

FIGS. 10A and 10B show an alignment of *Alg3* protein sequences from *A. niger* (SEQ ID
 NO: 2), *A. nidulans* (SEQ ID NO: 31), *Fusarium oxysporum* (SEQ ID NO: 32), *Neurospora crassa*
 (SEQ ID NO: 33), *S. cerevisiae* (SEQ ID NO: 34), *Arabidopsis thaliana* (SEQ ID NO: 35), and
Homo sapiens (SEQ ID NO: 36). The signs at the top of the alignment show: '-' the average
 20 weight of column pair exchanges is less than weight matrix mean value; '.' is less than mean value
 plus one SD; '+' is less than mean value plus two SD; and '*' is more than mean value plus two SD.

FIG. 11 shows an alignment of *Alg3* protein sequences from *A. niger* (top strand, amino
 acids 12-411 of SEQ ID NO: 2) and *S. cerevisiae* (bottom strand, amino acids 31-434 of SEQ ID
 NO: 34).

FIG. 12 shows an alignment of *LaeA* protein sequences from *A. nidulans* (top strand, amino
 acids 14-372 of SEQ ID NO: 41) and *A. niger* (bottom strand, amino acids 14-370 of SEQ ID NO:
 25 59).

FIG. 13 is a schematic diagram showing the construction of *pPTRpGPDALaeA* plasmid
 vector. Both the glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter and *LaeA* (loss of
 30 *afIR* expression A) gene coding sequence of genomic DNA were isolated by overlap PCR from
 pAN7-1 plasmid vector and *A. nidulans* with additions of HindIII restriction enzyme sites at 5'-end
 of *gpdA* promoter and 3'-end of *LaeA* gene, confirmed by DNA sequence, and ligated into pPTR1
 plasmid vector at HindIII restriction enzyme site.

FIG. 14 is a schematic illustrating a plasmid vector pRS426-*LaeA*, which contains the upstream region of *pyrG* gene of *A. niger*, the coding region of *LaeA* gene under the control of *gpdA* promoter and transcriptional terminator of *trpC* gene from *A. nidulans*, the pyrithiamine resistance (*ptrA*) gene from *A. oryzae*, and the downstream region of *pyrG* gene of *A. niger*. The unique restriction enzyme PmeI site was introduced at the both end of transgene expression fragment.

FIGS. 15A and 15B show the results of polymerase chain reaction (PCR) analysis of *LaeA* gene insertion in the transgenic *A. niger* genome of heterologous expression of *A. nidulans* *LaeA* gene. (FIG. 15A) PCR products of *A. oryzae ptrA* gene detected in selected single spore colony isolate (SCI) of *LaeA* gene transgenic mutants and parent kusA and *Alg3Δ* are control strains. (FIG. 15B) PCR products of transgene expression DNA fragment including the *gpdA* promoter, *LaeA* coding region and *trpC* gene transcriptional terminator. The SCI-1 to SCI-4 is the individual single spore colony of *LaeA* gene transgenic mutants and parent kusA and *Alg3Δ* are control strains. Lambda DNA marker is the restriction fragment of BstEII restriction enzyme.

FIG. 16 is a bar graph showing the results of citric acid production after 10 days in culture of parent strain (kusA), *alg3Δ* mutant (*Alg3*), and over-expression of *LaeA* gene in *alg3Δ* (*LaeA*-1 and *LaeA*-2) mutants. The data for each strain is the average of three replicates.

FIG. 17A is a schematic diagram showing the construction of the *pBSK-LaeA* deletion plasmid vector for *A. niger*. The DNA fragments of the 5' and 3' ends of *A. niger LaeA* (lack of aflR expression) gene and the *hph* (hygromycin phosphotransferase) expression cassette were isolated from *A. niger* genomic DNA (*LaeA*) or pCB1003 plasmid vector DNA by PCR with oligonucleotide pairs 1 and 2; 3 and 4; and 5 and 6. The PCR DNA fragments were assembled together by Gibson assembly cloning kit in pBSK backbone vector prepared by HindIII/PstI double digestion. The plasmid DNA fragment containing the *LaeA* gene deletion cassette was prepared by restriction enzyme digestion with restriction endonucleases of HindIII and XbaI and used for *A. niger* transformation.

FIG. 17B is a schematic diagram showing the construction of the *pRS426-A. niger LaeA* complementation plasmid vector for *A. niger*. The *LaeA* gene containing both its promoter and transcriptional terminator was isolated by PCR with oligonucleotide pair 9 and 10 from *A. niger* genomic DNA. The PCR fragment was cloned into the plasmid vector pRSB426-*LaeA* prepared by restriction endonuclease HindIII digestion and Klenow treatment with blunt-end ligation. The new plasmid DNA vector contains the upstream region of the *pyrG* gene of *A. niger*, the entire region of *A. niger LaeA* gene, the transcriptional terminator of the *trpC* gene from *A. nidulans*, the pyrithiamine resistance (*ptrA*) gene from *A. oryzae*, and the downstream region of the *pyrG* gene of

A. niger. The unique restriction endonuclease PmeI site was introduced at both ends of the transgene expression fragment.

FIG. 18 shows a digital image of the results of PCR analysis of the *LaeA* gene deletion in the transgenic *A. niger* genome by homologous recombination at the *pyrG* locus. The PCR products corresponding to the 5' end of the *A. niger* *LaeA* gene and part of the *hph* expression cassette were amplified with oligonucleotide pair 7 and 8, and were detected in selected single spore colony isolate (SCI) of *LaeA* gene deletion transgenic mutants.

FIG. 19 shows a digital image of the results of PCR analysis of the *LaeA* gene complementation (*cLaeA*) in the genetic background of *LaeA* deletion mutant or *A. nidulans* *LaeA* over-expression (*LaeA*) in the genetic background of *11414kusA* of those transgenic *A. niger* genome by homologous recombination. PCR products of *A. oryzae* *ptrA* gene detected in selected single spore colony isolate (SCI) of *LaeA* gene transgenic mutants and parent *kusA* (negative) and plasmid DNA of pRS426-*laeA* (positive) represent control DNA.

FIG. 20 is a bar graph showing the results of citric acid production after 5 days in culture of the parent strain (*kusA*), the *LaeA* Δ mutant, *A. niger* *LaeA* gene complementation in the *LaeA* Δ mutant (*cLaeA* Δ), and over-expression of *A. nidulans* *LaeA* gene in the parent (*KusA*) strain. The data for each strain is the average of at least three biological replicates.

FIGS. 21A is a diagram illustrating the *LaeA* deletion construct in which the *LaeA* coding region was replaced by the bacterial hygromycin B phosphotransferase (*hph*) gene.

Oligonucleotides 1546, 108, 107 and 1553 (see Table 6) were used for *LaeA* gene deletion confirmation in *A. terreus*.

FIG. 21B shows the genomic PCR confirmation of selected *LaeA* deletion mutants with oligonucleotide pair 1546/107 or 108/1553.

FIG. 22A is a diagram illustrating *LaeA* complementation in *A. terreus* *LaeA* deletion mutants. The complementation transgene fragment was targeted upstream of the *LaeA* gene and the bacterial bleomycin resistance gene (*ble*) was used for transgenic strain selection. The insertion of *LaeA* complementation was confirmed by oligonucleotide pair 1556/107.

FIG. 22B shows gel electrophoresis of genomic PCR product corresponding to *ble* and part of *hph*.

FIG. 23 is a graph showing itaconic acid production in selected strains of parent, *LaeA* deletion (*LaeA* Δ) and *LaeA* complementation (*cLaeA* Δ) mutants after 4 days of culture in itaconic acid production medium.

FIG. 24A shows a diagram of the transgene over-expression cassette of the *A. nidulans* *LaeA* gene under the control of the *A. nidulans* *gpdA* promoter and TrpC terminator with a locus

target of 2.5 kb of the *A. terreus* *LaeA* gene. The pyrithiamine resistance gene (*ptrA*) of *A. oryzae* was used for transgene expression selection. Oligonucleotides 1563, 1198 and 1564 (see Table 6) were used for genomic PCR to confirm the gene insertion.

FIG. 24B shows gel electrophoresis of PCR products corresponding to the oligonucleotide pairs 1198/1564 and 1563/1564 in selected transgenic strains.

FIG. 25 is a bar graph showing itaconic acid production in selected *A. terreus* transgenic strains with the *A. nidulans* *LaeA* over-expression transgene after 5 days of culture in itaconic acid production medium.

FIGS. 26A-26D depict transgene construction and provide confirmation of transformed *A. terreus* strains. FIG. 26A is a diagram of the *A. terreus* *alg3* gene deletion construct with the *hph* marker gene. P1 to P8 depict the location of oligonucleotides used for DNA fragment isolation and gene deletion confirmation. *Bgl*III and *Nco*I restriction sites are indicated. FIG. 26B is an agarose gel electrophoresis image of PCR products amplified using the P7/P8 oligonucleotide pair. *Alg3* gene deletion was confirmed in *A. terreus* strains 3 and 4. FIG. 26C is an agarose gel electrophoresis image of PCR products amplified using the P7/P8 oligonucleotide pair. *Alg3* gene deletion was confirmed in various *laeA* over-expression mutant strains (OE-*laeA*-7, OE-*laeA*-9 and OE-*laeA*-13). FIG. 26D is an agarose gel electrophoresis image of PCR products from selected *alg3* deletion strains digested with either *Bgl*III or *Nco*I restriction endonucleases.

FIG. 27 is a series of images of stereo microscopic observation of the parent strain, selected *alg3* deletion mutant strains (*alg3Δ*-1 and *alg3Δ*-2) and selected *laeA* over-expression mutant strains (OE-*laeA*-7, OE-*laeA*-9, OE-*laeA*-13, OE-*laeA*-7+*alg3Δ*, OE-*laeA*-9+*alg3Δ* and OE-*laeA*-13+*alg3Δ*) after 6 days of culture in itaconic acid production media. The results demonstrated that the *alg3* deletion reduced the pellet size and filamentous growth of *A. terreus*.

FIG. 28 is a graph showing itaconic acid production in the parent strain and different transgenic mutant strains grown in itaconic acid production medium for 6 days. The *alg3Δ*-1 and *alg3Δ*-2 strains have an *alg3* deletion in an *A. terreus* wild type background. OE-*laeA*7, OE-*laeA*9, and OE-*laeA*13 are the selected strains with *laeA* gene over-expression in an *A. terreus* wild type background. OE-*laeA*7+*alg3Δ*-1, OE-*laeA*7+*alg3Δ*-2, OE-*laeA*9+*alg3Δ*-1, OE-*laeA*13+*alg3Δ*-1, and OE-*laeA*13+*alg3Δ*-2 have an *alg3* deletion in a *laeA* over-expression background.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but

the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file, created on May 17, 2016, 157 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NOS: 1 and 2 are exemplary *Alg3* nucleic acid and protein sequences, respectively, from *A. niger*.

SEQ ID NOS: 3 and 4 are exemplary *Alg3* nucleic acid and protein sequences, respectively, from *A. oryzae*.

SEQ ID NOS: 5-30 show exemplary primer sequences.

SEQ ID NOS: 31-36 are exemplary *Alg3* protein sequences from *A. nidulans*, *Fusarium oxysporum*, *Arabidopsis thaliana*, *Neurospora crassa*, *S. cerevisiae*, and *Homo sapiens*, respectively.

SEQ ID NO: 37 is an exemplary *Aspergillus nidulans* glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter sequence.

SEQ ID NOS: 38 and 39 are exemplary forward and reverse primers, respectively, that can be used to isolate or amplify an *A. nidulans gpdA* promoter.

SEQ ID NOS: 40 and 41 are exemplary *Aspergillus nidulans* methyltransferase (*LaeA*) coding and protein sequences, respectively.

SEQ ID NOS: 42 and 43 are exemplary forward and reverse primers, respectively, that can be used to isolate or amplify an *A. nidulans LaeA* sequence.

SEQ ID NO: 44 is the nucleic acid sequence of the pGPDA-*LaeA* fragment described in FIG. 13 and Example 5.

SEQ ID NO: 45 is the upstream region of *A. niger pyrG* gene.

SEQ ID NO: 46 is the *trpC* transcriptional terminator of *A. nidulans*.

SEQ ID NO: 47 is the pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*.

SEQ ID NO: 48 is the downstream region of *A. niger pyrG* gene.

SEQ ID NOS: 49 and 50 are exemplary forward and reverse primers, respectively, that can be used to isolate or amplify an upstream region of *A. niger pyrG*.

SEQ ID NOS: 51 and 52 are exemplary forward and reverse primers, respectively, that can be used to isolate or amplify the *trpC* transcriptional terminator of *A. nidulans*.

SEQ ID NOS: 53 and 54 are exemplary forward and reverse primers, respectively, that can be used to isolate or amplify *ptrA* of *Aspergillus oryzae*.

SEQ ID NOS: 55 and 56 are exemplary forward and reverse primers, respectively, that can be used to isolate or a downstream region of *A. niger pyrG*.

SEQ ID NO: 57 is the nucleic acid sequence of the transgene fragment described in FIG. 13 and Example 6.

SEQ ID NOS: 58 and 59 are exemplary *Aspergillus niger* *LaeA* coding and protein sequences, respectively.

SEQ ID NO: 60 is the nucleic acid sequence of the transgene fragment used to complement the *alg3* Δ mutant with the original *alg3* gene at *pyrG* locus.

SEQ ID NO: 61 is the nucleic acid sequence of the 5' end of the *A. niger* *LaeA* gene.

SEQ ID NO: 62 is the nucleic acid sequence of the 3' end of the *A. niger* *LaeA* gene.

SEQ ID NO: 63 is the nucleic acid sequence of the *hph* expression cassette.

SEQ ID NO: 64 is the nucleic acid sequence of the *A. niger* *LaeA* gene.

SEQ ID NO: 65 is the nucleic acid sequence of the *LaeA* deletion cassette.

SEQ ID NO: 66 is the nucleic acid sequence of the *LaeA* complementation construct.

SEQ ID NOS: 67-100 are oligonucleotide primers.

SEQ ID NO: 101 is the nucleotide sequence of the 1546/1548 fragment.

SEQ ID NO: 102 is the nucleotide sequence of the 1549/1550 fragment.

SEQ ID NO: 103 is the nucleotide sequence of the 1551/1553 fragment.

SEQ ID NO: 104 is the nucleotide sequence of the 1547/1552 fragment.

SEQ ID NO: 105 is the nucleotide sequence of the 1554/1555 fragment.

SEQ ID NO: 106 is the nucleotide sequence of the 1556/1557 fragment.

SEQ ID NO: 107 is the nucleotide sequence of the 1558/1559 fragment.

SEQ ID NO: 108 is the nucleotide sequence of the 1561/1562 fragment.

SEQ ID NO: 109 is the nucleotide sequence of the 1563/1564 fragment.

SEQ ID NO: 110 is the nucleotide sequence of the 1565/1566 fragment.

SEQ ID NOS: 111-118 are oligonucleotide primers.

SEQ ID NO: 119 is the nucleotide sequence of the 5'-*alg3* fragment.

SEQ ID NO: 120 is the nucleotide sequence of the *hph* fragment.

SEQ ID NO: 121 is the nucleotide sequence of the 3'-*alg3* fragment.

SEQ ID NO: 122 is the nucleotide sequence of the pRF-Hu2 vector digested with BglII/SacI.

DETAILED DESCRIPTION

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Definitions of common terms in molecular biology may be found in Benjamin Lewin,

Genes V, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

The singular terms “a,” “an,” and “the” include plural referents unless context clearly indicates otherwise. Similarly, the word “or” is intended to include “and” unless the context clearly indicates otherwise. Hence “comprising A or B” means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All references and GENBANK™ Accession numbers mentioned herein are incorporated by reference (the sequence available on November 30, 2011). The materials, methods, and examples are illustrative only and not intended to be limiting.

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

Alg3 (dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase): Also known as asparagine-linked glycosylation 3 and α -1,3-mannosyltransferase. *Alg3* encodes an enzyme which catalyzes the addition of the first dol-p-man derived mannose in an α -1,3 linkage to Man5GlcNAc2-PP-Dol. The term *Alg3* (or Alg3) includes any *Alg3* gene (such as a fungal *Alg3* sequence), cDNA, mRNA, or protein, that is an *Alg3* involved in catalyzing the addition of the first dol-p-man derived mannose in an α -1,3 linkage to Man5GlcNAc2-PP-Dol, and when genetically inactivated results in a fungus that has an ability to produce more citric acid than the parent strain (such as at least 20%, at least 30%, at least 50%, at least 60%, or at least 70% more than a parent strain under the same growing conditions).

Alg3 sequences are publicly available for many species of *Aspergillus*. For example, GENBANK™ Accession Nos: XM_001823992.2 and XP_001824044 disclose *Aspergillus oryzae* RIB40 *Alg3* nucleic acid and protein sequences, respectively; GENBANK™ Accession Nos: XM_001398659.2 and XP_001398696.2 disclose *Aspergillus niger* CBS 513.88 *Alg3* nucleic acid and protein sequences, respectively (SEQ ID NOS: 1 and 2); and GENBANK™ Accession Nos: XM_748359.1 and XP_753452 disclose *Aspergillus fumigatus* Af293 *Alg3* nucleic acid and protein sequences, respectively. Additional exemplary *Alg3* sequences are provided in SEQ ID NOS: 1-4 and 31-36. However, one skilled in the art will appreciate that in some examples, an *Alg3* sequence can include variant sequences (such as allelic variants and homologs) that retain *Alg3* activity but

when genetically inactivated in *Aspergillus* results in a fungus that has an ability to produce more citric acid than the parent strain (such as at least 20%, at least 30%, at least 50%, at least 60%, or at least 70% more under the same growing conditions), or an ability to produce more itaconic acid (particularly when combined with upregulation of *LaeA*) than the parent strain (such as at least 20%, at least 30%, at least 50%, at least 60%, or at least 70% more under the same growing conditions).

***Aspergillus terreus*:** A fungus found in soil and vegetation. *A. terreus* is found worldwide, but is more prevalent in warmer climates, such as tropical and subtropical regions. This fungus is commonly used in industry to produce important organic acids, such as itaconic acid and *cis*-aconitic acid, and was the initial source for the drug mevinolin (lovastatin), a drug for lowering serum cholesterol (Bennett, "An Overview of the Genus *Aspergillus*," pages 1-17, *Aspergillus Molecular Biology and Genomics*, edited by Masayuki Machida and Katsuyi Gomi, Caister, Academic Press, 2010).

Detectable: Capable of having an existence or presence ascertained. For example, production of citric acid is detectable if the signal generated is strong enough to be measurable.

Genetic enhancement or up-regulation: When used in reference to the expression of a nucleic acid molecule, such as a gene, refers to any process which results in an increase in production of a gene product. A gene product can be RNA (such as mRNA, rRNA, tRNA, and structural RNA) or protein. Examples of processes that increase transcription include those that facilitate formation of a transcription initiation complex, those that increase transcription initiation rate, those that increase transcription elongation rate, those that increase processivity of transcription and those that relieve transcriptional repression (for example by blocking the binding of a transcriptional repressor). Gene up-regulation can include inhibition of repression as well as stimulation of expression above an existing level. Examples of processes that increase translation include those that increase translational initiation, those that increase translational elongation and those that increase mRNA stability. In one example, additional copies of genes are introduced into a cell in order to increase expression of that gene in the resulting transgenic cell.

Gene up-regulation includes any detectable increase in the production of a gene product. In certain examples, production of a gene product increases by at least 1.5-fold, at least 2-fold, or at least 5-fold), such as *LaeA*. For example, a genetic enhancement of a *LaeA* gene in *Aspergillus* (e.g., *A. niger* or *A. terreus*) results in an *Aspergillus* strain having increased levels of the *LaeA* protein relative to the parent strain, which can increase the ability of the fungus to produce more citric acid or itaconic acid. Genetic enhancement is also referred to herein as "enhancing or increasing expression."

Genetic inactivation or down-regulation: When used in reference to the expression of a nucleic acid molecule, such as a gene, refers to any process which results in a decrease in production of a gene product. A gene product can be RNA (such as mRNA, rRNA, tRNA, and structural RNA) or protein. Therefore, gene down-regulation or deactivation includes processes that decrease transcription of a gene or translation of mRNA.

For example, a mutation, such as a substitution, partial or complete deletion, insertion, or other variation, can be made to a gene sequence that significantly reduces (and in some cases eliminates) production of the gene product or renders the gene product substantially or completely non-functional. For example, a genetic inactivation of an *Alg3* gene in *Aspergillus* (e.g., *A. niger* or *A. terreus*) results in *Aspergillus* having a non-functional or non-existent (e.g., non-detectable) *Alg3* protein, which results in an ability of the fungus to produce more citric acid. Genetic inactivation is also referred to herein as “functional deletion”. In one example, a genetic inactivation of an *Alg3* gene in *Aspergillus* (e.g., *A. niger* or *A. terreus*) in combination with genetic upregulation of *LaeA*, results in *Aspergillus* results in an ability of the fungus to produce more itaconic acid, than with either alone.

Heterologous: Derived from separate genetic sources or species. For example, an *LaeA* gene that is heterologous to *A. terreus* is an *LaeA* gene from a species other than *A. terreus*.

Isolated: To be significantly separated from other agents. An “isolated” biological component (such as a nucleic acid molecule or protein) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component occurs, for example, other chromosomal and extra-chromosomal DNA and RNA, and proteins. Nucleic acid molecules and proteins which have been “isolated” include nucleic acid molecules and proteins purified by standard purification methods. The term also embraces nucleic acid molecules and proteins prepared by recombinant expression in a host cell as well as chemically synthesized proteins and nucleic acids. Samples of isolated biological components include samples of the biological component wherein the biological component represents greater than 90% (for example, greater than 95%, such as greater than 98%) of the sample.

An “isolated” microorganism (such as an *Alg3A* strain of *Aspergillus*) has been substantially separated or purified away from microorganisms of different types, strains, or species.

Microorganisms can be isolated by a variety of techniques, including serial dilution and culturing and resistance to certain chemicals.

Itaconic acid: An unsaturated dicarbonic acid; also known as 2-methylidenebutanedioic acid. Itaconic acid can be produced by the fermentation of carbohydrates using *Aspergillus terreus*.

Itaconic acid can be used as a monomer for the production of a number of different products, including resins, plastics, acrylate latexes, paints and synthetic fibers.

Itaconic acid production medium: Media containing in grams per liter (g/l): glucose, 100; ammonium sulfate, 2.36; potassium phosphate dibase, 0.11; magnesium sulfate heptahydrate, 2.08; calcium chloride dihydrate, 0.13; sodium chloride, 0.074; copper sulfate pentahydrate 2×10^{-4} ; ferrous sulfate heptahydrate, 5.5×10^{-3} ; manganese chloride tetrahydrate, 7×10^{-4} ; and zinc sulfate heptahydrate, 1.3×10^{-3} .

LaeA (loss of *aflR* expression A): *LaeA* encodes a protein which regulates secondary metabolite production in *Aspergillus*. The term *LaeA* (or *LaeA*) includes any *LaeA* gene (such as a fungal *LaeA* sequence), cDNA, mRNA, or protein, that is an *LaeA* involved in secondary metabolite production. In some embodiments, when *LaeA* expression is increased, for example in combination with a genetically inactivated *Alg3* gene, it results in a fungus that has an ability to produce more citric acid than the parent strain (such as at least 20%, at least 30%, at least 40%, 50%, at least 60%, or at least 70% more than a parent strain under the same growing conditions). In some embodiments, when *LaeA* expression is increased, for example in *A. terreus*, it results in a fungus that has an ability to produce more itaconic acid than the parent strain (such as at least 20%, at least 30%, at least 40%, 50%, at least 60%, or at least 70% more than a parent strain under the same growing conditions).

LaeA sequences are publicly available for many species of *Aspergillus*. For example, GENBANK™ Accession Nos: AB267276 and BAF74528.1 disclose *Aspergillus oryzae* *LaeA* nucleic acid and protein sequences, respectively; GENBANK™ Accession No. EHA27020.1 discloses an exemplary *Aspergillus niger* ATCC1015 *LaeA* protein sequence, a parent strain of 11414kusA (other exemplary sequences are provided in SEQ ID NOS: 58 and 59); GENBANK™ Accession No: CBF88745 discloses an *Aspergillus nidulans* *LaeA* protein sequence; and GENBANK™ Accession Nos: AY422723 and AAR01218 disclose *Aspergillus fumigatus* *LaeA* nucleic acid and protein sequences, respectively. Additional exemplary *LaeA* sequences are provided in SEQ ID NOS: 40-41 and 58-59. However, one skilled in the art will appreciate that in some examples, an *LaeA* sequence can include variant sequences (such as allelic variants and homologs) that retain *LaeA* activity and when genetically up-regulated in *Aspergillus* (for example in combination with *Alg3Δ*) results in a fungus that has an ability to produce more citric acid and/or itaconic acid than the parent strain (such as at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, or at least 70% more under the same growing conditions).

Mutation: A change in a nucleic acid sequence (such as a gene sequence) or amino acid sequence, for example as compared to a nucleic acid or amino acid sequence present in a wild-type

or native organism. In particular examples, a mutation is introduced into an *Alg3* gene in *Aspergillus*. Mutations can occur spontaneously, or can be introduced, for example using molecular biology methods. In particular examples, a mutation includes one or more nucleotide substitutions, deletions, insertions, or combinations thereof. In particular examples, the presence of one or more mutations in a gene can significantly inactivate that gene.

Operably linked: A first nucleic acid sequence is operably linked to a second nucleic acid sequence where the first nucleic acid sequence is joined in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

Promoter: An array of nucleic acid sequences that governs transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences to initiate gene transcription. A promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription.

Recombinant: A recombinant nucleic acid molecule or protein is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. In particular examples, this artificial combination is accomplished by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acids, for example, by genetic engineering techniques such as those described in Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 3d ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001. The term recombinant includes nucleic acid molecules that have been altered solely by addition, substitution, or deletion of a portion of the nucleic acid molecule.

Sequence identity/similarity: The identity/similarity between two or more nucleic acid sequences, or two or more amino acid sequences, is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences are. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the percentage, the more similar the sequences are.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988; Higgins & Sharp, *Gene*, 73:237-44, 1988; Higgins & Sharp, *CABIOS* 5:151-3,

1989; Corpet *et al.*, *Nuc. Acids Res.* 16:10881-90, 1988; Huang *et al.* *Computer Appls. in the Biosciences* 8, 155-65, 1992; and Pearson *et al.*, *Meth. Mol. Bio.* 24:307-31, 1994. Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

5 The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site.

10 BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. To compare two nucleic acid sequences, the options can be set as follows: -i is set to a file containing the first nucleic acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second nucleic acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastn; -o is set to any desired file name (e.g., C:\output.txt); -q is set to -1; -r is set to 2; and all
15 other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two sequences: C:\Bl2seq -i c:\seq1.txt -j c:\seq2.txt -p blastn -o c:\output.txt -q -1 -r 2.

 To compare two amino acid sequences, the options of Bl2seq can be set as follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file
20 containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\Bl2seq -i c:\seq1.txt -j c:\seq2.txt -p blastp -o c:\output.txt. If the two compared sequences share homology, then the designated output file will
25 present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

 Once aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is presented in both sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the
30 sequence set forth in the identified sequence, or by an articulated length (e.g., 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, a nucleic acid sequence that has 1166 matches when aligned with a test sequence having 1554 nucleotides is 75.0 percent identical to the test sequence (*i.e.*, $1166 \div 1554 * 100 = 75.0$). The percent sequence identity value is rounded to the

nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 are rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 are rounded up to 75.2. The length value will always be an integer. In another example, a target sequence containing a 20-nucleotide region that aligns with 20 consecutive nucleotides from an identified sequence as follows contains a region that shares 75 percent sequence identity to that identified sequence (*i.e.*, $15 \div 20 * 100 = 75$).

For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost of 1). Homologs are typically characterized by possession of at least 70% sequence identity counted over the full-length alignment with an amino acid sequence using the NCBI Basic Blast 2.0, gapped blastp with databases such as the nr or swissprot database. Queries searched with the blastn program are filtered with DUST (Hancock and Armstrong, 1994, *Comput. Appl. Biosci.* 10:67-70). Other programs use SEG. In addition, a manual alignment can be performed. Proteins with even greater similarity will show increasing percentage identities when assessed by this method, such as at least 75%, 80%, 85%, 90%, 95%, or 99% sequence identity.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode identical or similar (conserved) amino acid sequences, due to the degeneracy of the genetic code. Changes in a nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid molecules that all encode substantially the same protein. Such homologous nucleic acid sequences can, for example, possess at least 60%, 70%, 80%, 90%, 95%, 98%, or 99% sequence identity determined by this method.

One of skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is possible that strongly significant homologs could be obtained that fall outside the ranges provided.

Transformed: A cell, such as a fungal cell, into which a nucleic acid molecule has been introduced, for example by molecular biology methods known in the art. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including, but not limited to transfection with viral vectors, conjugation, transformation with plasmid vectors, and introduction of naked DNA by chemical-mediated, electroporation, lipofection, and biolistic particle delivery.

Overview

Disclosed herein is the finding that overexpression of the *LaeA* gene in *A. terreus* results in a significant increase in production of itaconic acid. In particular, *A. terreus* transformed with a

nucleic acid construct comprising an *Aspergillus* species *LaeA* gene produces significantly more itaconic acid relative to *A. terreus* that is not transformed with the construct. Also disclosed herein is the finding that *A. terreus* overexpressing the *LaeA* gene in combination with having a genetic inactivation of the *Alg3* gene produces higher levels of itaconic acid than *A. terreus* that only
 5 overexpresses *LaeA*.

Provided herein are isolated *A. terreus* fungi transformed with a heterologous nucleic acid construct comprising an *Aspergillus* species *LaeA* (loss of aflR expression A) gene. Expression of *LaeA* is increased in the transformed fungus compared to an *A. terreus* fungus that is not transformed with the heterologous nucleic acid construct.

10 In some embodiments, the heterologous nucleic acid construct comprises a heterologous *LaeA* gene, a heterologous promoter, a heterologous transcription terminator, a heterologous selective marker gene, or any combination thereof.

In some embodiments, the *Aspergillus* species *LaeA* gene is a homologous *LaeA* gene (*i.e.* an *A. terreus* *LaeA* gene). In other embodiments, the *LaeA* gene is a heterologous *LaeA* gene, such
 15 as an *LaeA* gene from *A. nidulans*, *A. niger*, *A. oryzae*, *A. fumigatus* or another *Aspergillus* species. In some examples, the *A. nidulans* *LaeA* gene encodes a protein having at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 41. In non-limiting examples, the *A. nidulans* *LaeA* gene encodes a protein comprising the amino acid sequence of SEQ ID NO: 41. In some examples,
 20 the *A. niger* *LaeA* gene encodes a protein having at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identity to the amino acid sequence of SEQ ID NO: 59. In non-limiting examples, the *A. niger* *LaeA* gene encodes a protein comprising the amino acid sequence of SEQ ID NO: 59.

In some embodiments, the heterologous nucleic acid molecule comprises a promoter
 25 operably linked to an *Aspergillus* species *LaeA* gene, a transcription terminator and/or a selective marker gene. In particular examples, the promoter is a heterologous promoter, such as, but not limited to, the *A. nidulans* *gpdA* promoter, or any other promoter that is functional in *Aspergillus* (*e.g.*, the *actA*, *ubi4*, *Arsa-7*, *A-37*, *Brsa-109*, *gpdA*, *pyrG* or *tef1* α promoters from *A. niger*). In other particular examples, the *Aspergillus* species *LaeA* gene is a heterologous *LaeA* gene, such as,
 30 but not limited to, an *A. nidulans*, *A. niger*, *A. oryzae*, or *A. fumigatus* *LaeA* gene. In other particular examples, the transcription terminator is a heterologous transcription terminator, such as, but not limited to, the *A. nidulans* *TrpC* transcription terminator. As an alternative to *trpC*, other transcriptional terminators can be used, such as promoters which include a transcriptional terminator (*e.g.*, *actA*, *Arsa7*, *Arsa-37*, *gpdA*, *pyrG*, *tef1* α or *ubi4*). In yet other particular

examples, the selective marker is a heterologous selective marker gene, such as a heterologous antibiotic resistance gene. In specific examples, selective marker gene is an *A. oryzae* pyrithiamine resistance (*ptrA*) gene. Other selective marker genes include, for example, the hygromycin B phosphotransferase (*hph*) gene, the *bar* gene (which confers glufosinate resistance), or bleomycin resistance gene (*ble*), or auxotrophic markers, such as *pyrG*.

In specific non-limiting examples, the heterologous nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 109.

In some embodiments, the isolated *A. terreus* fungus further includes a genetic inactivation of an endogenous dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase (*Alg3*) gene, wherein the *Alg3* gene is genetically inactivated by complete or partial deletion mutation or by insertional mutation. In particular examples, the *Alg3* gene is genetically inactivated by complete deletion mutation.

Further provided herein is a method of making itaconic acid. In some embodiments, the includes culturing an isolated *A. terreus* fungus transformed with a heterologous nucleic acid molecule comprising an *Aspergillus* species *LaeA* gene under conditions that permit the fungus to make itaconic acid. Expression of *LaeA* is increased in the transformed fungus compared to an *A. terreus* fungus that is not transformed with the heterologous nucleic acid construct. In some embodiments, the fungus is cultured in itaconic acid production medium, which contains grams per liter (g/l): glucose, 100; ammonium sulfate, 2.36; potassium phosphate dibase, 0.11; magnesium sulfate heptahydrate, 2.08; calcium chloride dihydrate, 0.13; sodium chloride, 0.074; copper sulfate pentahydrate 2×10^{-4} ; ferrous sulfate heptahydrate, 5.5×10^{-3} ; manganese chloride tetrahydrate, 7×10^{-4} ; and zinc sulfate heptahydrate, 1.3×10^{-3} .

In some embodiments of the methods of making itaconic acid, the heterologous nucleic acid construct comprises a heterologous *LaeA* gene, a heterologous promoter, a heterologous transcription terminator, a heterologous selective marker gene, or any combination thereof.

In some embodiments of the methods, the *Aspergillus* species *LaeA* gene is a homologous *LaeA* gene (i.e. an *A. terreus* *LaeA* gene). In other embodiments, the *LaeA* gene is a heterologous *LaeA* gene, such as an *LaeA* gene from *A. nidulans*, *A. niger*, *A. oryzae*, *A. fumigatus* or another *Aspergillus* species. In some examples, the *A. nidulans* *LaeA* gene encodes a protein having at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 41. In non-limiting examples, the *A. nidulans* *LaeA* gene encodes a protein comprising the amino acid sequence of SEQ ID NO: 41. In some examples, the *A. niger* *LaeA* gene encodes a protein having at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identity to the

amino acid sequence of SEQ ID NO: 59. In non-limiting examples, the *A. niger* *LaeA* gene encodes a protein comprising the amino acid sequence of SEQ ID NO: 59.

In some embodiments of the method, the heterologous nucleic acid molecule comprises a promoter operably linked to an *Aspergillus* species *LaeA* gene, a transcription terminator and/or a selective marker gene. In particular examples, the promoter is a heterologous promoter, such as, but not limited to, the *A. nidulans* *gpdA* promoter. In other particular examples, the *Aspergillus* species *LaeA* gene is a heterologous *LaeA* gene, such as, but not limited to, an *A. nidulans*, *A. niger*, *A. oryzae*, or *A. fumigatus* *LaeA* gene. In other particular examples, the transcription terminator is a heterologous transcription terminator, such as, but not limited to, the *A. nidulans* *TrpC* transcription terminator. In yet other particular examples, the selective marker is a heterologous selective marker gene, such as a heterologous antibiotic resistance gene. In specific examples, the selective marker gene is an *A. oryzae* pyrithiamine resistance (*ptrA*) gene. In specific non-limiting examples, the heterologous nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 109.

In some embodiments of the methods, the *A. terreus* fungus further includes a genetic inactivation of an endogenous *Alg3* gene, wherein the *Alg3* gene is genetically inactivated by complete or partial deletion mutation or by insertional mutation. In some examples, the *Alg3* gene is genetically inactivated by complete deletion mutation.

Also provided by the present disclosure are compositions that include isolated *A. terreus* fungi transformed with a heterologous nucleic acid construct. The compositions may further include growth medium. Further provided by the present disclosure are kits that include isolated *A. terreus* fungi transformed with a heterologous nucleic acid construct, such as a kit that includes a medium for culturing, storing, or growing the fungus, and/or antibiotics (for selection).

This disclosure also provides the first demonstration that genetic inactivation of *Alg3*, a gene involved in protein N-linked glycosylation, can result in substantial improvement of citric acid production in *A. niger*, while the total biomass is similar to the parent strain. The core oligosaccharide Glc3Man9GlcNAc2 is synthesized by a series of membrane-bound glycosyltransferases, which begins on the cytoplasmic side of the membrane of the endoplasmic reticulum (ER) and flips into the luminal side of the ER membrane to complete its synthesis. The lipid-linked core Glc3Man9GlcNAc2 is subsequently transferred to a nascent protein in the ER, where the glycoproteins are folded and then shuttled to the Golgi for additional, but divergent processing. The *Alg3* gene encodes the enzyme α -1,3-mannosyltransferase that converts Man5GlcNAc2-Dol-PP to Man6GlcNAc2-Dol-PP on the ER membrane of the luminal side. Provided herein is a homolog of *Saccharomyces cerevisiae* *Alg3* identified from *Aspergillus niger* (e.g., see SEQ ID NOS: 1 and 2).

It is shown herein that genetic inactivation of *Alg3* in *A. niger* resulted in a significant reduction of growth on complete medium (CM) and potato dextrose agar medium (PDA), but no effect on minimal medium (MM). The *Alg3* deletion also caused the substantial reduction in spore production of *A. niger* on CM, but no significant change on the PDA. When the spores were germinated in CM or PDA liquid culture medium, the *Alg3* Δ strain showed pronounced delay in spore germination. This growth phenotype is similar to the mutants with defects in signal transduction pathways observed in *A. nidulans* and *A. niger* (Fillinger *et al.*, *Mol. Microbiol.* 44(4):1001-16, 2002; Saudohar *et al.*, *Microbiol.* 148(8):2635-45, 2002; Xue *et al.*, *Eukaryot Cell* 3(2):557-60, 2004). Deletion of *pkaA*, *cycA* or *schA/pkaA* in *A. nidulans* substantially reduces its growth on CM medium plates and spore germination rate in MM liquid culture medium (Fillinger *et al.*, *Mol. Microbiol.* 44(4):1001-1016, 2002) and similar growth phenotypes were observed in the strains with the deletion of *pkaR*, *pkaC* or double deletion of *pkaR/pkaC* in *A. niger* (Saudohar *et al.*, *Microbiol.* 148(8):2635-2645, 2002). However, functional deletion of the MAP kinase *SakA* in *A. fumigatus* delays the spore germination in liquid CM, but stimulates spore germination in MM liquid medium (Xue *et al.*, *Eukaryot Cell* 3(2): 557-560, 2004).

Furthermore, the *Alg3* deletion reduced the overall growth on citric acid production (CAP) medium plates at different pHs. In contrast, the *Alg3* deletion triggered early spore germination and substantially improved spore germination rate in CAP liquid culture medium. Citric acid production in CAP liquid culture medium was significantly improved in *A. niger*. When the *alg3* Δ mutant was complemented with the original *alg3* gene at *pyrG* locus (FIG. 8A; SEQ ID NO: 60), its transcription levels was similar to parent strain with the cycle threshold (Ct) values about 23, while the Ct value for *alg3* Δ mutant was 30.8 in CAP liquid culture conditions, which was determined by real-time reverse-transcription PCR. Consequently, the citric acid production in the resulted complemented mutant strains was similar to the parent strain as shown in FIG. 8B. The results shown herein demonstrate the involvement of *Alg3* on the growth and development and citric acid production in *A. niger*.

It is proposed that inactivation of *Alg3* influences the N-glycosylation of those proteins involving in signal transduction pathways. The N-glycosylation consensus sequence (N-glycosite) for N- glycosylation in those proteins from the signal transduction pathways was observed. Most of those proteins contained 1 to 7 N-glycosites, such as, 6 N-glycosites found in *sskB* (map kinase kinase), 7 in *Ste11/SteC*, 5 in *acyA*, 5 in *rgsA*, 6 in *rgsC*, 4 in *gprA*, 4 in *pkaC2*, 4 in *flbA* and 3 in *G β* . Comparison of these results with previous studies indicates that the effects of the *Alg3* deletion on spore germination and growth may be regulated by altering the N-glycosylation in those proteins involved in signal transduction pathways in *A. niger*.

When the *Alg3Δ* strain was grown on CM medium, spore production of *Alg3Δ* mutants was dramatically reduced as compared to the parent strain, while maintaining a similar level when grown on PDA medium. This phenotype of sporulation production may be influenced by both endogenous and exogenous factors. For example, protein glycosylation was greatly influenced by culture conditions in filamentous fungi, such as fully glycosylated Cel7A only isolated from MM culture medium (Stals *et al.*, *Glycobiology* 14(8):725-737, 2004). In addition, higher amounts of proteases were secreted by the *Alg3Δ* strain than the parent in liquid MM culture supplemented 1g/l yeast extract, which further influenced nutrient uptakes, cellular formation and overall N-glycosylation. This would alter the yield and N-glycosylation in G protein system in *A. niger*, where G protein signaling is crucial for detection of major environmental stimuli for food acquisition, asexual sporulation, and spore germination (Chang *et al.*, *Genetics* 167(3):305, 2004; Li *et al.*, *Annu. Rev. Microbiol.* 61:423-452, 2007).

The spores of parent strain germinated more slowly and had a lower germination rate than the *Alg3Δ* strain in CAP liquid culture medium, which contains limited nitrogen source (3.1 g/l of NH_4NO_3), similar to MM. A similar phenotype was observed when the stress activating kinase, a MAP kinase, was deleted in *A. fumigatus* (*SakAΔ* strain) and grown in MM liquid culture medium (Xue *et al.*, *Eukaryot Cell* 3(2): 557-560, 2004). The spore germination of *SakAΔ* strain was dramatically influenced by nitrogen sources. For example, similar rates of spore germination between parent and *SakAΔ* strains were observed on MM containing 10 mM NH_4Cl or 10 mM Pro, while the spore germination rates of *SakAΔ* strain was much higher than the parent strain in the MM culture medium containing 10 mM NaNO_3 , NaNO_2 , or Phe. In addition, the CAP medium contains high level of glucose and low pH, which contributes additional stresses to *A. niger* growth. Although the *Alg3Δ* strain had earlier and higher germination in CAP medium, its biomass formation was less than the parent strain at early stages. The dried biomass yields for both parent and *Alg3Δ* strains were similar after growth in CAP medium for four and half days. However, more citric acid was produced by the *Alg3Δ* strain than the parent strain. This indicates more glucose was directly converted to citric acid by influence citric acid metabolism and reduction of glucose consumption for complex N-glycan formation and sequentially for other cellular metabolisms.

This disclosure also provides the first demonstration that genetic inactivation of *Alg3*, in combination with an increase in expression of the loss of *aflR* expression A (*LaeA*) gene, can result in substantial improvement of citric acid production in *A. niger*. It is proposed that increased expression of *LaeA* can also improve citric acid production in *A. niger* or other filamentous fungi. To increase expression of *LaeA* in fungal cells, a transgene was generated and expressed in *A. niger*

as follows. The *LaeA* gene of *A. nidulans* was operably controlled by glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter and *trpC* transcriptional terminator (*TtrpC*) of *A. nidulans*. This chimeric gene was flanked with the upstream of *A. niger pyrG* gene, the pyrithiamine resistance (*ptrA*) gene of *A. oryzae* and the downstream of *A. niger pyrG* gene. The transgene expression
 5 fragment containing the chimeric gene was used to transform the protoplasts of *alg3Δ* mutants of *A. niger*.

The present disclosure also describes the generation of an *A. niger* strain having a deletion of the *LaeA* gene (*LaeAΔ*), as well as an *A. niger LaeAΔ* strain complemented with a transgene encoding *LaeA* (*clLaeAΔ*) at the *pyrG* locus. These strains were used to evaluate the role of *LaeA*
 10 expression on citric acid production in *Aspergillus*. The data disclosed herein demonstrates that deletion of the *LaeA* gene in *A. niger* (*LaeAΔ*) results in a loss of citric acid production in culture. When the original *A. niger LaeA* gene was used for complementation in the *LaeAΔ* mutant (*clLaeAΔ*) at the *pyrG* locus, citric acid production was partially recovered, which indicates the importance of the chromosomal location of *LaeA* gene. When *A. nidulans LaeA* was over-
 15 expressed in the *A. niger* parent strain, citric acid production was higher than the parent strain. These results indicate that *LaeA* enhances citric acid production in *A. niger*.

In summary, the deletion of *Alg3*, increasing expression of *LaeA*, or both, can be used to increase citric acid production in fungi (such as filamentous fungi, *e.g.*, *A. niger*). In addition, deletion of *Alg3* alters the overall N-glycosylation and further influences the spore germination,
 20 filamentous growth, sporulation and other organic acid production in *A. niger*.

***Alg3Δ* Fungi**

The present disclosure provides isolated fungi having its *Alg3* gene inactivated, wherein such inactivation results in increased citric acid production by the fungi. Such fungi are referred to
 25 herein as *Alg3Δ* fungi. It is disclosed herein that genetic inactivation of *Alg3* results in *Aspergillus* fungi that can increase citric acid production as compared to *Aspergillus* having a native *Alg3* sequence.

Contemplated herein are isolated fungi containing a genetic inactivation of a dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase gene (*Alg3*). Any fungus can be used,
 30 such as any genus or variety of *Aspergillus*. In particular examples, the disclosed *Aspergillus* fungus is *A. niger*, such as *Aspergillus niger* strain 11414 (American Type Culture Collection (ATCC) No. 11414; NRRL 2270); 1015 (ATCC No. 1015; NRRL 328, CBS 113.46); NRRL 3 (ATCC No. 9029, CBS 120.49, N400); NRRL 3122 (ATCC No. 22343); or 11414*KusA*-. In other

specific examples, the *Aspergillus* is *A. aculeatus*, *A. awamori*, *A. carbonarius*, *A. wentii*, *A. foetidus*, *A. oryzae*, *A. terreus*, or *A. fumigatus*.

In addition, any method for genetic inactivation can be used, as long as the expression of the gene is significantly reduced or eliminated, or the function of the expressed protein is significantly reduced or eliminated. In particular examples, the *Alg3* gene is genetically inactivated by complete or partial deletion mutation or by insertional mutation. In some examples genetic inactivation need not be 100% genetic inactivation. In some embodiments, genetic inactivation refers to at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95% gene or protein inactivation. The term “reduced” or “decreased” as used herein with respect to a cell and a particular gene or protein activity refers to a lower level of activity than that measured in a comparable cell of the same species. For example, a particular fungi lacking *Alg3* activity has reduced *Alg3* activity if a comparable fungi not having an *Alg3* genetic inactivation has detectable *Alg3* activity.

Alg3 sequences are disclosed herein and others are publicly available, for example from GENBANK™ or EMBL. In some examples, the *Alg3* gene functionally deleted encodes a protein having at least 80%, at least 90%, at least 95%, at least 97%, or at least 98% sequence identity to SEQ ID NO: 2, 4, 31, 32, 33, 34, 35, or 36. In some examples, the *Alg3* gene functionally deleted comprises at least 80%, at least 90%, at least 95%, at least 97%, or at least 98% sequence identity to SEQ ID NO: 1 or 3 or nucleotides 1186-2582 of SEQ ID NO: 1.

The inactivation of *Alg3* results in many phenotypes in the fungi. For example, *Alg3Δ* mutants can have one or more of the following phenotypes: slower growth on citric acid production (CAP) medium, earlier spore germination in CAP medium (for example germination in at least 3 hours, at least 4 hours, or at least 5 hours after inoculation, such as within 3 hours of inoculation), increased spore germination rate in CAP medium, increased citric acid production in CAP medium, slower growth on complete medium (CM) or potato dextrose (PDA) medium, delay initiation of spore germination in CM or PDA medium, reduced sporulation on CM, or combinations thereof.

Such changes (such as increases or decreases) can be relative to a fungi having a wild-type *Alg3* gene, such as a parental strain (*e.g.*, *A. niger* strain 11414*KusA*), grown under the same conditions as the *Alg3Δ* mutant. In some examples, an increased germination rate is germination of at least 20%, at least 25%, or at least 30% of the spores from an *Alg3Δ* fungus have germinated 8 hours after inoculation in CAP medium (such as 20% to 35%, such as 32%), as compared to no more than 20%, no more than 15%, or no more than 10% (such as 5 to 15%, or 10%) for *A. niger* strain 11414*KusA*. In some examples, an increased germination rate is germination of at least 80%,

at least 85%, or at least 90% of the spores from an *Alg3A* fungus have germinated 15 hours after inoculation in CAP medium (such as 80% to 95%, such as 90%), as compared to no more than 60%, no more than 65%, or no more than 75% (such as 55 to 65%, or 60%) for *A. niger* strain 11414*KusA*. In some examples, increased citric acid production in CAP medium is an increase of
5 at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 65%, or at least 70%, by an *Alg3A* fungus as compared to *A. niger* strain 11414*KusA*. In some examples, reduced sporulation on complete medium is a reduction of sporulation by at least 20%, at least 30%, at least 40%, at least 50%, or at least 60%, (such as a 40% to 60% reduction) by an *Alg3A* fungus as compared to *A. niger* strain 11414*KusA*.

10 One skilled in the art will appreciate that additional genes can also be inactivated, wherein the additional genes may or may not provide additional enhancement of citric acid production to the fungus. In one example *KusA* (e.g., GENBANK™ Accession No. EF061656) is also genetically inactivated.

Also provided by the present disclosure are compositions that include isolated *Alg3A* fungi,
15 such as a growth medium. Also provided by the present disclosure are kits that include isolated *Alg3A* fungi, such as a kit that includes a medium for culturing, storing, or growing the fungus. Exemplary mediums include solid medium (such as those containing agar, for example CM, PDA or MM) and liquid media (such as a fermentation broth, such as CM, MM, or CAP medium).

A. Methods of functionally deleting genes

20 As used herein, an “inactivated” or “functionally deleted” gene means that the gene has been mutated, for example by insertion, deletion, or substitution (or combinations thereof) of one or more nucleotides such that the mutation substantially reduces (and in some cases abolishes) expression or biological activity of the encoded gene product. The mutation can act through affecting transcription or translation of the gene or its mRNA, or the mutation can affect the
25 polypeptide product itself in such a way as to render it substantially inactive.

Genetic inactivation of one or more genes (which in some examples is also referred to as functional deletion) can be performed using any conventional method known in the art. In one example, a strain of *Aspergillus* is transformed with a vector which has the effect of down-regulating or otherwise inactivating an *Alg3* gene. This can be done by mutating control elements
30 such as promoters and the like which control gene expression, by mutating the coding region of the gene so that any protein expressed is substantially inactive, or by deleting the *Alg3* gene entirely. For example, an *Alg3* gene can be functionally deleted by complete or partial deletion mutation (for example by deleting a portion of the coding region of the gene) or by insertional mutation (for example by inserting a sequence of nucleotides into the coding region of the gene, such as a

sequence of about 1-5000 nucleotides). Thus, the disclosure in some examples provides transformed fungi that include at least one exogenous nucleic acid molecule which genetically inactivates an *Alg3* gene (such as a nucleic acid sequence encoding SEQ ID NO: 2 or 4). In one example, such a transformed cell produces more citric acid, for example relative to a comparable
5 fungus with a native *Alg3* sequence.

In particular examples, an insertional mutation includes introduction of a sequence that is in multiples of three bases (*e.g.*, a sequence of 3, 9, 12, or 15 nucleotides) to reduce the possibility that the insertion will be polar on downstream genes. For example, insertion or deletion of even a single nucleotide that causes a frame shift in the open reading frame, which in turn can cause
10 premature termination of the encoded *Alg3* polypeptide or expression of a substantially inactive polypeptide. Mutations can also be generated through insertion of foreign gene sequences, for example the insertion of a gene encoding antibiotic resistance (such as hygromycin or bleomycin).

In one example, genetic inactivation is achieved by deletion of a portion of the coding region of the *Alg3* gene. For example, some, most (such as at least 50%) or virtually the entire
15 coding region can be deleted. In particular examples, about 5% to about 100% of the gene is deleted, such as at least 20% of the gene, at least 40% of the gene, at least 75% of the gene, or at least 90% of the *Alg3* gene.

Deletion mutants can be constructed using any of a number of techniques known in the art. In one example, allelic exchange is employed to genetically inactivate one or more genes in
20 *Aspergillus*. A specific example of such a method is described in Example 2 below.

In one example, a strategy using counterselectable markers can be employed which has been utilized to delete genes. For a review, see Reyrat *et al.* (*Infect. Immun.* 66:4011-4017, 1998). In this technique, a double selection strategy is employed wherein a plasmid is constructed encoding both a selectable and counterselectable marker, with flanking DNA sequences derived
25 from both sides of the desired deletion. The selectable marker is used to select for fungi in which the plasmid has integrated into the genome in the appropriate location and manner. The counterselectable marker is used to select for the very small percentage of fungi that have spontaneously eliminated the integrated plasmid. A fraction of these fungi will then contain only the desired deletion with no other foreign DNA present.

In another technique, the *cre-lox* system is used for site specific recombination of DNA (for example see Steiger *et al.*, *Appl. Environ. Microbiol.* 77(1):114, 2011). The system includes 34 base pair lox sequences that are recognized by the bacterial *cre* recombinase gene. If the lox sites are present in the DNA in an appropriate orientation, DNA flanked by the lox sites will be excised by the *cre* recombinase, resulting in the deletion of all sequences except for one remaining copy of
30

the lox sequence. Using standard recombination techniques, the targeted gene of interest (*e.g.*, *Alg3*) can be deleted in the *Aspergillus* genome and to replace it with a selectable marker (for example a gene coding for kanamycin resistance) that is flanked by the lox sites. Transient expression (by electroporation of a suicide plasmid containing the *cre* gene under control of a promoter that functions in *Aspergillus*) of the *cre* recombinase should result in efficient elimination of the lox flanked marker. This process will produce a mutant containing the desired deletion mutation and one copy of the lox sequence.

In another method, an *Alg3* gene sequence in the *Aspergillus* genome is replaced with a marker gene, such as green fluorescent protein, β -galactosidase, or luciferase. In this technique, DNA segments flanking a desired deletion are prepared by PCR and cloned into a suicide (non-replicating) vector for *Aspergillus*. An expression cassette, containing a promoter active in *Aspergillus* and the appropriate marker gene, is cloned between the flanking sequences. The plasmid is introduced into wild-type *Aspergillus*. Fungi that incorporate and express the marker gene are isolated and examined for the appropriate recombination event (replacement of the wild type *Alg3* gene with the marker gene).

Thus, for example, a fungal cell can be engineered to have a disrupted *Alg3* gene using common mutagenesis or knock-out technology. (Methods in Yeast Genetics (1997 edition), Adams, Gottschling, Kaiser, and Sterns, Cold Spring Harbor Press, 1998; Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97: 6640-5, 2000; and Dai *et al.*, *Appl. Environ. Microbiol.* 70(4):2474-85, 2004). Alternatively, antisense technology can be used to reduce or eliminate the activity of *Alg3*. For example, a fungal cell can be engineered to contain a cDNA that encodes an antisense molecule that prevents *Alg3* from being translated. The term "antisense molecule" encompasses any nucleic acid molecule or nucleic acid analog (*e.g.*, peptide nucleic acids) that contains a sequence that corresponds to the coding strand of an endogenous *Alg3* gene. An antisense molecule also can have flanking sequences (*e.g.*, regulatory sequences). Thus, antisense molecules can be ribozymes or antisense oligonucleotides. A ribozyme can have any general structure including, without limitation, hairpin, hammerhead, or axehead structures, provided the molecule cleaves RNA. Further, gene silencing can be used to reduce the activity of *Alg3*.

B. Measuring gene inactivation

A fungus having an inactivated *Alg3* gene can be identified using any method known in the art. For example, PCR and nucleic acid hybridization techniques, such as Northern and Southern analysis, can be used to confirm that a fungus has an inactivated *Alg3* gene. Alternatively, real-time reverse transcription PCR (qRT-PCR) can be used for detection and quantification of targeted messenger RNA, such as mRNA of *Alg3* gene in the parent and mutant strains as grown at the same

culture conditions. Immunohistochemical and biochemical techniques can also be used to determine if a cell expresses *Alg3* by detecting the expression of the *Alg3* peptide encoded by *Alg3*. For example, an antibody having specificity for *Alg3* can be used to determine whether or not a particular fungus contains a functional nucleic acid encoding *Alg3* protein. Further, biochemical techniques can be used to determine if a cell contains a particular gene inactivation by detecting a product produced as a result of the expression of the peptide. For example, structural determination of N-glycans excised from glycoproteins can indicate that a fungal cell contains an inactivated *Alg3* gene. In addition, measurements of sporulation, germination, secondary metabolite production, and citric acid production can be measured using the methods described herein.

C. Measuring citric acid production

Methods of determining whether a genetic inactivation of *Alg3* in *Aspergillus* increases citric acid production, for example relative to the same strain with a native *Alg3* sequence (such as a parental strain), are routine in the art. Although particular examples are disclosed herein, the methods are not limiting.

For example, production of citric acid by *Aspergillus* (such as an *Alg3A* strain) can be measured using a spectrophotometric assay. In one example citric acid production can be determined with an endpoint spectrophotometric enzyme assay (for example see, Bergmeyer, H. U. 1985. Metabolites 2: tri- and dicarboxylic acids, purines, pyrimidines and derivatives, coenzymes, inorganic compounds, p. 5-10. In Citric acids. VCH Publishers, Weinheim, Germany). Citric acid can also be measured by liquid chromatography (LC) or high-performance liquid chromatography (HPLC) methods.

D. Alg3 sequences

Alg3 protein and nucleic acid sequences are publicly available and specific examples are provided herein. In addition, *Alg3* sequences can be identified using routine molecular biology methods.

Examples of *Alg3* nucleic acid sequences shown in SEQ ID NOS: 1 and 3. However, the disclosure also encompasses variants of SEQ ID NOS: 1 and 3 which retain the ability to encode an *Alg3* protein. One skilled in the art will understand that variant *Alg3* nucleic acid sequences can be inactivated. Variant sequences may contain a single insertion, a single deletion, a single substitution, multiple insertions, multiple deletions, multiple substitutions, or any combination thereof (e.g., single deletion together with multiple insertions). In addition, the degeneracy of the code permits multiple nucleic acid sequences to encode the same protein. For example, FIG. 9 shows an alignment of *Alg3* nucleic acid sequences from *A. niger* (nucleotides 1986-2518 of SEQ ID NO: 1) and *A. oryzae* (nucleotides 643-1175 of SEQ ID NO: 3), which permits one to identify

nucleotides that can tolerate substitution (*e.g.*, those that are not conserved between species) and those that may not (*e.g.*, those that are conserved between species). Such nucleic acid molecules can share at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to any known *Alg3* nucleic acid sequence, such as SEQ ID NO: 1 or 3
 5 or nucleotides 1186-1306, 1393-1916 and 1989 -2582 of SEQ ID NO: 1.

Examples of *Alg3* protein sequences shown in SEQ ID NOS: 2, 4, 31, 32, 33, 34, 35, and 36. However, the disclosure also encompasses variants SEQ ID NOS: 2, 4, 31, 32, 33, 34, 35, and 36 which retain *Alg3* activity. One skilled in the art will understand that variant *Alg3* enzyme sequences can be inactivated. Variant sequences may contain a single insertion, a single deletion, a
 10 single substitution, multiple insertions, multiple deletions, multiple substitutions, or any combination thereof (*e.g.*, single deletion together with multiple insertions). Such polypeptides share at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to an *Alg3* sequence, such as SEQ ID NO: 2, 4, 31, 32, 33, 34, 35, or 36.

Variant sequences can be identified, for example by aligning known *Alg3* sequences. For
 15 example, FIGS. 10A and 10B show the alignment of seven different *Alg3* sequences from different organisms. In addition, FIG. 11 shows a detailed alignment of *Alg3* protein sequences from *A. niger* (amino acids 12-411 of SEQ ID NO: 2) and *S. cerevisiae*, indicating amino acids that are identical, conserved (+) or not conserved (space). Based on these alignments, variants of *Alg3* sequences can be identified. For example, amino acid residues that are conserved between
 20 organisms are ones that should not be substituted (such as amino acids M50, T70, Y81, Q100 and D150 based on the numbering for *A. niger*), while amino acid residues that are not conserved between organisms are ones likely to tolerate substitution (such as amino acids R8, L160, S395 and N405 based on the numbering for *A. niger*). Similarly, amino acid positions in FIGS. 10A and 10B indicated with different amino acids at the same position are ones likely to tolerate substitution,
 25 while positions with the same amino acid (*) are not.

In some examples, an *Alg3* sequence that is to be genetically inactivated encodes or includes one or more conservative amino acid substitutions. A conservative amino acid substitution is a substitution of one amino acid (such as one found in a native sequence) for another amino acid having similar biochemical properties. Typically, conservative substitutions have little
 30 to no impact on the activity of a resulting peptide. In one example, an *Alg3* sequence (such as any of SEQ ID NOS: 2, 4, 31, 32, 33, 34, 35, or 36) includes one or more amino acid substitutions (for example at 1, 2, 5 or 10 residues). Examples of amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions include: Ser for Ala; Lys for Arg; Gln or His for Asn; Glu for Asp; Ser for Cys; Asn for Gln; Asp for Glu; Pro

for Gly; Asn or Gln for His; Leu or Val for Ile; Ile or Val for Leu; Arg or Gln for Lys; Leu or Ile for Met; Met, Leu or Tyr for Phe; Thr for Ser; Ser for Thr; Tyr for Trp; Trp or Phe for Tyr; and Ile or Leu for Val. Further information about conservative substitutions can be found in, among other locations in, Ben-Bassat *et al.*, (*J. Bacteriol.* 169:751-7, 1987), O'Regan *et al.*, (*Gene* 77:237-51, 1989), Sahin-Toth *et al.*, (*Protein Sci.* 3:240-7, 1994), Hochuli *et al.*, (*Bio/Technology* 6:1321-5, 1988), WO 00/67796 (Curd *et al.*) and in standard textbooks of genetics and molecular biology.

The *Alg3* gene inactivated in a fungus, in particular examples, includes a sequence that encodes an Alg3 protein having at least at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to an Alg3 sequence, such as SEQ ID NO: 2, 4, 31, 32, 33, 34, 35, or 36, wherein the protein can catalyze the addition of the first dol-p-man derived mannose in an α -1,3 linkage to Man5GlcNAc2-PP-Dol. In a specific example, the *Alg3* gene inactivated in a fungus encodes an Alg3 protein shown in SEQ ID NO: 2, 4, 31, 32, 33, 34, 35, or 36.

The *Alg3* gene that is to be inactivated in a fungus, in particular examples, includes a sequence having at least at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to an *Alg3* nucleic acid sequence, such as SEQ ID NO: 1 or 3 or nucleotides 1186-1306, 1393-1916 and 1989 -2582 of SEQ ID NO: 1, and encode an Alg3 protein that can catalyze the addition of the first dol-p-man derived mannose in an α -1,3 linkage to Man5GlcNAc2-PP-Dol. In a specific example, the *Alg3* gene inactivated in a fungus is shown in SEQ ID NO: 2 or 4.

One skilled in the art will appreciate that additional Alg3 sequences can be identified using any method such as those described herein. For example, *Alg3* nucleic acid molecules that encode an Alg3 protein can be identified and obtained using common molecular cloning or chemical nucleic acid synthesis procedures and techniques, including PCR. In addition, standard nucleic acid sequencing techniques and software programs that translate nucleic acid sequences into amino acid sequences based on the genetic code can be used to determine whether or not a particular nucleic acid has any sequence homology with known Alg3 sequences. Sequence alignment software such as MEGALIGN (DNASTAR, Madison, WI, 1997) can be used to compare various sequences.

In addition, nucleic acid hybridization techniques can be used to identify and obtain a nucleic acid molecule that encodes an Alg3 protein. Briefly, any known *Alg3* nucleic acid molecule, or fragment thereof, can be used as a probe to identify similar nucleic acid molecules by hybridization under conditions of moderate to high stringency. Such similar nucleic acid molecules

then can be isolated, sequenced, and analyzed to determine whether the encoded protein is an Alg3 protein.

Any method can be used to introduce an exogenous nucleic acid molecule into a fungal cell, for example to genetically inactivate *Alg3*. For example, chemical mediated-protoplast transformation, electroporation, *Agrobacterium*-mediated transformation, fusion of protoplasts, and biolistic delivery are common methods for introducing nucleic acid into fungal cells. (See, *e.g.*, Ito *et al.*, *J. Bacteriol.* 153:163-8, 1983; Durrens *et al.*, *Curr. Genet.* 18:7-12, 1990; Sambrook *et al.*, *Molecular cloning: A laboratory manual*, Cold Spring Harbour Laboratory Press, New York, USA, third edition, 2001; and Becker and Guarente, *Methods in Enzymology* 194:182-7, 1991. An exogenous nucleic acid molecule contained within a particular cell of the disclosure can be maintained within that cell in any form. For example, exogenous nucleic acid molecules can be integrated into the genome of the cell or maintained in an episomal state. That is, a cell can be a stable or transient transformant.

Fungi with Increased *LaeA* Expression or Increased *LaeA* Expression and *Alg3* Deletion

The present disclosure provides isolated fungi having increased *LaeA* expression, wherein such increased expression or activity (for example in combination with an *Alg3* functional inactivation, *Alg3Δ*) results in increased citric acid production by the fungi. Such fungi are referred to herein as increased *LaeA* fungal strains. It is disclosed herein that increased expression of *LaeA* (for example in combination with genetic inactivation of *Alg3*, *Alg3Δ*) results in *Aspergillus* fungi that can increase citric acid production as compared to *Aspergillus* having native levels of expression.

Contemplated herein are isolated fungi having increased *LaeA* activity/expression, for example in combination with a genetic inactivation of *Alg3*. Any fungus can be used, such as any genus or variety of *Aspergillus*. In particular examples, the *Aspergillus* fungus is *A. niger*, such as *Aspergillus niger* strain 11414 (American Type Culture Collection (ATCC) No. 11414; NRRL 2270); 1015 (ATCC No. 1015; NRRL 328, CBS 113.46); NRRL 3 (ATCC No. 9029, CBS 120.49, N400); NRRL 3122 (ATCC No. 22343); or 11414*KusA*-. In other specific examples, the *Aspergillus* is *A. aculeatus*, *A. awamori*, *A. carbonarius*, *A. wentii*, *A. foetidus*, *A. fumigatus*, *A. oryzae*, or *A. terreus*.

The present disclosure also provides isolated *A. terreus* fungi having increased *LaeA* expression, wherein such increased expression or activity results in increased itaconic acid production by the fungi. It is disclosed herein that increased expression of *LaeA* results in *A.*

terreus fungi that can increase itaconic acid production as compared to *A. terreus* having native levels of *LaeA* expression. Contemplated herein are isolated *A. terreus* fungi transformed with a nucleic acid construct comprising an *Aspergillus* species *LaeA* gene. The *LaeA* gene can be from any species of *Aspergillus*. In some examples, the *LaeA* gene is an *A. niger*, *A. nidulans*, *A.*

5 *aculeatus*, *A. awamori*, *A. carbonarius*, *A. wentii*, *A. foetidus*, *A. fumigatus*, *A. oryzae*, or *A. terreus* *LaeA* gene.

Any method for genetic enhancement or up-regulation can be used, as long as the expression of the gene and/or gene product is significantly increased, or the function of the expressed protein is significantly increased. In particular examples, *LaeA* gene expression is up-regulated by transformation of the fungi with one or more copies of a *LaeA* coding or genomic
10 sequence (which can be a native or non-native *LaeA* sequence). In some embodiments, up-regulation refers to an increase in gene or protein expression of at least 20%, at least 40%, at least 50%, at least 100%, at least 150%, at least 200%, at least 300%, or at least 500%, for example relative to the parental fungal strain without the additional copies of an *LaeA* gene. The term
15 “increased” or “up-regulated” as used herein with respect to a cell and a particular gene or protein activity refers to a higher level of activity than that measured in a comparable cell of the same species. For example, a particular fungi having increased or up-regulated *LaeA* activity has increased *LaeA* activity if a comparable fungi having native *LaeA* activity has less detectable *LaeA* activity (for example as measured by gene or protein expression).

20 *LaeA* sequences are disclosed herein and others are publicly available, for example from GENBANK™ or EMBL. In some examples, the *LaeA* gene upregulated encodes a protein having at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity to SEQ ID NO: 41 or SEQ ID NO: 59. In some examples, the *LaeA* gene upregulated comprises at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at
25 least 97%, at least 98% or at least 99% sequence identity to SEQ ID NO: 40 (e.g., nt 1-236 and 367-1252 of SEQ ID NO: 41) or SEQ ID NO: 58 (e.g., nt 1-230 and 373-1267 of SEQ ID NO: 58).

Increasing *LaeA* activity (for example in combination with genetic inactivation of *Alg3*) results in many phenotypes in the fungi. For example, such recombinant fungi exhibit increased citric acid production in CAP medium. Such increases can be relative to a fungi having a native or
30 wild-type level of *LaeA* (or *LaeA* and *Alg3*) gene or protein expression, such as a parental strain (e.g., *A. niger* strain 11414*KusA*), grown under the same conditions as the fungi with increased *LaeA* activity (or increased *LaeA* activity and decreased *Alg3* activity). In some examples, increased citric acid production in CAP medium is an increase of at least 20%, at least 30%, at least 50%, at least 60%, at least 65%, or at least 70%, by such a recombinant fungus as compared to *A.*

niger strain 11414*KusA*. In some examples, recombinant fungi with increased *LaeA* activity (for example in combination with genetic inactivation of *Alg3*) have increased sporulation relative to *A. niger* *Alg3Δ* on MM, accumulate red color pigments to *A. niger* strain 11414*KusA* on complete medium, or both. In other examples, increasing *LaeA* activity in *A. terreus* fungi results in an increase in itaconic acid production in itaconic acid production media. Such increases can be relative to *A. terreus* fungi having a native or wild-type level of *LaeA* gene or protein expression, such as a parental strain *A. terreus* strain grown under the same conditions as the fungi with increased *LaeA* activity. In some examples, increased itaconic acid production is an increase of at least 20%, at least 30%, at least 50%, at least 60%, at least 65%, or at least 70%, as compared to a parental strain.

One skilled in the art will appreciate that additional genes can also be inactivated or upregulated, wherein the additional genes may or may not provide additional enhancement of citric acid production or itaconic acid production to the fungus. In one example *KusA* (*e.g.*, GENBANK™ Accession No. EF061656) is also genetically inactivated.

Also provided by the present disclosure are compositions that include isolated *LaeA* up-regulated fungi, such as a growth medium. Also provided by the present disclosure are kits that include isolated *LaeA* up-regulated fungi, such as a kit that includes a medium for culturing, storing, or growing the fungus. Exemplary mediums include solid medium (such as those containing agar, for example CM, PDA or MM) and liquid media (such as a fermentation broth, such as CM, MM, or CAP medium).

A. Methods of up-regulating gene and/or protein expression

As used herein, an “activated” or “up-regulated” gene means that expression of the gene or gene product (*e.g.*, protein) has been up-regulated, for example by introduction of additional copies of the appropriate gene or coding sequence into the fungus (or other common molecular biology methods), such that the introduced nucleic acid sequence is expressed, resulting in increased expression or biological activity of the encoded gene product.

Increasing expression of one or more genes (which in some examples is also referred to as up-regulation) can be performed using any conventional method known in the art. In one example, a strain of *Aspergillus* is transformed with a vector which has the effect of up-regulating or otherwise activating a *LaeA* gene (such as a native or non-native *LaeA* gene). This can be done by introducing one or more *LaeA* coding sequences (such as a gene sequence), whose expression is controlled by elements such as promoters and the like which control gene expression, by introducing a nucleic acid sequence which itself (or its encoded protein) can increase *LaeA* protein activity in the fungus, or by introducing another molecule (such as a protein or antibody) increases

LaeA protein activity in the fungus. For example, a *LaeA* gene can be up-regulated by introduction of a vector that includes one or more *LaeA* sequences (such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 *LaeA* sequences or copies of such sequences) into the desired fungus. In some examples, such *LaeA* sequences are from different fungal species, can be multiple copies from a single species, or combinations thereof, such as *LaeA* sequences from at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 different fungal species. In some examples, the *LaeA* sequence(s) introduced into the fungus is optimized for codon usage. Thus, the disclosure in some examples provides transformed fungi that include at least one exogenous nucleic acid molecule which includes a *LaeA* gene or coding sequence (such as a nucleic acid sequence encoding SEQ ID NOs: 41 or 59), for example in combination with *Alg3A*. In one example, such transformed cells produce more citric acid, for example relative to a comparable fungus with a native *LaeA* sequence (or a native *LaeA* sequence combined with a native *Alg3* sequence).

In another technique, the *cre-lox* system is used for site specific recombination of DNA (for example see Steiger *et al.*, *Appl. Environ. Microbiol.* 77(1):114, 2011). The system includes 34 base pair lox sequences that are recognized by the bacterial *cre* recombinase gene. If the lox sites are present in the DNA in an appropriate orientation, DNA flanked by the lox sites will be excised by the *cre* recombinase, resulting in the deletion of all sequences except for one remaining copy of the lox sequence. Using standard recombination techniques, the targeted gene of interest (*e.g.*, *LaeA*) can be deleted in the *Aspergillus* genome and replaced with one or more copies of a non-native *LaeA* sequence (for example in *A. niger*, replacing one or both *A. niger* *LaeA* sequences with one or more, or combination of, *LaeA* sequences from *A. nidulans*, *A. flavus*, *fusarium oxysporum*, *penicillium chrysogenum*, which have high secondary metabolite production) flanked by the lox sites. Transient expression (by electroporation of a suicide plasmid containing the *cre* gene under control of a promoter that functions in *Aspergillus*) of the *cre* recombinase should result in efficient elimination of the lox flanked marker. This process will produce a fungus containing the desired insertion mutation and one copy of the lox sequence.

In one example, one or more *LaeA* genes are introduced into fungal cells by chemical mediated protoplast transformation in combination of yeast-gap repairing method for transgene expression construction.

In one example, a transgene is generated and expressed in the desired fungal cell, such as an *Alg3Δ* cell, to increase *LaeA* expression. For example, such a transgene can include a *LaeA* genomic or cDNA sequence (such as one having at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to any known *LaeA* sequence, such as SEQ ID NO: 40 or 58), for example operably linked to a promoter, such as a

glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter or other promoter, such as one that has high activity in CAP culture medium, for example a polyubiquitin promoter, *Arsa-7*, and *A-37* from *A. niger*. In one example, the promoter has at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 37. In one example, the promoter comprises or consists of the sequence shown in SEQ ID NO: 37. In some examples, the transgene further includes *pyrG* upstream and downstream sequences (for example that are at the 5'- and 3'-end, respectively, of the transgene). The *pyrG* gene in *A. niger* is mutated and has lost its original functions. Thus, other non-essential gene loci can be used as long as it is not influenced by the native neighbor genes. In one example, the *pyrG* upstream and downstream sequences have at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 45 and 48, respectively. In one example, the *pyrG* upstream and downstream sequences comprise or consist of the sequence shown in SEQ ID NO: 45 or 48, respectively. In some examples, the transgene further includes a *trpC* transcriptional terminator sequence of *A. nidulans*, for example downstream of the *LaeA* sequence. As an alternative to *trpC*, other transcriptional terminators can be used, such as promoters which include a transcriptional terminators (*e.g.*, *Arsa7*, *Arsa-37*, polyubiquitin (*ubi4*)). In one example, the *trpC* transcriptional terminator has at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 46. In one example, the *trpC* transcriptional terminator comprises or consists of the sequence shown in SEQ ID NO: 46. In some examples, the transgene further includes a *ptrA* sequence, for example downstream of the *trpC* transcriptional terminator sequence. As an alternative to *ptrA*, the bleomycin gene or bar gene can be used. In one example, the *ptrA* sequence has at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 47. In one example, the *ptrA* sequence comprises or consists of the sequence shown in SEQ ID NO: 47. In one example, the transgene comprises a sequence having at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 44 or 57. In one example, the transgene comprises or consists of the sequence shown in SEQ ID NO: 44 or 57.

Thus, for example, a fungal cell can be engineered to have increased copies of *LaeA* using common recombinant technology methods.

B. Measuring gene activation or up-regulation

A fungus having an activated or up-regulated *LaeA* gene can be identified using any method known in the art. For example, PCR and nucleic acid hybridization techniques, such as Northern, RT-PCR, and Southern analysis, can be used to confirm that a fungus has an up-regulated *LaeA* gene, such as an increase in the *LaeA* copy number. Immunohistochemical and biochemical

techniques can also be used to determine if a cell expresses *LaeA* by detecting the expression of the *LaeA* peptide encoded by *LaeA*. For example, an antibody having specificity for *LaeA* can be used to determine whether or not a particular fungus has increased *LaeA* protein expression. Further, biochemical techniques can be used to determine if a cell has increased *LaeA* expression by detecting a product produced as a result of the expression of the peptide. For example, measurement of secondary metabolites can indicate that a fungal cell contains an up-regulated *LaeA* gene. In addition, measurements of citric acid production can be measured using the methods described herein.

C. Measuring citric acid production

Methods of determining whether a genetic up-regulation of *LaeA* (alone or in combination with inactivation of *Alg3*) in *Aspergillus* increases citric acid production, for example relative to the same strain with a native *LaeA* sequence, *Alg3* sequence, or both (such as a parental strain), are routine in the art. Although particular examples are disclosed herein (see above and in the examples below), the methods are not limiting.

D. Measuring itaconic acid production

Methods of determining whether a genetic up-regulation (*e.g.* overexpression) of *LaeA* in *Aspergillus terreus* increases itaconic acid production, for example relative to the same strain that does not overexpress *LaeA* (such as a parental strain), are routine in the art. Although particular examples are disclosed herein (see Example 14), the methods are not limiting. For example, production of itaconic acid by *A. terreus* can be performed as previously described by Dickman (*Analytical Chem* 24: 1064-1066, 1952), or using the modified method described in Example 14.

E. *LaeA* sequences

LaeA protein and nucleic acid sequences are publicly available and specific examples are provided herein. In addition, *LaeA* sequences can be identified using routine molecular biology methods.

Examples of *LaeA* nucleic acid sequences shown in SEQ ID NOs: 40 and 58. However, the disclosure also encompasses variants of SEQ ID NOs: 40 and 58 (such as the coding regions nt 1-236 and 367-1252 of SEQ ID NO: 41 and nt 1-230 and 373-1267 of SEQ ID NO: 58) which retain the ability to encode a *LaeA* protein. One skilled in the art will understand that variant *LaeA* nucleic acid sequences can be used to increase expression of *LaeA*. Variant sequences may contain a single insertion, a single deletion, a single substitution, multiple insertions, multiple deletions, multiple substitutions, or any combination thereof (*e.g.*, single deletion together with multiple insertions). In addition, the degeneracy of the code permits multiple nucleic acid sequences to encode the same protein. Thus, in one example, a *LaeA* sequence having at least 80%, at least 85%,

at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to any known *LaeA* sequence, such as SEQ ID NO: 40 or SEQ ID NO: 58 (such as the coding regions nt 1-236 and 367-1252 of SEQ ID NO: 41 and nt 1-230 and 373-1267 of SEQ ID NO: 58) can be expressed in a fungal cell to increase *LaeA* expression in the fungal cell.

For example, FIG. 12 shows an alignment of *LaeA* protein sequences from *A. nidulans* (aa 14-372 of SEQ ID NO: 41) and *A. niger* (aa 14-370 of SEQ ID NO: 59), which permits one to identify amino acids that can tolerate substitution (*e.g.*, those that are not conserved between species) and those that may not (*e.g.*, those that are conserved between species). Based on these alignments, variants of *LaeA* sequences can be identified. For example, amino acid residues that are conserved between organisms are ones that should not be substituted (such as amino acids S16, M42, and P52 based on the numbering for *A. nidulans*), while amino acid residues that are not conserved between organisms are ones likely to tolerate substitution (such as amino acids T15, S44, and N325 based on the numbering for *A. nidulans*). Similarly, amino acid positions in FIG. 12 indicated with a space are ones likely to tolerate substitution, while positions with the same amino acid are not.

Such protein molecules can share at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to any known *LaeA* nucleic acid sequence, such as SEQ ID NOs: 41 and 59, and such variants can be used to increase *LaeA* activity in a fungal cell. One skilled in the art will understand that variant *LaeA* enzyme sequences can be used to increase *LaeA* activity in a fungal cell. Variant sequences may contain a single insertion, a single deletion, a single substitution, multiple insertions, multiple deletions, multiple substitutions, or any combination thereof (*e.g.*, single deletion together with multiple insertions).

In some examples, a *LaeA* sequence whose expression is to be up-regulated encodes or includes one or more conservative amino acid substitutions. A conservative amino acid substitution is a substitution of one amino acid (such as one found in a native sequence) for another amino acid having similar biochemical properties. Typically, conservative substitutions have little to no impact on the activity of a resulting peptide. In one example, a *LaeA* sequence (such as any of SEQ ID NOs: 41 and 59) includes one or more amino acid substitutions (for example at 1, 2, 5 or 10 residues). Examples of amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions include those discussed above for Alg3.

The *LaeA* gene up-regulated in a fungus, in particular examples, includes a sequence that encodes a *LaeA* protein having at least at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to an *LaeA* sequence, such as

SEQ ID NO: 41 or SEQ ID NO: 59, wherein the protein can regulate secondary metabolite production in *Aspergillus*. In a specific example, the *LaeA* gene up-regulated in a fungus encodes a *LaeA* protein shown in SEQ ID NO: 41 or 59.

The *LaeA* gene up-regulated in a fungus, in particular examples, includes a sequence having at least at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to a *LaeA* nucleic acid sequence, such as SEQ ID NO: 40 or SEQ ID NO: 58 (or to the coding regions nt 1-236 and 367-1252 of SEQ ID NO: 41 or nt 1-230 and 373-1267 of SEQ ID NO: 58), and encodes a *LaeA* protein which can regulate secondary metabolite production in *Aspergillus*. In a specific example, the *LaeA* gene upregulated in a fungus is shown in SEQ ID NO: 40 or SEQ ID NO: 58 (or includes the coding regions nt 1-236 and 367-1252 of SEQ ID NO: 41 or nt 1-230 and 373-1267 of SEQ ID NO: 58).

One skilled in the art will appreciate that additional *LaeA* sequences can be identified and obtained using any method such as those described herein. For example, *LaeA* nucleic acid molecules that encode a *LaeA* protein can be identified and obtained using common molecular cloning or chemical nucleic acid synthesis procedures and techniques, including PCR. In addition, standard nucleic acid sequencing techniques and software programs that translate nucleic acid sequences into amino acid sequences based on the genetic code can be used to determine whether or not a particular nucleic acid has any sequence homology with known *LaeA* sequences. Sequence alignment software such as MEGALIGN (DNASTAR, Madison, WI, 1997) can be used to compare various sequences.

In addition, nucleic acid hybridization techniques can be used to identify and obtain a nucleic acid molecule that encodes a *LaeA* protein. Briefly, any known *LaeA* nucleic acid molecule, or fragment thereof, can be used as a probe to identify similar nucleic acid molecules by hybridization under conditions of moderate to high stringency. Such similar nucleic acid molecules then can be isolated, sequenced, and analyzed to determine whether the encoded protein is a *LaeA* protein. The gene specific oligonucleotide pair can also be designed, synthesized and used for real-time RT-PCR to quantify the *LaeA* gene transcription level.

Any method can be used to introduce an exogenous nucleic acid molecule into a fungal cell, for example to genetically enhance *LaeA* expression. For example, chemical mediated-protoplast transformation, electroporation, *Agrobacterium*-mediated transformation, fusion of protoplasts, and biolistic delivery are common methods for introducing nucleic acid into fungal cells. (See, *e.g.*, Ito *et al.*, *J. Bacteriol.* 153:163-8, 1983; Durrens *et al.*, *Curr. Genet.* 18:7-12, 1990; Sambrook *et al.*, *Molecular cloning: A laboratory manual*, Cold Spring Harbour Laboratory Press, New York, USA, second edition, 1989; and Becker and Guarente, *Methods in Enzymology* 194:182-7, 1991). An

exogenous nucleic acid molecule contained within a particular cell of the disclosure can be maintained within that cell in any form. For example, exogenous nucleic acid molecules can be integrated into the genome of the cell or maintained in an episomal state. That is, a cell can be a stable or transient transformant.

F. *LaeA* deletion and complementation

Disclosed herein is a *LaeA* deletion cassette for generating a fungus, such an *Aspergillus* species fungus, having a deletion in the *LaeA* gene. A schematic of the *LaeA* deletion cassette is shown in FIG. 17A. To generate the deletion cassette, DNA fragments comprising the 5' and 3' ends of the *A. niger* *LaeA* gene (SEQ ID NOs: 61 and 62, respectively) were isolated from *A. niger* genomic DNA and the *hph* expression cassette (SEQ ID NO: 63) (*hph*) was isolated from pCB1003 plasmid vector DNA by PCR. The PCR DNA fragments were assembled in the pBSK backbone vector to produce the *LaeA* deletion cassette (SEQ ID NO: 65).

Provided herein is a nucleic acid molecule comprising a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 65. In some embodiments, the nucleic acid molecule comprises or consists of the nucleotide sequence of SEQ ID NO: 65. Further provided is an isolated fungus transformed with a nucleic acid molecule comprising a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 65. In some embodiments, the isolated fungus is transformed with a nucleic acid molecule comprising or consisting of the nucleotide sequence of SEQ ID NO: 65. In some embodiments, the fungus is a species of *Aspergillus*, such as *A. niger*.

Further provided herein are isolated fungi (such as filamentous fungi) having a gene inactivation (also referred to as a gene deletion) of a *LaeA* gene. Any strain of fungi can be used, such as a filamentous fungi, for example *A. niger* or particular strains thereof (for example *A. niger* strain 11414 or 11414*KusA*). In particular examples, the *LaeA* gene is genetically inactivated by complete or partial deletion mutation or by insertional mutation. In some examples genetic inactivation need not be 100% genetic inactivation. In some embodiments, genetic inactivation refers to at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95% gene or protein inactivation. The term “reduced” or “decreased” as used herein with respect to a cell and a particular gene or protein activity refers to a lower level of activity than that measured in a comparable cell of the same species. For example, a particular fungi lacking *LaeA* activity has reduced *LaeA* activity if a comparable fungi not having an *LaeA* genetic inactivation has detectable *LaeA* activity. In some embodiments, the isolated fungi having a gene inactivation are generated using the *LaeA* deletion construct set forth herein as SEQ ID NO: 65.

Also provided herein is a *LaeA* complementation construct for complementing expression of *LaeA* in fungi having a deleted *LaeA* gene. A schematic of the *LaeA* complementation construct is shown in FIG. 17B. To generate the complementation construct, the *LaeA* gene containing both its promoter and transcriptional terminator was isolated by PCR from *A. niger* genomic DNA. The PCR fragment was cloned into the plasmid vector pRSB426-*LaeA*. The new plasmid DNA vector contained the upstream region of the *pyrG* gene of *A. niger*, the entire region of *A. niger* *LaeA* gene, the transcriptional terminator of the *trpC* gene from *A. nidulans*, the pyrithiamine resistance (*ptrA*) gene from *A. oryzae*, and the downstream region of the *pyrG* gene of *A. niger* (SEQ ID NO: 66).

Provided herein is a nucleic acid molecule comprising a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 66. In some embodiments, the nucleic acid molecule comprises or consists of the nucleotide sequence of SEQ ID NO: 66. Further provided is an isolated fungus transformed with a nucleic acid molecule comprising a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 66. In some embodiments, the isolated fungus is transformed with a nucleic acid molecule comprising or consisting of the nucleotide sequence of SEQ ID NO: 66. In some embodiments, the fungus is a species of *Aspergillus*, such as *A. niger*.

Further provided herein are isolated fungi (such as filamentous fungi) having a gene inactivation (also referred to as a gene deletion) of a *LaeA* gene and further transformed by a nucleic acid construct comprising a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 66. In some embodiments, the isolated fungus is transformed with a nucleic acid molecule comprising or consisting of the nucleotide sequence of SEQ ID NO: 66. In some embodiments, the fungus is a species of *Aspergillus*, such as *A. niger*. Any strain of fungi can be used, such as a filamentous fungi, for example *A. niger* or particular strains thereof (for example *A. niger* strain 11414 or 11414*KusA*).

Production of Citric Acid Using *Alg3A* Mutants, Fungi with Increased *LaeA* Expression, or Both

The fungi provided herein, namely *Alg3A* fungi, up-regulated *LaeA* fungi, and fungi with both *Alg3A* and up-regulated *LaeA*, can be used to produce citric acid, as well as derivatives thereof such as hydroxycitric acid (for example for medical applications). Such fungi can be from any species, such as *Aspergillus* or *Rhizopus* cells. For example, the disclosure provides methods of making citric acid, which can include culturing *Alg3A* fungi, up-regulated *LaeA* fungi, or fungi with

both *Alg3A* and up-regulated *LaeA*, under conditions that permit the fungus to make citric acid, for example in CAP medium.

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) combines a pleasant taste with low toxicity and palatability and is a ubiquitous food additive. It is also able to complex heavy metal ions, like iron and copper, and is therefore applied in the stabilization of oils and fats or ascorbinic acid during metal ion-catalyzed oxidation reactions. Consequently, it is today one of the bulk products produced by fermentation, most of which occurs with the fungus *Aspergillus niger*, although a small portion is also produced by fermentation with yeast, such as *Candida oleophila* and *Candida lipolytica*.

Citric acid production generally requires a unique combination of several unusual nutrient conditions (*e.g.*, excessive concentrations of carbon source, H⁺, and dissolved oxygen, or suboptimal concentrations of certain trace metals and phosphate), which synergistically influence the yield of citric acid. Table 1 below shows the environmental parameters that influence citric acid accumulation.

Table 1: Parameters that influence citric acid accumulation by *A. niger*

Parameter	Requirement for citric acid accumulation
Carbon source concentration	Higher than 50 g/l
Carbon source type	Enable rapid catabolism
Nitrogen source	Consumption leads to some decrease in pH
Phosphate concentration	Suboptimal
Aeration	In excess
Trace metal ions	Limiting, especially Mn ²⁺
pH	Below pH 3

Methods of making citric acid, which can include culturing *Alg3A* fungi, up-regulated *LaeA* fungi, or fungi with both *Alg3A* and up-regulated *LaeA*, under conditions that permit the fungus to make citric acid, are provided. In general, the culture media and/or culture conditions can be such that the fungi grow to an adequate density and produce citric acid efficiently. In one example the *Alg3A* fungi, up-regulated *LaeA* fungi, or fungi with both *Alg3A* and up-regulated *LaeA*, are cultured or grown in a liquid medium that includes sucrose and/or glucose as the carbon source, for example at a concentration of at least 50g/liter, such as at least 100 g/l, or at least 140 g/l. Thus, a fungus within the scope of the disclosure in some examples can utilize a variety of carbon sources. In one example the *Alg3A* fungi, up-regulated *LaeA* fungi, or fungi with both *Alg3A* and up-

regulated *LaeA*, are cultured or grown in a liquid medium that includes a very small amount of manganese, such as less than 100 parts per billion (ppb), less than 50 ppb, less than 20 ppb, less than 15 ppb, for example 5 ppb to 15 ppb or 10 ppb to 15 ppb, such as 5, 10, 13, 15 or 20 ppb. In one example the *Alg3A* fungi, up-regulated *LaeA* fungi, or fungi with both *Alg3A* and up-regulated *LaeA*, are cultured or grown in a liquid medium having an initial pH of less than 3, such as less than 2.5, for example about pH 1.8 to 3, 1.8 to 2.5, 1.8 to 2.2, 1.9 to 2.1, for example pH 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8 or 2.9. In some examples the *Alg3A* fungi, up-regulated *LaeA* fungi, or fungi with both *Alg3A* and up-regulated *LaeA*, are cultured or grown in a liquid medium at about 25 to 35°C (such as 28 to 32°C, or 30°C) with rotation of 180 to 300 rpm.

In a specific example, the *Alg3A* fungi, up-regulated *LaeA* fungi, or fungi with both *Alg3A* and up-regulated *LaeA*, are grown in citric acid production (CAP) medium. In a specific example, the CAP medium includes 140 g of glucose/liter, 3.1 g of NH_4NO_3 /liter, 0.15 g of KH_2PO_4 /liter, 0.15 g of NaCl /liter, 2.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ /liter, 6.6 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ /liter, and 0.1 mg of FeCl_3 /liter adjusted to about pH 2 with 4 M H_2SO_4 . Cations can be removed from the glucose solution by ion exchange on Dowex 50W-X8, 100/200-mesh, H cation exchange resin (Fisher Scientific, Pittsburgh, Pa.) prior to adding the other nutrient components. The manganese concentration in the medium can be adjusted by the addition of appropriate volumes of a stock solution of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (10 mM). In one example, the manganese concentration is less than 50 ppb, such as less than 20 ppb, for example 5 to 15 ppb, such as 10 ppb.

Methods of culturing *Aspergillus* to enable citric acid production are well known in the art. In one example, the fungi are grown in culture containers (such as baffled flasks, and in some examples are silanized (5% solution of dichlorodimethylsilane in heptane (Sigma, St. Louis, MO)). The *Alg3A* fungi, up-regulated *LaeA* fungi, or fungi with both *Alg3A* and up-regulated *LaeA*, provided herein can be grown in CAP media containing low amounts of Mn^{2+} (e.g., 10 ppb) at 30°C with rotation (e.g., 200 to 250 rpm) for at least 3 days (e.g., 3 to 7 days). Each culture container is inoculated with spores (such as at least 10^6 spores/ml) and incubated for at least 12 or at least 15 hours at 30°C and 200 to 250 rpm to obtain properly pelleted morphology.

In one example, the *Alg3A* fungi, up-regulated *LaeA* fungi, or fungi with both *Alg3A* and up-regulated *LaeA*, produce more citric acid than a corresponding fungus with wild-type *Alg3*. In specific examples, the *Alg3A* fungi, up-regulated *LaeA* fungi, or fungi with both *Alg3A* and up-regulated *LaeA*, produce at least 25 g/l of citric acid (for example at least 30 g/l, at least 32 g/l, at least 35 g/l, at least 40 g/l, at least 42 g/l, at least 45 g/l, at least 50 g/l, at least 52 g/l or at least 55 g/l), for example after at least 4 days (such as at least 5 days, at least 6 days, at least 7 days, at least

8 days, or at least 10 days, such as after 4 to 6 days, 8 to 10 days, or 4 to 5 days) when grown in CAP medium at 30°C with 200 rpm shaking.

In some examples, the method further includes isolating the citric acid made by the *Alg3A* fungi, up-regulated *LaeA* fungi, or fungi with both *Alg3A* and up-regulated *LaeA*. Once produced, any method can be used to isolate the citric acid. For example, common separation techniques can be used to remove the fungal biomass from the culture medium, and common isolation procedures (e.g., filtration, distillation, precipitation, electrodialysis, and ion-exchange procedures) can be used to obtain the citric acid from the broth (such as a fungi-free broth). In addition, the citric acid can be isolated from the culture medium after the citric acid production phase has been terminated.

Example 1

Materials and Methods

This example describes methods used in the experiments described in Examples 2-5 below.

Strains and media. The *Escherichia coli* strains Top10 and *Saccharomyces cerevisiae*

strain YVH10 were used as hosts for routine cloning and gap repair experiments. *A. niger* strain ATCC 11414 (American Type Culture Collection, Rockville, MD), was grown on potato dextrose agar plates (PDA) and complete medium (CM) agar plates at 30°C for culture maintenance and spore preparation, respectively. The mutant strain *Aspergillus niger* 11414*KusA* was generated by the deletion of *kusA* in *A. niger* strain 11414 by the replacement with *A. fumigatus* *pyrG* gene, which encodes the ortholog of the ku70 protein that involves in the non-homologous end joining pathway of DNA repair for the integration of a DNA fragment into the genome in other eukaryotes, and was confirmed by Southern blotting analysis. The 11414*kusA* strain with high rate of homologous replacement was mainly used as a parent strain. The cultures on PDA or complete medium (CM) agar plates were incubated for four days at 30°C and the spores were harvested by washing with sterile 0.8% Tween 80 (polyoxyethylenesorbitan monooleate). The CM medium contains 20 g of D-glucose/liter, 5 g yeast extract/liter, 2 g trypticase peptone/liter, 1 g casamino acids/liter, 6 g NaNO₃/liter, 0.52 g KCl/liter, 0.52 g MgSO₄ • 7H₂O/liter, 1.52 g KH₂PO₄/liter, 36.7 mg ZnSO₄ • 7H₂O/liter, 18.3 mg H₃BO₃/liter, 8.3 mg MnCl₂ • 4H₂O/liter, 8.3 mg FeSO₄7H₂O, 2.8 mg CoCl₂ • 6H₂O/liter, 2.7 mg CuSO₄ • 5H₂O/liter, 2.5 mg Na₂MoO₄ • 2H₂O/liter, 83.3 mg Na₂EDTA/liter, 1 mg biotin/liter, 1 mg pyridoxin/liter, 1 mg thiamine/liter, 1 mg riboflavin/liter, 1 mg p-aminobenzoic acid/liter and 1 mg nicotinic acid/liter. The PDA medium contains 4 g/liter potato starch and 20 g/liter dextrose. Conidia were enumerated with a hemacytometer. Aliquots of the resulting spore suspension (1 × 10⁹ spores/ml) were used to inoculate baffled-flask liquid cultures. The citric acid production (CAP) medium contained 140 g/l of glucose, 3.1 g/l NH₄NO₃,

0.15 g/l KH_2PO_4 , 0.15 g/l NaCl, 2.2 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.6 mg/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.1 mg/l FeCl_3 adjusted to pH 2.1 with 4 M H_2SO_4 . Cations were removed from the glucose solution by ion-exchange on Dowex 50W-X8, 100-200 mesh, H cation exchange resin (Fisher Scientific, Pittsburgh, PA) prior to adding the other nutrient components.

- 5 **Culture methods.** Glass baffled-flasks of 250 ml or 1000 ml were silanized by rinsing in a 5% solution of dichlorodimethylsilane in heptane (Sigma, St. Louis, MO) to minimize leaching of metals. For citric acid production tests, 1×10^6 spores/ml of parent or mutant strains were grown in 80 ml CAP media containing 10 ppb Mn^{2+} in 250 ml baffled flasks or 220 ml CAP media in 1000 ml baffled flasks at 30°C and 200 rpm. Samples for citric acid analysis were taken at intervals.
- 10 The biomass of transgenic clones and parent stain were prepared from 2 ml CM station cultures with proper antibiotics and grown in 16 × 125 mm glass culture-tubes at 30°C without shaking. The biomass formed on the surface of the culture medium was collected, frozen immediately in liquid nitrogen and dried in the lyophilizer.

- Dried biomass measurement.** After proper cultivation, the cell mass from citric acid
- 15 production culture was collected by centrifugation at room temperature and 4500x g for 5 min in Sorvall floor centrifuge with swinging-bucket rotor. The cell mass was then transferred onto the Whatman Grade No 1 filter paper or left in centrifuge tubes for freeze-drying. The biomass was then dried in high temperature oven at 80°C or freeze-dried in the lyophilizer. Prior to being used, the centrifuge tube or Whatman filter paper was weighted and re-weighted after the biomass was
- 20 completely dried.

- Total genomic DNA isolation for PCR and Southern blotting analysis.** Total genomic DNA was isolated from *A. niger* according to the SDS extraction method described previously by Dellaporta *et al.* (*Plant Molecular Biology Reporter* 1(4):19-21, 1983) with some modifications. Briefly, fungal biomass from 2ml station cultures was looped and transferred into a 1.5 ml
- 25 microcentrifuge tube. A needle size hole on the cap was punched with 18 gauge needle. The tube was immediately frozen in liquid N_2 for 5 minutes and biomass in the tube was dried in a VirTis benchtop manifold freeze dryer (SP Scientific, Gardiner, NY) overnight. The dried biomass and two 3.5 mm diameter glass beads were transferred into the 2ml polypropylene microvial, where biomass was pulverized into fine power with Mini-Beadbeater-8 (Bio Spec Products Inc.,
- 30 Bartlesville, OK) for one minute. Then, 500µl of 60°C extraction buffer and 80µl of 15% SDS were added into the microcentrifuge tube and incubated at 65°C for at least 30 minutes with occasionally swirling to mix. Two-hundred microliters of 5M potassium acetate was added, mixed and incubated on ice for 30 minutes. The supernatant was collected by centrifugation at 12,000 g for 10 minutes at 4°C and transferred into the new microcentrifuge tube. The total nucleic acids

were precipitated with 780 µl of 2-propanol for 30 minutes at -20°C and centrifuged at 12,000 g for 10 minutes. The nucleic acids were re-suspended in 200 µl 50TE buffer containing 2 µl RNase (10 µg/µl stock solution) and incubated in Eppendorf thermomixer at 50°C and 500 rpm for 30 minutes. The proteins and cell debris was removed by being added and well mixed 20 µl 3M sodium acetate and equal volume of phenol:chloroform and centrifuged at 15,000 g for 5 minutes. The supernatant was transferred to new DNase-free microcentrifuge tube containing 220 µl of 2-propanol, mixed well and incubated at room temperature for 5 minutes. The genomic DNA was pelleted by centrifugation at 15,000 g for 10 minutes and washed with 500 µl of 70% ethanol. The genomic DNA was re-suspended in 80 µl 10 mM TrisHCl (pH8.0) buffer and determined with Qubit fluorometer (Invitrogen, Carisbad, CA). One microgram of total genomic DNA was digested with restriction endonuclease BamH and SacII. The genomic DNA fragments were separated in 1% agarose gel electrophoretically and transferred onto the zeta-probe membrane (BioRad) with alkaline capillary transfer method. A 3.8 kb genomic DNA fragment containing the *Alg3* sequence was used for preparation of the biotin-labeled probe. The genomic DNA in Zeta-probe membrane was hybridized with the biotin-labeled probe overnight in 60°C hybridization oven. The genomic DNA on hybridized membrane was visualized with North2South chemiluminescent detection kit (Pierce Protein Research Products, Rockford, IL).

Spore production and germination. The spore production on the PDA or CM agar plates described above was excised with plastic closures of culture tubes in 27 mm diameter and transferred into the 50 ml centrifuge tubes containing 25 ml 0.8% tween 80. The spores were released from the agar surface by scraping with plastic loops and vortexed with vortex mixer at top speed. The spores were diluted properly and enumerated with a hemacytometer. The spore production in a unit area (cm²) was determined. For spore germination, 1x10⁵ spores per well were added into each well of 24 well Schwarz sensoplate and incubated in the microscopic incubator with temperature control at 30°C. The spore germination was automatically imaged hourly for 24 hours through the Olympus inverted system microscope (Olympus America Inc., Center Valley, PA, USA). The spore germination was visualized with Adobe Photoshop CSS (San Jose, CA) and counted manually.

Citric acid measurements. Citric acid concentrations were determined with an end-point spectrophotometric enzyme assay as described in the instruction from the manufacturer (R-Biopharm AG/Roche, Darmstadt, Germany) with a proper dilution.

Table 2 shows oligonucleotides used in the methods.

Table 2. Oligonucleotides

Name	Sequence	Product size (kb)
<u>Alg3Δ construction</u>		
Alg3-ForScr	CGGTTTCCCTTCAGTTTCCAGT (SEQ ID NO: 5)	
Alg3-1	GTAACGCCAGGGTTTTCCCAGTCACGACGTCAATACTTCTCTCCCTCC (SEQ ID NO: 6)	1.06
Alg3-2	ATCCACTTAACGTTACTGAAATCTCCAAC <u>TTTCATGGACACACACAGACC</u> (SEQ ID NO: 7)	
Hph-F	GGTCTGTGTGTGTCCATGAAGTTGGAGATTTCAGTAACGTTAAGTGGAT (SEQ ID NO: 8)	1.49
Hph-R	GCTACTACTGATCCCTCTGCGTCGGAGACAGAAGATGATATTGAAGGAG (SEQ ID NO: 9)	1.03
Alg3-3	CTCCTTCAATATCATCTTCTGTCTCCGACGCAGAGGGATCAGTAGTAGC (SEQ ID NO: 10)	
Alg3-4	GCGGATAACAATTTACACAGGAAACAGCCGTGAGAGGTTTGTAGTACG (SEQ ID NO: 11)	
Alg3-RevScr	AAGCTGAGAGCGACATCTTCA (SEQ ID NO: 12)	
hyg-RevScr	GTACTTCTACACAGCCATCGGTCCA (SEQ ID NO: 13)	
hyg-ForScr	GTACTTCTACACAGCCATCGGTCCA (SEQ ID NO: 14)	
<u>Alg3Δ + Alg3 construction</u>		
pryGScr	TCTGCTGTCTTGCAATGAGGTCCTT (SEQ ID NO: 15)	
pyrGScr	Agcgtaggacaagggtcgtctctgt (SEQ ID NO: 16)	2.34
5-pyrG5F	GTAACGCCAGGGTTTTCCCAGTCACGAC <u>Gtttaaac</u> ATGCATCATTCTCCC GCTTTGT (SEQ ID NO: 17)	1.69
5-pyrG3R	agaaagagtcaccgggtcacGacatcgccaatcacctcaatcac (SEQ ID NO: 18)	1.47
ble5F	gtgattgaggtgattggcgatgtCgtgaccgggtgactctttct (SEQ ID NO: 19)	1.23

Name	Sequence	Product size (kb)
Ble3R	<u>TCCAACCTTG TAGCAACCAAGCTTCGAGCGTCCCAAACCT</u> (SEQ ID NO: 20)	
Alg3-5F1	<u>AGGTTTGGGACGCTCGAAGCTTTGGTTGCTACAAGGTTGGA</u> (SEQ ID NO: 21)	
Alg3-3R1	TCAAGTAGAGCACAGCAAATAGTATCTGA (SEQ ID NO: 22)	
Alg3-5F2	<u>TCAGATACTATTTGCTGTGCTCTACTTGA</u> (SEQ ID NO: 23)	
Alg3-3R2	ttgatccttgtgccacaccaTCCTACGTGGTCATCGATACCA (SEQ ID NO: 24)	
3-pyrG5F	<u>TGGTATCGATGACCACGTAGGA</u> ttggtgtggcacaaggatcaa (SEQ ID NO: 25)	
3-pyrG3R	GCGGATAACAATTTACACAGGAAACAGCgttttaaactgtgccagtcaattgtccgaagt (SEQ ID NO: 26)	
Alg3Seq-1	TACAGACGCGTGACGCATGT (SEQ ID NO: 27)	
Alg3seq-2	TGCTATTGTCCACAGATACCGAGA (SEQ ID NO: 28)	
Alg3seq-3	GAGCTAACCAGACAGTTCATGT (SEQ ID NO: 29)	
Alg3seq-4	Tcgtcgtaccgcattgatacct (SEQ ID NO: 30)	

Example 2

Genetic Inactivation of Alg3 in *A. niger*

This example describes methods used to genetically clone and then inactivate *Alg3* in *A. niger* strain 11414*KusA*. Based on these teachings, one skilled in the art will appreciate that *Alg3* can be similarly inactivated in other strains of *Aspergillus*.

Alg3 has been identified and characterized in *Arabidopsis thaliana*, *Homo sapiens*, *Pichia pastoris*, *Trypanosoma brucei* and *Saccharomyces cerevisiae* (see for example Korner *et al.*, *EMBO J.* 18(23): 6816-6822, 1999; Davidson *et al.*, *Glycobiology* 14(5):399-407, 2004; Manthri *et al.*, *Glycobiology* 18(5): 367-383, 2008; Kajiura *et al.*, *Glycobiology* 20(6):736-751, 2010). A database search based on the amino acid sequence of *S. cerevisiae* *Alg3* identified a putative α -1,3-mannosyltransferase gene in JGI (DOE Joint Genome Institute)-*A. niger* genome database (jgiAspni5|42720). The *A. niger* *Alg3* gene contains two introns and its 1400 bp open reading frame (nt 1186-1306, 1393-1916 and 1989-2582 of SEQ ID NO: 1) encodes a protein consisting of 413 amino acids (SEQ ID NO: 2), which contains one potential N-glycosite at the amino acid position 374. The predicted *Alg3* amino acid sequence has 39% sequence identity to the *S. cerevisiae* *Alg3*.

Alg3 was functionally inactivated in *A. niger* using a gene deletion vector constructed by yeast gap repairing approach. The 5'- and 3'-end of the hygromycin marker (*hph*) gene was flanked with about 1 kb upstream and downstream fragments of *Alg3* coding region that were isolated by PCR from *A. niger* genomic DNA. The DNA sequence of the upstream and downstream fragments was confirmed by DNA sequencing analysis. The *Alg3* in *A. niger* was deleted by homologous replacement with hygromycin marker (*hph*) gene in the *kusA* deletion background of *A. niger*, where the *kusA* gene, encoding the ortholog of the Ku70 protein in other eukaryotes, was deleted for dramatically improved homologous integration efficiency. FIGS. 1A and 1B show the predicted restriction enzyme digestion patterns of genomic DNA of the parent and mutant strains with BamHI and SacII. FIG. 1C shows the Southern blotting analysis of the digested genomic DNA of parent and mutant strains. The results confirm that the *Alg3* coding region in *A. niger* was replaced by the hygromycin selection marker gene (*hph*) in the *Alg3*Δ strains.

Example 3

Effects of *Alg3* Deletion on *A. niger* Growth and Development

This example describes methods used to determine the effect genetically inactivating *Alg3* in *A. niger*.

It was previously demonstrated that the deletion of *Alg3* in different organisms causes underglycosylation, but no obvious phenotype changes were observed at the selected culture condition in those studies (Aebi *et al.*, *Glycobiology* 6(4):439-444, 1996; Korner *et al.*, *EMBO J.* 18(23): 6816-6822, 1999; Davidson *et al.*, *Glycobiology* 14(5):399-407, 2004; Manthri *et al.*, *Glycobiology* 18(5): 367-383, 2008; Kajiura *et al.*, *Glycobiology* 20(6):736-751, 2010).

The effects of the *Alg3* deletion were examined on CM, PDA and MM plates. As exhibited in FIGS. 2A-I, the *Alg3*Δ strain grew much slower than the parent strain when grown on either CM or PDA medium plate, but there was no significant difference between the *Alg3*Δ mutants and parent strain when grown on the MM medium plate. When both *Alg3*Δ strain and parent strain were grown in the liquid culture of CM and PDA, the initiation of spore germination of *Alg3*Δ strain was pronouncedly delayed (FIG. 3A), but the spore germination rate was not affected by the *Alg3* deletion (FIG. 3B). These results demonstrate that deletion of *Alg3* has significant effects on *A. niger* growth on nutrient rich media.

The effects of *Alg3* deletion on spore production on both CM and PDA plates were also examined. The *Alg3* deletion had a substantial reduction of sporulation on CM medium plate, while no obvious difference was exhibited on PDA plate (FIG. 4). The spore production at given area was enumerated with hemocytometer (Table 3). The average spore production of *Alg3*Δ was

2.64x10⁷ spores/cm², about 40% of parent strain (6.44x10⁷ spores/cm²) on CM medium plates, while average spore production per a square millimeter was similar between *Alg3Δ* mutant (7.72x10⁷ spores/cm²) and parent strains (7.89x10⁷ spores/cm²) on PDA medium plates.

5 **Table 3. Average spore production in PDA and CM media plates (x10⁷ sp/cm²). This was averaged from four cuts and 3 replicate counting.**

Strain	CM pates	PDA
11414-kusA	6.44±1.24	7.72±1.78
<i>Alg3Δ</i>	2.64±0.49	7.89±1.18

Example 4

Effects of *Alg3* Deletion on Spore Germination, Growth and Citric Acid Production

10 This example describes methods used to measure spore germination, growth, and citric acid production in the *Alg3Δ A. niger* strain generated in Example 1. Based on these teachings, one skilled in the art will appreciate that spore germination, growth, and citric acid production can be similarly measured in other *Alg3Δ* strains of *Aspergillus*.

15 *A. niger* strain ATCC11414 is a strain developed for industrial production of citric acid. *A. niger* morphology plays a role in citric acid production. The fungal morphology affect overall molecular regulation in response to the endogenous and exogenous factors, which include the regulations of transcription, post-transcription, translation and post-translation. Therefore, the effects of *Alg3* deletion on *A. niger* growth on CAP agar plates at different pHs or in CAP liquid culture conditions was determined.

20 FIG. 5 shows the effects of *Alg3* deletion in fungi grown on CAP medium plates at different pH conditions after 28 hours in culture. When the *Alg3Δ* strain was grown on the CAP medium plates at pH 1.8, its growth was relatively slower than the parent strain, where its colonies were much smaller than the parent strain. At pH 2.1, the *Alg3Δ* strain formed a less tight pellet than that of parent strain. Growth of the *Alg3Δ* strain on the CAP medium at pH 4.5 and pH 7.0 was affected
25 more profoundly than that of parent strain, where the *Alg3Δ* strain formed thinner layer of biomass on the agar plates than that of parent strain. These results show that the *Alg3* deletion reduces the normal growth of *A. niger* at different levels on CAP culture medium plates at different pHs.

30 The spore germination of the *Alg3Δ* and parent strains in CAP liquid culture medium was also examined by using automated microscopic imaging, enumerating the germination manually in a same visual unit area, and expressing spore germination as a percentage of total spores at the same visual unit area. FIG. 6 shows the different dynamics of spore germination between the

Alg3Δ and parent strains. The spore germination of *Alg3Δ* strain began as early as 3 hours after spore inoculation, while the parent strain was not initiated until 6 hours after spore inoculation. After 8 hours inoculation, more than 32% spores of *Alg3Δ* mutant germinated, while only 10% of parent strain spores did (FIG. 6A, top panels; and FIG. 6B). After 15 hours growth in CAP liquid medium, more than 90% spores of *Alg3Δ* strain germinated, while only 50% spores of parent strain did (FIG. 6A, bottom panels; and FIG. 6B). After 24 hours of growth in the liquid culture, about 75% spores of parent strain germinated, while the *Alg3Δ* strain achieved 94% of germination rate (FIG. 6B). The *Alg3* deletion leads to earlier germination and a higher germination rate than parent strain.

The effect of *Alg3* deletion on citric acid production was determined in CAP flask cultures. FIG. 7 shows the time course of citric acid production in CAP liquid medium. The yield of citric acid production was similar between the *Alg3Δ* mutant and parent strain in 2 days culture. After 3 days culture, the average citric acid production by *Alg3Δ* mutants was 8.8 g/l citric acid, while the parent strain only produced 5.8 g/l. After 4.5 days of culture, the parent strain accumulated 18.8 g/l citric acid and the *Alg3Δ* mutant produced 33.3 g/l citric acid (more than 70% higher than the parent strain). Thus, the *Alg3* deletion substantially improves the citric acid production in *A. niger*.

The effect of *Alg3* deletion on citric acid production was also examined by complementation of its original gene into the *alg3Δ* mutant. FIG. 8B shows the citric acid production in CAP liquid medium after 10 days of culture. The yield of citric acid production was similar between the *alg3Δ* complemented (*cAlg3Δ*) mutant and parent strain, but much lower than the *alg3Δ* mutant in 10 days culture. After 10 days culture, the average citric acid production by *Alg3Δ* mutants was 46.1 g/l citric acid, while the parent and *cAlg3Δ* strain only produced 34.8 and 29.4 g/l, respectively.

Example 5

pPTRpGPDA*LaeA* plasmid vector construction

This example describes methods used to generate the pPTRpGPDA*LaeA* plasmid vector (FIG. 13). One skilled in the art will appreciate that although *gpdA* and *LaeA* sequences were used from *Aspergillus nidulans*, one skilled in the art will appreciate that variants of these sequences can be used in the fungi and methods provided herein, such as *gpdA* and *LaeA* sequences from other *Aspergillus* species. In one example, a *gpdA* sequence having at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to SEQ ID NO: 37 is used in the fungi and methods provided herein.

The *Aspergillus nidulans* glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter (SEQ ID NO: 37) was isolated from the pAN8-1 plasmid DNA using the primer set *gpdA5F/gpdA3R* (*gpdA5F*: CGCAGATCTC AAGCTGTAAG GATTTTCGGCA SEQ ID NO: 38; *gpdA3R*: CACCGGGCCC ATCTCAAACA TTGTGATGTC TGCTCAAGCG SEQ ID NO: 39) and the *LaeA* coding sequence of genomic DNA from *A. nidulans* (SEQ ID NO: 40) obtained by PCR using *LaeA5F/LaeA3R* (*LaeA5F*: CGCTTGAGCA GACATCACAA TGTTTGAGAT GGGCCCGGTG; SEQ ID NO: 42; *LaeA3R*: CGCAGATCTG AGGATTATGA GAAGGGAGC; SEQ ID NO: 43).

The DNA fragment of pGPDA and *LaeA* was filled together by overlap PCR and a HindIII restriction enzyme site was introduced at both 5'- and 3'-end of the DNA fragment. The DNA fragment (SEQ ID NO: 44) and pPTR1 plasmid DNA were cut with Hind III and ligated together by a quick DNA ligation kit at 25°C for 30 min. The ligated plasmid DNA was transferred into the Top10 *E. coli* competent cells by lithium acetate mediated transformation. The transformed bacterial colonies were screened for the DNA fragment insertion by PCR with the primers *gpdA5F* (SEQ ID NO: 38) and *LaeA3R* (SEQ ID NO: 43). The plasmid DNA for the selected transformed colonies was prepared for restriction enzyme confirmation and further expression vector construction.

Example 6

pRS426-*LaeA* vector construction

This example describes methods used to generate a transgene containing *A. niger LaeA* (FIG. 14).

PCR was performed to isolate DNA fragments of *A. niger pyrG* upstream region (SEQ ID NO: 45), *trpC* transcriptional terminator of *A. nidulans* (SEQ ID NO: 46), pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae* (SEQ ID NO: 47), and *A. niger pyrG* downstream region (SEQ ID NO: 48), using primers *pyrGU5F/PTRU3R* (*pyrGU5F*: GTAACGCCAG GGTTTTCCCA GTCACGACGT TTAAACATGC ATCATTCTCC CGCTTTGT, SEQ ID NO: 49; *pyrGU3R*: TGCCGAAATC CTTACAGCTT GAAGCTTCAT CGCCAATCAC CTCAATCAC, SEQ ID NO: 50), *Trp5F/Trp3R* (*Trp5F*: AGCTCCCTTC TCATAATCCT CAAGCTTGGA CCGATGGCTG TGTAGAAGT, SEQ ID NO: 51; *Trp3R*: CGTAATCAAT TGCCCGTCTG TCAGAGAGCG GATTCTCAG TCTCGT; SEQ ID NO: 52), *PTR5F/PTR3R* (*PTR5F*: ACGAGACTGA GGAATCCGCT CTCTGACAGA CGGGCAATTG ATTACG, SEQ ID NO: 53; *PTR3R*: ACAGCAGTGC TTATCTGCGA TGACGAGCCG CTCTTGATC TTTGT, SEQ ID NO: 54) and *PyrGD5F/PTRD3R* (*pyrGD5F*: ACAAAGATGC AAGAGCGGCT CGTCATCGCA

GATAAGCACT GCTGT; SEQ ID NO: 55, pyrGD3R: TGAGACGCTG TTTCACCGAG TACATCGCCA ATCACCTCAA TCAC, SEQ ID NO: 56), respectively.

As shown in FIG. 14, the DNA fragment of pGDPA*LaeA* (SEQ ID NO: 44) was isolated from pPTRpGDPA*LaeA* (FIG. 13) by HindIII digestion. The yeast gap repairing vector pRS426 was double digested with restriction enzyme HindIII and XhoI. Hundred nanograms of each DNA fragment generated from PCR (*i.e.*, SEQ ID NOs: 45-56) or restriction enzyme digestions were used for *S. cerevisiae* transformation. The gap repairing plasmid DNA in the total *S. cerevisiae* genomic DNA was isolated by transferred into the Top10 *E. coli* cells.

The transformed plasmid DNA was confirmed by PCR and digested with PmeI. The PmeI DNA fragment (SEQ ID NO: 57) was used for *A. niger* transformation.

One skilled in the art will appreciate that although the *pyrG* upstream and downstream sequences, *trpC* transcriptional terminator sequence, and *ptrA* sequence used were from particular organisms, one skilled in the art will appreciate that variants of these sequences can be used in the fungi and methods provided herein, such as those from other *Aspergillus* species. In one example, *pyrG* upstream and downstream sequences having at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to SEQ ID NO: 45 and 48 are used in the fungi and methods provided herein. In one example, a *trpC* transcriptional terminator sequence having at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to SEQ ID NO: 46 is used in the fungi and methods provided herein. In one example, a *ptrA* sequence having at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to SEQ ID NO: 47 is used in the fungi and methods provided herein.

Example 7

Expression of pGDPA*LaeA*-Containing Transgene in *A. niger* alg3Δ

This example describes methods used to introduce pGDPA*LaeA* (SEQ ID NO: 57) into *A. niger*.

The originally transformed *A. niger* colonies were picked from the minimal medium plates with 0.1 µg/ml pyrithiamine hydrobromide selection on minimal medium agar plates without thiamine supplementation. The single spore colonies were picked for spore production after the initial transformant spores were grown on the same selection medium plates. The biomass was harvested from the single spore colony isolates grown in minimal medium with pyrithiamine selection and dried in the VirTis bench top freeze dryer. The genomic DNA was prepared for PCR confirmation of pGDPA*LaeA* insertion in transgenic *A. niger*. As shown in FIG. 15A, the primer set of PTR5F (SEQ ID NO: 53) and PTR3R (SEQ ID NO: 54) was used to confirm the presence of

A. oryzae pyrithiamine resistance gene (*ptrA*) in transgenic *A. niger* with the expected size of 2kb PCR DNA fragment. As shown in FIG. 15B, the primer set *LaeA5F* (SEQ ID NO: 42) and *TRP3R* (SEQ ID NO: 52) was used to demonstrate that the transgene *A. nidulans LaeA* was under the control of *gdpA* promoter and *trpC* transcriptional terminator of *A. nidulans* with the expected 3.4 kb PCR fragment size. The genomic DNA of parent strain and the plasmid DNA of transgene vector carrying the pGDPA*LaeA* fragment were used for negative and positive references.

Example 8

Increased Production of Citric Acid

This example describes methods used to demonstrate that citric acid production was increased in the presence of increased expression of *LaeA*, alone or in combination with deletion of *Alg3*.

Citric acid was produced as described in Example 1.

As shown in FIG. 16, of citric acid production was increased in the *Alg3Δ* mutant (*Alg3*), and in the mutants over-expressing *LaeA* in *Alg3Δ* (*LaeA*-1 and *LaeA*-2), as compared to the parent strain (*kusA*).

Example 9

pBSK-*LaeAΔ* plasmid vector construction

This example describes methods used to generate the pBSK-*LaeAΔ* plasmid vector (FIG. 17A). The 5' (SEQ ID NO: 61) and 3' (SEQ ID NO: 62) ends of the *Aspergillus niger LaeA* gene were isolated from *A. niger* genomic DNA by PCR using oligonucleotide sets D1 & D2, and D5 & D6 (see Table 4 below). The *hph* expression cassette was isolated from plasmid vector DNA of pCB1003 (SEQ ID NO: 63) by PCR using the oligonucleotide sets D3 & D4. The DNA fragments were assembled together into the backbone plasmid vector of pBSK linearized with restriction endonucleases of both *HindIII* and *PstI* using the Gibson assembly cloning kit. The assembled plasmid DNA was transferred into the Top10 *E. coli* competent cells by lithium acetate mediated transformation. The transformed bacterial colonies were screened for the DNA fragment insertion by restriction endonuclease digestion of *PvuII* and *XhoI*. The plasmid DNA for the transformed colonies was prepared and digested with endonucleases of *HindIII* and *XbaI* for further *LaeA* deletion in *A. niger*.

Table 4: Oligonucleotide Primers for *A. niger* *LaeA* Deletion

Name	Sequence	SEQ ID NO:
D1	gtcgacgggtatcgataAGCTTCAAGACAGCGGCTGCAA	67
D2	gccgaccgTGGTAGAGATACAGGGGTTTC	68
D3	ctctaccaCGGTCGGCATCTACTCTATTC	69
D4	gcgactgaGCTGGAGCTAGTGGAGGT	70
D5	gctccagcTCAGTCGCATCTTTCACTG	71
D6	atccccggggtgcaTGGTAGGCTGTCTCAAGG	72
SC7	ACTCGCAGCAGAGATGCCATCT	73
SC8	CGTTATGTTTATCGGCACTTTGCAT	74

Example 10**pRS426-*A. niger* *LaeA* complementation plasmid vector construction**

- 5 This example describes methods used to generate the pBSK-*LaeAΔ* plasmid vector (FIG. 17B). The entire *LaeA* gene (2.34kb; SEQ ID NO: 64) containing 740 bp of promoter region and 330 bp of transcriptional terminator region was isolated from *A. niger* genomic DNA by PCR using oligonucleotide set CP9 & CP10 (see Table 5 below). The DNA fragment was ligated into the plasmid DNA of pRS426-*A. nidulans* *LaeA* over-expression vector at a HindIII restriction
- 10 endonuclease site after the pGPDA*LaeA* fragment in the vector was removed by agarose gel separation. The assembled plasmid DNA was transferred into the Top10 *E. coli* competent cells by lithium acetate mediated transformation. The transformed bacterial colonies were screened for the DNA fragment insertion by restriction endonuclease digestion of BamHI and XhoI. The plasmid DNA for the transformed colonies was prepared and digested with PmeI restriction endonuclease
- 15 for *A. niger* *LaeA* expression at pyrG locus to complement the *LaeA* deletion in *A. niger* *LaeAΔ* mutants.

Table 5: Oligonucleotide Primers for *A. niger* *LaeA* Complementation

Name	Sequence	SEQ ID NO:
CP9	aggtgattggcgatgaTGCCGTTCAAGCTGTCTGC	75
CP10	acagccatcgggtccaTCTCTCCTCGTAACGCCTG	76
SC11	<u>ggatagaatcgggtgccgctgatct</u>	77
SC12	gagaacctggcaccgaaggt	78

Example 11

Deletion of *A. niger* *LaeA* gene in *A. niger*

This example describes methods used to introduce the HindIII/XbaI DNA fragment contain the *LaeA* deletion cassette (SEQ ID NO: 65) into *A. niger*.

5 The originally transformed *A. niger* colonies were picked from the minimal medium plates with 100 µg/ml Hygromycin B selection on minimal medium agar plates. The single spore colonies were picked for spore production after the initial transformant spores were grown on the same selection medium plates. The biomass was harvested from the single spore colony isolates grown in complete medium with Hygromycin B selection and dried in the VirTis bench top freeze
10 dryer. The genomic DNA was prepared for PCR confirmation of the replacement of *LaeA* gene coding region in transgenic *A. niger*. As shown in FIG. 18, the primer set SC7 and SC8 (see Table 4) was used to confirm the *hph* replacement of the *LaeA* gene in transgenic *A. niger* with the expected 2kb PCR DNA fragment. The genomic DNA of parent strain was used for negative and positive references.

Example 12

Expression of pGDPA*LaeA*-Containing Transgene in *A. niger*

This example describes methods used to introduce the *A. niger* *LaeA* gene into the *pyrG* locus in the *A. niger* *LaeAΔ* mutant to complement the loss of *LaeA* function with PmeI DNA
20 fragment (SEQ ID NO: 66).

The originally transformed *A. niger* colonies were picked from the minimal medium plates with 0.1 µg/ml pyrithiamine hydrobromide selection on minimal medium agar plates without thiamine supplementation. The single spore colonies were picked for spore production after the initial transformant spores were grown on the same selection medium plates. The biomass was
25 harvested from the single spore colony isolates grown in minimal medium with pyrithiamine selection and dried in the VirTis bench top freeze dryer. The genomic DNA was prepared for PCR confirmation of *LaeA* gene insertion in transgenic *A. niger*. As shown in FIG. 19, the primer set of PTR5F (SEQ ID NO: 53) and PTR3R (SEQ ID NO: 54) was used to confirm the presence of *A. oryzae* pyrithiamine resistance gene (*ptrA*) in transgenic *A. niger* with the expected size of 2kb PCR
30 DNA fragment. The genomic DNA of the parent strain and the plasmid DNA of the transgene vector carrying the *A. oryzae* pyrithiamine resistance gene (*ptrA*) fragment were used for negative and positive references.

Example 13

The effects of *LaeA* gene deletion and complementation and over-expression of *A. nidulans* *LaeA* gene on citric acid production

This example describes methods used to demonstrate that citric acid production in *A. niger* mutant strains was significantly influenced by perturbing the *LaeA* gene expression in *A. niger*. At least three individual clones per mutant strain were selected for spore production on complete medium plates at 30°C for 4 days. The spores were counted with a hemocytometer. Citric acid production culture was initiated by inoculation with a 1×10^6 spore/ml of 75 ml citric acid production culture medium in a 250 ml baffled Erlenmeyer glass flask siliconized with a 5% solution of dichlorodimethylsilane in hexane solvent. The culture was maintained at 30°C and 220 rpm in shaker incubator for 5 days. One microliter of culture was harvested and briefly spun down in a microcentrifuge at full speed. The supernatants of cultures were diluted 200-fold with dH₂O prior to citric acid measurement. The citric acid in the supernatant was quantified with the citric acid assay kit from R-Biopharm AG. The detailed procedure for the citric acid assay was performed essentially according to the manufacturer's description. The results in FIG. 20 show that deletion of the *LaeA* gene in *A. niger* (*LaeA*Δ) led to loss of citric acid production in citric acid production culture. When the original *A. niger* *LaeA* gene was used for complementation in the *LaeA*Δ mutant (*cLaeA*Δ) at the *pyrG* locus, the citric acid production was partially recovered, which indicates the importance of chromosome location of *LaeA* gene. When *A. nidulans* *LaeA* was over-expressed in the *A. niger* parent strain (*LaeA*), the citric acid production was higher than the parent strain. This indicates that the *LaeA* is involved in citric acid production in *A. niger*.

Example 14

Enhancement of itaconic production by *LaeA* complementation

A. terreus growth conditions

All strains were maintained on potato dextrose agar (PDA), which is composed of 39 g/L of Difco Potato Dextrose Agar (BD, USA). Spore inocula were grown on PDA and harvested after 5 days. The transformant was selected for either hygromycin or bleomycin resistance on minimum media (MM, 50 ml 20x nitrate salts, 1 ml 1000x trace elements, 1 ml 1000x vitamin stock, 10 g glucose, pH 6.5, 100 μg/ml hygromycin or 250 μg/ml bleomycin). The 20x NO₃ salts contains in one liter H₂O: Na₂NO₃, 120g; KCL, 10.4 g; MgSO₄.7H₂O, 10.4 g; KH₂PO₄, 30.4 g. The 1000x vitamin solution (yellow) contains in 100 ml H₂O: biotin, 0.01g; pyridoxine-HCl, 0.01 g; thiamine-HCl, 0.01 g; riboflavin, 0.01 g; para-amino benzoic acid, 0.01 g; and nicotinic acid (or niacin), 0.01 g, filtered and stored at 4°C. The 1000x trace elements contains per 100 ml H₂O: ZnSO₄.7H₂O, 2.2

g; H₃BO₃, 1.1 g; MnCl₂·4H₂O, 0.5 g; FeSO₄·7H₂O, 0.5 g; CoCl₂·6H₂O, 0.17 g; CuSO₄·5H₂O, 0.16 g; Na₂MoO₄·2H₂O, 0.15 g; Na₂EDTA, 5 g, which is added in the order, boil and cool to room temperature and adjusted the pH to 6.5 with KOH. For pyrithiamine marker selection, the thiamine-HCl was eliminated from minimum media.

About 1x10⁶ spores/ml were used to inoculate 50 ml of itaconic acid production media in 250 ml glass flask. The itaconic acid production medium essentially followed the description of Riscaldati *et al.* (*J Biotechnol* 83:219-230, 2000). Itaconic acid production medium contains in grams per liter (g/l): glucose, 100; ammonium sulfate, 2.36; potassium phosphate dibase, 0.11; magnesium sulfate heptahydrate, 2.08; calcium chloride dihydrate, 0.13; sodium chloride, 0.074; copper sulfate pentahydrate 2x10⁻⁴; ferrous sulfate heptahydrate, 5.5x10⁻³; manganese chloride tetrahydrate, 7x10⁻⁴; and zinc sulfate heptahydrate, 1.3x10⁻³. The flask cultures were shaken at 30°C and 150 rpm. At least three biological replicates for each strain were used for each experiment.

Itaconic acid measurement

The itaconic acid method followed the previous description by Dickman (*Analytical Chem* 24: 1064-1066, 1952) with some modification. The reaction volume was scaled down to 1.4 ml for each reaction. Ten to 250 µl of culture supernatant or itaconic acid standard dissolved in dH₂O were used. The proper amount of culture supernatant or itaconic acid standard was added into 690 to 930 µl dH₂O in 1.5 ml microcentrifuge tube. Then, 60 µl of freshly prepared metaphosphoric acid (0.425 g/ml) was added into the tube, mixed well, and incubated at 4°C for 10 minutes. Next, 400 µl of freshly prepared potassium permanganate (5 mM) from stock solution (100 mM) was added, mixed well and incubated in the dark at room temperature for 10 minutes. Finally, the absorbency of the reaction mixture was determined at 530 nm spectrophotometrically.

LaeA deletion construct

The DNA fragments corresponding to the upstream and downstream regions of the *LaeA* gene in *A. terreus* and *hph* were isolated with oligonucleotide pairs 1546/1548, 1551/1553 and 1549/1550, respectively (see Table 6 for oligonucleotide sequences). These three DNA fragments (SEQ ID NOS: 89-91) were fused together by double-joint PCR (Yu, *et al.*, *Fungal Genetics and Biology* 41: 973-981, 2004). The whole *LaeA* deletion fragment was isolated by PCR with oligonucleotide pair 1547/1552 (see Table 6 and SEQ ID NO: 104). FIG. 21A depicts the *LaeA* deletion construct. FIG. 21B shows genomic PCR confirmation of selected *LaeA* deletion mutants with oligonucleotide pair 1546/107 or 108/1553.

***LaeA* complementation construct**

The *LaeA* complementation construct was prepared for targeting upstream of the *LaeA* locus in *A. terreus* using the Gibson Assembly method. The DNA fragments of the whole *LaeA* gene, ble selection marker gene, and *hph* gene used for gene deletion were isolated by PCR with oligonucleotide pairs 1554/1555, 1556/15557, and 1558/1559, respectively (see Table 6). In order to isolate the whole transgene cassette, an *Xba*I restriction endonuclease site was introduced into the upstream fragment of 1554/1555. The three DNA fragments (SEQ ID NOs: 107-109) were then assembled into the cloning vector of pBluescript SK(-). FIG. 22A is a diagram illustrating the *LaeA* complementation constructs. The insertion of *LaeA* complementation was confirmed by oligonucleotide pair 1556/107. FIG. 22B shows gel electrophoresis of genomic PCR products corresponding to ble and part of *hph*.

***LaeA* over-expression construct**

The *Aspergillus nidulans* *LaeA* gene under the control of *A. nidulans* *gpdA* promoter and *TrpC* transcription terminator, and the selection marker gene of pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*, were isolated from the transgene expression construct used for *A. niger* transformation (as described in Example 6) with oligonucleotide pair 1563 and 1564 (Table 6). Due to the extremely high homologous replacement rate in *A. terreus*, the *laeA* overexpression transgene cassette was designed to replace the 127 bp genomic DNA fragment of the non-gene region located at 1571 bp upstream in *A. terreus* *laeA* coding gene sequence. The corresponding DNA fragments flanked to the both ends of *LaeA* expression cassette for homologous replacement were isolated by PCR with oligonucleotide pairs 1561/1562 and 1565/1566 (Table 6). The three DNA fragments (SEQ ID NOs: 96-98) were fused together in pBluescript SK(-) by Gibson Assembly. The restriction endonuclease site for *Pme*I was introduced into the 5' end of the upstream region fragment of 1561/1562 (SEQ ID NO: 96) and the 3' end of the downstream region fragment of 1565/1566 (SEQ ID NO: 110), which enables the isolation of whole transgene expression construct. FIG. 24A is a diagram of the transgene over-expression cassette of the *A. nidulans* *LaeA* gene. Oligonucleotides 1563, 1198 and 1564 (see Table 6) were used for genomic PCR to confirm the gene insertion. FIG. 24B shows gel electrophoresis of PCR products corresponding to the oligonucleotide pairs 1198/1564 and 1563/1564 in selected transgenic strains.

A. terreus protoplast isolation and transformation

A. terreus (10^6 conidia/ml) was inoculated into 100 ml of complete media containing in g/l: D-glucose, 10; peptone, 2; yeast extract, 1; casamino acids, 1; 20x nitrate salts, 50 ml; trace elements, 1 ml; vitamin solution, 1 ml (pH6.5) or MM without thiamine-HCl. The inoculated culture was shaken at 150 rpm and 30°C for 16-22 hours. The mycelia were harvested by filtering the culture through Miracloth and rinsed well with sterile dH₂O. The mycelia (mass of approximately several bean sizes) were digested with 20 ml of 20 mg/ml lysing enzyme (L1412, Sigma) that was dissolved in osmotic washing buffer (0.5 M KCl, 10 mM sodium phosphate, pH 5.8) for approximately 2-3 hours at 80 rpm and 30°C. Protoplasts were filtered through sterile miracloth into a 50 ml sterile centrifuge tube and centrifuged for 10 minutes at 1000 x g and 4°C. Protoplasts were washed twice with 20 ml washing solution (0.6M KCl, 0.1 M Tris-HCl, pH 7.0) and once in 10 ml conditioning solution (0.6M KCl, 50 mM CaCl₂, 10 mM Tris-HCl, pH 7.5).

For transformation, 1-2 µg DNA was added to 2×10^7 protoplasts in 0.1 ml conditioning solution. After addition of 25 µl PEG solution, the protoplasts were incubated on ice for 20 minutes. An additional 500 µl PEG solution was carefully added, mixed and incubated on ice for additional 5 minutes. Finally, 1 ml cold conditioning solution was added and mixed by inverting the tube several times. About 300-500 µl of transformed protoplasts were pipetted into 12 ml melted MM agar (MM+0.6M KCl+1.5% agar +antibiotics) tubes at 50°C. The transformed protoplasts were mixed well by inverting the tubes 3-4 times and poured directly onto the 100 mm petri dishes. The petri dishes were incubated overnight at 30°C. An additional 8 ml of regular MM agar with proper antibiotics was overlaid and further incubated at 30°C for approximately 3-4 days. The transformed colonies were picked and further grown on 2 ml MM agar gel for spore production.

Table 6. Oligonucleotides

Oligo	DNA sequence	SEQ ID NO:
107	GTACTTCTACACAGCCATCGGTCCA	79
108	CGTTATGTTTATCGGCACTTTGCAT	80
1546	ACAGGTACTTCCATCTTGTACTGGT	81
1547	<u>TCtctccaacgtccgatct</u>	82
1548	acctcactagctccagcaagccgaacagaggtaaagacga	83
1549	tcgtctttacctctgttcggcttgctggagctagtggaggtca	84
1550	taccaacgtgcgaccattTCTcggtcggcatctactctattcct	85

Oligo	DNA sequence	SEQ ID NO:
1551	<u>aggaatagagtagatgccgaccg</u> AGAaaatggtcgcacgttggtta	86
1552	AAGCGTCTCTTTTCCTGGGTCTT	87
1553	TGCCAGTTCTGTTGGACATCTCT	88
1554	tcgaggtcgacggtatcgatatctagaACAGGTACTTCCAT CTTGTAC	89
1555	ggtcactgtTCCTGGCAGCTGACATTG	90
1556	ctgccaggaACAGTGACCGGTGACTCT	91
1557	aagcagcagatACGACCGTTGATCTGCTTG	92
1558	acggtcgtATCTGCTGCTTGGTGCAC	93
1559	actagtggatccccgggctgcagCGGTCGGCATCTACTC TATTC	94
1561	cgaggtcgacggtatcgataGTTTAAACCTCCCAGGTA CCGACTAAC	95
1562	ctcaatcacaGATCATGTTTGGGTGGGTTC	96
1563	aaacatgacTG TGATTGAGGTGATTGGCG	97
1564	ctctgtgcctACAGCAGTGCTTATCTGCGATG	98
1565	gcactgtgtAGGCACAGAGTAACAGGTAGGTAG ACAG	99
1566	agtggatccccgggctgcaGTTTAAACTCCCACGCAC GAAAGCAACT	100

Results

Itaconic acid production in selected *LaeA* deletion (*LaeA*Δ) and *LaeA* complementation (*cLaeA*Δ) mutants of *A. terreus* was tested after four days of culture in itaconic acid production medium. The parental *A. terreus* strain was also tested as a control. As shown in FIG. 23, deletion of *LaeA* significantly decreased the production of itaconic acid. However, complementation of the *LaeA* gene in *A. terreus* enabled itaconic acid production to similar levels observed in the *A. terreus* parent.

An additional study was performed to test the effect of overexpression of the *LaeA* gene in *A. terreus*. FIG. 25 is a graph showing itaconic acid production in selected *A. terreus* transgenic strains having the *A. nidulans* *LaeA* over-expression transgene following five days of culture in itaconic acid production medium. Also shown in FIG. 25 is the percentage of itaconic acid

production relative to the parent strain. The results demonstrated that overexpression of *LaeA* in *A. terreus* led to a significant increase in itaconic acid production relative to itaconic acid production in the parental strain.

5

Example 15

Itaconic acid production in wild-type *A. terreus*, *alg3* deletion mutants and *LaeA* overexpression mutants

An *alg3* gene deletion transgene construct (FIG. 26A) was assembled in a T-DNA binary vector for *Agrobacterium*-mediated transformation in *A. terreus* and mutants of *laeA* over-
 10 expression. The DNA fragments of 5'-*alg3* upstream (SEQ ID NO: 119), *hph* (SEQ ID NO: 120) and 3'-*alg3* downstream (SEQ ID NO: 121) were isolated by PCR with oligonucleotide pairs P1/P2, P3/P4 and P5/P6, respectively. The three DNA fragments were assembled into a T-DNA binary vector of pRF-Hu2 digested with restriction endonucleases *Bgl*III and *Sac*I (SEQ ID NO: 122) by Gibson Assembly cloning kit (New England Biolabs).

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The genomic DNA of the parent and transgenic strains were isolated by CTAB method. The *alg3* deletion in those strains were confirmed by PCR with oligonucleotide pair P7/P8 (FIGS. 26B and 26C), which are located outside the transgene deletion fragments of 5'- and 3'-*alg3*. The DNA fragments were then digested with restriction enzymes *Bgl*III or *Nco*I and visualized by agarose gel electrophoresis (FIG. 26D). Itaconic acid in the culture broths of various parent and
 20 transgenic strains were determined by the permanganate method of Dickman (1952).

Table 7. Oligonucleotides

Oligo	DNA sequence	SEQ ID NO:
P1	gcactagtcgggggatcctctaAGGACAATTGATCTCGATCG	111
P2	Tgacctccactagctccagc TAAAGTGAGCGTTGATGCAACTGATGTGGC	112
P3	GCCACATCAGTTGCATCAACGCTCACTTTAgctggagctagtggaggtca	113
P4	GGACCTAGACTTGTCTAGCGATGATGTTTGCcggtcggcatctactctatt	114
P5	aatagagtagatgccgaccg GCAAACATCATCGCTGACAAGTCTAGGTCC	115
P6	cgacggccagtgaattcgagctTCGACATTCCAGTACTGCAG	116
P7	GGACTTGTGTTTTCGGGTATCA	117
P8	CTGCATAGGAACGAACAGCCTAA	118

Results

Following 6 days of culture in itaconic production media, the parent strain, selected *alg3* deletion mutant strains (*alg3Δ-1* and *alg3Δ-2*) and selected *laeA* over-expression mutant strains (OE-*laeA-7*, OE-*laeA-9*, OE-*laeA-13*, OE-*laeA-7+alg3Δ*, OE-*laeA-9+alg3Δ* and OE-*laeA-13+alg3Δ*), were evaluated by stereo microscopic observation (FIG. 27). The results demonstrated that *alg3* deletion reduced the pellet size and filamentous growth of *A. terreus*.

Itaconic acid production by the parent strain and different transgenic mutant strains was measured following 6 days of culture in itaconic acid production medium (FIG. 28). The *alg3Δ-1* and *alg3Δ-2* strains have an *alg3* deletion in an *A. terreus* wild type background; OE-*laeA7*, OE-*laeA9*, and OE-*laeA13* are *laeA* over-expression strains in an *A. terreus* wild type background; and OE-*laeA7+alg3Δ-1*, OE-*laeA7+alg3Δ-2*, OE-*laeA9+alg3Δ-1*, OE-*laeA13+alg3Δ-1*, and OE-*laeA13+alg3Δ-2* have an *alg3* deletion in a *laeA* over-expression background. The results demonstrated that overexpression of *laeA* and deletion of *alg3* individually increased itaconic acid production, while strains having an *alg3* deletion combined with overexpression of *laeA* exhibited the highest levels of itaconic acid production (FIG. 28).

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only examples of the disclosure and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

CLAIMS

1. An isolated *Aspergillus terreus* fungus transformed with a heterologous nucleic acid construct comprising an *Aspergillus* species *LaeA* (loss of aflR expression A) gene, wherein
5 expression of *LaeA* is increased in the transformed fungus compared to an *A. terreus* fungus that is not transformed with the heterologous nucleic acid construct.

2. The isolated *A. terreus* fungus of claim 1, wherein the heterologous nucleic acid construct comprises a heterologous *LaeA* gene, a heterologous promoter, a heterologous
10 transcription terminator, a heterologous selective marker gene, or any combination thereof.

3. The isolated *A. terreus* fungus of claim 2, wherein the heterologous *LaeA* gene is an *A. nidulans* or an *A. niger* *LaeA* gene.

4. The isolated *A. terreus* fungus of claim 3, wherein the *A. nidulans* *LaeA* gene
15 encodes a protein having at least 80% sequence identity to the amino acid sequence of SEQ ID NO: 41.

5. The isolated *A. terreus* fungus of claim 4, wherein the *A. nidulans* *LaeA* gene
20 encodes a protein comprising the amino acid sequence of SEQ ID NO: 41.

6. The isolated *A. terreus* fungus of claim 3, wherein the *A. niger* *LaeA* gene encodes a protein having at least 80% identity to the amino acid sequence of SEQ ID NO: 59.

7. The isolated *A. terreus* fungus of claim 6, wherein the *A. niger* *LaeA* gene encodes a protein comprising the amino acid sequence of SEQ ID NO: 59.

8. The isolated *A. terreus* fungus of any one of claims 1-7, wherein the heterologous nucleic acid molecule comprises a promoter operably linked to an *Aspergillus* species *LaeA* gene, a
30 transcription terminator and a selective marker gene.

9. The isolated *A. terreus* fungus of claim 8, wherein:
the promoter comprises the *A. nidulans* *gpdA* promoter;
the *Aspergillus* species *LaeA* gene is an *A. nidulans* *LaeA* gene;

the transcription terminator comprises the *A. nidulans* *TrpC* transcription terminator; or
the selective marker gene comprises the *A. oryzae* pyrithiamine resistance (*ptrA*) gene.

10. The isolated *A. terreus* fungus of claim 8 or claim 9, wherein the heterologous
5 nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 109.

11. The isolated *A. terreus* fungus of any one of claims 1-10, further comprising a
genetic inactivation of an endogenous dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl
mannosyltransferase (*Alg3*) gene, wherein the *Alg3* gene is genetically inactivated by complete or
10 partial deletion mutation or by insertional mutation.

12. The isolated *A. terreus* fungus of claim 11, wherein the *Alg3* gene is genetically
inactivated by complete deletion mutation.

13. A method of making itaconic acid, comprising culturing an isolated *Aspergillus*
terreus fungus transformed with a heterologous nucleic acid molecule comprising an *Aspergillus*
species *LaeA* (loss of aflR expression A) gene under conditions that permit the fungus to make
itaconic acid, wherein expression of *LaeA* is increased in the transformed fungus compared to an *A.*
terreus fungus that is not transformed with the heterologous nucleic acid construct, thereby making
20 itaconic acid.

14. The method of claim 13, wherein the fungus is cultured in itaconic acid production
media.

15. The method of claim 13 or claim 14, wherein the heterologous nucleic acid construct
comprises a heterologous *LaeA* gene, a heterologous promoter, a heterologous transcription
terminator, a heterologous selective marker gene, or any combination thereof.

16. The method of claim 15, wherein the heterologous *Aspergillus* species *LaeA* gene is
30 an *A. nidulans* or an *A. niger* *LaeA* gene.

17. The method of claim 16, wherein the *A. nidulans* *LaeA* gene encodes a protein
having at least 80% sequence identity to the amino acid sequence of SEQ ID NO: 41, or the *A.*

niger LaeA gene encodes a protein having at least 80% identity to the amino acid sequence of SEQ ID NO: 59.

18. The method of claim 17, wherein the *A. nidulans LaeA* gene encodes a protein comprising the amino acid sequence of SEQ ID NO: 41, or the *A. niger LaeA* gene encodes a protein comprising the amino acid sequence of SEQ ID NO: 59.

19. The method of any one of claims 13-18, wherein the heterologous nucleic acid molecule comprises a promoter operably linked to an *Aspergillus* species *LaeA* gene, a transcription terminator and a selective marker gene.

20. The method of claim 19, wherein:
the promoter comprises the *A. nidulans gpdA* promoter;
the *Aspergillus* species *LaeA* gene is an *A. nidulans* or an *A. niger LaeA* gene;
the transcription terminator comprises the *A. nidulans TrpC* transcription terminator; or
the selective marker gene comprises the *A. oryzae* pyrithiamine resistance (*ptrA*) gene.

21. The method of claim 19 or claim 20, wherein the nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 109.

22. The method of any one of claims 13-21, wherein the *A. terreus* fungus further comprises a genetic inactivation of an endogenous dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase (*Alg3*) gene, wherein the *Alg3* gene is genetically inactivated by complete or partial deletion mutation or by insertional mutation.

23. The method of claim 22, wherein the *Alg3* gene is genetically inactivated by complete deletion mutation.

FIG. 1A

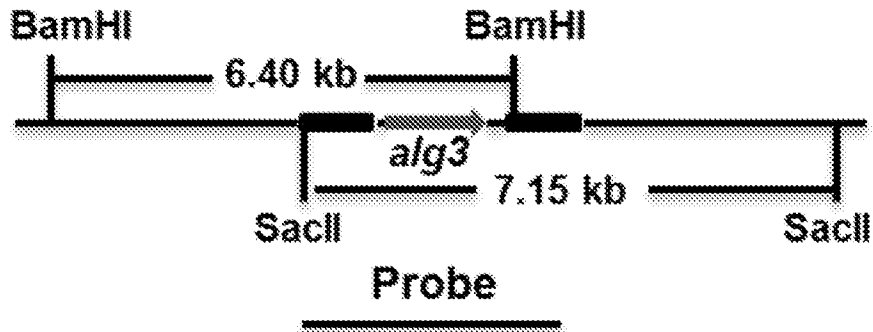


FIG. 1B

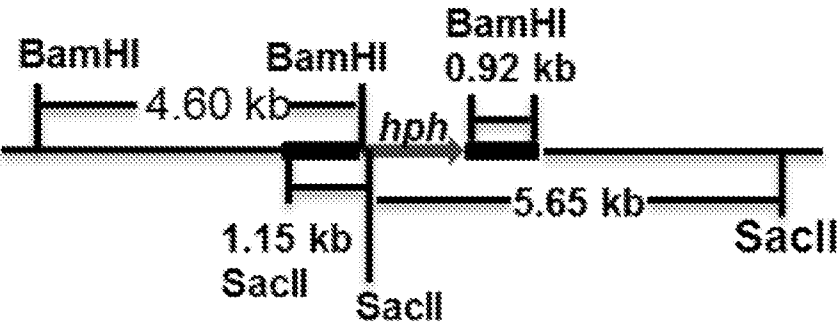
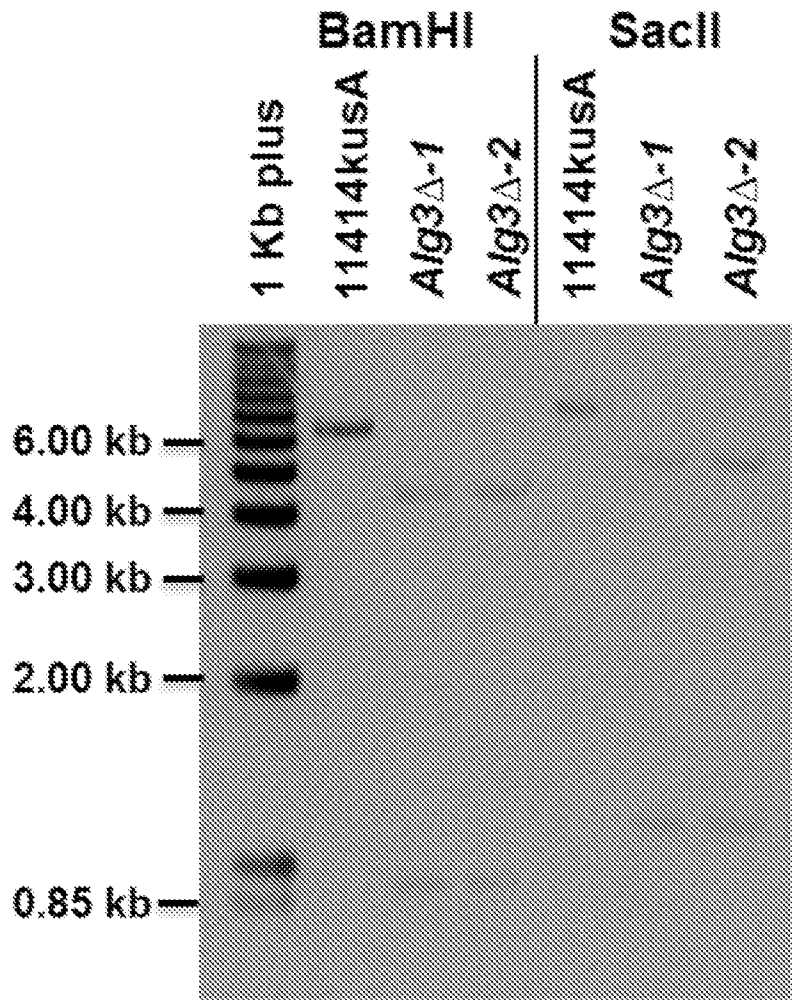


FIG. 1C



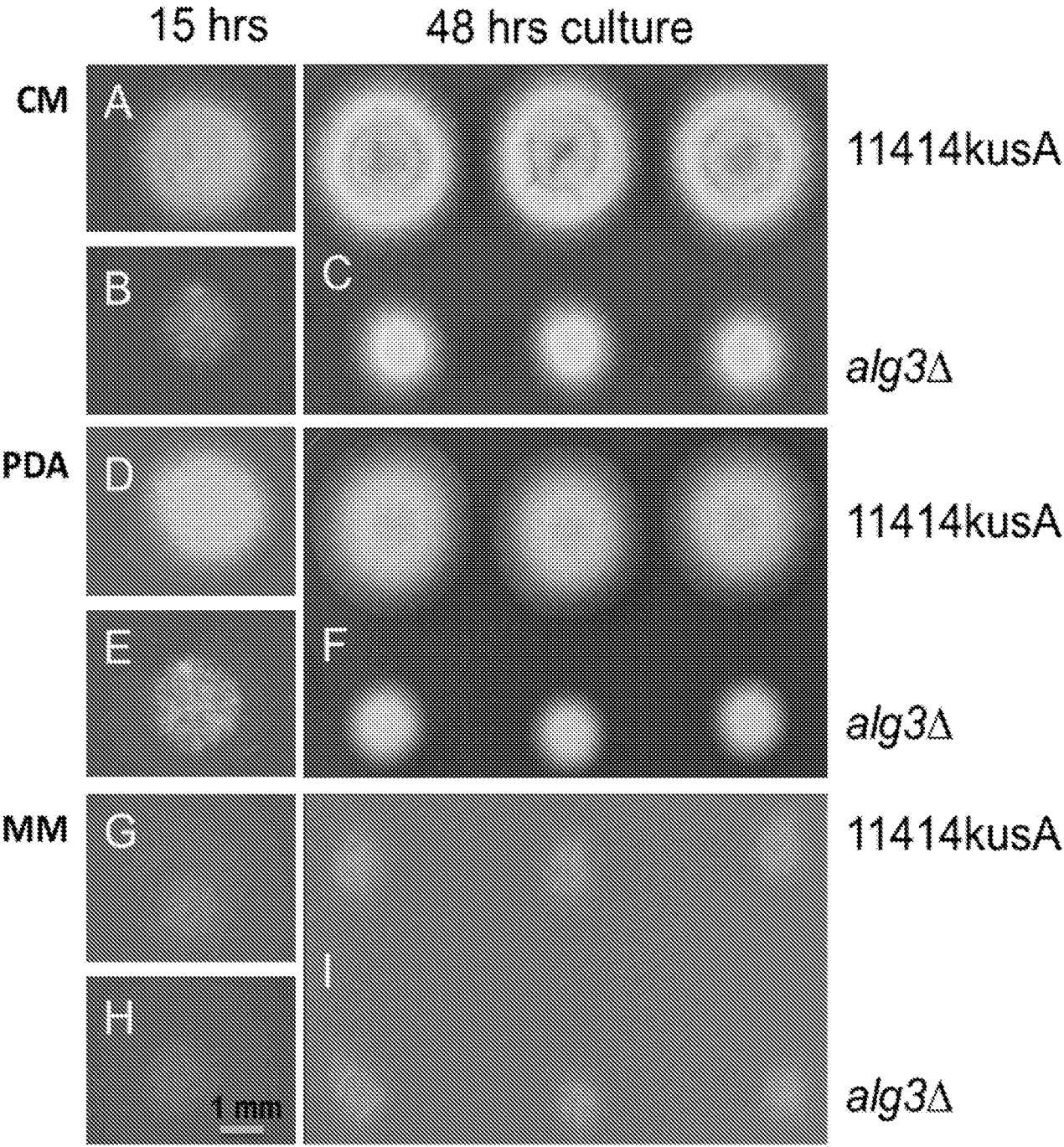


FIG. 2

FIG. 3A

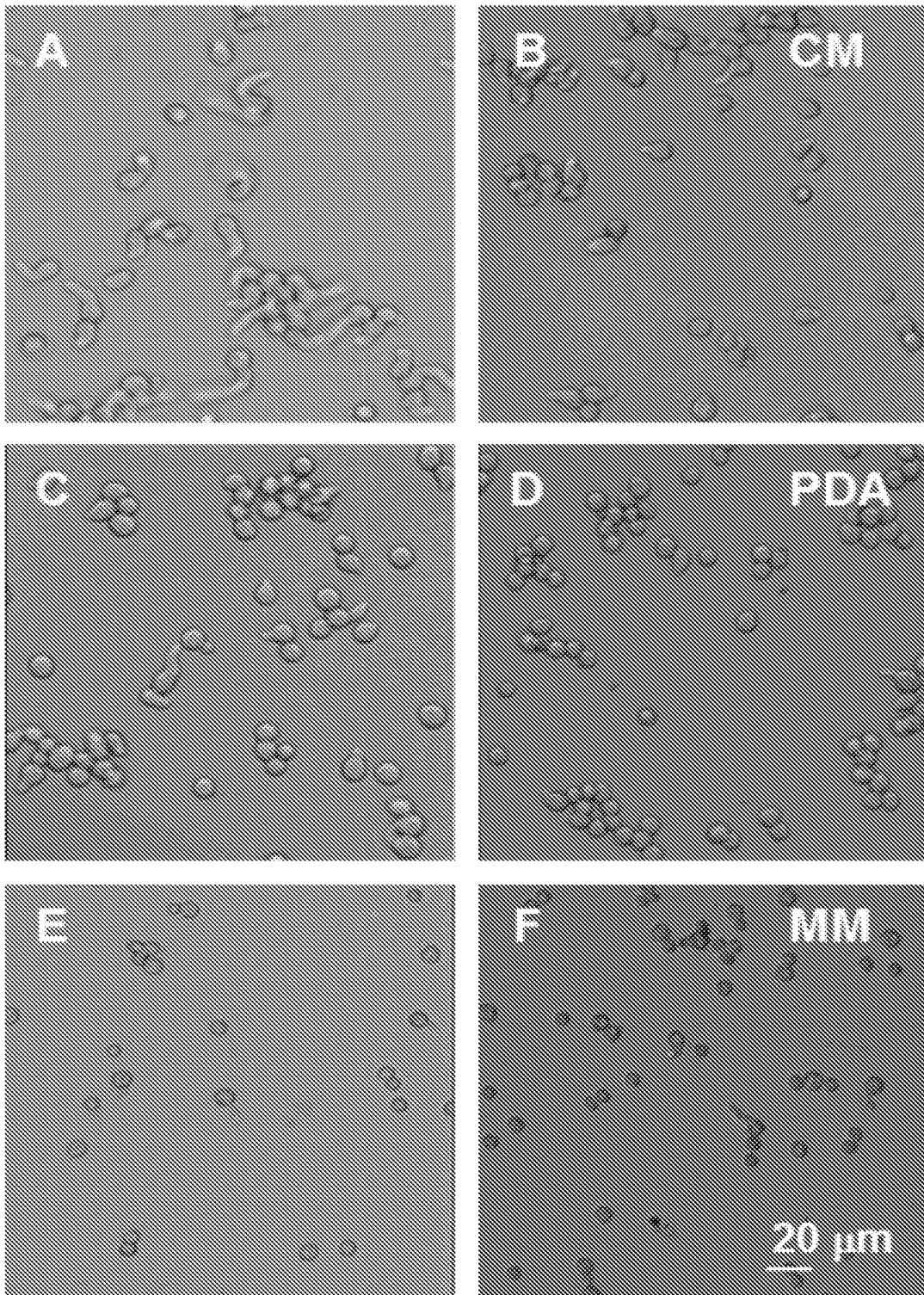
*11414kusA**alg3Δ*

FIG. 3B

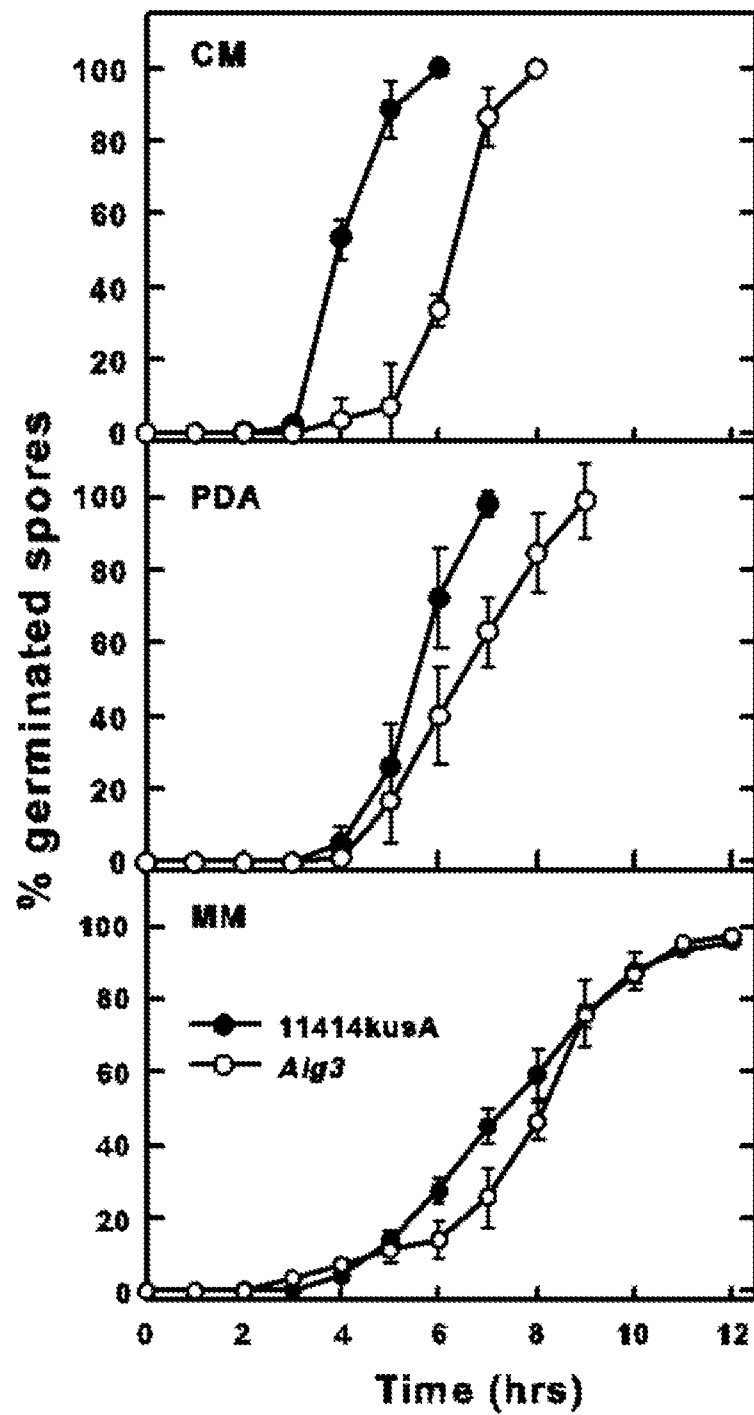
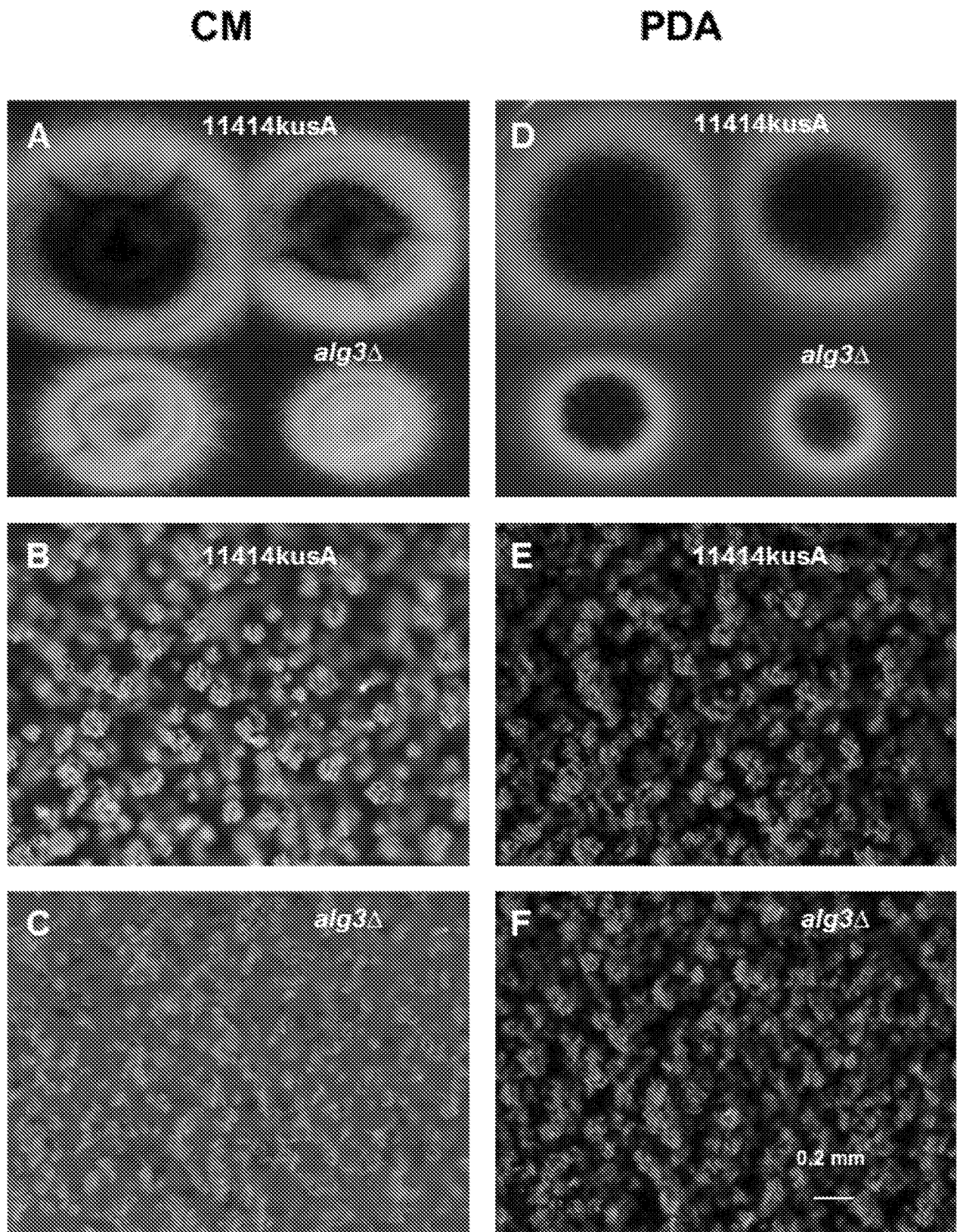


FIG. 4



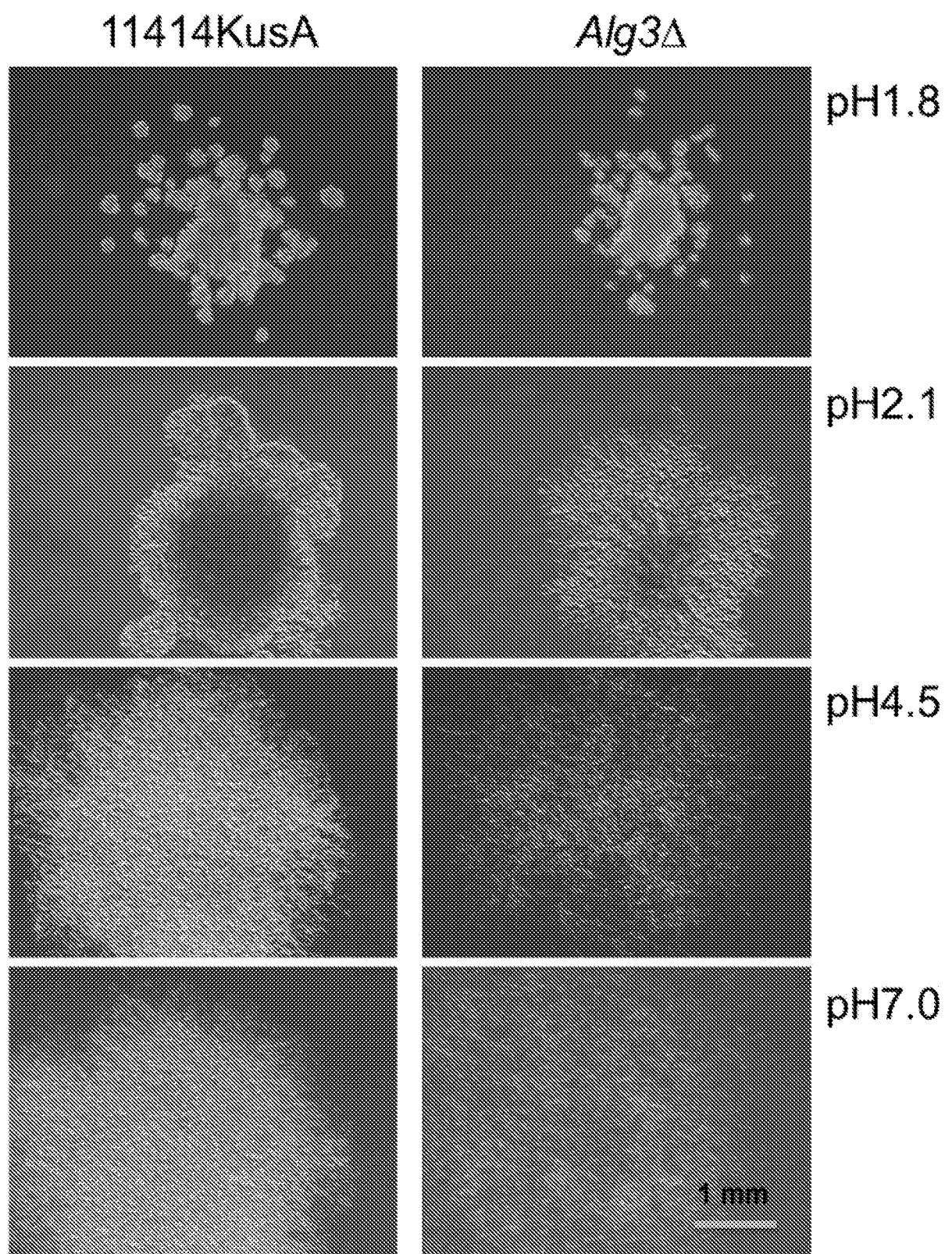
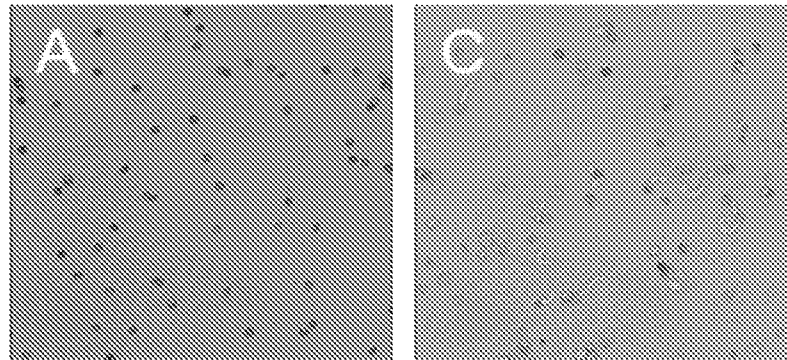


FIG. 5

FIG. 6A *11414kusaA* *Alg3Δ*
8 hrs after inoculation



15 hrs after inoculation

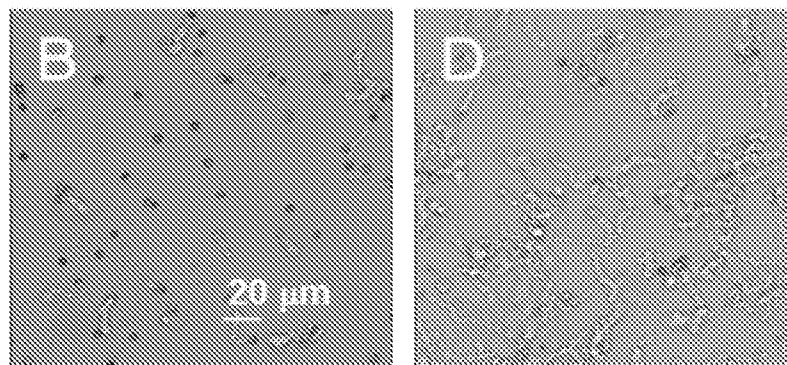
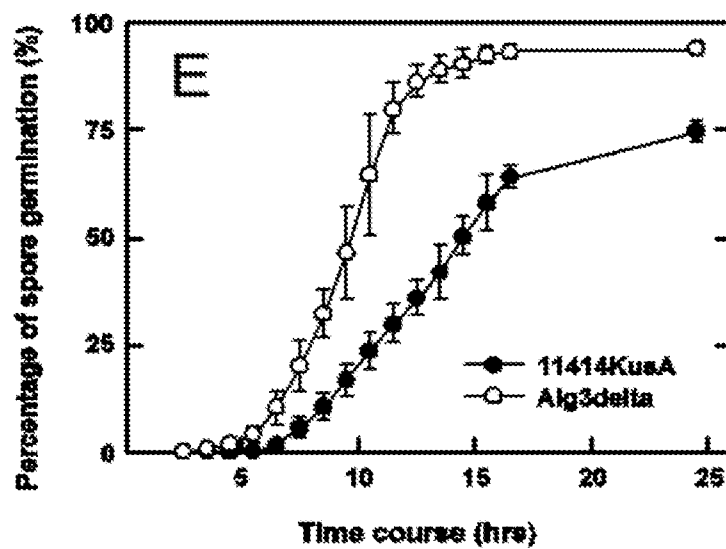


FIG. 6B



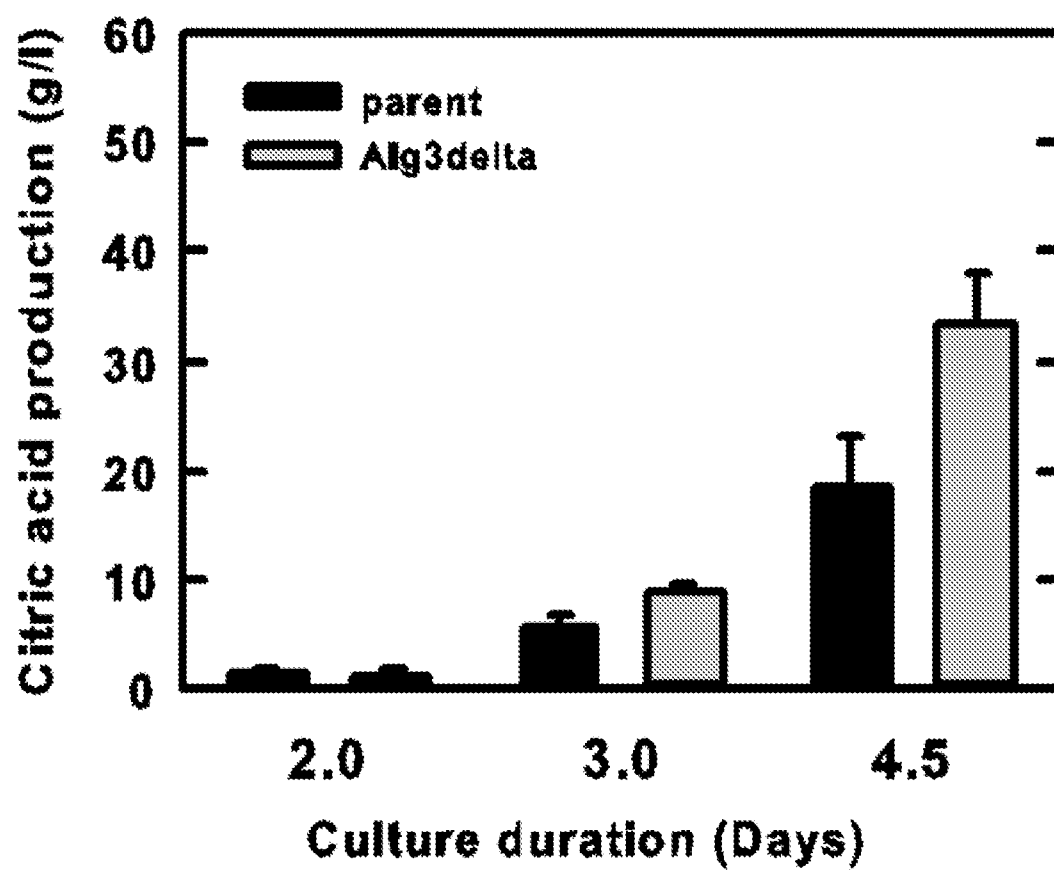


FIG. 7

Transgene fragment for complemented transformation

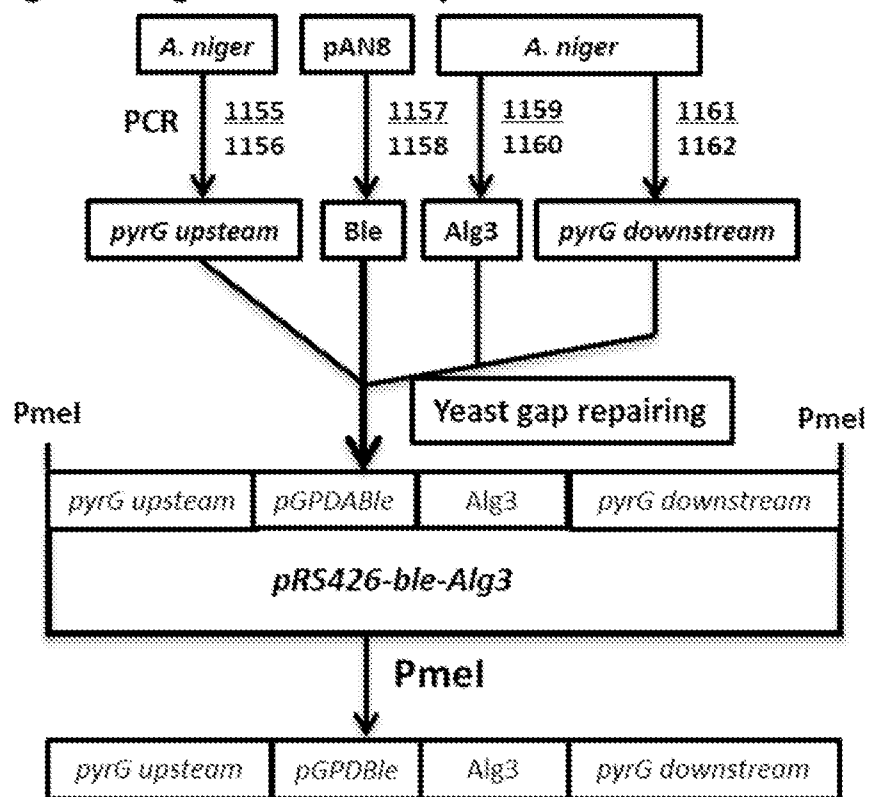


FIG. 8A

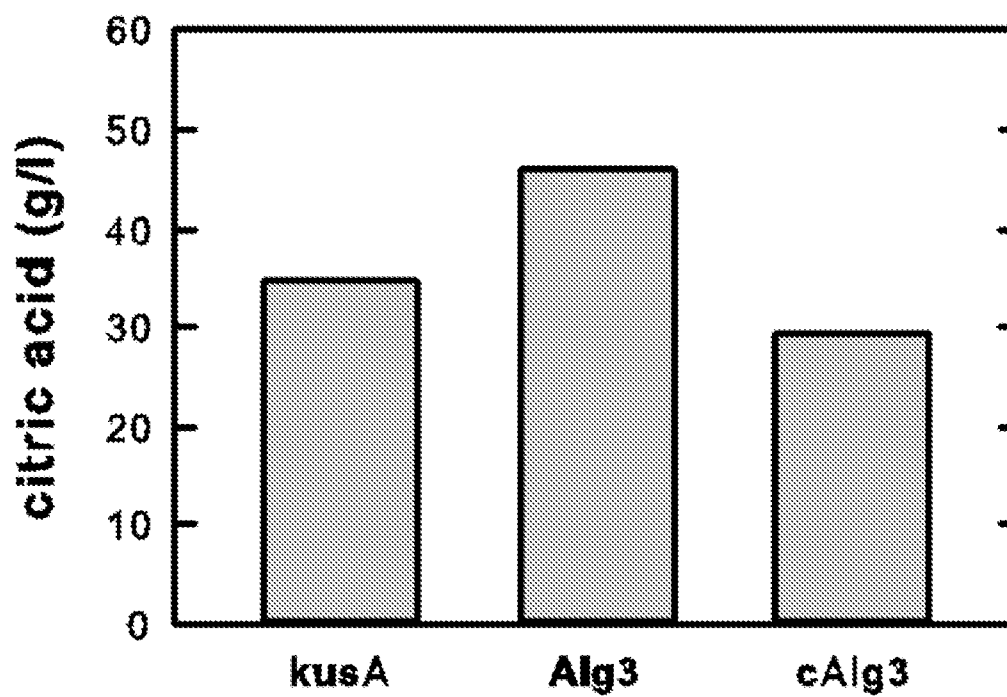


FIG. 8B

FIG. 9

GENE ID: 5996303 AOR_1_556094 | dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl
mannosyltransferase [Aspergillus oryzae RIB40] (10 or fewer PubMed links)

Score = 137 bits (74), Expect = 2e-28
Identities = 400/553 (72%), Gaps = 40/553 (7%)
Strand=Plus/Plus

```

Query   801   CAGGTTTTACTCGCGATACCGTTCCTACAAAACAACCCGGCGGGGTATC-TCTCGCGGGC   859
          ||||| ||| ||||| || ||||| ||||| || || || || |||||
Sbjct   643   CAGGTTCTACTAGCGATTCCCTTCCTACAGGGTAACCCCATAGGATA-CGTCGCGCGGGC   701

Query   860   GTTCGAGCTAACCAGACAGTTCATGTTTAAATGGACAGTCAATTGGAGATTTGTTGGCGA   919
          || ||| | || ||||| ||||| ||||| ||||| ||||| || |||
Sbjct   702   CTTTGAGTTGACTAGACAGTTTATGTTCAAATGGACTGTCAATTGGAGGTTTGTGGGTGA   761

Query   920   AGAAGTATTCTTAT-CTAAGAGCTTTTCCCTGGCATTGCT-GGCCGTCCACATTGTGCTG   977
          ||| | ||| ||| || || ||||| || || || || || || || || || ||
Sbjct   762   AGACTTGTTCTATCCAAACAG-TTTTCTCTAGCCTTACTAGG-TTGCATATTTTCTG   819

Query   978   CTAGGCG-CT-TTGGCCGTCACCTGGTTGGCTGA-GATAC-TCCAGG-TCTAGCTTGCTG   1032
          || ||| | ||||| || || ||||| || || || || || || || || || ||
Sbjct   820   CT-GG-GATTATTGTTACCACAGGCTGG-TTACG-GCCGT-CAGGATCTAACGTCCCTG   874

Query   1033  -CGTTCATTCCGAATCTGCTAGC-GGGTCGACATCGCACAGT-GTCCCTCCCCAAACCT   1089
          | ||| | |||| || || | || || || ||||| || || || || || ||
Sbjct   875   AC-TTCCTCCGGAGCCTACT-CCAAGGACGCCAACGCACCGTGGT-GCTTTCTAAGTCTT   931

Query   1090  ACATCATGAGCGTGATGCTCTCGTCTCTGACAG-TTGGCTTGTTGTGCGCAAGGTCCCTT   1148
          ||| |||| ||||| || || || || || || ||||| ||||| ||||| |||||
Sbjct   932   TCATAATGACCGTGATGTTGACATCGCTGGC-GATCGGGTTGTTGTGCGCAAGGTCCCTT   990

Query   1149  CATTACCAATTCTTCGCCTACCTCTCCTGGGCGACACCCCTT-CCTCCTCTGGCGCGCAGG   1207
          ||||| ||||| ||||| ||||| ||||| || || || || || ||||| || ||
Sbjct   991   CATTACCAATTCTTGCCTATCTCTCCTGGGCTACGCC-TTGCCTTCTCTGGCGGGCTCG   1049

Query   1208  GTTTCATCCAATC-TTGCTGTAC-CTTATCTGGGCTA-TGCAAGAGTGGGCTTGGAACA-   1263
          || ||||| ||| || || || || ||||| || || || ||||| |||||
Sbjct   1050  GCTCCATCCGATCCTTA-TATATGCG-ATCTGGGC-ACTACAGGAGTGGGCTTGGAATGT   1106

Query   1264  CATTCCCCAGCACCAAC-CTCAGTTCATCATT-GTTGTCTCTCACTTGCTACCCAGAG   1321
          || || || ||||| || ||||| || || ||||| ||||| ||||| |||||
Sbjct   1107  C-TACCCAAGCACCAATGC-CAGTTCT-TCGGTCGTTGTCTTCTCACTTGCTGTTTTCAG-G   1162

Query   1322  TTT-CGGCGTCCT   1333
          ||| ||| |||||
Sbjct   1163  TTTTCGGTGTCTCT   1175

```

[illegible]

FIG. 10B

```

**++++*****+*+.*+*****+.....++.....*..++++...*++*+*****..**
Aspergillus niger 322) VGLLCARSLHYQFFAYLSWATPFLWRAGFHPI---LLYLIWAMQEWAWNTFPSTNLSSI
Aspergillus nid. 322) VGLLCARSLHYQFFAYLSWATPFLWQAGYHPI---LVYALWLQEWAWNVYPSTNLSSA
Fusarium oxy. 341) IGLLFARSLHYQFYAYLAWATPYLLWRATEDPV---IVAIWAAQEWAWNVYPSTDLSST
Neurospora cra. 349) VGLLFARSLHYQFYAYVAWSTPFLWRAGLHPV---LVYLLWAVHEWAWNVFPSTPASSA
Saccharomyces cer. 342) IGMCFSRSLHYQFYVWYFHTLPYLLWSGGVKKLARLLRVLLGLIELSWNTYPSTNYSSL
Arabidopsis tha. 345) IGIVFARSLHYQFYSWYFYSLPYLLWRTPF-PT--WLRIMFLGIELCWNVYPSTPSSSG
Homo sap. 343) IGICFSRSLHYQFYVWYFHTLPYLLWAMPARWLTHLLRLLVLGLIELSWNTYPSTSCSSA

.+.....+++.+.... .....+.+.+.-
Aspergillus niger 379) IVVLSLATQSFGLANSASA-FYTMRSNPSGKEHNQ--
Aspergillus nid. 379) AVVLLGAQVLGVLVNRDRA-FPSSPPTPKAQHVQ--
Fusarium oxy. 398) IAVNTMLATVVLVYLG TARR-AVPAPAAQVGNVDDKNk
Neurospora cra. 406) VVVGVLGVTVAGVWFGAREEwEPGMKSSSKKEEAAMR-
Saccharomyces cer. 402) SLHVCHLIILLCLWLNPNPA-SPSHRSENKAKSH----
Arabidopsis tha. 402) LLLCLHLIILVGLWLAPSVD-PYQLKEHPKSQIHKKA-
Homo sap. 403) ALHICHAVILLQLWLGPQPF--PKSTQHSK-KAH-----

```

FIG. 11

Score = 279 bits (713), Expect = 2e-87, Method: Compositional matrix adjust.
Identities = 159/405 (39%), Positives = 239/405 (59%), Gaps = 6/405 (1%)

Query	12	FNPRHTKWMAPLLVLGDAFLCALIIWKVPYTEIDWATYMQQISLYLSGERDYTLIRGSTG	71
		F P +T + +L + + +I KV YTEIDW YM ++ ++G DYT ++G TG	
Sbjct	31	FKPEYTLTAVLWFLFLEIAINIWVIQKVSYTEIDWKAYMDEVEGVINGTYDYTQLKGD TG	90
Query	72	PLVYPAAHVYSY TALYHLTDEGRDIFFGQILFAVLYLITLVVVLCCY-RQSGAPPYLLPL	130
		PLVYPA VY +T LY+LTD G +I GQ +FAV YLI L++V+ Y R PPY+	
Sbjct	91	PLVYPAGFVYIFTGLYYLTDHGHNIRLGQYVFAVSYLINLLLVMRIYHRTKKVPPYVFFF	150
Query	131	LVL-SKRLHSVYVLRFLFNDGLAALAMWVAILLFMNRKWTAAVAVWSTGVAIKMTLLLLAP	189
		+ S R+HS+++LRLFND +A + + AI LF++ +WT A++S V++KM +LL AP	
Sbjct	151	ICCASYRIHSIFILRLFNDPVAMMLCFGAINLFLDGRWTLGCALYSLAVSVKMNVLLFAP	210
Query	190	AIAVVTVLSLSLGP SVGLGVLA VLVQVLLAIPFLQNNPAGYLSRAFELTRQFMFKWTVNW	249
		+ + + L ++ L ++Q++L +PFL NP GY+SRAF+L RQF+FKWTVNW	
Sbjct	211	GLLFLLLCEFG LWKTLPR LALCAVIQLVLGLPFLLVNPGYVSRAFDLGRQFLFKWTVNW	270
Query	250	RFVGEVFLSKSFSLALLAVHIVLLGAFVAVTGWLRYSRSSLPAFIRNLLAGRHRTVSLPK	309
		RF+ E+VFL++ F LALL HI L FA+ W R S SS+ +++ + +	
Sbjct	271	RFLPEDVFLNRYFHLALLLAHITTTLLLFALKRWKR-SGSSIWTILKDPSEKETAHKVNA	329
Query	310	PYIMSVMLSSLTVGLLCARSLHYQFFAYLSWATPFLWWRAGFHP---ILLYLIWAMQEWA	366
		++ ++ +S +G+ +RSLHYQF+ + P+LLW G +L LI + E +	
Sbjct	330	DQMV LILFTSNFIGMCF SRSLHYQFYVWYFHTLPYLLWSGGVKKLARLLRVLILGLIELS	389
Query	367	WNTFPSTNLSSIIIVVLSLATQSFGVLANSASAFYTMRSNPSGKEH	411
		WNT+PSTN SS+ + + + N A + RS K H	
Sbjct	390	WNTYPSTNYSSLSLHVCHLIILLCLWLNPNPASPSHRSENKAKSH	434

FIG. 12

A. nidulans

Query 1 MTSPAHHYSYHSPTSSDRGRSRQNSDAMDIIQSITEREPATR-----YAVAGGPAPWN 53

M SP N+YSY S D GRSRQNSDAMD I T +EP Y GGPA +

Sbjct 14 MASPNNNNYSYQGIESYDSGRSRQNSDAMDIIHVITAQEPPREPPDNDPYDGHGGPAGTS 73

A. niger

Query 54 RNGSPSMSPINSENRNQFHEENGRTYHGFRRGMYFLPCDEQEQRDLDFHKLFTVARVSES 113

P R F+EENGRTYHG+RRG+Y LPCDEQEQRDLDFHKLFTVAR+SES

Sbjct 74 HYSKPP-----NRWLFYEENGRTYHGYRRGVYPLPCDEQEQRDLDFHKLFTVARMSES 127

Query 114 LIYAPHPTNGRFLDLGCGTGIWAIEVANKYPDAFVAGVDLAPIQPPNHPKNCEFYAPFDF 173

LIYAPHP NGRFLDLGCGTGIWAI+VA+KYP+AFVAGVDLAPIQPPNHP NCEFYAPFDF

Sbjct 128 LIYAPHPPNGRFLDLGCGTGIWAIDVAHKYPNAFVAGVDLAPIQPPNHPDNCEFYAPFDF 187

Query 174 EAPWAMGEDSWDLIHLQMCGCSVMGWPNLYRRIFAHLRPGAWFEQVEIDFEPRCDDRSLD 233

EAPW +GE+SWDLIHLQMCGCSV+GW NLY+RI HL+PGAWFEQVEIDFEPRCDDRSL+

Sbjct 188 EAPWTLGENSWDLIHLQMCGCSVLGWQONLYKRILRHLQPGAWFEQVEIDFEPRCDDRSLN 247

Query 234 GTALRHWYDCLKQATAETMRPIAHSSRDTIKDLQDAGFTEIDHQIVGLPLNPWHQDEHER 293

G ALR WY LKQAT +TMRPIAHSSRDTI+ L++AGFT+IDHQ+VGLPLNPWH+DEHE+

Sbjct 248 GLALREWYQYLKQATQDTMRPIAHSSRDTIRHLEEAGFTQIDHQMVGGLPLNPWHRDEHEQ 307

Query 294 KVARWYNLAVSESIENLSLAPFSRVYRWPLERIQQLAADVKSEAFNKEIHAYNILHIYQA 353

KVARWYNLA+SESIE LSLAPFSR++ W L+RI+Q+ A+VKS+AFNKEIHAYNILHIYQA

Sbjct 308 KVARWYNLAISESIETLSLAPFSRIFHWDLDRIRQITA EVKSQAFNKEIHAYNILHIYQA 367

Query 354 RKP 356

RKP

Sbjct 368 RKP 370

FIG. 13

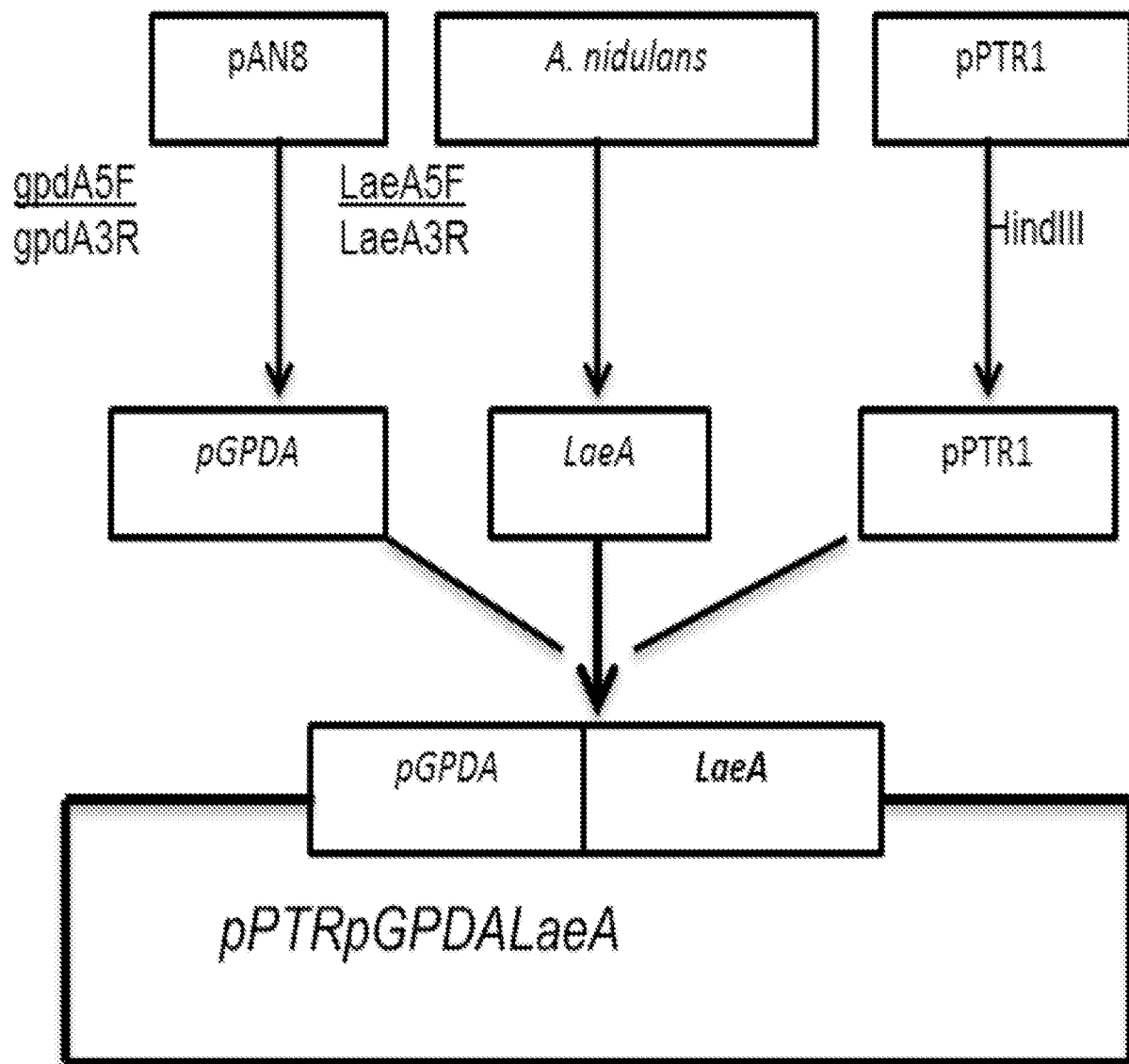


FIG. 14

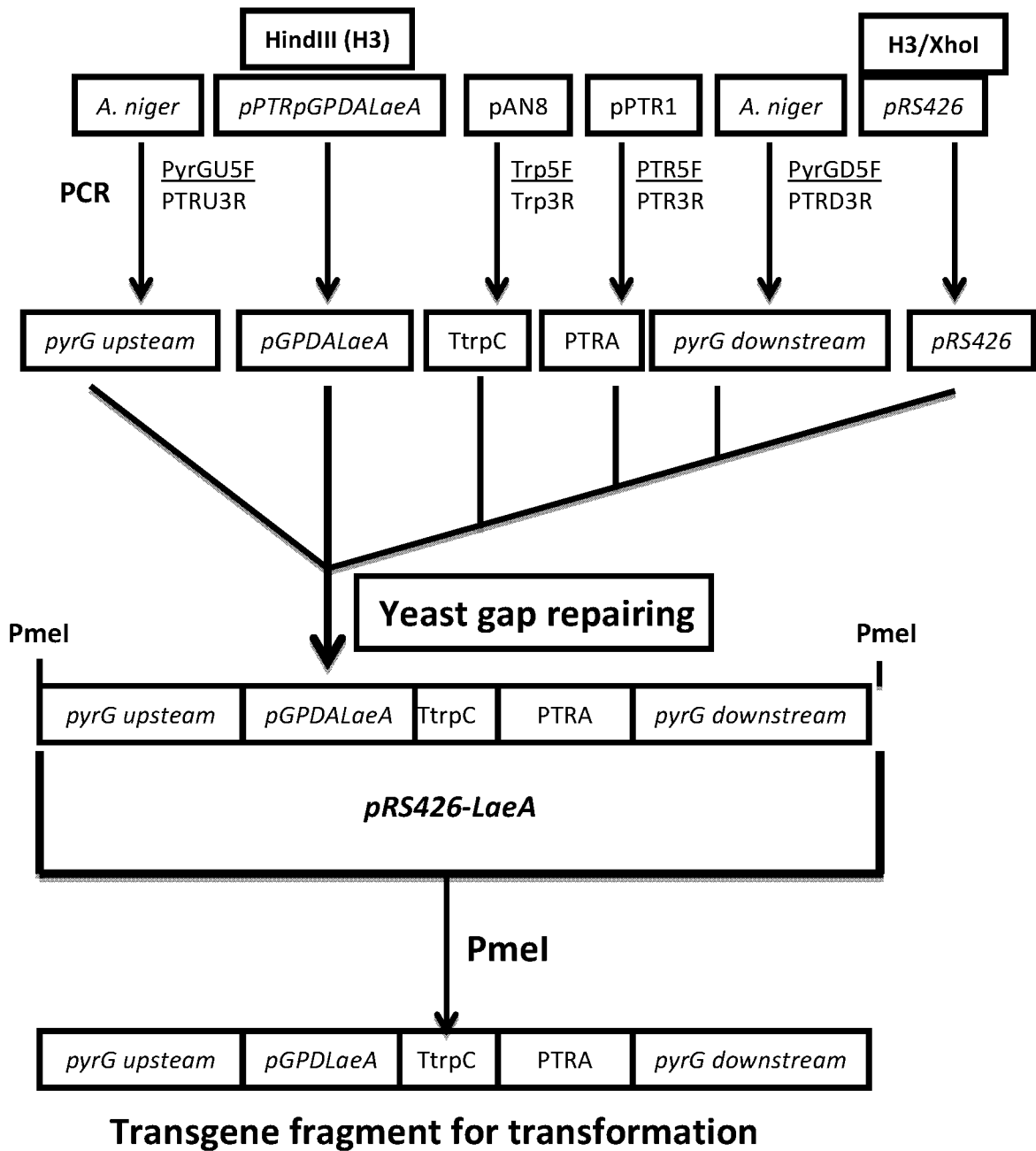


FIG. 15A. Oligo primers:PTR5F/PTR3R

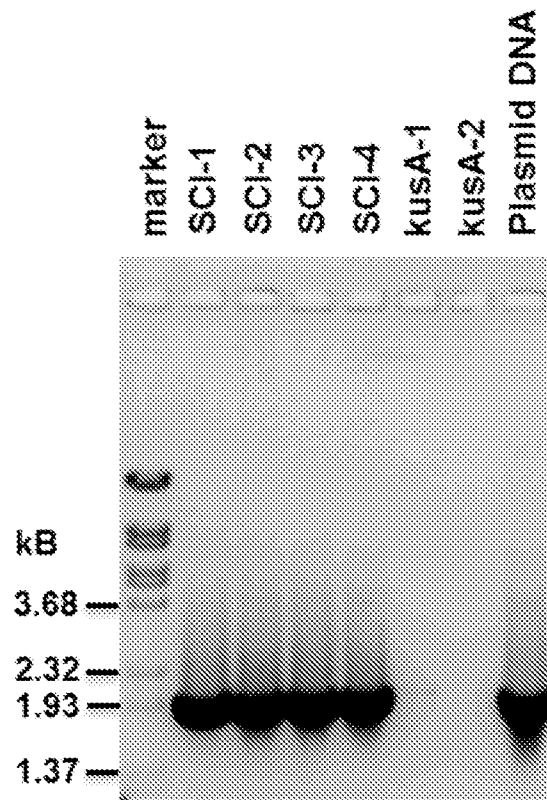
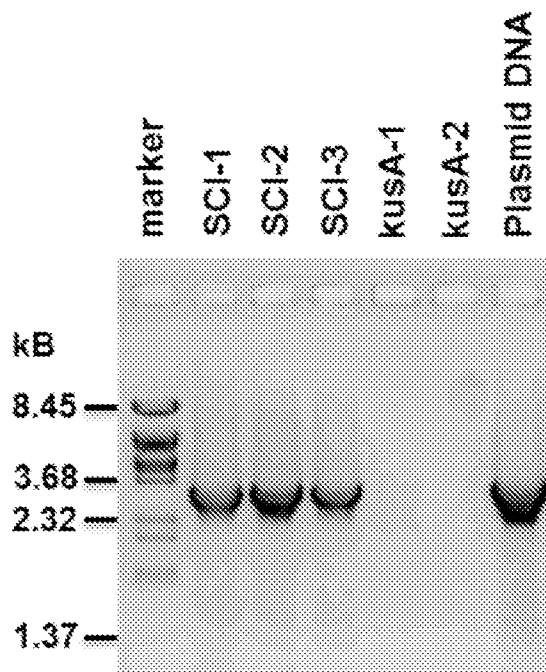


FIG. 15B. Oligo primers:LaeA5F/TRP3R



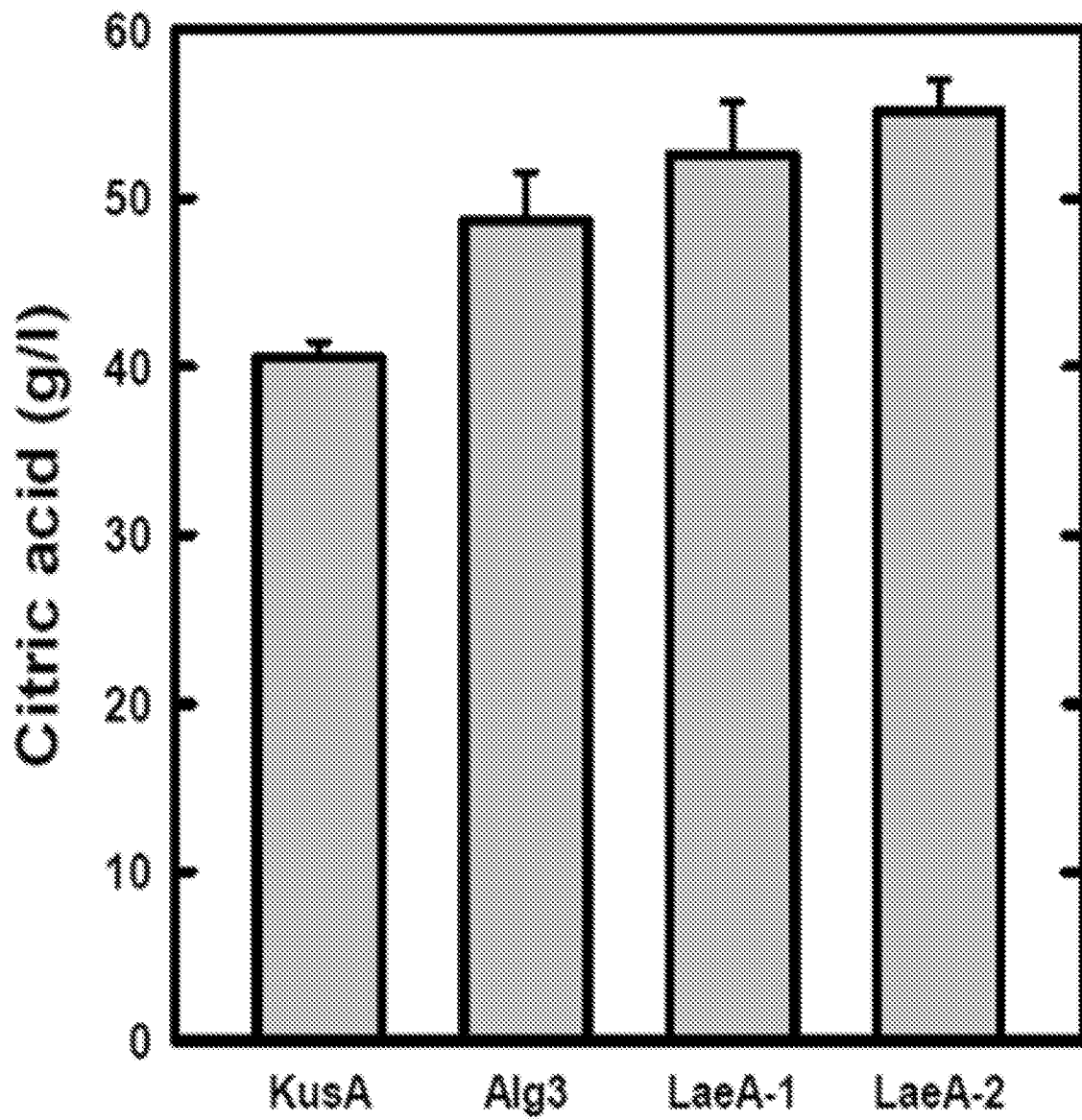


FIG. 16

FIG. 17A

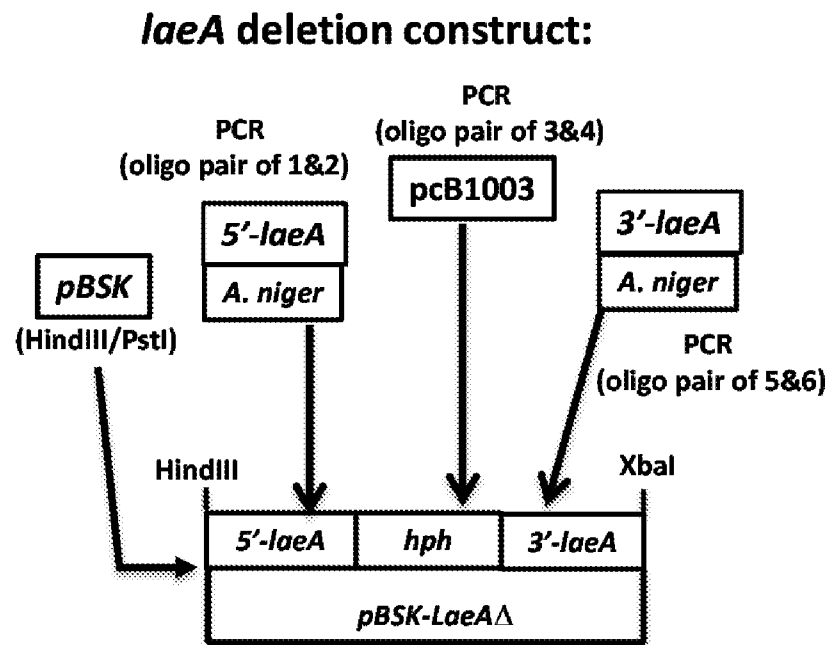


FIG. 17B

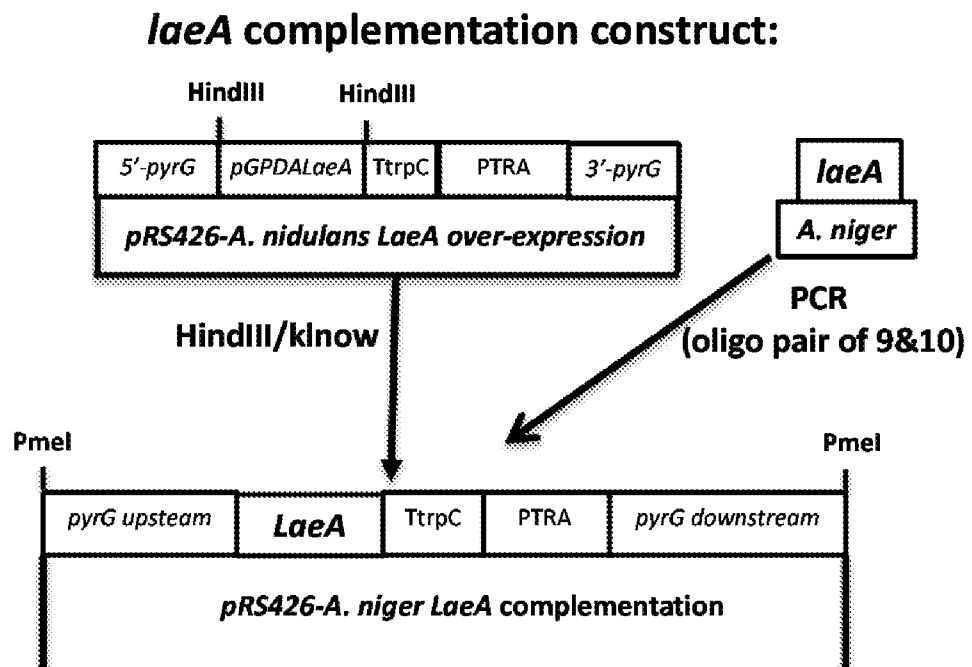
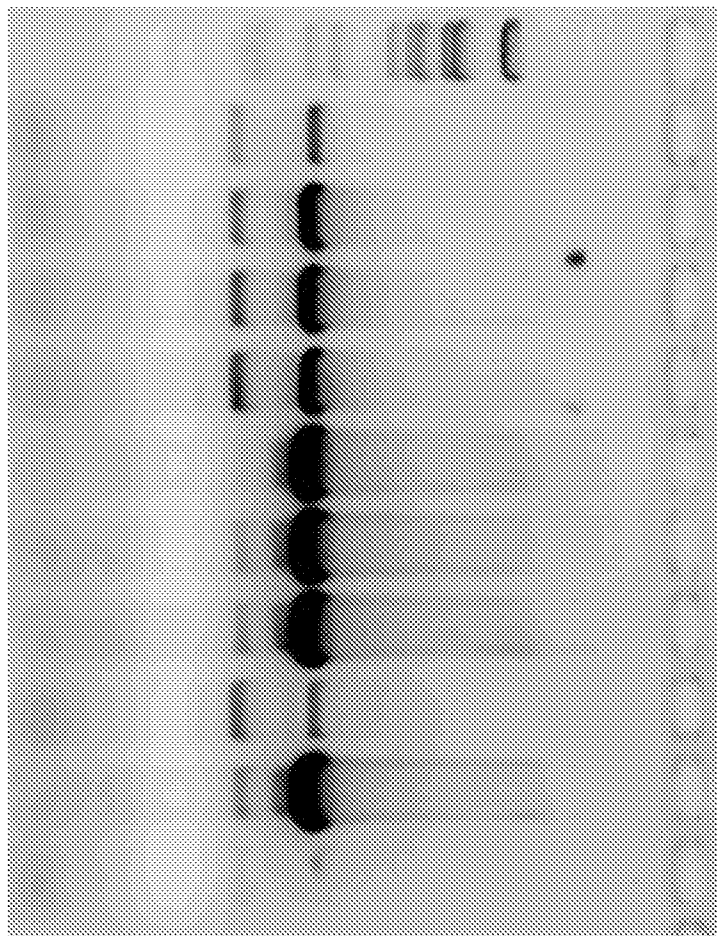


FIG. 18

laeA gene deletion



Bλ

Clone-1

Clone-2

Clone-3

Clone-4

Clone-5

Clone-6

Clone-7

Clone-8

Clone-9

parent

FIG. 19

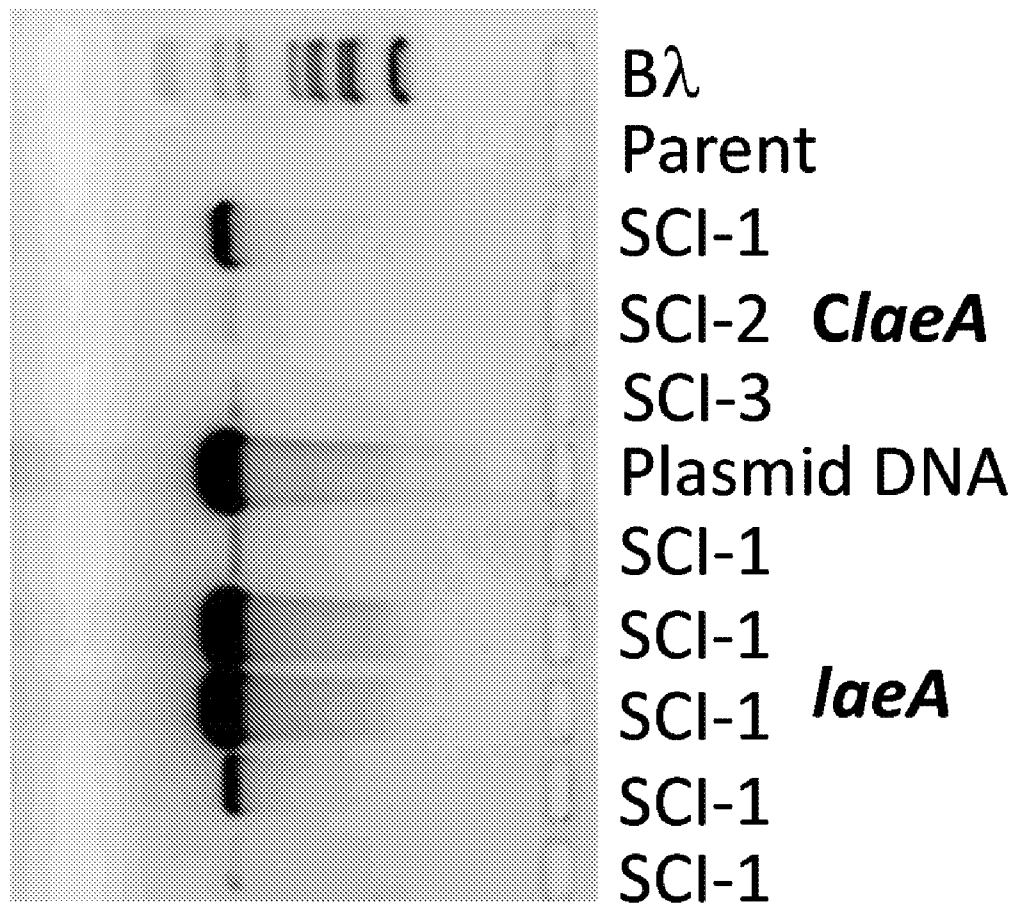


FIG. 20

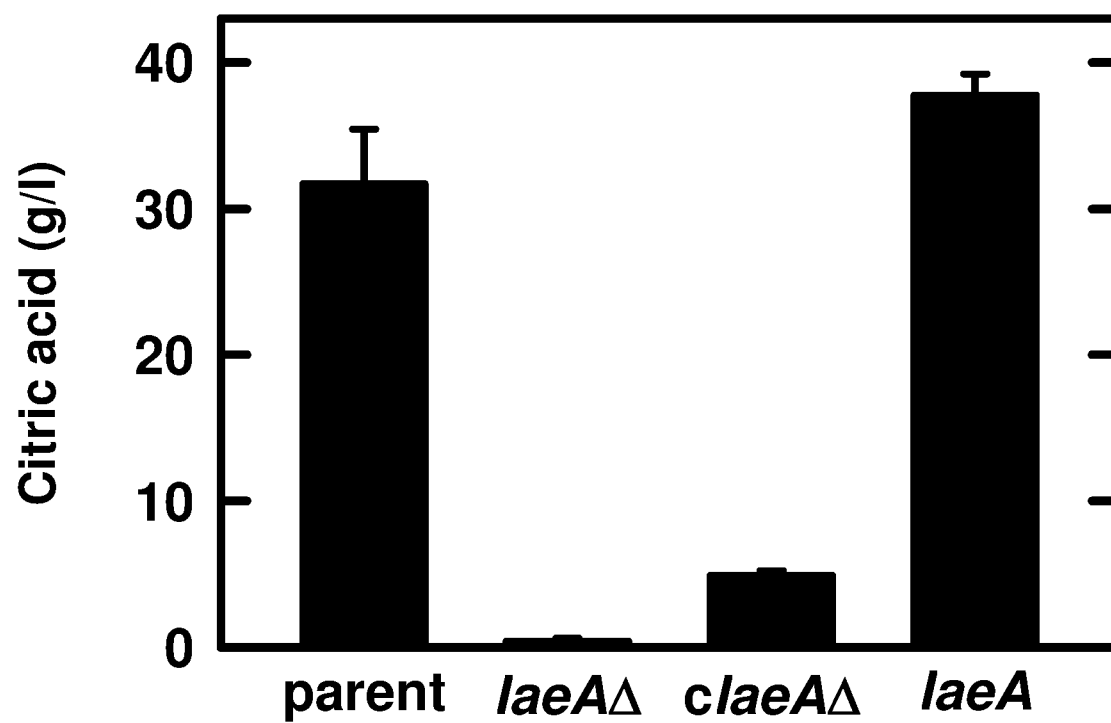


FIG. 21A

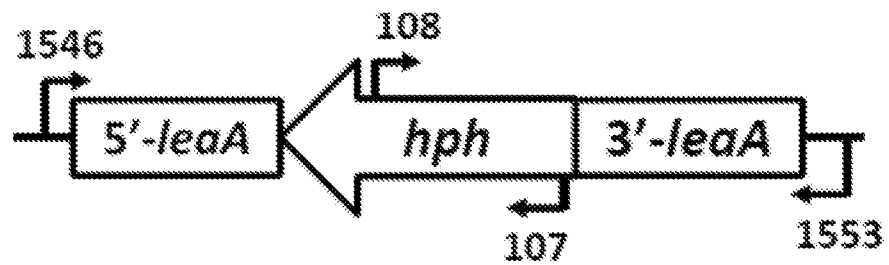


FIG. 21B

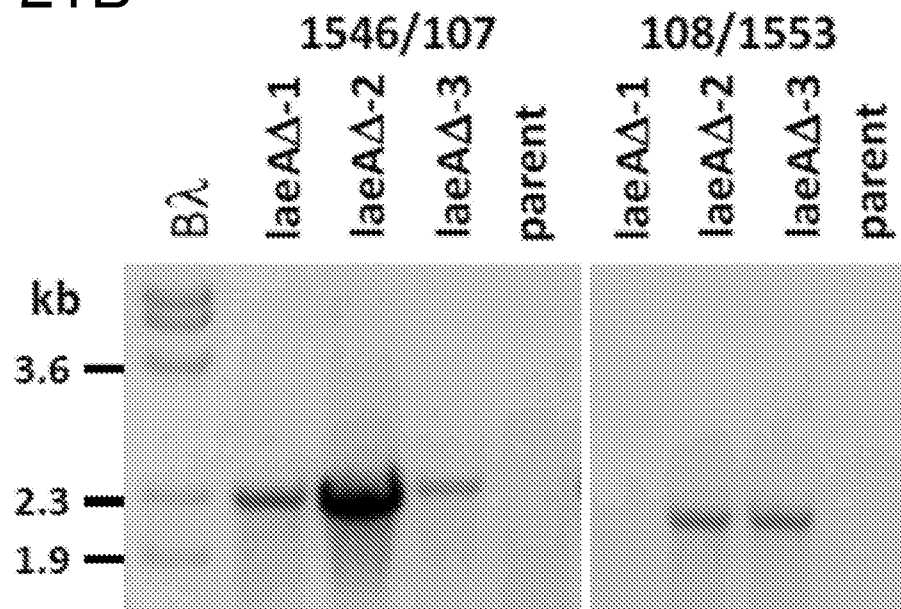


FIG. 22A

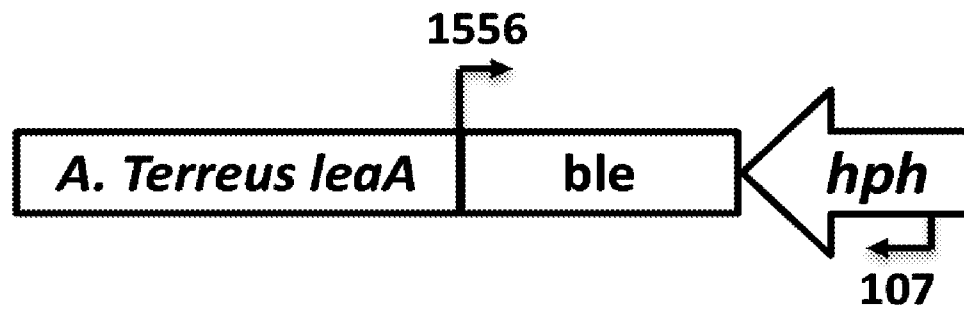


FIG. 22B

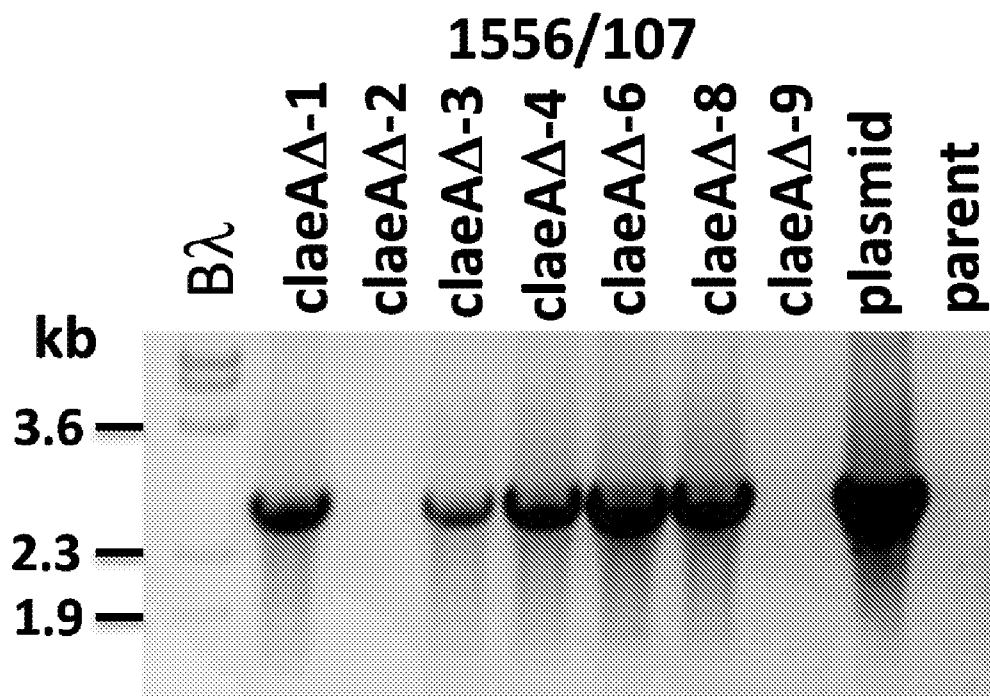


FIG. 23

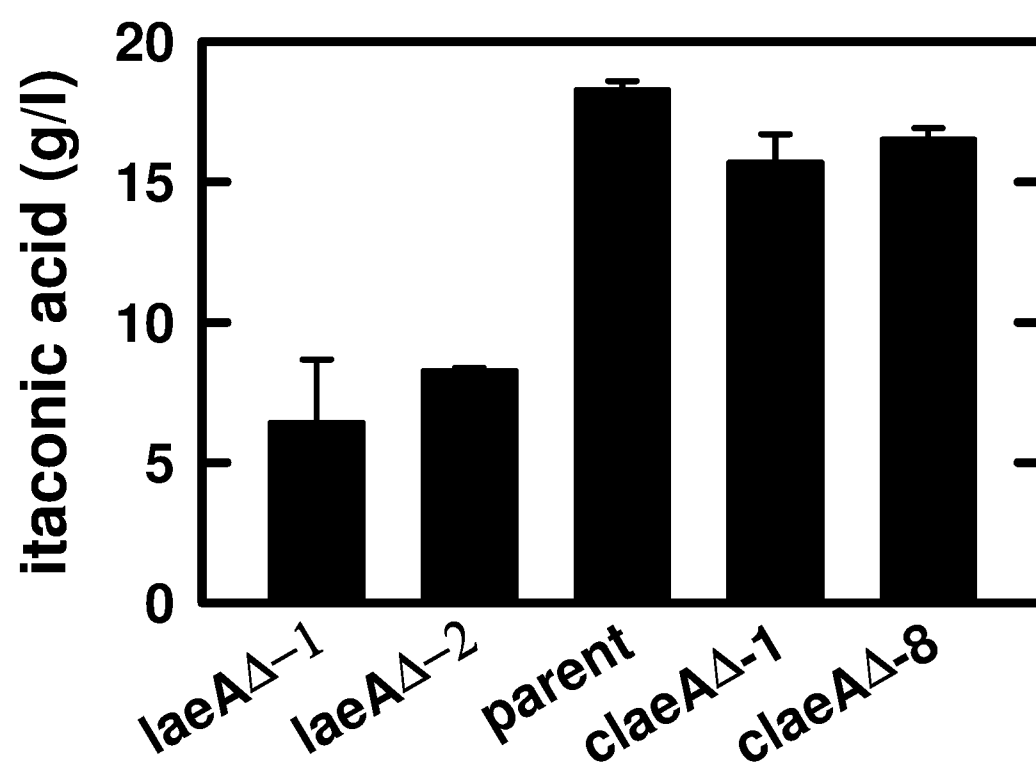


FIG. 24A

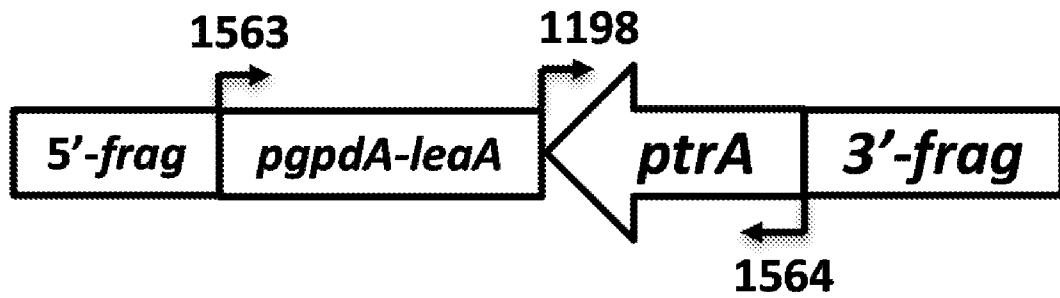


FIG. 24B

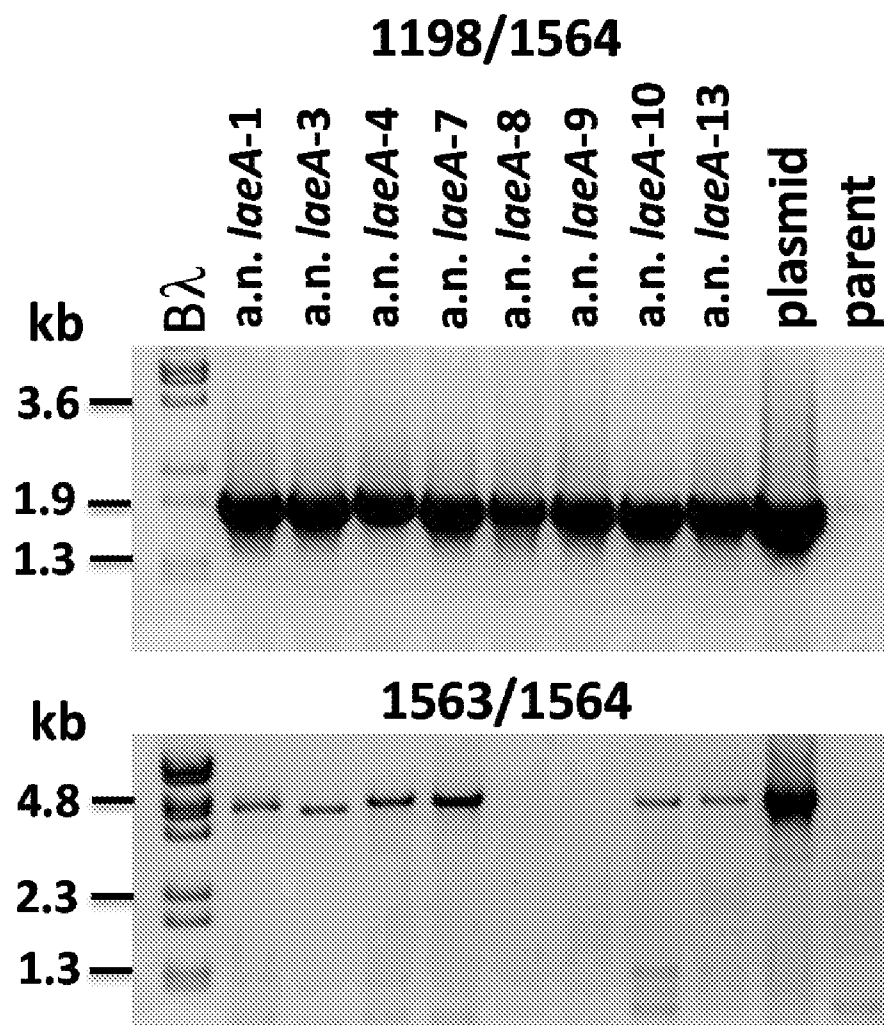
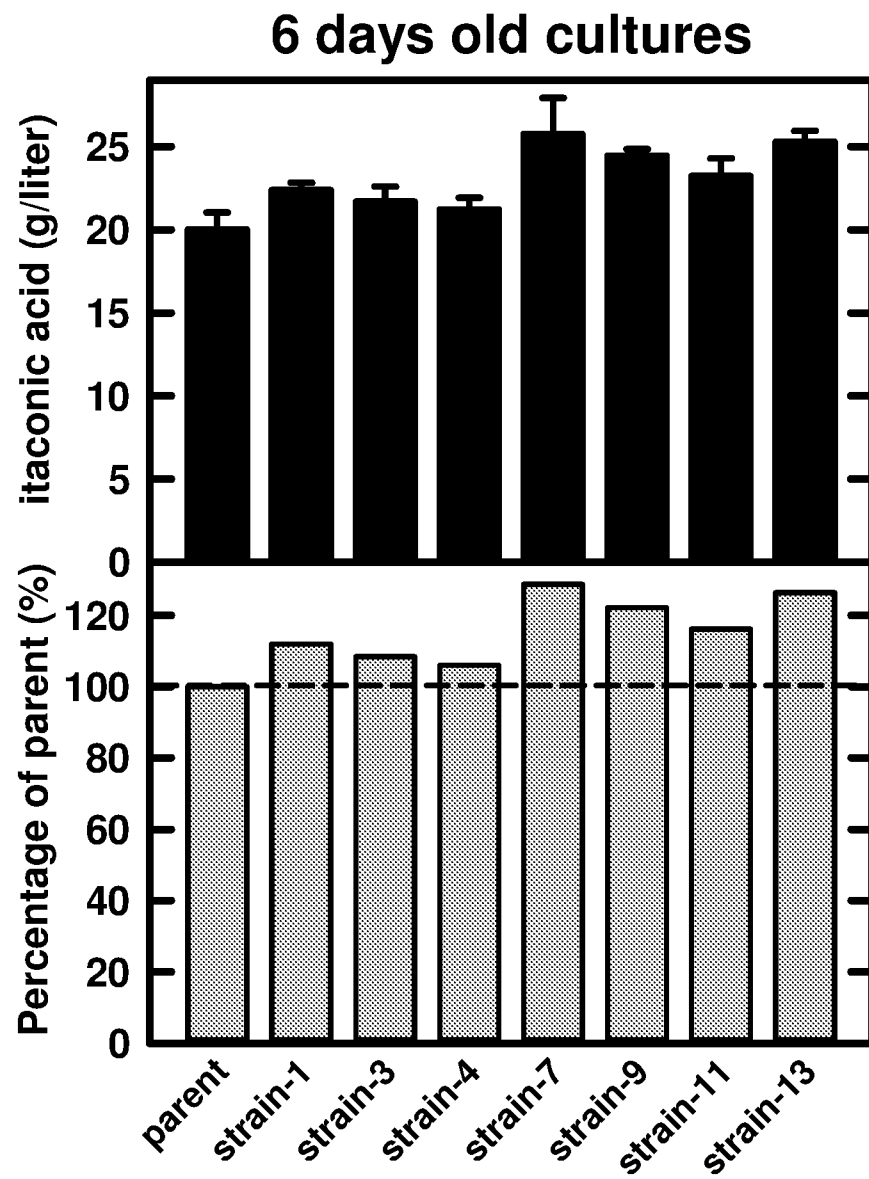
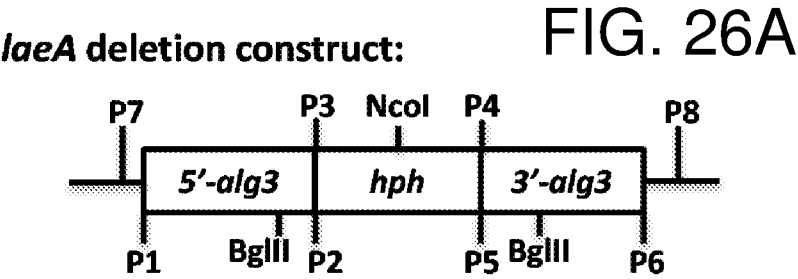
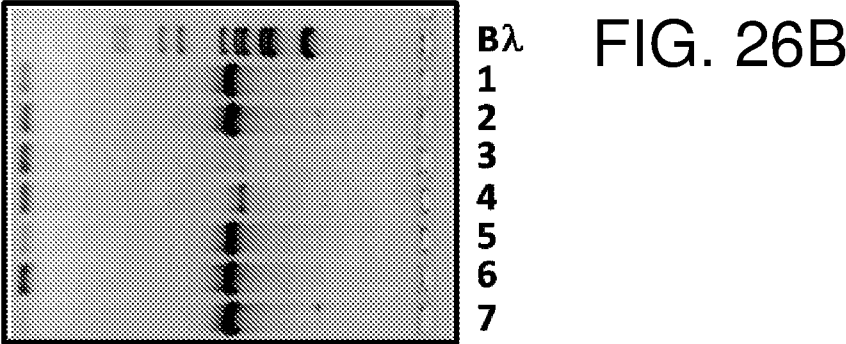


FIG. 25



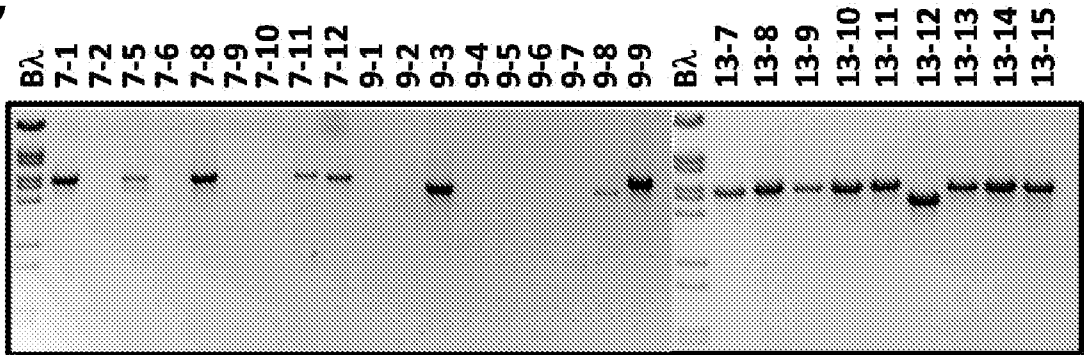


PCR with oligo pair of P7&P8 for genomic DNA of *alg3Δ* mutants



PCR with oligo pair of P7&P8 for genomic DNA of *alg3Δ* mutants in *laeA* over-expression backgrounds

FIG. 26C



PCR products digested with BglII or NcoI

FIG. 26D

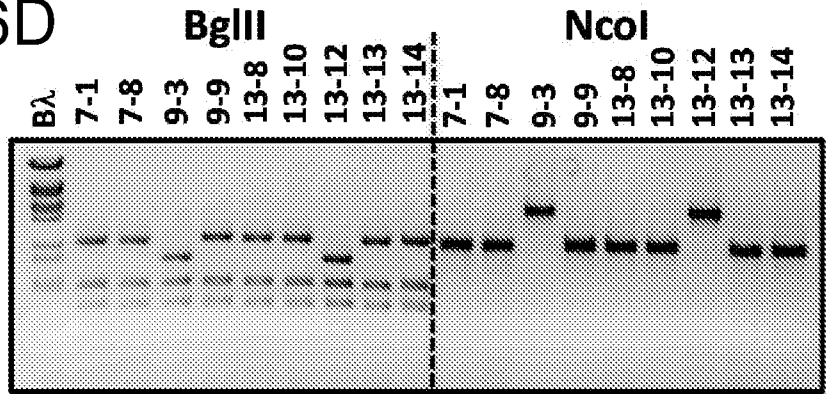


FIG. 27

**Stereo microscopic observation in various strains
of *A. terreus* after 6 days culture**

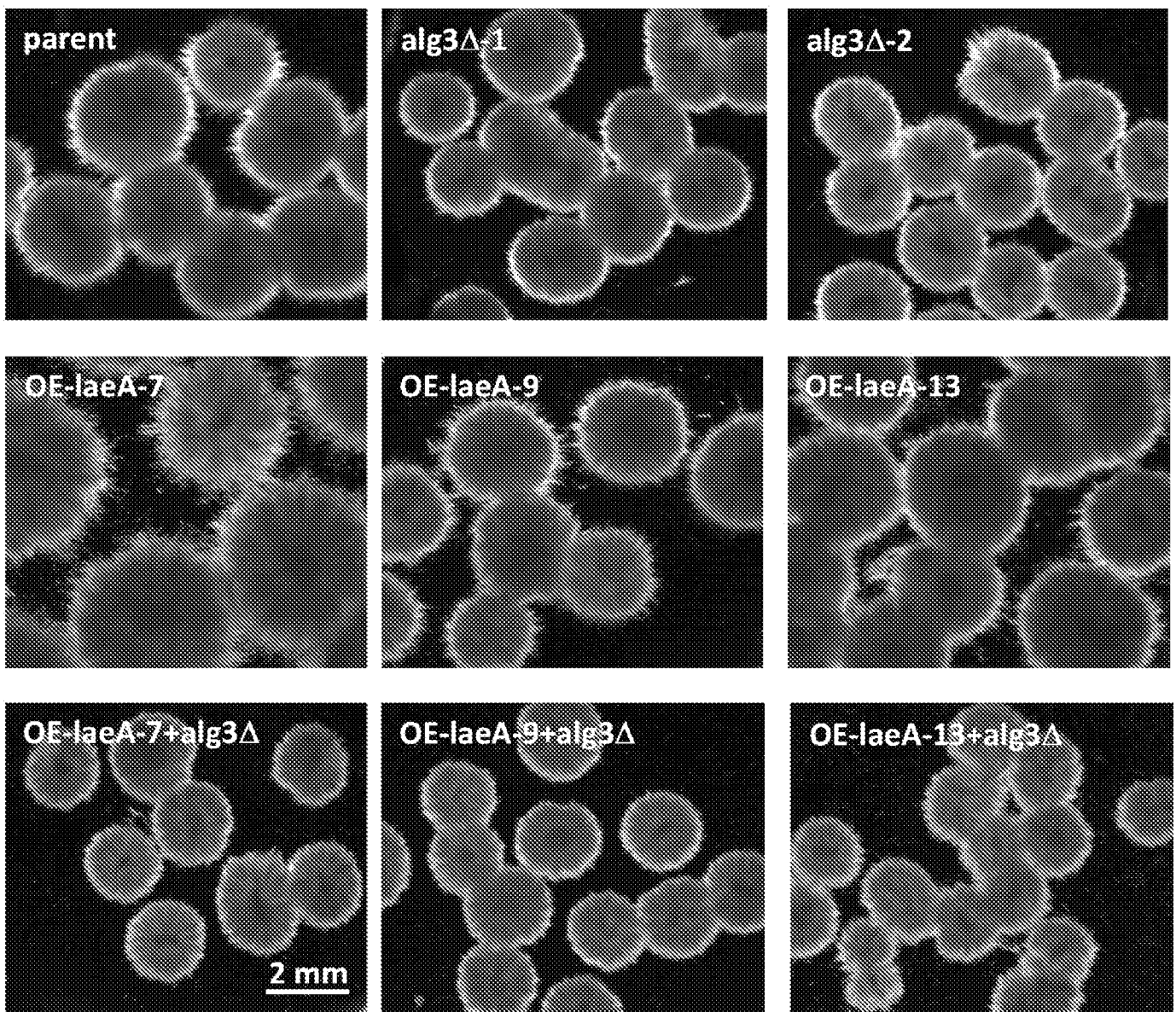
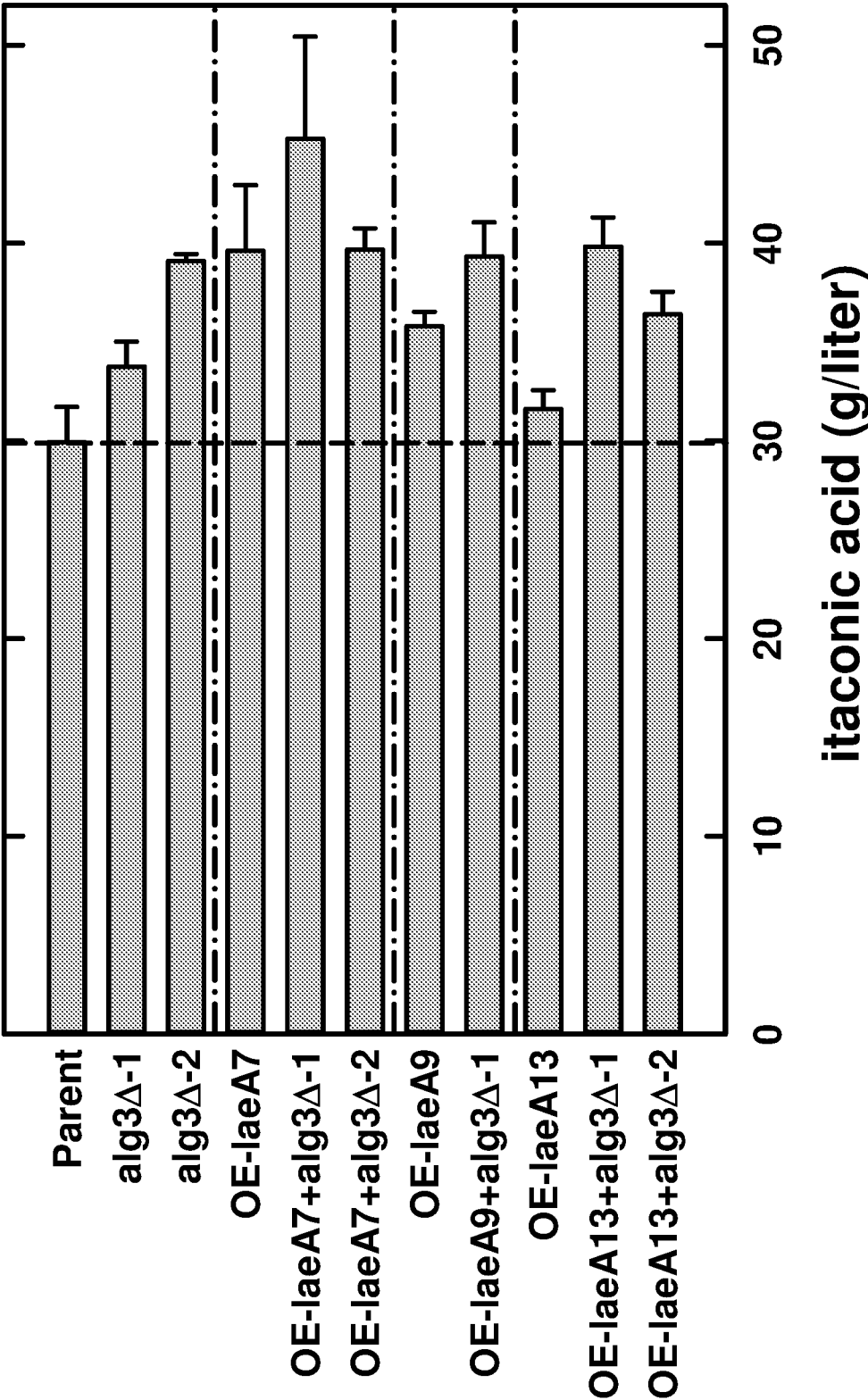


FIG. 28
Itaconic acid production in *A. terreus* after 6 days culture



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/46122

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/46122

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 10-12, 19-23
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/46122

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12 P 7/44, C07 K 14/38 (2016.01)

CPC - C12P 7/44, C07K 14/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - C12 P 7/44, C07 K 14/38 (2016.01)

USPC - C12P 7/44, C07K 14/38

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/142, 435/254.3

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

pubWEST; PatBase; Google Scholar

search terms - LaeA, loss of aflR expression A, terreus, itaconic acid, citric acid, fungus, fungi, methylenesuccinic acid, methylene succinic acid, cultur*, distil*

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 2013/0137150 A1 (Battelle Memorial Institute et al.) 30 May 2013 (30.05.2013) abstract; para [0112]; [0113]; [0133]-[0144]; [0161]; [0164]; SEQ ID NOs: 41, 59.	1-9 ----- 13-18
Y	BENTLEY et al. "BIOSYNTHESIS OF ITACONIC ACID IN ASPERGILLUS TERREUS III. THE PROPERTIES AND REACTION MECHANISM OF cis-ACONITIC ACID DECARBOXYLASE." Journal of Biological Chemistry 226.2 (1957): 703-720. entire document, especially abstract, p. 704-705.	13-18

☐ Further documents are listed in the continuation of Box C.
 ☐

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

30 September 2016

Date of mailing of the international search report

31 OCT 2016

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774