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(54) Title: INCREASED VEGETATIVE GROWTH OF FUNGI

(57) Abstract: The present invention relates to the field of microbiology, specifically the field of fungi, especially Basidiomycetes.

Increased vegetative growth of fungi.

FIELD OF THE INVENTION

The present invention relates to the field of microbiology, specifically the field of fungi, especially Basidiomycetes.

BACKGROUND OF THE INVENTION

Filamentous fungi grow by means of tip-growing hyphae that branch subapically. As a result, a network of hyphae is formed that is called mycelium. The hyphae feed on organic material such as plant waste. To this end, they penetrate their substrate while secreting enzymes that degrade the plant polymers into molecules that can be taken up to serve as nutrients. Hyphal penetration of the substrate is facilitated by turgor pressure generated by the cytoplasm and by the rigidity of the surrounding cell wall. The composition of fungal cell walls is dynamic and varies between species, strains, environmental conditions (Bowman and Free 2006), and developmental stage (Wessels 1994). In general, fungal cell walls consist of (glyco)proteins and polysaccharides. Glucan and chitin are the most abundant polysaccharides in the cell wall. Chitin provides rigidity to the cell wall. It is composed of β-1-4 linked N-acetylglucosamine and its microfibrils form inter-chain hydrogen bonds (Rudall and Kenchington 1978; Ruiz-Herrera 1992; Kameda et al. 2005). Chitin accounts for 1-2 % of the cell wall dry mass of *Saccharomyces cerevisiae* (Klis et al. 2002), while it can make up 30% of the cell wall of filamentous ascomycetes (de Nobel et al. 2000). Glucan is predominantly present in the cell wall as linear chains of (1-3)-β-linked glucose that provide elasticity (Klis et al. 2002). Other glucans like (1-6)-β-glucan, (1-3)(1-4)-β-glucan (Inoue et al. 1996; Douglas 2001), (1-3)(1-6)-β-glucan, and (1-3)-α-glucan (Beauvais et al. 2013; Latge and Beauvais 2014) may also be present within the cell wall. The cell wall of the vegetative mycelium of *Schizophyllum commune*, as an example of a basidiomycete, consists of glucose (67.6%), N-acetylglucosamine (12.5%), mannose (3.4%), xylose (0.2%), amino acids (6.4%), and lipids (3.0%) (Sietsma and Wessels, 1977). These monomers make up chitin, glucan, and proteins as well as other, not yet identified cell wall constituents. The outer layer of the cell wall is a water-soluble mucilage consisting of (1,3)-β-linked glucose units with branches of single (1,6)-β-linked glucose molecules at every third glucose along the chain (Wessels et al., 1972). This mucilage, known as schizophyllan,

is also secreted into the culture medium. An alkali-soluble glucan consisting of (1,3)-α-linked glucose units, known as S-glucan, is located beneath the mucilage and accounts for about half of the thickness of the water-insoluble portion of the wall. The inner layer of the cell wall consists of an alkali-insoluble glucan, known as R-glucan, and chitin (Wessels, 1994). The R-glucan was found to be a highly branched (1,3)(1,6)-β-glucan. Part of this highly insoluble glucan has structural similarity to schizophyllan. Most of the R-glucan is linked to chitin (Sietsma and Wessels, 1981) via basic amino acids and N-acetylglucosamine (Sietsma and Wessels, 1977).

Mycelium initially grows vegetative but at a certain moment asexual or sexual development is initiated. Thus, vegetative growth occurs when the mycelium is not yet differentiated to allow asexual or sexual development and / or when environmental conditions suppress these developmental processes. Strains in which genes (e.g. regulatory genes) have been inactivated may be unable to develop reproductive structures and may therefore always grow vegetative. It should be noted that during asexual or sexual development vegetative growth may continue.

Schizophyllum commune is an example of a model system to study sexual development in filamentous fungi and in particular that of basidiomycetes. The life cycle of *S. commune* starts with a monokaryotic (i.e. homokaryotic) mycelium that results from the germination of a basidiospore. Monokaryotic mycelia are sterile and always grow vegetative. A fertile dikaryon (i.e. heterokaryon) is formed upon fusion of two monokaryons with compatible mating types. Blue light is required to initiate fruiting in the dikaryotic mycelium (Perkins and Gordon 1969), whereas high CO_2 levels repress this developmental program (Niederpruem 1963; Raudaskoski and Viitanen 1982). So, in the dark and / or at high CO_2 levels the dikaryon grows vegetative, while at ambient CO_2 levels and in the light fruiting bodies are formed. Initiation of fruiting body formation by *S. commune* starts with asymmetrical colony growth. This means that vegetative growth is reduced; in some parts stronger than in other parts of the colony. Asymmetrical colony growth is followed by aggregation of aerial hyphae, and subsequent formation of primordia. These primordia can develop into fruiting bodies that form basidia within the hymenium. Karyogamy, meiosis, and one round of mitosis occur in the basidia, resulting in haploid, binucleate basidiospores.

The fruiting bodies of *S. commune* are an example of a mushroom. Mushrooms are the most conspicuous fungal structures. Mushrooms may be defined as a fleshy, spore-

bearing fruiting body of a fungus, typically produced above ground on soil or on its food source. A typical example of a "mushroom" is the cultivated white button mushroom, *Agaricus bisporus*, hence the word mushroom is most often applied to those fungi (*Basidiomycota*, *Agaricomycetes*) that have a stem (stipe), a cap (pileus), and gills (lamellae, sing. lamella) on the underside of the cap. Mushrooms may also have pores instead of lamellae. The word "mushroom" is also used for a wide variety of fungal fruiting bodies that produce sexual spores and that either or not have stems, and the term is used even more generally, to describe both the fleshy fruiting bodies of some *Ascomycota* and the woody or leathery fruiting bodies of some *Basidiomycota*. Forms deviating from the standard morphology usually have more specific names, such as “bracket”, "puffball", "stinkhorn", and "morel", and gilled mushrooms themselves are often called "agarics" in reference to their similarity to *Agaricus* or their place *Agaricales*. Mushrooms are *inter alia* used as a food source and for their therapeutic compounds (Kües and Liu 2000).

Regulation of mushroom formation has been studied in *S. commune* (Ohm et al., 2010b, 2011; 2013). The blue light receptor complex consists of Wc-1 that has a blue light sensing domain and the transcription factor Wc-2. Inactivation of *wc-1* and / or *wc-2* results in a blind phenotype. Dikaryotic colonies of the homozygous deletion strains grow symmetrically in blue light (similar to dark-grown wild-type dikaryons) and do not produce aggregates, primordia, and fruiting bodies. Deletion of the homeodomain gene *hom2* and the DNA binding Bright domain protein gene *briI* shows a similar phenotype. In contrast, inactivation of the zinc finger transcription factor gene *fst4* results in dikaryons that still grow irregular in the light under low CO_2 conditions but aggregates, primordia, and fruiting bodies are not produced. Strains in which the Cys2His2 zinc finger protein gene *c2h2* has been inactivated are arrested at the aggregate stage (Ohm et al. 2011). On the other hand, deletion strains of *fst3*, *gat1* or *hom1* form smaller fruiting bodies but in higher numbers. The zinc finger protein Fst3 was proposed to play a role in repression of outgrowth of fruiting bodies from primordia. On the other hand, Gat1, a GATA type zinc finger protein, and Hom1, a homeodomain protein, have been proposed to play a role in expansion of the fruiting body. Homologues of the *S. commune* transcription factors have been identified in *Agaricus bisporus*, *Laccaria bicolor* and *Coprinopsis cinerea*. Expression studies suggest the existence of a core regulatory program for fruiting body development in basidiomycetes (Ohm et al. 2010b; Morin et

al. 2012; Plaza et al. 2014). Variations in expression of these genes would explain species-specific morphology and environmental sensing, i.e. phenotypical features during growth under fruiting conditions. In addition to understanding of species-specific morphology and environmental sensing, it would be of great value if modulators of vegetative growth would be known since all art cited here above relates to phenotypical features during growth under fruiting conditions.

SUMMARY OF THE INVENTION

In an aspect, the present invention provides for a fungus derived from a parental fungus, wherein said fungus exhibits:

- a decreased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of: Wc-1, Wc-2, Hom2, Fst4 and Tea1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions, and/or has
- an increased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of Bri1 and Hom1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

wherein the decreased and/or increased expression level results in increased vegetative growth of said fungus compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions. Preferably, the fungus according to claim 1, wherein the fungus is a filamentous fungus. Preferably, the fungus is an Ascomycete or a Basidiomycete, preferably a Basidiomycete. More preferably, the fungus is a mushroom forming Basidiomycete. Preferably, at least the expression level of a polynucleotide encoding Fst4 is decreased compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions, and wherein the expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of: Wc-1, Wc-2, Hom2, and Tea1 is decreased, and/or wherein the expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of Bri1 and Hom1 is increased. Preferably, said fungus comprising a polynucleotide encoding a compound of interest.

The invention further provides a method of culturing a fungus according to any one of the preceding claims, comprising culturing said fungus under conditions conducive to the production of said fungus and, optionally, isolating and/or purifying said fungus.

The invention further provides a method for the production of a fungus and/or a mushroom, comprising contacting a fungus according to the invention with a substrate and with a fungus able to produce mushrooms, wherein said fungus according to the invention grows more rapidly in the substrate. The fungus able to produce mushrooms may either or not fuse with the fungus according to the invention.

The invention further provides for a method for the production of a compound of interest comprising culturing a fungus according to the invention under conditions conducive to the production of the compound of interest and, optionally, isolating and/or purifying the compound of interest.

The invention further provides for a method for the degradation of organic material comprising contacting a fungus according to the invention with an organic material and applying conditions suitable for the degradation of the organic material to the fungus contacted with the organic material.

The invention further provides for a method for the degradation of inorganic material comprising, contacting a fungus according to the invention with an inorganic material and applying conditions suitable for the degradation of the inorganic material to the fungus contacted with the inorganic material.

The invention further provides for a method for the production of a composite material, comprising contacting a fungus according to the invention with an organic and/or inorganic material and applying conditions suitable for the production of the composite material consisting of fungal mycelium and the organic and/or inorganic material.

The invention further provides for a method for the production of fungal mycelium comprising contacting a fungus according to the invention with an organic material and applying conditions suitable for the production of the fungal mycelium. Preferably, the composite material or the fungal mycelium is a construction material, an isolation material, a packaging material, a textile, a container, a material or device used for medical applications, a material or device used in horticulture, or a decoration material.

The invention further provides for a composite material or a fungal mycelium obtained or obtainable by the method here above.

DETAILED DESCRIPTION OF THE INVENTION

Surprisingly, it has now been demonstrated that several fungal genes involved in fruiting body development or in a broader context in sexual or asexual development are also involved in the modulation of vegetative growth of fungi, even under non-fruiting conditions, and thereby in determining the mechanical properties of the mycelium. Previously, several regulatory genes (i.e. *wc-1, wc-2, hom2, fst4*) have been described that are involved in mushroom development as well as asymmetrical growth during initiation of fruiting, (Ohm, 2010c; Ohm et al., 2013). However, it was unexpected that vegetative growth was also suppressed by these regulators under non-fruiting conditions.

Accordingly, in a first aspect the present invention provides a fungus derived from a parental fungus, wherein said fungus exhibits:

- a decreased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of: Wc-1, Wc-2, Hom2, Fst4 and Tea1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions, and/or has
- an increased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of Bri1 and Hom1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

wherein the decreased and/or increased expression level results in increased vegetative growth of said fungus compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions. Said fungus is herein referred to as a fungus according to the invention. Said polynucleotide is herein referred to as a polynucleotide according to the invention and said polypeptide is herein referred to as a polypeptide according to the invention.

Vegetative growth or vegetative propagation of a fungus is herein defined as any growth or propagation that does not involve the sexual or asexual stages of development. In filamentous fungi, hyphae are the main mode of vegetative growth, and are collectively called a mycelium.

Increased vegetative growth is herein defined as that more biomass is produced by the fungus according to the invention in a specified time unit, such as but not limited to an hour, a day, two days, three days, four days, five days or a week, compared to the parental fungus said fungus according to the invention is derived from when both are cultured and

assayed under identical conditions. The person skilled in the art knows how to measure the production of biomass. The produced biomass can e.g. be expressed in dry weight.

The term "expression" is to be construed as to include any step involved in the production of the peptide or polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification and, optionally, secretion.

A decreased expression level of a polynucleotide encoding a polypeptide is herein defined as a decreased activity of the polypeptide in the fungus according to the invention compared to the activity of the polypeptide in the parental fungus said fungus according to the invention is derived from. It follows that an increased expression level of a polynucleotide encoding a polypeptide is herein defined as an increased activity of the polypeptide in the fungus according to the invention compared to the activity of the polypeptide in the parental fungus said fungus according to the invention is derived from. Higher or lower activity of the polypeptide may be due to a respective higher or lower specific activity of the polypeptide (i.e. a higher or lower activity for the same amount of polypeptide) or may be higher or lower activity due to a respective higher or lower amount of the polypeptide (i.e. the specific activity of the polypeptide is the same where the amount is different). A combination of both higher amount and higher specific activity or lower amount and lower specific activity is also within the scope of the invention. The person skilled in the art knows how to determine polypeptide activity. An increased expression level or a decreased expression level may be achieved by any means known to the person skilled in the art. Increased expression may be achieved by recombinant techniques such as but not limited to overexpression by using a stronger promoter and/or introducing multiple copies of the gene to be overexpressed. Overexpression may also be achieved by other methods such as for example by increasing mRNA stability or by introducing introns. Increased expression may also be achieved by non-recombinant means, e.g. by classical mutagenesis of a parental fungus, preferably followed by screening for a mutant with increased expression level of the polypeptide. Likewise, decreased expression may be achieved by recombinant techniques such as but not limited to underexpression by using a weaker promoter or by removing one or multiple copies of the gene from the parental fungal cell. Decreased expression may also be achieved by other methods such as for example by decreasing mRNA stability, introduction of an inactivation construct and RNAi. Decreased

expression may also be achieved by non-recombinant means, e.g. by classical mutagenesis of a parental fungus, preferably followed by screening for a mutant with decreased expression level of the polypeptide. Alternatively or in combination with any of the herein mentioned ways of obtaining a fungus according to the invention, one may cross natural or non-natural isolates of fungi with different degrees of expression of a polynucleotide according to the invention.

Preferably, increased expression level of a polynucleotide encoding a polypeptide in a fungus according to the invention means at least 10%, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or at least 1000% higher expression level as compared to the expression level of the corresponding polypeptide in the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions. Likewise, decreased expression level of a polynucleotide encoding a polypeptide in a fungus according to the invention means at least 10%, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or at least 100% lower expression level as compared to the expression level of the corresponding polypeptide in the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions.

A “nucleic acid molecule” or “polynucleotide” (the terms are used interchangeably herein) is represented by a nucleotide sequence.

A “polypeptide” as used herein refers to any peptide, oligopeptide, polypeptide, gene product, expression product, or protein. A polypeptide is comprised of consecutive amino acids. The term "polypeptide" encompasses naturally occurring or synthetic molecules. A “polypeptide” is represented by an amino acid sequence.

Operably linked is defined herein as a configuration in which a control sequence is appropriately placed at a position relative to the nucleotide sequence coding for the polypeptide of the invention such that the control sequence directs the production of the polypeptide of the invention in a fungal cell and/or in a mushroom.

Expression will be understood to include any step involved in the production of the polypeptide including, but not limited to transcription, post-transcriptional modification, translation, post-translational modification and secretion.

Control sequence is defined herein to include all components, which are necessary or advantageous for the expression of a polypeptide. At a minimum, the control sequences include a promoter and transcriptional and translational stop signals.

As used herein, the term "promoter" refers to a nucleic acid fragment that functions to control the transcription of one or more genes or nucleic acids, located upstream with respect to the direction of transcription of the transcription initiation site of the gene, and is related to the binding site identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one skilled in the art to act directly or indirectly to regulate the amount of transcription from the promoter. Within the context of the invention, a promoter preferably ends at nucleotide -1 of the transcription start site (TSS).

A promoter is preferably capable of driving expression of the nucleotide sequence in a fungus and/or in a mushroom. Preferred promoters include: promoters that are constitutively expressed such as that of glyceraldehyde-3-phosphate-dehydrogenase (gpd) of *Schizophyllum commune* (Harmsen et al., 1992) and inducible promoters.

The polypeptide according to the invention, encoded by the polynucleotide according to the invention, is a polypeptide selected from the group consisting of Wc-1, Wc-2, Hom2, Fst4, Tea1, Bri1 and Hom1. The polynucleotide encoding the polypeptide preferably is a polynucleotide native (also referred to as wild-type) to the fungus according to the invention. Alternatively, the polynucleotide is heterologous to the fungus according to the invention. The polynucleotide may e.g. be a mutant, variant and/or may be codon optimized. The term "heterologous" in the context of a polynucleotide or a polypeptide as used herein refers to a polynucleotide or polypeptide that does not naturally occur in a host cell. With respect to a heterologous polynucleotide, the sequence has a portion which is not native to the cell in which it is expressed. With respect to a heterologous polypeptide, the sequence has a portion which is not native to the cell in which it is expressed.

The properties and activities of the polypeptides according to the invention are extensively described in the examples herein.

A polypeptide according to the invention may be Wc-1. Wc-1 has been studied in *Schizophyllum commune* and is part of the blue light receptor complex. A preferred Wc-1 is a polypeptide that has at least 40% sequence identity with SEQ ID NO: 1 and/or comprises a domain that has at least 50% sequence identity with SEQ ID NO: 24, and has Wc-1 activity.

A polypeptide according to the invention may be Wc-2. Wc-2 has been studied in *Schizophyllum commune* and is a transcription factor part of the blue light receptor complex. A preferred Wc-2 is a polypeptide that has at least 40% sequence identity with SEQ ID NO: 2 and/or comprises a domain that has at least 50% sequence identity with SEQ ID NO: 25, and has Wc-2 activity.

A polypeptide according to the invention may be Hom2. Hom2 has been studied in *Schizophyllum commune* and is a transcription factor homeodomain protein and functions in early stages of mushroom development. A preferred Hom2 is a polypeptide that has at least 40% sequence identity with SEQ ID NO: 3 and/or comprises a domain that has at least 50% sequence identity with SEQ ID NO: 26, and has Hom2 activity.

A polypeptide according to the invention may be Fst4. Fst4 has been studied in *Schizophyllum commune* and is a zinc-finger transcription factor and functions in early stages of mushroom development . A preferred Fst4 is a polypeptide that has at least 40% sequence identity with SEQ ID NO: 4 and/or comprises a domain that has at least 50% sequence identity with SEQ ID NO: 27, and has Fst4 activity.

A polypeptide according to the invention may be Tea1. Tea1 has been studied in *Schizophyllum commune* and is a TEA/ATTS domain transcription factor and functions downstream of the blue light receptor complex and of Hom2 and Fst4. A preferred Tea1 is a polypeptide that has at least 40% sequence identity with SEQ ID NO: 5 and/or comprises a domain that has at least 50% sequence identity with SEQ ID NO: 28, and has Tea1 activity.

A polypeptide according to the invention may be Bri1. Bri1 has been studied in *Schizophyllum commune* and is a transcription factor, a DNA binding Bright domain protein. A preferred Bri1 is a polypeptide that has at least 40% sequence identity with SEQ ID NO: 6 and/or comprises a domain that has at least 50% sequence identity with SEQ ID NO: 29, and has Bri1 activity.

A polypeptide according to the invention may be Hom1. Hom1 has been studied in *Schizophyllum commune* and is a transcription factor homeodomain protein. A preferred Hom1 is a polypeptide that has at least 40% sequence identity with SEQ ID NO: 7 and/or comprises a domain that has at least 50% sequence identity with SEQ ID NO: 30, and has Hom1 activity.

A polypeptide according to the invention preferably has at least 40% sequence identity with the respective amino acid sequence as described here above or comprises a domain

that has at least 50% sequence identity with the respective amino acid sequence as described here above. More preferably, a polypeptide according to the invention has a sequence identity of at least 42%, at least 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or most preferably 100% sequence identity with the respective amino acid sequence as described here above and or comprises a domain that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or most preferably 100% sequence identity with the respective amino acid sequence as described here above. Preferably, sequence identity is determined by comparing the whole length of the sequences as identified herein.

A polypeptide according to the invention preferably is a polypeptide native (also referred to as wild-type) to the fungus according to the invention. Alternatively, the polypeptide is heterologous to the fungus according to the invention and may e.g. be mutant or variant.

"Sequence identity" is herein defined as a relationship between two or more amino acid (polypeptide or protein) sequences or two or more nucleic acid (nucleotide, polynucleotide) sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between amino acid or nucleic acid sequences, as the case may be, as determined by the match between strings of such sequences "Identity" can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heine, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48:1073 (1988).

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include e.g. the GCG program package (Devereux et al., 1984). BestFit, BLASTP, BLASTN, and FASTA (Altschul, et al., 1990). The BLAST X program is publicly available from NCBI and other sources

(BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894). The well-known Smith Waterman algorithm may also be used to determine identity.

Preferred parameters for polypeptide sequence comparison include the following: Algorithm: Needleman and Wunsch (1970); Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff (1992); Gap Penalty: 12; and Gap Length Penalty: 4. A program useful with these parameters is publicly available as the "Ogap" program from Genetics Computer Group, located in Madison, WI. The aforementioned parameters are the default parameters for amino acid comparisons (along with no penalty for end gaps).

Preferred parameters for nucleic acid comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison matrix: matches=+10, mismatch=0; Gap Penalty: 50; Gap Length Penalty: 3. Available as the Gap program from Genetics Computer Group, located in Madison, Wis. Given above are the default parameters for nucleic acid comparisons.

Optionally, in determining the degree of amino acid similarity, the skilled person may also take into account so-called "conservative" amino acid substitutions, as will be clear to the skilled person. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulphur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Substitutional variants of the amino acid sequence disclosed herein are those in which at least one residue in the disclosed sequences has been removed and a different residue inserted in its place. Preferably, the amino acid change is conservative. Preferred conservative substitutions for each of the naturally occurring amino acids are as follows: Ala to ser; Arg to lys; Asn to gln or his; Asp to glu; Cys to ser or ala; Gln to asn; Glu to asp; Gly to pro; His to asn or gln; Ile to leu or val; Leu to ile or val; Lys to arg; gln or glu; Met to leu or ile; Phe to met, leu or tyr; Ser to thr; Thr to ser; Trp to tyr; Tyr to trp or phe; and, Val to ile or leu.

A fungus according to the invention may be any fungus known to the person skilled in the art. Preferably, the fungus according to the invention is a filamentous fungus. A preferred filamentous fungus is a strain selected from the group of filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth et al., In, Ainsworth and Bisby's Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK). Such filamentous fungal strains include, but are not limited to, strains of *Acremonium, Agaricus, Aspergillus, Aureobasidium, Chrysosporium, Coprinus, Cryptococcus, Filibasidium, Fusarium, Humicola, Magnaporthe, Mortierella, Mucor, Myceliophthora, Neocallimastix, Neurospora, Paecilomyces, Penicillium, Piromyces, Phanerochaete, Pleurotus, Schizophyllum, Talaromyces, Rasamsonia, Thermoascus, Thielavia, Tolypocladium, Trametes,* and *Trichoderma*. A further preferred filamentous fungal host cell according to the present invention is from a genus selected from the group consisting of *Acremonium, Aspergillus, Chrysosporium, Myceliophthora, Penicillium, Talaromyces, Rasamsonia, Thielavia, Fusarium* and *Trichoderma*; more preferably from a species selected from the group consisting of *Aspergillus niger*, *Acremonium alabamense, Aspergillus awamori, Aspergillus foetidus, Aspergillus sojae*, *Aspergillus fumigatus, Talaromyces emersonii*, *Rasamsonia emersonii, Aspergillus oryzae*, *Chrysosporium lucknowense, Fusarium oxysporum*, *Mortierella alpina*, *Myceliophthora thermophila, Trichoderma reesei, Thielavia terrestris*, and *Penicillium chrysogenum*.

A further preferred fungus is a fungus which produces a mushroom, preferably a mushroom that is attractive to be produced or which is suspected to be attractive to produce a substance or material of interest. Preferably, the fungus according to the invention is a *Basidiomycete* or an *Ascomycete*, preferably a *Basidiomycete*, more preferably a mushroom forming *Basidiomycete,* preferably a Polyporales or an Agaricales or a Schizophyllaceae, more preferably *Schizophyllum*. Further preferred is a *Schizophyllum commune*, more preferably *Schizophyllum commune* strain 4-8 (FGSC#9210). Preferred *Agaricales* are for example members of the genus *Agaricus* (e.g. Agaricus *bisporus*), *Pleurotus* (e.g. *Pleurotus ostreatus*), *Lentinus* (e.g. *Lentinus edodus*) *and Armillaria* (e.g. *Armillaria bulbosa*). Preferred Polyporales are species belonging to the genus *Trametes* (e.g. *Trametes versicolor*), and *Phanerochaete* (e.g. *Phanerochaete chrysosporium*)

Preferably, in a fungus according to the invention, a modification in view of the parental fungus is derived from can be a homozygous or a heterozygous modification; preferably such modification is a homozygous modification. A fungus according to the invention may be monokaryotic, dikaryotic, homokaryotic, heterokaryotic, or haploid, diploid, or aneuploid.

Preferably, in the fungus according to the invention, at least the expression level of a polynucleotide encoding Fst4 is decreased compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions, and the expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of: Wc-1, Wc-2, Hom2, and Tea1 is decreased, and/or the expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of Bri1 and Hom1 is increased. A preferred fungus according to the invention is a fungus wherein at least the expression level of a polynucleotide encoding Fst4 and of a polynucleotide encoding Hom2 is decreased compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions. Preferred fungi according to the invention are the ones provided in the examples herein.

A fungus according to the invention can conveniently be used for the production of a compound of interest. Accordingly, a preferred fungus according to the invention comprises a polynucleotide encoding a compound of interest. The fungus according to the invention may have been modified in order to be able to produce said compound of interest. The compound of interest may be any compound that can be produced by a fungus according to the invention. Such substance includes, but is not limited to, a protein, a polypeptide and a primary or secondary metabolite. A protein or polypeptide in this context may a pharmaceutical protein or polypeptide and/or a protein or polypeptide for interest for food, feed, or non-food, non-feed applications. Such compound may be native (endogenous) for the fungus according to the invention or may be foreign (heterologous) to the fungus according to the invention.

In an embodiment, a fungus according to the invention comprises a nucleic acid construct comprising a polynucleotide encoding the compound of interest to be produced. In this case, the compound of interest may be a polypeptide. Alternatively, a polypeptide encoded by the polynucleotide may be involved in the production or synthesis of a compound of interest. Also encompassed by the invention is a fungus which has been further modified by increasing/decreasing the expression level of a polypeptide known

to be involved in the production of the compound of interest. In an aspect, the invention provides for a method for the production of a compound of interest comprising culturing a fungus according to the invention under conditions conducive to the production of the compound of interest and, optionally, isolating and/or purifying the compound of interest.

In a further aspect, the invention provides for a method of culturing a fungus according to the invention, comprising culturing said fungus under conditions conducive to the production of said fungus and, optionally, isolating and/or purifying said fungus. Methods to culture fungi such as a fungus according to the invention are known to the person skilled in the art. Culturing may be performed in any scale from lab scale (in a petri dish or flask with a volume of tens of microliters to several litres) to industrial scale surface culture and industrial scale submerged culture (several litres to a several hundred cubic metres).

A fungus according to the invention can conveniently be combined with another fungus according to the invention or with a wild-type fungus; such wild-type fungus may e.g. be a fungus that is able to produce mushrooms. The fungi according to the invention may conveniently be used in the methods described in WO2011130247, which is herein incorporated by reference. Accordingly, in a further aspect, the invention provides for a method for the production of a fungus and/or a mushroom, comprising contacting a fungus according to the invention with a substrate and with a fungus able to produce mushrooms, wherein said fungus according to the invention grows more rapidly in the substrate (i.e. having increased vegetative growth as defined previously herein). The fungi used may either or not fuse with each other.

In an embodiment of the method according to this aspect of the invention, there is provided a method for the production of mushrooms, comprising:

- seeding a lower nutrient substrate layer with a first filamentous fungal inoculant which is a fungus according to the invention, so that said substrate becomes colonized with the inoculant according to the invention,
- overlaying said nutrient substrate with an upper water-holding substrate layer that has been inoculated with a second filamentous fungal inoculant, and allowing fruiting bodies, preferably mushrooms, to form,

wherein the increased vegetative growth of the first filamentous fungus inoculant results in increased growth of the fruiting bodies and wherein the second filamentous fungal inoculant preferably is a wild-type fungus able to produce fruiting bodies, preferably mushrooms.

In an embodiment of the method according to this aspect of the invention, there is provided a method of modulating the activity of a protein in a fruiting body, preferably a mushroom, of a filamentous fungus comprising:

- seeding a lower nutrient substrate layer with a first filamentous fungal inoculant which is a fungus according to the invention, so that said substrate becomes colonized with the inoculant, wherein said first filamentous fungal inoculant comprises and expresses a heterologous polynucleotide construct,
- overlaying said nutrient substrate with an upper water-holding substrate layer that has been inoculated with a second filamentous fungal inoculant, and allowing fruiting bodies, preferably mushrooms, to form,

wherein the polynucleotide construct from the first filamentous fungal inoculant construct provides modulation of the activity of a protein in the formed fruiting bodies, wherein the protein is a heterologous or an endogenous protein, and wherein the second filamentous fungal inoculant preferably is a wild-type fungus able to produce fruiting bodies, preferably mushrooms.

In an embodiment of the method according to this aspect of the invention, there is provided a method of modulating the activity of a protein in a filamentous fungus comprising:

- seeding a lower nutrient substrate layer with a first filamentous fungal inoculant which is a fungus according to the invention, so that said substrate becomes colonized with the inoculant, wherein said first filamentous fungal inoculant comprises and expresses a heterologous polynucleotide construct,
- overlaying said nutrient substrate with an upper water-holding substrate layer that has been inoculated with a second filamentous fungal inoculant, and allowing the second filamentous fungal inoculant to grow and colonize the substrate,

wherein the polynucleotide construct from the first filamentous fungal inoculant construct provides modulation of the activity of a protein in the mycelium of the second filamentous fungal inoculant, wherein the protein is a heterologous or an endogenous protein, and wherein the second filamentous fungal inoculant preferably is a wild-type

fungus able to produce fruiting bodies, preferably mushrooms. Herein a fungal inculant may be any part of the fungus that can used as an inoculant, such as but not limited to mycelium or a spore

In a further aspect, the invention provides for a method for the degradation of organic material comprising contacting a fungus according to the invention with an organic material and applying conditions suitable for the degradation of the organic material to the fungus contacted with the organic material. The organic material may be any material that can be used as a nutrient source by the fungus, such as but not limited to material originating from vegetative or reproductive parts of plants, animals, fungi, bacteria or protists such as algae; materials of plants such as saw dust e.g. from oak, beech, birch; cotton; bamboo; straw such as that of rice or wheat; coffee; tea; grape; molasses and silage. The organic material may be used as such or may be pre-treated such as by enzymes, chemicals and/or heat to improve the availability of the nutrients to the fungus according to the invention. The person skilled in the art is aware of pretreatment methods and of conditions suitable for the degradation of the organic material.

In a further aspect, the invention provides for a method for the degradation of an inorganic material comprising contacting a fungus according to the invention with an inorganic material and applying conditions suitable for the degradation of the inorganic material to the fungus contacted with the inorganic material. The inorganic material may be any inorganic material that can be used as a nutrient source by the fungus or that can be at least partly degraded or can be modified by the fungus according to the invention, such as but not limited to a plastic (such a polycarbonate, a polyethylene and a polypropylene), an aromatic hydrocarbon, a polyaromatic hydrocarbon, a dioxin and a furan. The inorganic material may be used as such or may be pre-treated such as by enzymes, chemicals and/or heat to improve the availability of the nutrients to the fungus according to the invention. The person skilled in the art is aware of pretreatment methods and of conditions suitable for the degradation of the inorganic material.

Within the scope of the invention is a combination of the two aspects here above; i.e. a method for the degradation of an inorganic material as well as an organic material.

In a further aspect, the invention provides for a method for the production of a composite material, comprising contacting a fungus according to the invention with an organic

and/or inorganic material and applying conditions suitable for the production of the composite material consisting of fungal mycelium and the organic and/or inorganic material. In the method for the production of a composite material, a mold may be used to shape the composite material and/or to control the conditions for the production of the composite material. Preferably, the composite material or the fungal mycelium is a construction material, an isolation material, a packaging material, a textile, a container, a material or device used for medical applications, a material or device used in horticulture, or a decoration material. The invention further provides for a composite material obtained or obtainable by the method according to this aspect. The invention further provides for a composite material comprising a fungus or a fungal mycelium according to the invention.

In a further aspect, the invention provides for a method for the production of fungal mycelium comprising contacting a fungus according to the invention with an organic material and applying conditions suitable for the production of the fungal mycelium. In the method for the production of a fungal mycelium, a mold may be used to shape the fungal mycelium and/or to control the conditions for the production of the fungal mycelium. Preferably, the fungal mycelium is used as a construction material, an isolation material, a packaging material, a textile, a container, a material or device used for medical applications, a material or device used in horticulture, or a decoration material; either or not with chemical (e.g. acid treatment), biological (e.g. enzyme treatment) or physical (e.g. heat treatment or using high pressure) pre- or post-treatments. The invention further provides for a fungal mycelium obtained or obtainable by the method according to this aspect.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

The word “about” or “approximately” when used in association with a numerical value (e.g. about 10) preferably means that the value may be the given value (of 10) more or less 0.1% of the value.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

Table 1. Sequences

SEQ ID NO	Gene / Polypeptide	Sequence
1	Wc-1	See sequence listing
2	Wc-2	See sequence listing
3	Hom2	See sequence listing
4	Fst4	See sequence listing
5	Tea1	See sequence listing
6	Bri1	See sequence listing
7	Hom1	See sequence listing
8	PCR primer Δ2519514ufw	GGCCTAATAGGCCTAGAATGCGCTCTCCGTC
9	PCR primer Δ2519514urv	GGCCTCGCAGGCCAGGGAGGATGACGCAAAG
10	PCR primer Δ2519514dfw	GGCCTGCGAGGCCGTCCGTGTTCTTGGATAC
11	PCR primer Δ2519514drv	GGCCTATTAGGCCGTGCGTTGTTTCGTTTCC
12	PCR primer 2519514 ufcfw	TCCACGCTGGCTGAATAG
13	PCR primer 2519514 dfcrv	TCGATGTGAGGTACTGTC
14	PCR primer Nourdelrev	TAAGCCGTGTCGTCA
15	PCR primer sc3tersqf	CCGGGAATTCCAGAT
16	PCR primer 2519514 ifw	CCGACTTCGATATCACTC
17	PCR primer 2519514 irv	TCGGCGATGCAAGAAGTC
18	PCR primer tea1 comp fw	CATGGCTAGCAGGTGATGCAGCGCGACGATAG
19	PCR primer tea1 comp rv	GGATCCTTAGATCATGAAAGCGCCGCC
20	PCR primer	GGCCTAATAGGCCCTGTCACGCACCAGTACG

	Δ2703923ufw	
21	PCR primer Δ2703923urv	GGCCTCGCAGGCCGGGCGAACGTGAGATAAG
22	PCR primer Δ2703923dfw	GGCCTGCGAGGCCGCTGTGGACGGTCTTAAC
23	PCR primer Δ2703923drv	GGCCTATTAGGCCCATTGCACGAGTCCATTC
24	Wc-1 sensory domain	LLEKTPDFVHVVSLKGSFLYVAPTVKRVLGYDPSELVGKGLSDICH PADVVPLMRELKEASSTGTESSGVTSASAGASKASAQQDPSMPRTV DLLFRAQMKTGRYVWMECRGRLHVEPGKGRKAIMLSGR
25	Wc-2 DNA binding domain	FTKRKRWADLLVTELADAIILVLGVPNPKILYCGAAVEELLGWRDT DVIDLDLTELM
26	Hom2 DNA binding domain	DYRTFFPYQPNEVKHRRRTTAVQLKVLEGIFKTETKPNAALRNKLA VQLEMTARGVQVWFQNRRAKEKLKASK
27	Fst4 DNA binding domain	LSPRRLALLLMVLSIGSLVDLKRPLGYLSAEAYHHLARASVCEIPL MEEPDFDTVHALFFMIWYHLIFSDNRKALGYAWNLLGFVAKLVQGV HRETSGSSKLIPEESERRRNIFWELLNLDYRMSLTLGRPPSIS
28	Tea1 DNA binding domain	SVTGRKAWKTMKGKGEAVWPPHLEKALLKGLAAYEPDTKSTKALNR FPRRNRFISEYIYQRTGEWRTAKQVSSRLQQLK
29	Bri1 DNA binding domain	RRKIEYVPFAREVDTFGGRDLAALEKYAEEARRRPIRDFNDWGNID VDHLIMSLRSRVATELSYALTTLSMLSAMR
30	Hom1 DNA binding domain	KKKRKRADANQLRVLNDVYMRTAFPSTEERHQLAKQLDMSPRSVQI WFQNKRQAMRSTNRQ

FIGURE LEGENDS

Figure 1. Biomass of 6-day-old dark-grown agar cultures of the wild-type dikaryon (H4-8) and dikaryotic transcription factor deletion strains using glucose as carbon source.

Figure 2. Biomass of 6-day-old dark-grown cultures of the wild-type dikaryon (H4-8) and dikaryotic transcription factor deletion strains that had been grown on agar medium (A) or as liquid shaken cultures (B) using glucose as a carbon source.

Figure 3. Biomass of 6-day-old dark-grown agar cultures of the wild-type dikaryon (H4-8) and dikaryotic transcription factor deletion strains using 4% xylose (A), 3.4% sucrose (B), and 1% pectin (C) as carbon source.

Figure 4. Fruiting body development of the wild-type dikaryon (A,D), Δ*bri1*Δ*bri1* (B, E), and Δ*hom1*Δ*hom1* (C, F) after 7 (A-C) and 15 (D-F) days of growth.

Figure 5. Principal component analysis of expression profiles of wild-type (H4-8) and deletion strain dikaryons during aggregation (A) and fruiting body formation (B).

Figure 6. Venn diagrams showing overlapping differentially expressed transcription factor genes in Δ*fst3*Δ*fst3,* Δ*hom1*Δ*hom1,* and Δ*gat1*Δ*gat1* (A) and Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4* (B) when compared to the wild-type aggregate and fruiting body stage, respectively.

Figure 7. Expression of *wc-1* (a), *wc-2* (b), *hom2* (c), *fst4* (d), *c2h2* (e), *fst3* (f), *gat1* (g), *hom1* (h), *bri1* (i), *tea1* (j), and *c2h2d* (k) in dikaryotic transcription factor deletion strains when compared to the wild-type during aggregation (A) and fruiting body formation (B).

Figure 8. Aerial growth of dikaryotic wild-type colonies (H4-8) (A) and Δ*tea1* (B, C, D). Strain Δ*tea1* produced less dense and high aerial hyphae when transferred to light (B) and only forms small clusters of fruiting bodies (C and D). Arrow indicates the transition from dense aerial hyphae production to thinner aerial hyphae production upon light induction.

Figure 9. Regulatory model of activation and repression of vegetative growth and initiation and maturation of fruiting body formation in *S. commune*. Transcription factor genes control both vegetative growth and fruiting body development (A) and influence each other's expression levels (B).

Figure 10. *S. commune* wild-type, Δhom2, and Δfst4 were grown at 30 °C as a thin layer in a Petri-dish in the light or in the dark at ambient or high CO_2 for 3 days.

Medium was introduced underneath the colony and growth of the floating mycelium was prolonged for 5 days. Initial Young's modulus in MPa (A), Maximum tensile strength at breaking in MPa (B), and elongation at breaking in % (C) of mycelia were determined and are depicted. Asterisks indicate * $p \leq 0.05$, ** $p \leq 0.005$ and *** $p \leq 0.0005$.

EXAMPLES

The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

Unless stated otherwise, the practice of the invention will employ standard conventional methods of molecular biology, virology, microbiology or biochemistry. Such techniques are described in Sambrook *et al.* (1989) Molecular Cloning, A Laboratory Manual (2nd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press; in Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual,* Third Edition, Cold Spring Harbor Laboratory Press, NY; in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology, Current Protocols*, USA; and in Volumes I and II of Brown (1998) *Molecular Biology LabFax*, Second Edition, Academic Press (UK); *Oligonucleotide Synthesis* (N. Gait editor); *Nucleic Acid Hybridization* (Hames and Higgins, eds.).

Example 1. Transcription factors of *Schizophyllum commune* that link vegetative growth and mushroom formation

Abstract

Transcription factors have been identified that are involved in mushroom formation in *Schizophyllum commune*. The DNA binding Bright domain protein BriI and the homeodomain protein Hom1 are involved in late stages of mushroom development, while the blue light receptor transcription factor Wc-2, the homeodomain protein Hom2, and the zinc-finger transcription factor Fst4 function in early stages of mushroom development. Here it is shown that BriI and Hom1 also stimulate vegetative growth, while biomass formation is repressed by Wc-2, Hom2, and Fst4. The Δ*bri1*Δ*bri1* and the Δ*hom1*Δ*hom1* strains formed up to 0.6 fold less biomass when compared to the wild-

type. In contrast, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4* strains formed up to 2.8 fold more biomass. RNA sequencing showed that repression of vegetative growth correlates with decreased expression of genes involved in carbohydrate metabolism. The TEA/ATTS domain transcription factor gene *tea1* was also downregulated in the Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2* and Δ*fst4*Δ*fst4* strains. The Δ*tea1*Δ*tea1* strain produced 1.3 fold more biomass than the wild-type and was severely affected in fruiting body development. Together, these data show that transcription factors Wc-2, Hom2, Fst4, and Tea1 link mushroom initiation and repression of vegetative growth.

Introduction

Mushrooms are the most conspicuous fungal structures. They are used as a food source or for their therapeutic compounds (Kües and Liu 2000). The formation of mushrooms involves a complex developmental program. *Coprinopsis cinerea* and *Schizophyllum commune* are the model systems to study this program (Kües and Navarro-González 2015). The life cycle of *S. commune* starts with a monokaryotic mycelium that results from the germination of a basidiospore. A fertile dikaryon is formed upon fusion of two monokaryons with compatible mating types. Blue light is required to initiate fruiting in the dikaryotic mycelium (Perkins and Gordon 1969), whereas high CO_2 levels repress this developmental program (Niederpruem 1963; Raudaskoski and Viitanen 1982). Initiation of mushroom formation starts with asymmetrical colony growth, followed by aggregation of aerial hyphae, and subsequent formation of primordia. These primordia can develop into fruiting bodies that form basidia within the hymenium. Karyogamy, meiosis, and one round of mitosis occur in the basidia, resulting in haploid, binucleate basidiospores.

Regulation of mushroom formation has been studied in *S. commune*. The blue light receptor complex consists of Wc-1 that has a blue light sensing domain and the transcription factor Wc-2. Inactivation of *wc-1* and / or *wc-2* results in a blind phenotype (Ohm et al. 2013). Dikaryotic colonies of the homozygous deletion strains grow symmetrically in blue light (similar to dark-grown wild-type dikaryons) and do not produce aggregates, primordia, and fruiting bodies. Deletion of the homeodomain gene *hom2* and the DNA binding Bright domain protein gene *briI* shows a similar phenotype (Ohm et al. 2011). In contrast, inactivation of the zinc finger transcription factor gene *fst4* results in dikaryons that still grow irregular in the light under low CO_2 conditions

but aggregates, primordia, and fruiting bodies are not produced (Ohm et al. 2011). Strains in which the Cys2His2 zinc finger protein gene *c2h2* has been inactivated are arrested at the aggregate stage (Ohm et al. 2011). On the other hand, deletion strains of *fst3*, *gat1* or *hom1* form smaller fruiting bodies but in higher numbers (Ohm et al. 2011). The zinc finger protein Fst3 was proposed to play a role in repression of outgrowth of fruiting bodies from primordia. On the other hand, Gat1, a GATA type zinc finger protein, and Hom1, a homeodomain protein, have been proposed to play a role in expansion of the fruiting body. Homologues of the *S. commune* transcription factors have been identified in *Agaricus bisporus*, *Laccaria bicolor* and *C. cinerea*. Expression studies suggest the existence of a core regulatory program for fruiting body development in basidiomycetes (Ohm et al. 2010b; Morin et al. 2012; Plaza et al. 2014). Variations in expression of these genes would explain species-specific morphology and environmental sensing.

In this study, it is shown that Wc-1, Wc-2, Hom2, and Fst4 not only initiate mushroom formation but also repress vegetative growth. On the other hand, BriI and Hom1 were shown to stimulate biomass formation. Whole genome expression analysis indicates that repression of vegetative growth is the result of down-regulation of genes involved in carbohydrate metabolism. Expression analysis also revealed that the TEA/ATTS domain transcription factor gene *tea1* is down regulated in Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, and Δ*fst4*Δ*fst4*. Inactivation of *tea1* resulted in increased vegetative growth, and severely reduced formation of fruiting bodies. These data indicate that Tea1 functions downstream of the blue light receptor complex, Hom2 and Fst4.

Experimental procedures

Culture conditions and strains

The compatible *S. commune* strains H4-8 (*mat*A43*mat*B41; available *inter alia* at the fungal [http://www.fgsc.net/], accession number FGSC 9210) (Ohm et al. 2010b) and H4-8b (*mat*A41*mat*B43) (Ohm et al. 2010a), their derived dikaryotic deletion strains Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2*, Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1*, Δ*bri1*Δ*bri1*, Δ*gat1*Δ*gat1* (Ohm et al. 2010b; 2011, 2013), as well as Δ*hom2*Δ*hom2*Δ*fst4*Δ*fst4* and Δ*hom2*Δ*hom2*Δ*fst3*Δ*fst3* were used in this study. The Δ*ku80* H4-8 strain (de Jong et al. 2010) was used for gene inactivation. Strains were grown in the dark or in the light (1200 lux white LED light; Conrad Electronic, Hirschau, Germany) at 25 °C on minimal medium (MM) containing 1% glucose and 1.5% agar, if

applicable (van Peer et al. 2009). Liquid shaken cultures were inoculated with a mycelial homogenate (van Wetter et al. 2000) and grown at 250 rpm in 250 ml Erlenmeyers containing 100 ml MM. Agar cultures were inoculated with a point inoculum taken from the periphery of a 7-day-old colony. To assess growth on other carbon sources, glucose was replaced for 4% xylose, 3.4% sucrose, or 1% pectin.

Gene inactivation

Deletion vectors for *tea1* (Protein ID 2519514; http://genome.jgi-psf.org/Schco3) and *c2h2d* (Protein ID 2703923) were constructed using pDelcas that contains a nourseothricine and a phleomycin resistance cassette (Ohm et al. 2010a). Upstream and downstream flanks of *tea1* and *c2h2d* were cloned at either site of the nourseothricine resistance cassette. To this end, the flanks were amplified by PCR using Taq polymerase and H4-8 chromosomal DNA as template. The 906 bp upstream flank and the 946 bp downstream flank of *tea1* were amplified using the primer combination Δ9519514ufw/Δ9519514urv and Δ9519514dfw/Δ9519514drv, respectively (Table 1). Primer pair combinations Δ2703923ufw/Δ2703923urv and Δ2703923dfw/Δ2703923drv were used to amplify the 897 bp upstream and 975 bp downstream flank of *c2h2d*, respectively (Table 1). The PCR products were cloned into pGEM-T Easy (Promega, Madison, USA). The upstream flanks were retrieved from the resulting constructs using Van91I and introduced into the Van91I site of pDelcas, resulting in pDel-2519514-UF and pDel-2703923-UF. The downstream flanks were retrieved from the pGEM-T easy derived constructs using SfiI and introduced into the SfiI site of pDel_2519514-UF and pDel_2703923-UF. This resulted in the knock-out constructs pDelcas-2519514 and pDelcas-2703923.

Transformation

Deletion constructs were introduced in H4-8Δ*ku80*. Transformation was done as described (van Peer et al. 2009). $1 \cdot 10^7$ protoplasts were incubated with 20 µg vector DNA and regenerated overnight without antibiotic. Selection took place for 4 days at 30 °C on MM plates containing 8 µg ml^{-1} nourseothricin. Transformants were transferred to a second selection plate containing 20 µg ml^{-1} phleomycin to distinguish between homologous and ectopic integrations. Gene deletion was confirmed by PCR using primers outside the flanks and inside the nourseothricin cassette. Primer pairs

Δ2519514ufcfw/nourdelrev and Δ2519514dfcrv/sc3tersqf were used to screen for *tea1* deletion (Table 1), while primer pairs c2h2D UFCFW/nourdelrev and c2h2D DFCRV/sc3tersqf were used to confirm *c2h2d* deletion (Table 1).

Biomass of colonies

Colonies were grown as liquid shaken cultures or on agar medium on a PC-membrane (diameter 76 mm, pore size 0.1 μm; Osmonics, GE Water Technologies). Mycelium of liquid cultures was separated from the medium using Miracloth filter (Merck Millipore, Billerica, USA). Mycelium was freeze-dried and weighed. Statistical analyses was done with an independent sample t-test (p-value ≤ 0.05) using IBM SPSS 20.

Whole genome expression analysis

The wild type dikaryon and strains Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2*, Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1*, Δ*bri1*Δ*bri1,* and Δ*gat1*Δ*gat1* were grown for 5 days in the dark at 22˚C, after which they were transferred to the light. Colonies (biological duplicates) were harvested at the moment the wild-type dikaryon formed aggregates (day 8) or fruiting bodies (day 12). H4-8 colonies were also harvested at the moment they were transferred to the light (day 5) and when they had formed primordia (day 10). Mycelium was frozen in liquid nitrogen and homogenized using the TissueLyser II (Qiagen, Düsseldorf, Germany). RNA was extracted using TriZol (Life technologies, Carlsbad, USA) and purified using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany). Quality of RNA was checked using the BioAnalyzer and sent to ServiceXS (Leiden, the Netherlands) for Illumina Next Generation Sequencing.

RNA-Seq Analysis Pipeline

The RNA-Seq pipeline used the STAR aligner (Dobin et al. 2013) to align the 100 bp paired end reads to the *S. commune* v3.0 genome (http://genome.jgi-psf.org/Schco3/Schco3.home.html). The size of introns was limited to a maximum of 1500 bp based on the largest intron sizes in the genome annotation.

Abundance estimation and differential expression were performed by Cufflinks version 2.1.1 (Trapnell et al. 2012a), and Cuffdiff (Trapnell et al. 2012b). Enrichments of GO terms were analysed within sets of differentially expressed genes. Proteins annotated to

contain a DNA-binding or regulatory protein domain were defined as transcription factors (Ohm et al. 2010b).

Results

Genes involved in mushroom development also control vegetative growth

Dikaryotic colonies of wild-type H4-8, Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*bri1*Δ*bri1,* Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2*, Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1*, and Δ*gat1*Δ*gat1* were grown for 6 days on PC membranes on glucose MM in the dark. The Δ*hom2*Δ*hom2* strain formed 1.4-fold more biomass when compared to H4-8, while Δ*bri1*Δ*bri1* and Δ*hom1*Δ*hom1* formed 0.4 and 0.6-fold less biomass, respectively (Figure 1). Biomass formation of the other strains was not significantly different from H4-8. Strains Δ*hom2*Δ*hom2*Δ*fst3*Δ*fst3* and Δ*hom2*Δ*hom2*Δ*fst4*Δ*fst4* also formed more biomass than the wild-type. Biomass of both strains was similar to that of Δ*hom2*Δ*hom2* in the case of the agar cultures (Figure 2A). Notably, the Δ*hom2*Δ*hom2*Δ*fst4*Δ*fst4* strain formed 1.7 fold more biomass in liquid shaken cultures when compared to Δ*hom2*Δ*hom2* (Figure 2B). These data show that *bri1* and *hom1* stimulate vegetative growth, while *hom2* and *fst4* repress biomass formation of dikaryotic strains when glucose is used as a carbon source.

Biomass formation of Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2* and Δ*fst4*Δ*fst4* was assessed on xylose, pectin, and sucrose (Figure 3). The Δ*fst4*Δ*fst4* strain formed 2.8-fold more biomass on xylose when compared to H4-8 (Figure 3A). Moreover, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4* formed more biomass on sucrose (1.2, 1.4, and 1.6-fold, respectively) and pectin (1.3, 1.4, and 1.6-fold, respectively) when compared to H4-8 (Figure 3BC). Together, these data show that Wc-2, Hom2 and Fst4 repress vegetative growth on different carbon sources.

Reduced vegetative growth of the Δ*bri1*Δ*bri1* and Δ*hom1*Δ*hom1* strains may slow down fruiting body development. Therefore, fruiting was monitored after 7 days (Ohm et al. 2011) and 15 days of culturing. The Δ*hom1*Δ*hom1* strain formed more but smaller mushrooms both after 7 days and 15 days (Figure 4). In contrast, Δ*bri1*Δ*bri1* had not formed fruiting bodies after 7 days but did so after 15 days showing that fruiting in this strain is delayed and not abolished as reported previously (Ohm et al. 2011).

Genome-wide expression analysis

RNA composition of wild-type H4-8 was determined during vegetative growth in the dark, after transfer to the light, and during aggregate, primordium, and fruiting body formation. Expression of *wc-1*, *wc-2*, *hom2*, *fst4*, *fst3*, *gat1,* and *bri1* changed less than 2-fold during development when compared to the vegetative mycelium grown in the dark (Table 2). In contrast, *c2h2* and *hom1* expression increased gradually with a maximum fold change of 4.7 and 2, respectively, during the fruiting body stage.

Expression profiles of wild type, Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2*, Δ*fst3*Δ*fst3*, Δ*gat1*Δ*gat1*, Δ*hom1*Δ*hom1,* and Δ*bri1*Δ*bri1* strains were compared at the moment the wild-type formed aggregates (day 8) and fruiting bodies (day 12). Principal component analysis of the RNA profiles of 8-day-old colonies revealed a first and second component explaining 38 and 29% of the variation, respectively. Two distinct clusters were observed (Figure 5A). The first cluster consisted of Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2,* and Δ*hom2*Δ*hom2* that are all affected in early stages of fruiting body development. The second cluster consisted of Δ*gat1*Δ*gat1* and Δ*fst3*Δ*fst3* that are affected in late stages of development. The other deletion strains did not cluster but rather showed a gradual change in expression. Principal component analysis of the RNA profiles of 12-day-old colonies revealed a first and second component explaining 72 and 7% of the variation, respectively. In this case, Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2,* Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4* clustered, whereas the other strains clustered with the wild-type (Figure 5B).

The number of up- and down-regulated genes were between 86 and 1392 and 131 and 1463, respectively, when expression of the 8- and 12-day-old cultures of the deletion strains was compared with the wild type (Table 3). Enriched functional categories in the upregulated genes of 8-day-old colonies of Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4* were mainly linked to carbohydrate metabolism, when compared to the 8-day-old wild-type colonies (Table 4). Downregulated functional groups were linked to energy transfer in these strains. Functional groups involved in energy transfer and carbohydrate metabolism were upregulated in 12-days-old colonies of Δ*wc-1*Δ*wc-1* and Δ*wc-2*Δ*wc-2* (Table 5). Downregulated genes were enriched for nucleosome, catalytic and oxidoreductase activity for Δ*wc-1*Δ*wc-1* and Δ*wc-2*Δ*wc-2*. The functional group ATPase activity was also overrepresented in the downregulated genes of Δ*wc-2*Δ*wc-2*. In 12-day-old colonies of Δ*hom2*Δ*hom2* functional groups involved in translation,

energy transfer, and carbohydrate metabolism were overrepresented in the upregulated genes, while functional groups involved in transport and energy transfer were overrepresented in the downregulated genes. In 12-days-old colonies of Δ*fst4*Δ*fst4* functional groups involved in carbohydrate processes and energy transfer were enriched in the upregulated genes. Groups involved in oxidoreductase activity, metabolic process and nucleosome, amongst others, were enriched in the downregulated genes. Functional categories involved in energy transfer and carbohydrate metabolism were upregulated in 8-day-old colonies of Δ*c2h2*Δ*c2h2*, while processes involved in cytoplasm, ATP binding, nucleosome and peptidase activity were enriched in the downregulated genes. In 12-day-old colonies of Δ*c2h2*Δ*c2h2* upregulated genes were enriched in functional groups related to energy transfer, carbohydrate metabolic process, and chitin catabolic process. Downregulated genes were enriched in groups involved in cell wall, catalytic activity, tryptophan and fatty acid synthesis. No functional groups were overrepresented in the upregulated genes in 8-day-old and 12-day-old colonies of Δ*hom1*Δ*hom1* but functional groups related to chitinase activity, carbohydrate metabolism and energy transfer were enriched in the downregulated genes. 12-day-old Δ*hom1*Δ*hom1* colonies showed overrepresentation of functional groups related to amino acid synthesis, oxidoreductase, and carbohydrate metabolism activity in the down regulated genes. The functional categories that were enriched in the upregulated genes of 8-day-old colonies of Δ*fst3*Δ*fst3* and Δ*gat1*Δ*gat1* were mainly involved in energy transfer, tryptophan metabolism, and cell wall processes. Genes involved in energy transfer and carbohydrate metabolism were enriched in the down-regulated genes. Groups involved in carbohydrate metabolism, methyltransferase, and leucine synthesis were enriched in the upregulated genes in 12-days-old colonies of Δ*fst3*Δ*fst3*. Downregulated genes were mainly enriched in cell wall processes and chitinase activity. Functional groups were upregulated for sphingolipid metabolism in 12-days-old colonies of Δ*gat1*Δ*gat1*. Downregulated genes were mainly enriched for translation and cell wall. No functional groups were overrepresented in the upregulated genes of 8-day-old and 12-day-old colonies of the Δ*bri1*Δ*bri1* strain. In contrast, functional groups related to metabolic process, carbohydrate metabolism, transcription and cell wall were overrepresented in the downregulated genes of 8-day-old colonies. Downregulated genes in 12-day-old Δ*bri1*Δ*bri1* colonies were mainly enriched for carbohydrate metabolism, hydrolase activity and transcription repressor activity.

Between 1 and 42 transcription factor genes were exclusively ≥ 2-fold differentially expressed in one of the deletion strains when compared to the wild-type (Figure 6). Expression of *hom1* and *c2h2* had decreased in 12-day-old Δ*wc-1*Δ*wc-1* colonies. In 12-day-old colonies of Δ*wc-2*Δ*wc-2* *hom2* expression was increased, while *c2h2* was downregulated. Expression of *c2h2*, *gat1,* and *hom1* was downregulated in 12-day-old Δ*hom2*Δ*hom2* colonies. In 8-day-old Δ*fst4*Δ*fst4* colonies expression of *hom1* was downregulated and in 12-day-old colonies *hom2* expression increased, while *c2h2* and *hom1* expression decreased. In contrast, *hom1* levels were upregulated in 8-day-old Δ*fst3*Δ*fst3* colonies. Similarly, expression of *hom1* and additionally *c2h2* was increased in 8-day-old colonies of Δ*gat1*Δ*gat1*. In 8-day-old Δ*bri1*Δ*bri1* colonies *gat1* expression was increased, while *hom2* was increased in 12-day-old colonies (Figure 7).

Gene tea1 is involved in fruiting body development and represses vegetative growth

Transcription factor gene *tea1* (protein ID 2519514) showed ≥ 2-fold decreased expression in Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2,* Δ*hom2*Δ*hom2,* Δ*briI*Δ*briI,* and Δ*fst4*Δ*fst4* when compared to the wild-type during aggregation (8-day-old colonies), while it was upregulated ≥ 2-fold in Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1,* and Δ*gat1*Δ*gat1*. A ≥ 2-fold differential expression of *tea1* was not observed when the wild-type had formed fruiting bodies (12-day-old colonies), explained by reduced expression of *tea1* in 12-day-old wild-type colonies. Gene *c2h2d* (proteinID 2703923) was also downregulated in Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2,* Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4*, while it was upregulated ≥2-fold in Δ*gat1*Δ*gat1* during aggregation (Figure 7). Furthermore, expression of *c2h2d* was decreased >2-fold in 12-day-old colonies of Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2,* Δ*hom2*Δ*hom2,* Δ*fst4*Δ*fst4*, Δ*fst3*Δ*fst3,* and Δ*gat1*Δ*gat1*. Gene *c2h2d* has a predicted C2H2 DNA binding domain and *tea1* is a predicted TEA/ATTS transcription factor. Expression of *tea1* and *c2h2* in the wild-type strain peaked during primordia and fruiting body formation, respectively (Table 2).

Deletion constructs were made for *c2h2d* and *tea1*. No deletion strains were obtained for *c2h2d* after screening 100 transformants. In contrast, deletion of *tea1* in the H4-*8*Δ*ku80* *was* successful. PCR analysis confirmed inactivation of *tea1* in one of the transformants. The transformant was crossed with the compatible wild-type H4-8b and siblings were selected with a deleted *tea1* gene and an intact *ku80* gene. One of these siblings was crossed with a H4-8 strain to obtain a Δ*tea1* strain with compatible mating types. These

monokaryons were then crossed to obtain the Δ*tea1*Δ*tea1* strain. The Δ*tea1*Δ*tea1* strain showed a 1.3-fold increase in biomass when compared to the wild-type. This increase was similar to that of Δ*hom2*Δ*hom2* (Figure 1). Transfer to the light did not induce irregular vegetative growth as observed in the wild-type. Interestingly, newly formed light-exposed mycelium did not produce aerial hyphae (Figure 8B). This resulted in a distinct border between the dark-grown mycelium and mycelium grown in the light. Fruiting body formation was almost completely abolished. Instead of the typical ring of fruiting bodies, only local clusters of fully-developed mushrooms were formed (Figure 8CD). These clusters were made at random positions in the colony; they were not restricted to the center, or the periphery. Together, these data show that *tea1* represses vegetative growth and promotes fruiting body formation in the wild-type dikaryon.

Discussion

The transcription factor genes *wc-2*, *hom2*, *fst4*, *briI*, *c2h2*, *fst3*, *gat1*, and *hom1* have been reported to be involved in fruiting body formation in *S. commune* (Ohm et al. 2010b; 2011; 2013). We here showed that the homeodomain domain protein Hom2, the zinc finger transcription factor Fst4 and the blue light complex transcription factor Wc-2 are not only involved in early stages of fruiting body development but also repress vegetative growth. In contrast, the DNA binding BRIGHT domain protein BriI and the homeodomain protein Hom1 stimulated vegetative growth. The latter protein is involved in late stages of mushroom development, while BriI was shown not to be essential for fruiting, although in its absence fruiting is delayed. Based on phenotypic analysis, whole genome expression analysis of the deletion strains, and inactivation of gene *tea1* we propose a modified model for mushroom development (Figure 9).

The Δ*bri1*Δ*bri1* strain does not fruit after the standard growth period of 10 days (Ohm et al. 2011). However, we here showed that fully developed mushrooms had formed after 4 weeks. This shows that BriI is not required for fruiting. Delayed mushroom development may be the result of reduced growth speed. Lower biomass formation may well be explained by the fact that functional categories metabolic process, carbohydrate metabolism, catalytic activity, transcription, and cell wall are down-regulated in the deletion strain in 8-day-old colonies. 12-day-old colonies showed down-regulation of functional categories carbohydrate metabolism, hydrolase activity, and transcription repressor activity. As a consequence of reduced biomass formation, a quorum sensing

pathway may become activated at a later moment delaying the switch to fruiting body formation (Wösten and Willey 2000). Notably, BriI deletion has an effect on expression of *tea1* and of *gat1*. The repression of *tea1* may be a direct or an indirect effect due to its stimulatory effect on *gat1* expression. Together, BriI stimulates vegetative growth and has an effect on expression of the transcription factors *tea1* and *gat1* that are involved in mushroom formation (Figure 9).

Genes *hom2* and *wc-2* are involved in the switch from vegetative growth to fruiting. Inactivation of these genes abolishes early stages of fruiting body formation (Ohm et al. 2011; 2013) but also increased the vegetative growth rate. Strain Δ*wc-2*Δ*wc-2* formed more biomass on sucrose and pectin when compared to the wild-type, while Δ*hom2*Δ*hom2* formed more biomass on glucose, sucrose, and pectin. Expression of *hom2* and *wc-2* is rather constant during development, suggesting post-transcriptional regulation of these genes. In the case of Wc-2, this may be accomplished by the interaction with the blue light sensor Wc-1 (Ohm et al. 2013). Preliminary results indicate that Hom2 activity is regulated by phosphorylation of its Pka sites (data not shown). Increased growth rate was associated with enrichment of upregulated genes associated to carbohydrate metabolism in 8-day-old colonies, and of functional groups involved in carbohydrate metabolism and energy transfer in 12-day old colonies. Deletion of *wc-2* and *hom2* resulted in a ≥ 2-fold downregulation of *c2h2* in 12-day-old colonies. Downregulation was also observed in 8-day-old colonies, although the effect was less pronounced. Together, this confirms that Wc-2 and Hom2 stimulate *c2h2* expression (Ohm et al. 2011; 2013; Figure 9).

Gene *fst4* is constitutively expressed during the *S. commune* life cycle. Like Hom2 and Wc-2 it is involved in the switch from vegetative growth to fruiting. Strain Δ*fst4*Δ*fst4* grows irregular in the light like the wild-type but does not aggregate. It formed more biomass than the wild-type on xylose, sucrose, and pectin but not on glucose. This indicates that Fst4 and Hom2 represent different parts of the repression pathway of vegetative growth. In liquid shaken cultures with glucose as carbon source these pathways may merge explaining why Δ*hom2*Δ*hom2*Δ*fst4*Δ*fst4* formed more biomass than Δ*hom2*Δ*hom2*. Strain Δ*fst4*Δ*fst4* showed enrichment of carbohydrate metabolism in the upregulated genes of 8-day-old and 12-day-old-colonies similar to that observed in Δ*wc-2*Δ*wc-2* and Δ*hom2*Δ*hom2*. The fact that *fst4* expression is not affected in Δ*wc-2*Δ*wc-2* and Δ*hom2*Δ*hom2* strengthens the hypothesis that Fst4 and Hom2 represent

different pathways. Gene *fst4* stimulates *c2h2* like *hom2* and *wc-2* do. This indicates that Fst4, Hom2, and Wc-2 input are channeled into the fruiting pathway via *c2h2* (Figure 9B).

Transcription factor gene *tea1* was downregulated in Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*briI*Δ*briI*, and Δ*fst4*Δ*fst4* when compared to the aggregating wild-type (8-day-old colonies), while it was upregulated in Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1*, and Δ*gat1*Δ*gat1*. This indicates it is upregulated during early stages of development while it is repressed during late stages of mushroom formation. This agrees with the expression profile in the wild-type. The Δ*tea1*Δ*tea1* strain formed more biomass on glucose when compared to the wild-type. Moreover, it was severely affected in mushroom formation. Only local clusters of fully developed mushrooms were formed in the deletion strain. This phenotype may be explained by a reduced sensitivity of a signaling pathway leading to a developmental switch from "off" to "on".

Expression of *c2h2* increased >2-fold when RNA profiles of 5- and 8-day-old colonies were compared. The increased expression during the aggregation stage and further increased expression in primordia and fruiting bodies agrees with the phenotype of Δ*c2h2*Δ*c2h2* forming aggregates but not primordia and fruiting bodies (Ohm et al. 2011; Figure 9). C2H2 did not affect biomass formation implying that it is downstream of the switch between vegetative growth and mushroom development.

Deletion of *fst3*, *gat1* or *hom1* results in more, but smaller mushrooms (Ohm et al. 2011). In addition, Hom1 and Gat1 are involved in mushroom tissue formation (Ohm et al. 2011). Gene *fst3* is constitutively expressed and its expression is not affected by any of the other transcriptional regulators. This suggests that Fst3 is subject to post-transcriptional regulation. The fact that a higher number of genes are differentially expressed in 8-day-old colonies (wild-type forming aggregates) when compared to 12-day-old colonies (wild-type forming fruiting bodies) (i.e. 882 and 217 genes, respectively) suggest that Fst3 exerts its effect already early in mushroom development. Upregulated functional groups were involved in energy transfer and cell wall processes in 8-day-old colonies, while downregulated functional groups were involved in carbohydrate metabolism. In 12-day-old colonies these groups were regulated in the opposite direction. Expression of *gat1* was highest during fruiting body formation. It is repressed by Bri1 in 8-day-old colonies, while it is activated by Hom2 during fruiting body formation. Colonies of 8 days old were enriched for groups involved in energy

transfer and cell wall processes in upregulated genes, while groups involved in carbohydrate metabolism were downregulated. Groups involved in translation and cell wall were enriched in downregulated genes in 12-day-old colonies. Expression of *hom1* gradually increases upon progression of fruiting. Expression analysis showed that Wc-1, Hom2 and Fst4, and probably Wc-2, stimulate expression of Hom1. Notably, Hom1 stimulates formation of biomass of the vegetative mycelium. This suggests that Hom1 operates at two distinct stages of development (Figure 9). It may well be that Hom1 functions in mushroom formation by stimulation of biomass formation. This would explain the reduced size of the fruiting bodies in Δ*hom1*Δ*hom1*. Notably, both Fst3 and Gat1 repress *hom1* expression. Functional groups involved in chitinase activity, carbohydrate metabolism, and energy transfer were enriched for downregulated genes in 8-day-old colonies. Functional groups involved in amino acid synthesis were enriched for downregulated genes in 12-day-old colonies.

The model of development of *S. commune* may apply to mushroom forming basidiomycetes as well. Hom2, Hom1, Fst3, Fst4, C2H2, and Gat1 are basidiomycete-specific regulatory proteins (Todd et al. 2014). Homologues of these genes were identified in *L. bicolor* and *A. bisporus* (Ohm et al. 2010b; Morin et al. 2012). The homologues for *fst4*, *fst3*, *c2h2* and *hom1* were upregulated during sexual development in two *A. bisporus* varieties (Morin et al. 2012), while homologues of *hom2*, *fst4*, *c2h2*, *fst3*, *gat1* and *hom1* showed similar expression expression profiles in *L. bicolor* showed similarity to *S. commune* (Morin et al. 2012). Expression patterns of *c2h2*, *fst3*, *hom1* and *gat1* were also found to be similar in *C. cinerea* (Plaza et al. 2014).

This is the first time a direct link has been shown between repression of vegetative growth and induction of sexual reproduction. Previously, a link has been shown between vegetative growth and asexual development in *Aspergillus* (Krijgsheld et al. 2013). This link involves trimeric G-protein signaling. The activity of the Gα-subunit of *Aspergillus* is regulated by the FlbA protein (Yu et al. 1996). Inactivation of this gene results in a strain that cannot initiate asexual development. Notably, *S. commune* has a homologue of *flbA* called *thn*. Inactivation of this gene results in a strain unable to form fruiting bodies (Fowler and Mitton 2000). This suggests that similar signaling pathways are involved in the decision to stop vegetative growth and to invest in reproduction in ascomycetes and basidiomycetes. We also showed that genes involved in fruiting may stimulate vegetative growth. These genes are involved in late stages of development.

Table 2. Temporal expression of the blue light sensor gene *wc-1* and transcription factor genes involved in fruiting body development in the wild-type dikaryon. Values are expressed in FKPM. Green shaded boxes indicate a significant ≥2-fold up-regulation when compared to dark grown vegetative mycelium.

	Veg Myc	**Veg Ind**	**Aggregate**	**Primordia**	**Fruiting body**
wc-1	37.30	40.06	54.83	51.97	62.23
wc-2	38.29	46.19	39.88	61.66	47.47
hom2	107.64	135.84	115.99	104.15	58.63
tea1	9.39	14.73	18.88	62.40	10.48
fst4	112.88	159.43	140.70	178.65	119.08
c2h2	22.06	29.61	47.48	87.84	105.36
fst3	103.23	97.82	102.70	119.53	115.64
gat1	75.01	65.00	72.79	61.87	126.09
hom1	114.38	108.69	149.72	200.48	234.15
bri1	30.03	34.22	30.56	34.99	29.95
c2h2d	3.72	8.76	16.57	39.17	54.94

Table 3. Number of genes significantly up- and downregulated compared to wildtype at the moment the wild-type had formed aggregates (AG) and fruiting bodies (FB).

	Upregulated		Downregulated	
	AG	FB	AG	FB
Δ*wc1*Δ*wc1*	415	1195	431	1298
Δ*wc2*Δ*wc2*	375	1392	500	1421
Δ*hom2*Δ*hom2*	494	1267	652	1462
Δ*fst4*Δ*fst4*	1267	1150	1226	1330
Δ*c2h2*Δ*c2h2*	668	306	644	327
Δ*fst3*Δ*fst3*	400	86	482	131
Δ*hom1*Δ*hom1*	194	317	247	400
Δ*gat1*Δ*gat1*	480	662	556	991

Table 4. Enrichment of GO terms in up- and downregulated genes of 8-day-old colonies of Δ*wc-1*Δ*wc-1* and transcription factor deletion strains Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2*, Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1*, Δ*gat1*Δ*gat1*, and Δ*briI*Δ*briI* when compared to the aggregating wild-type strain.

Δwc-1Δwc-1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005975	carbohydrate metabolic process	0004497	oxidoreductase activity
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0015171	monooxygenase activity
0008643	carbohydrate transport	0006865	amino acid transmembrane transporter activity
0008643	L-arabinose isomerase activity	0016491	electron transport
0005351	sugar:hydrogen symporter activity	0005618	amino acid transport
0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide)	0006118	metabolic process
0005506	iron ion binding	0005199	glutathione transferase activity
0016614	oxidoreductase activity, acting on CH-OH group of donors	0004194	structural constituent of cell wall
0009082	branched chain family amino acid biosynthetic process	0006810	cell wall
0008812	choline dehydrogenase activity		

Δwc-2Δwc-2

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005975	carbohydrate metabolic process	0016491	oxidoreductase activity
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0004497	monooxygenase activity

0005506	iron ion binding	0043039	amino acid transmembrane transporter activity
0004497	monooxygenase activity	0006118	electron transport
0050381	unspecific monooxygenase activity	0006865	amino acid transport
0020037	heme binding	0008152	metabolic process
0016614	oxidoreductase activity, acting on CH-OH group of donors	0004364	glutathione transferase activity
0008812	choline dehydrogenase activity	0005199	structural constituent of cell wall
0009082	branched chain family amino acid biosynthetic process	0005618	cell wall
0006066	alcohol metabolic process	0050162	oxalate oxidase activity

Δhom2Δhom2

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005975	carbohydrate metabolic process	0004497	monooxygenase activity
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0006810	transport
0030246	carbohydrate binding	0020037	heme binding
0016491	oxidoreductase activity	0006118	electron transport
0003824	catalytic activity	0005215	transporter activity
0005215	transporter activity	0050381	unspecific monooxygenase activity
0008152	metabolic process	0005506	iron ion binding
0016021	integral to membrane	0015171	amino acid transmembrane transporter activity
0008422	beta-glucosidase activity	0006865	amino acid transport
0006118	electron transport	0016614	oxidoreductase activity, acting on CH-OH group of donors

Δfst4Δfst4

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005975	carbohydrate metabolic process	0005524	ATP binding
0008733	L-arabinose isomerase activity	0003677	DNA binding
0005351	sugar:hydrogen symporter activity	0000166	nucleotide binding
0008643	carbohydrate transport	0008152	metabolic process
0005215	transporter activity	0005737	cytoplasm
0006810	transport	0017111	nucleoside-triphosphatase activity
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0016491	oxidoreductase activity
0016021	integral to membrane	0003824	catalytic activity
0030246	carbohydrate binding	0016887	ATPase activity
0015171	amino acid transmembrane transporter activity	0005643	nuclear pore

Δc2h2Δc2h2

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005506	iron ion binding	0005737	cytoplasm
0004497	monooxygenase activity	0005524	ATP binding
0006118	electron transport	0008152	metabolic process
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0003824	catalytic activity
0020037	heme binding	0016491	oxidoreductase activity
0008733	L-arabinose isomerase activity	0044267	cellular protein metabolic process
0050381	unspecific monooxygenase activity	0000786	nucleosome
0005351	sugar:hydrogen symporter activity	0006334	nucleosome assembly

0050660	FAD binding	0004298	threonine endopeptidase activity
0008643	carbohydrate transport	0004299	proteasome endopeptidase activity

Δfst3Δfst3

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005199	structural constituent of cell wall	0005506	iron ion binding
0005618	cell wall	0004497	monooxygenase activity
0016491	oxidoreductase activity	0020037	heme binding
0008152	metabolic process	0005975	carbohydrate metabolic process
0050660	FAD binding	0006118	electron transport
0016614	oxidoreductase activity, acting on CH-OH group of donors	0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
0033754	indoleamine 2,3-dioxygenase activity	0050381	unspecific monooxygenase activity
0016717	oxidoreductase activity, acting on paired donors, with oxidation	0004568	chitinase activity
0020037	heme binding	0008843	endochitinase activity
0005215	transporter activity	0006032	chitin catabolic process

Δhom1Δhom1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
	NONE	0004568	chitinase activity
		0008843	endochitinase activity
		0004497	monooxygenase activity
		0016491	oxidoreductase activity

		0005975	carbohydrate metabolic process
		0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
		0008115	sarcosine oxidase activity
		0006118	electron transport
		0006032	chitin catabolic process
		0006725	aromatic compound metabolic process

Δgat1Δgat1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005199	structural constituent of cell wall	0005975	carbohydrate metabolic process
0005618	cell wall	0005506	iron ion binding
0016491	oxidoreductase activity	0020037	heme binding
0008152	metabolic process	0006118	electron transport
0004497	monooxygenase activity	0004497	monooxygenase activity
0050660	FAD binding	0005524	ATP binding
0020037	heme binding	0004316	3-oxoacyl-[acyl-carrier-protein] reductase activity
0033754	indoleamine 2,3-dioxygenase activity	0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
0016614	oxidoreductase activity, acting on CH-OH group of donors	0050381	unspecific monooxygenase activity
0006118	electron transport	0005215	transporter activity

Δbri1Δbri1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
	NONE	0008152	metabolic process
		0005975	carbohydrate metabolic process
		0003824	catalytic activity
		0000786	nucleosome
		0005215	transporter activity
		0005199	structural constituent of cell wall
		0006334	nucleosome assembly
		0005618	cell wall
		0006810	transport
		0016491	oxidoreductase activity

Table 5. Enrichment of GO terms in up- and downregulated genes of 12-day-old colonies of Δ*wc-1*Δ*wc-1* and transcription factor deletion strains Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2*, Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1*, Δ*gat1*Δ*gat1*, and Δ*briI*Δ*briI* when compared to the fruiting wild-type strain.

Δwc-1Δwc-1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005506	iron ion binding	0008152	metabolic process
0020037	heme binding	0016491	oxidoreductase activity
0004497	monooxygenase activity	0000786	nucleosome
0006118	electron transport	0006334	nucleosome assembly
0050381	unspecific monooxygenase activity	0003824	catalytic activity
0005975	carbohydrate metabolic process	0006810	transport

0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0016020	membrane
0005524	ATP binding	0004497	monooxygenase activity
0005215	transporter activity	0016021	integral to membrane
0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide)	0006118	electron transport

Δwc-2Δwc-2

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0020037	heme binding	0000786	nucleosome
0005506	iron ion binding	0006334	nucleosome assembly
0004497	monooxygenase activity	0003677	DNA binding
0050381	unspecific monooxygenase activity	0003824	catalytic activity
0005975	carbohydrate metabolic process	0008152	metabolic process
0006118	electron transport	0006260	DNA replication
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0042624	ATPase activity, uncoupled
0005215	transporter activity	0016491	oxidoreductase activity
0005524	ATP binding	0042623	ATPase activity, coupled
0006032	chitin catabolic process	0008186	RNA-dependent ATPase activity

Δhom2Δhom2

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0003735	structural constituent of ribosome	0006810	transport
0005840	ribosome	0005215	transporter activity
0006412	translation	0008152	metabolic process
0005975	carbohydrate metabolic process	0016020	membrane
0005506	iron ion binding	0016021	integral to membrane

0020037	heme binding	0016491	oxidoreductase activity
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0003824	catalytic activity
0005622	intracellular	0006865	amino acid transport
0004497	monooxygenase activity	0015171	amino acid transmembrane transporter activity
0006118	electron transport	0004497	monooxygenase activity

Δfst4Δfst4

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005975	carbohydrate metabolic process	0016491	oxidoreductase activity
0005215	transporter activity	0008152	metabolic process
0006508	proteolysis	0003824	catalytic activity
0005506	iron ion binding	0000786	nucleosome
0020037	heme binding	0030170	pyridoxal phosphate binding
0050381	unspecific monooxygenase activity	0006334	nucleosome assembly
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0006118	electron transport
0004497	monooxygenase activity	0006810	transport
0006118	electron transport	0008483	transaminase activity
0008236	serine-type peptidase activity	0005199	structural constituent of cell wall

Δc2h2Δc2h2

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005506	iron ion binding	0003824	catalytic activity
0020037	heme binding	0008152	metabolic process
0004497	monooxygenase activity	0030170	pyridoxal phosphate binding

0006118	electron transport	0005199	structural constituent of cell wall
0050381	unspecic monooxygenase activity	0005618	cell wall
0016711	flavonoid 3'-monooxygenase activity	0005992	trehalose biosynthetic process
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0005488	Binding
0005975	hydrolase activity, hydrolyzing O-glycosyl compounds	0004316	3-oxoacyl-[acyl-carrier-protein] reductase activity
0006032	carbohydrate metabolic process	0033754	indoleamine 2,3-dioxygenase activity
0005618	chitin catabolic process	0006164	purine nucleotide biosynthetic process

Δfst3Δfst3

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0004565	beta-galactosidase activity	0004568	chitinase activity
0009341	beta-galactosidase complex	0008843	endochitinase activity
0008168	methyltransferase activity	0005576	extracellular region
0005975	carbohydrate metabolic process	0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
0009316	3-isopropylmalate dehydratase complex	0006032	chitin catabolic process
0003861	3-isopropylmalate dehydratase activity	0005199	structural constituent of cell wall
0004035	alkaline phosphatase activity	0005618	cell wall
0005385	zinc ion transmembrane transporter activity	0030246	carbohydrate binding

0006829	zinc ion transport	0050381	unspecific monooxygenase activity
0005315	inorganic phosphate transmembrane transporter activity	0005975	carbohydrate metabolic process

Δhom1Δhom1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
	NONE	0003824	catalytic activity
		0016491	oxidoreductase activity
		0004316	3-oxoacyl-[acyl-carrier-protein] reductase activity
		0008152	metabolic process
		0006526	arginine biosynthetic process
		0016021	integral to membrane
		0003939	L-iditol 2-dehydrogenase activity
		0004932	mating-type factor pheromone receptor activity
		0003991	acetylglutamate kinase activity
		0004358	glutamate N-acetyltransferase activity

Δgat1Δgat1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0017059	serine C-palmitoyltransferase complex	0005840	ribosome
0004758	serine C-palmitoyltransferase activity	0003735	structural constituent of ribosome

0006412	translation
0005622	intracellular
0005839	proteasome core complex
0005199	structural constituent of cell wall
0005618	cell wall
0008152	metabolic process
0006118	electron transport
0006526	arginine biosynthetic process

Δbri1Δbri1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
	NONE	0005975	carbohydrate metabolic process
		0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
		0016491	oxidoreductase activity
		0004364	glutathione transferase activity
		0005618	cell wall
		0003824	catalytic activity
		0004568	chitinase activity
		0005199	structural constituent of cell wall
		0008843	endochitinase activity
		0016564	transcription repressor activity

Example 2: Fungal mycelium as a biomaterial

Abstract

Filamentous fungi colonize organic material such as plant waste by means of hyphae that grow at their tips and that branch subapically. As a result, a hyphal network is formed that is called mycelium and that has a fabric-like appearance. Here, material properties of mycelium of the mushroom forming fungus *Schizophyllum commune* were determined in relation to light conditions and the genetic background. Mycelium of liquid standing cultures of the wild-type strain H4-8b grown in the light or in the dark in sealed Petri dishes showed a Young's modulus of 801-897 MPa. Maximum tensile strength was between 12.1 and 13.5 MPa, while elongation at breaking ranged between 3.1 and 3.8 %. Mycelium of the Δ*hom2* strain showed similar mechanical strength as the wild-type in the dark and in the light as well as a similar Young's modulus in the dark. The Young's modulus of Δ*hom2* was however higher when compared to the wild-type strain in the light. Elongation of Δ*hom2* ranged between 1.3 and 2.0 %. The Δ*fst4* strain showed the highest Young's modulus and maximum strength in the light and in the dark with values of 1467-2015 MPa and 19.8 and 24.5 MPa, respectively. The elongation at breaking of the Δ*fst4* strain was between 1.7 and 2.1 %. Together, these data show that the transcription factor genes *hom2* and *fst4* that are involved in fruiting can also affect mechanical properties of the vegetative mycelium. The resulting Young's moduli are similar to those of thermoplastics showing the potential of fungal mycelium as a sustainable biomaterial. Overall, it has clearly been demonstrated that the fungi according to the invention have superior mechanical properties as compared to wild-type fungi.

Introduction

Filamentous fungi grow by means of hyphae that grow at their tips and that branch subapically. As a result, a network of hyphae is formed that is called mycelium. The hyphae feed on organic material such as plant waste. To this end, they penetrate their substrate while secreting enzymes that degrade the plant polymers into molecules that can be taken up to serve as nutrients. Hyphal penetration of the substrate is facilitated by the turgor pressure generated by the cytoplasm and by the rigidity of the surrounding cell wall. The composition of fungal cell walls is dynamic and varies between species, strains,

environmental conditions (Bowman and Free 2006), and developmental stage (Wessels 1994).

Schizophyllum commune is a model for mushroom forming fungi (Ohm et al. 2010b). Its life cycle starts with a monokaryotic (i.e. homokaryotic) mycelium that results from the germination of a basidiospore. Monokaryotic mycelia are sterile and always grow vegetative. A fertile dikaryon is formed upon fusion of two monokaryons with compatible mating types. Blue light is required to initiate fruiting in the dikaryotic (i.e heterokaryotic) mycelium (Perkins and Gordon 1969), whereas high CO_2 levels repress this developmental program (Niederpruem 1963; Raudaskoski and Viitanen 1982). So, in the dark and / or at high CO_2 levels the dikaryon grows vegetative, while at ambient CO_2 levels and in the light fruiting bodies are formed. Initiation of fruiting body formation by *S. commune* starts with asymmetrical colony growth, followed by aggregation of aerial hyphae, and subsequent formation of primordia. These primordia can develop into fruiting bodies that form basidia within the hymenium. Karyogamy, meiosis, and one round of mitosis occur in the basidia, resulting in haploid, binucleate basidiospores.

The cell wall of the vegetative mycelium of *Schizophyllum commune*, as an example of a basidiomycete, consists of glucose (67.6%), N-acetylglucosamine (12.5%), mannose (3.4%), xylose (0.2%), amino acids (6.4%), and lipids (3.0%) (Sietsma and Wessels, 1977). These monomers make up chitin, glucan, and proteins as well as other, not yet identified cell wall constituents. The outer layer of the cell wall is a water-soluble mucilage consisting of (1,3)-β-linked glucose units with branches of single (1,6)-β-linked glucose molecules at every third glucose along the chain (Wessels et al., 1972). This mucilage, known as schizophyllan, is also secreted into the culture medium. An alkali-soluble glucan consisting of (1,3)-α-linked glucose units, known as S-glucan, is located beneath the mucilage and accounts for about half of the thickness of the water-insoluble portion of the wall. The inner layer of the cell wall consists of an alkali-insoluble glucan, known as R-glucan, and chitin (Wessels, 1994). The R-glucan was found to be a highly branched (1,3)(1,6)-β-glucan. Part of this highly insoluble glucan has structural similarity to schizophyllan. Most of the R-glucan is linked to chitin (Sietsma and Wessels, 1981) via basic amino acids and N-acetylglucosamine (Sietsma and Wessels, 1977).

The rigidity of cell walls combined with the capacity to produce large networks in low value waste streams makes mycelium of interest as a biomaterial. We here studied

physical properties of mycelium of wild-type *S. commune* and of strains in which either *fst4* or *hom2* have been inactivated. Data show that transcription factor genes that are involved in fruiting can also affect mechanical properties of the mycelium.

Material and Methods

Strains and culture conditions

S. commune wild-type strain H4-8b (Ohm et al., 2010a) and its derivative strains Δ*fst4* (Ohm et al., 2010b) and Δ*hom2* (Ohm et al., 2011) were used in this study. Strains were grown on minimal medium (MM) (Dons et al. 1979) either or not solidified with 1.5 % agar. A quarter of a 7-day-old colony grown on agar medium was homogenized in 50 ml MM for 30 s at low speed using a Waring Blender (Waring Laboratory, Torrington, England). Static liquid cultures were inoculated by taking up 400 mg wet weight mycelial homogenate in a final volume of 6 ml MM and spreading it into a confluent layer in a 9 cm Petri dish. Cultures were grown at 30 °C in the light (450 Lux from fluorescent tubes, Osram, L36W/840 Lumilux Cool White, Munich, Germany) or in the dark. Cultures were sealed with a double layer of Parafilm and a layer of scotch tape to create high CO_2 conditions. After 3 days of incubation, 30 ml MM was applied underneath the mycelial mat. Growth was prolonged for 5 days at 30 °C using conditions described above.

Tensile measurements

- Young's modulus or elastic modulus: A number that depicts the elastic deformation of an object when a force is applied to it
- Maximum tensile strength: The amount of stress that a material can withstand before breaking
- Elongation at breaking: the elongation of an object (in %) at the moment the maximum tensile strength is reached and the object breaks
- Stress (mechanical): The force (N) that is applied to an object divided by the area (m^2) of the object to which the force is applied. (Stress is in MPa, $1Pa=1N/M^2$)
- Strain (mechanical): The relative change in length (t1) caused by the deformation to the original length (t0)

Static liquid cultures were dried at room temperature. Tensile measurements were performed on 5 rectangles (12 x 4 mm) of each biological duplicate. Thickness of the

mycelium was measured by High Accuracy length gauge (Heidenhain MT1281, Traunreut, Germany). Tensile measurements were performed using a Dynamic Mechanical Analyzer (DMA) Q800 TA instrument (TA Instruments, New Castle, DE, USA), equipped with an 18 N capacity load cell. The elasticity modulus (i.e. Young's modulus) (in MPa) was obtained by dividing the mechanical stress by the mechanical strain. The maximum strength (in MPa) was obtained from force per unit area, while elongation at breaking point (in %) was obtained by calculating the strain (in mm) at the moment of breaking compared to the original length of the samples (in mm).

Statistical analysis

Statistical analysis was performed with the software package IBM SPSS statistics 22.0 (IBM Corporation, Armonk, New York). Mechanical properties of mycelia were analysed by Independent T Tests (asterisks indicate * $p \leq 0.05$, ** $p \leq 0.005$ and *** $p \leq 0.0005$).

Results

The wild-type strain H4-8b and its derivatives Δ*fst4* and Δ*hom2* were grown as a thin layer in a Petri-dish in the light or in the dark at high CO_2. After 3 days liquid medium was introduced underneath the colony. Growth of the floating mycelium was prolonged for 5 days, after which the mycelial mats were dried. Mycelium of liquid standing cultures of the wild-type strain H4-8b grown in the light or in the dark in sealed Petri dishes showed a Young's modulus of 801-897 MPa (Figure 10). Maximum tensile strength was between 12.1 and 13.5 MPa, while elongation at breaking ranged between 3.1 and 3.8 %. Mycelium of the Δ*hom2* strain showed a similar maximum strength in the dark and in the light (with a minimum of 11.4 MPa when grown in the dark) and a similar Young's modulus in the dark. However, its Young's modulus was higher when compared to the wild-type strain in the light. Elongation of Δ*hom2* was between 1.3 and 2.0 % and thereby lower as that of the wild-type. The Δ*fst4* strain showed the highest Young's modulus and maximum strength in the light and in the dark with values of 1467-2015 MPa and 19.8 and 24.5 MPa. The elongation at breaking of the Δ*fst4* strain was between 1.7 and 2.1 % being lower than the wild-type.

Discussion

The oil-based economy should be transformed into a sustainable circular system to halt climate change, pollution and depletion of natural resources. This includes the use of sustainable and renewable energy and materials. The use of bio-based materials is part of this change. This includes a wide range of resources such as those of microbes, plants, macro-algae, shellfish and other animals. Waste streams can also be converted into biomaterials. For instance, biodegradable plastics can be made from a wide variety of organic materials such as starch, cellulose and lactic acid. We here used mycelium as a new source of sustainable biomaterials. Mycelium-based materials could replace existing non-sustainable materials or even enable development of novel materials.

The Young's modulus and strength of materials is highly variable. For instance, elastomers (e.g. synthetic rubber) are characterized by a Young's modulus $\leq$ 100 MPa and a maximum strength of 1-50 MPa, while steel and ceramics have an Young's modulus of 10-800 GPa and a strength of 100-2000 MPa (Ashby, 2005). Wood and thermoplastics (e.g. polyethylene, polypropylene, polyvinyl chloride, polystyrene, nylon, and Teflon) are characterized by intermediate Young's moduli and strength. These values are 1-10 GPa and 30-50 MPa for wood and 500-5000 MPa and 10-100 MPa for thermoplastics, respectively. The Young's modulus of mycelium as obtained in this study was in the range of 801-2015 MPa, while its strength ranged between 11.4-24.5 MPa depending on strain and growth conditions. Thus, mycelium has a Young's modulus and strength properties similar to that of thermoplastics.

Mycelium was grown in the light or the dark in sealed Petri dishes. Light did not impact elasticity and maximum strength of the wild-type mycelium. In the case of Δ*hom2* we did observe an effect for the Young's modulus, the maximum strength, and the elongation at breaking. These values were higher when the mycelium had grown in the light. Notably, the highest Young's modulus and maximum strength were obtained with strain Δ*fst4*. Also in this case the Young's modulus was higher in the light when compared to the dark. Yet, elongation at breaking and the maximum strength was similar when growth in the dark and light were compared.

Together, we have provided evidence that mycelium could replace non-sustainable materials. Fungi grow on low value waste streams and grow optimally at a temperature of 15-30 °C. The latter implies that heating of the growth facilities is minimum, if needed at all, to grow the fungal materials. We have shown that we can create mycelium

materials with different properties by changing the genetic make-up and light conditions during growth. Moreover, CO_2 levels, inoculum density, carbon source, pH, and growth temperature during growth may impact the mechanical properties of the mycelium. The latter three environmental conditions have been shown to impact cell wall composition of *S. cerevisae* and *Candida albicans* (Aguilar-Uscanga and Francois, 2003; Ene et al, 2012). On top of that, material properties of mycelium could be altered by posttreatment with plasticizers or crosslinker, thus increasing the palette of mycelium-based materials.

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CLAIMS

1. A fungus derived from a parental fungus, wherein said fungus exhibits a decreased expression of a polynucleotide encoding Fst4 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions, and

- a decreased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of: Wc-1, Wc-2, Hom2 and Tea1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions, and/or has
- an increased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of Bri1 and Hom1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

wherein the decreased and/or increased expression level results in increased vegetative growth of said fungus compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions.

2. A fungus according to claim 1, wherein the fungus is a filamentous fungus.

3. A fungus according to claim 1 or 2, wherein the fungus is an Ascomycete or a Basidiomycete, preferably a Basidiomycete.

4. A fungus according to claim 3, wherein the fungus is a mushroom forming Basidiomycete.

5. A fungus according to any one of the preceding claims, wherein at least the expression level of a polynucleotide encoding Fst4 and of a polypeptide encoding Hom2 is decreased compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions.

6. A fungus according to any one of the preceding claims, or
a fungus derived from a parental fungus, wherein said fungus exhibits:

- a decreased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of: Wc-1, Wc-2, Fst4, Hom2 and Tea1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions, and/or has
- an increased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of Bri1 and Hom1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

wherein the decreased and/or increased expression level results in increased vegetative growth of said fungus compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

wherein said fungus comprising a polynucleotide encoding a compound of interest.

7. A method of culturing a fungus according to any one of the preceding claims or a fungus derived from a parental fungus, wherein said fungus exhibits:

- a decreased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of: Wc-1, Wc-2, Fst4, Hom2 and Tea1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions, and/or has
- an increased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of Bri1 and Hom1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

wherein the decreased and/or increased expression level results in increased vegetative growth of said fungus compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

comprising culturing said fungus under conditions conducive to the production of said fungus and isolating and/or purifying said fungus, or

culturing said fungus on an industrial scale under conditions conducive to the production of said fungus and, optionally, isolating and/or purifying said fungus.

8. A method for the production of a fungus and/or a mushroom, comprising contacting a fungus according to any one of claims 1 – 6 or a fungus derived from a parental fungus, wherein said fungus exhibits:

- a decreased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of: Wc-1, Wc-2, Fst4, Hom2 and Tea1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions, and/or has
- an increased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of Bri1 and Hom1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

wherein the decreased and/or increased expression level results in increased vegetative growth of said fungus compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

with a substrate and with a fungus able to produce mushrooms, wherein said fungus according to any one of claims 1 – 6 exhibits increased vegetative growth in the substrate.

9. A method for the production of a compound of interest comprising culturing a fungus according to any one of claims 1 - 6 or a fungus derived from a parental fungus, wherein said fungus exhibits:

- a decreased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of: Wc-1, Wc-2, Fst4, Hom2 and Tea1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions, and/or has
- an increased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of Bri1 and Hom1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

wherein the decreased and/or increased expression level results in increased vegetative growth of said fungus compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

under conditions conducive to the production of the compound of interest and isolating and/or purifying the compound of interest.

10. A method for the degradation of organic material comprising contacting a fungus according to any one of claims 1 - 6 with an organic material and applying conditions suitable for the degradation of the organic material to the fungus contacted with the organic material.

11. A method for the degradation of inorganic material comprising, contacting a fungus according to any one of claims 1 - 6 with an inorganic material and applying conditions suitable for the degradation of the inorganic material to the fungus contacted with the inorganic material.

12. A method for the production of a composite material, comprising contacting a fungus according to any one of claims 1 – 6 or a fungus derived from a parental fungus, wherein said fungus exhibits:

- a decreased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of: Wc-1, Wc-2, Fst4, Hom2 and Tea1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions, and/or has
- an increased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of Bri1 and Hom1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

wherein the decreased and/or increased expression level results in increased vegetative growth of said fungus compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

with an organic and/or inorganic material and applying conditions suitable for the production of the composite material consisting of fungal mycelium and the organic and/or inorganic material.

13. A method for the production of fungal mycelium comprising contacting a fungus according to any one of claims 1 – 6 with an organic material and applying conditions suitable for the production of the fungal mycelium.

14. A method according to claim 12 or 13, wherein the composite material or the fungal mycelium is a construction material, an isolation material, a packaging material, a textile, a container, a material or device used for medical applications, a material or device used in horticulture, or a decoration material.

15. A composite material or a fungal mycelium obtained or obtainable by the method according to any one of claims 12 - 14.

16. A composite material comprising:

- a fungus according to any one of claims 1 – 6, or,
- a fungus derived from a parental fungus, wherein said fungus exhibits:
  - a decreased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of: Wc-1, Wc-2, Fst4, Hom2 and Tea1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions, and/or has
  - an increased expression level of at least one polynucleotide encoding a polypeptide selected from the group consisting of Bri1 and Hom1 compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

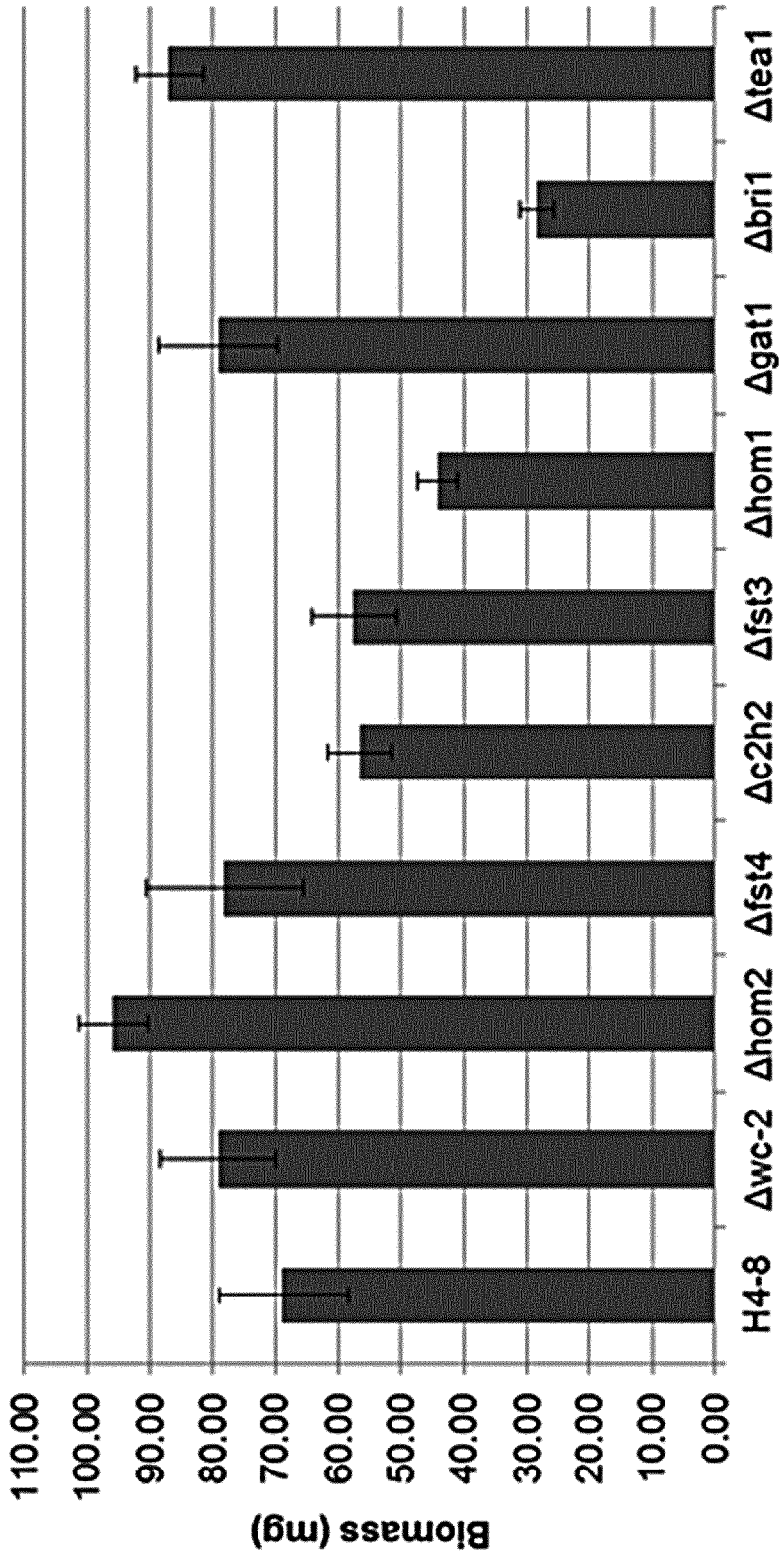
  wherein the decreased and/or increased expression level results in increased vegetative growth of said fungus compared to the parental fungus said fungus is derived from when both are cultured and assayed under identical conditions,

or,

- a fungal mycelium obtained or obtainable by the method according to any one of claims 12 – 14.

17. A composite material according to claim 16, wherein composite material is a construction material, an isolation material, a packaging material, a textile, a container, a material or device used for medical applications, a material or device used in horticulture, or a decoration material.

Fig. 1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100.00
90.00
80.00
70.00
60.00
50.00
40.00
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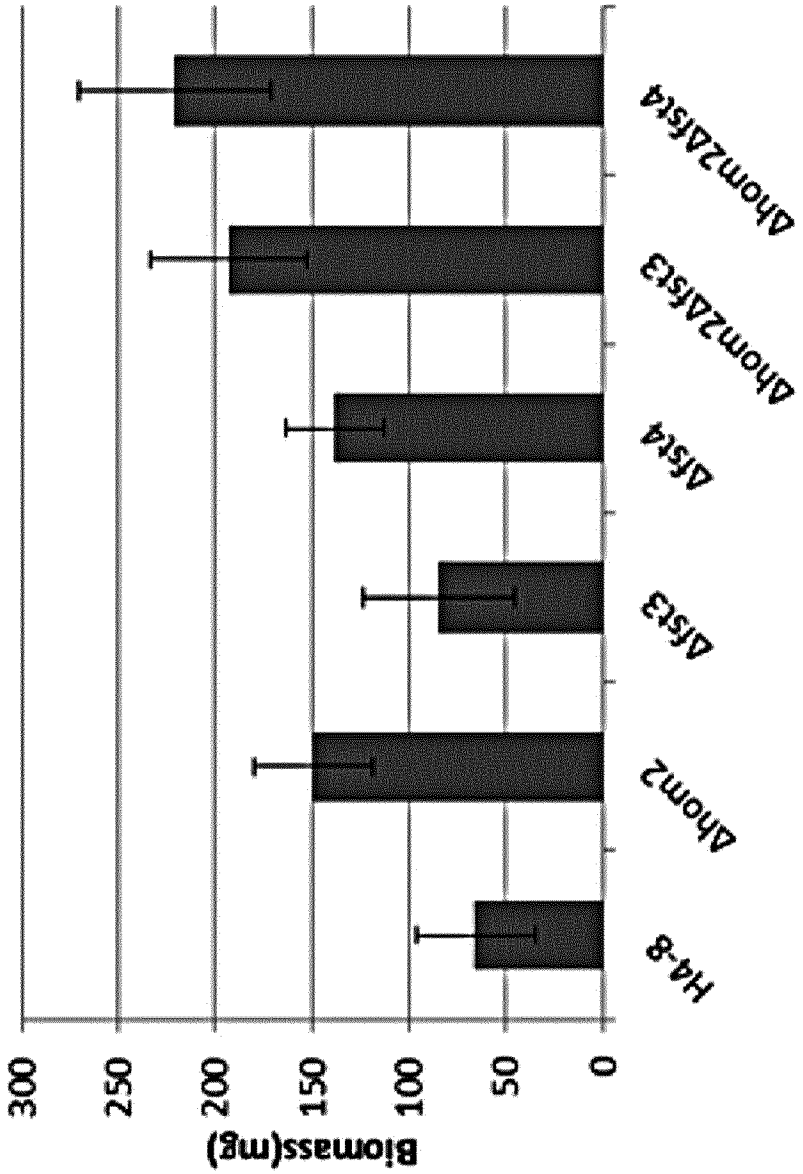
20.00
10.00
0.00
H4-8
Δwc-2
Δhom2
Δfst4
Δc2h2
Δfst3
Δhom1
Δgat1
Δbri1
Δtea1


Biomass(mg)
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200
150
100
50
0
H4-8
Δhom2
Δfst3
Δfst4
Δhom2Δfst3
Δhom2Δfst4

Fig. 2A

Fig. 2B

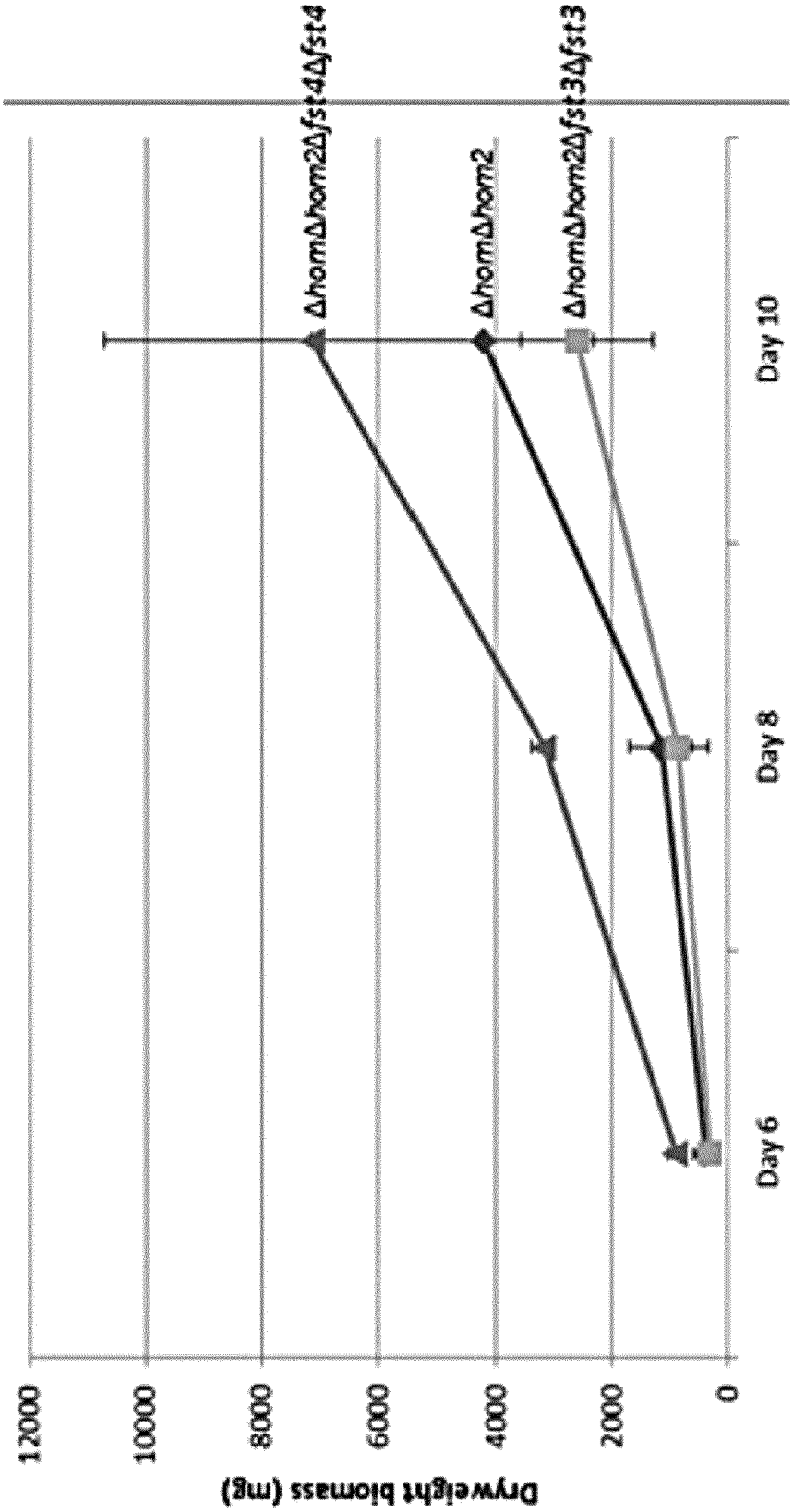

Δhom-Δhom2Δfst4Δfst4
Δhom-Δhom2
Δhom-Δhom2Δfst3Δfst3
Day 10
Day 8
Day 6
Dryweight biomass (mg)
12000
10000
8000
6000
4000
2000
0

Fig. 3A

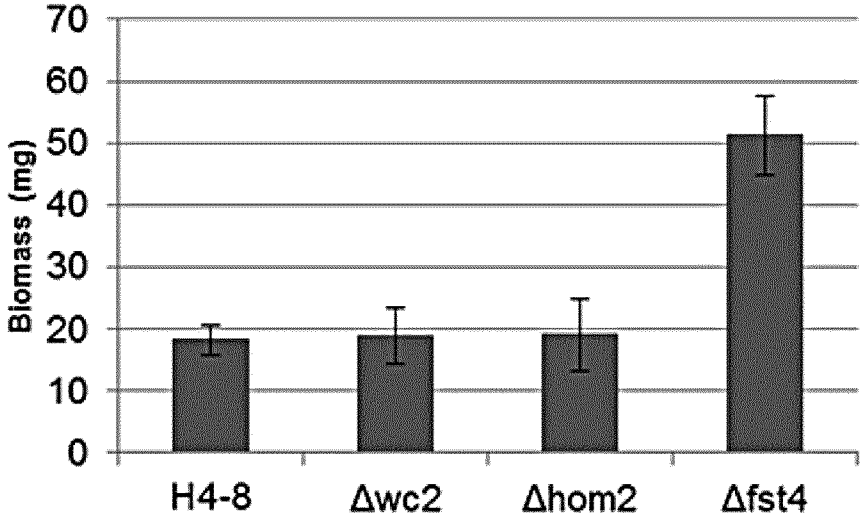


Fig. 3B

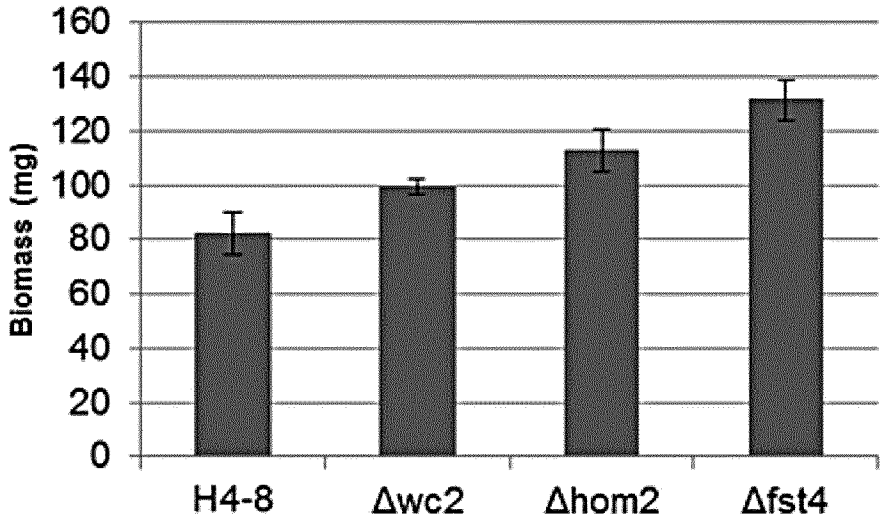
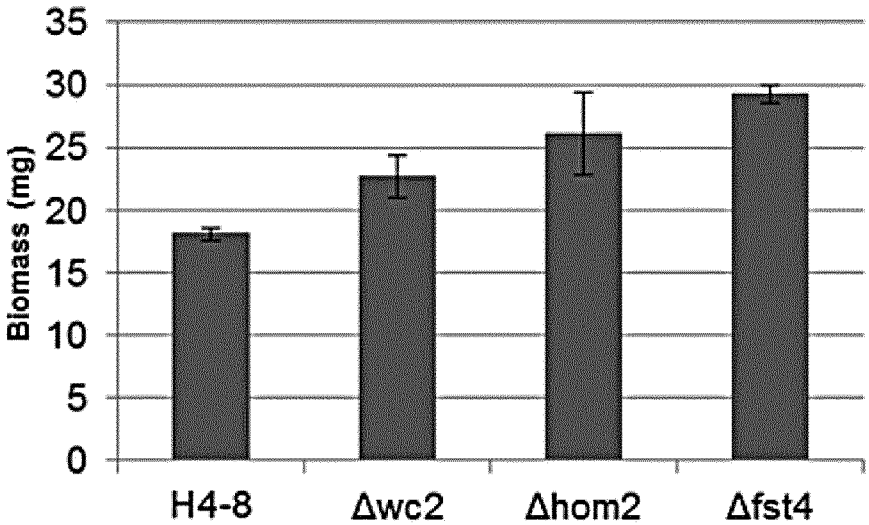


Fig. 3C



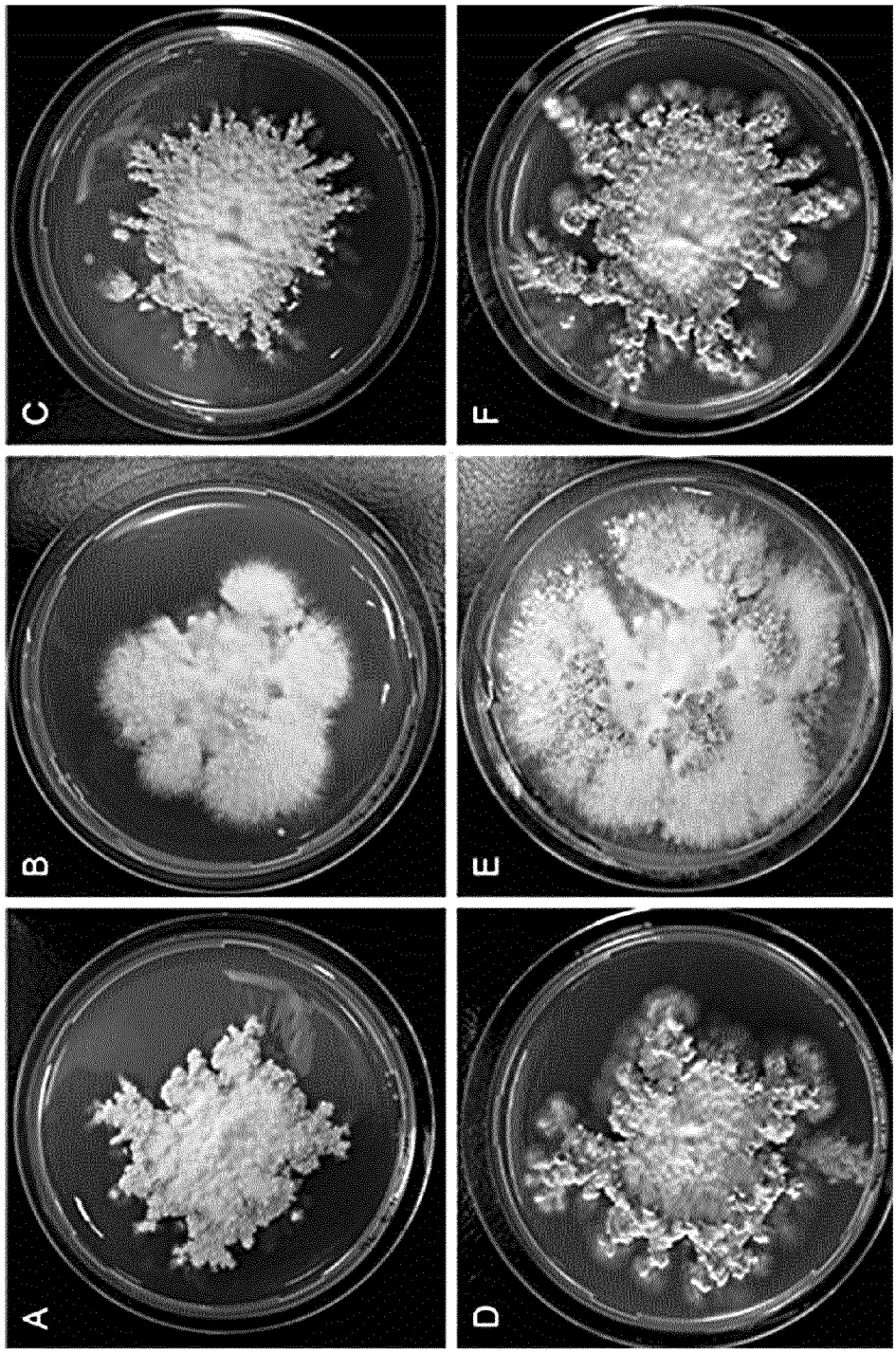
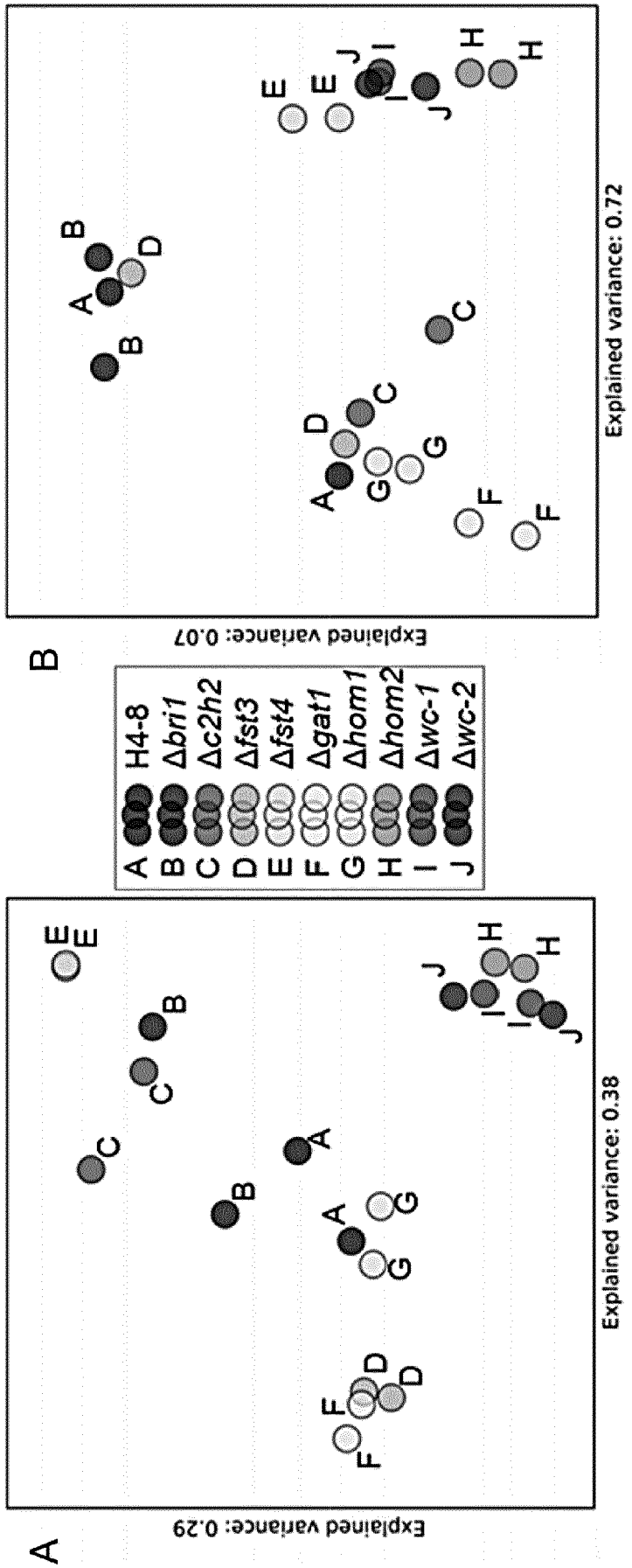
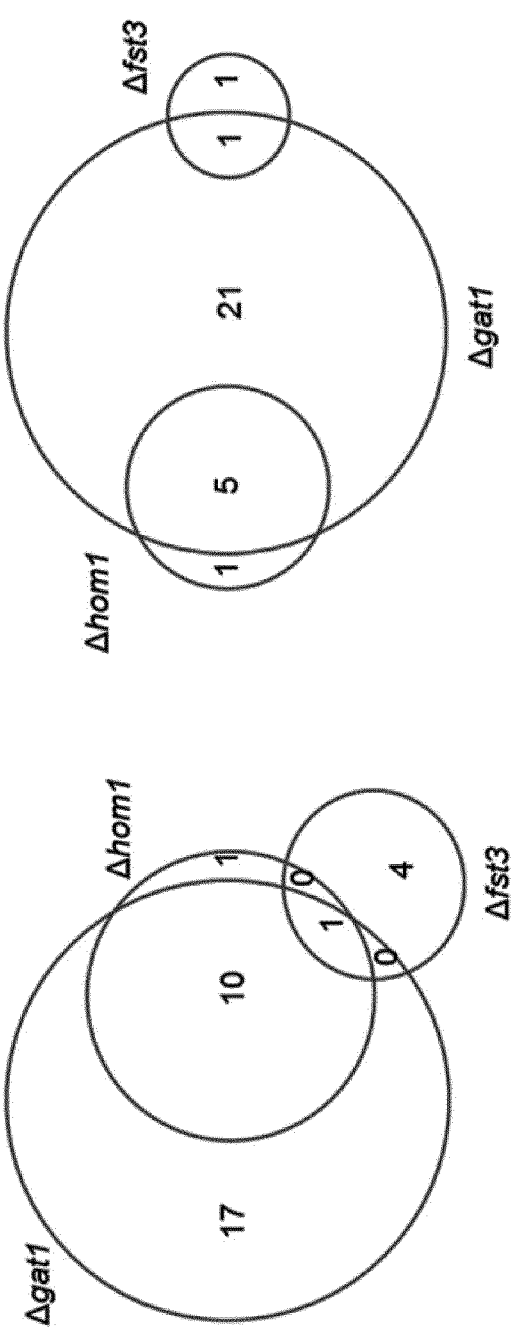


Fig. 4

Fig. 5


A
Explained variance: 0.29
Explained variance: 0.38
B
Explained variance: 0.07
Explained variance: 0.72
A H4-8
B Δbri1
C Δc2h2
D Δfst3
E Δfst4
F Δgat1
G Δhom1
H Δhom2
I Δwc-1
J Δwc-2


Δfst3
Δhom1
Δgat1
1
1
1
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1
Δgat1
Δhom1
Δfst3
17
10
1
0
1
0
4

Fig. 6A

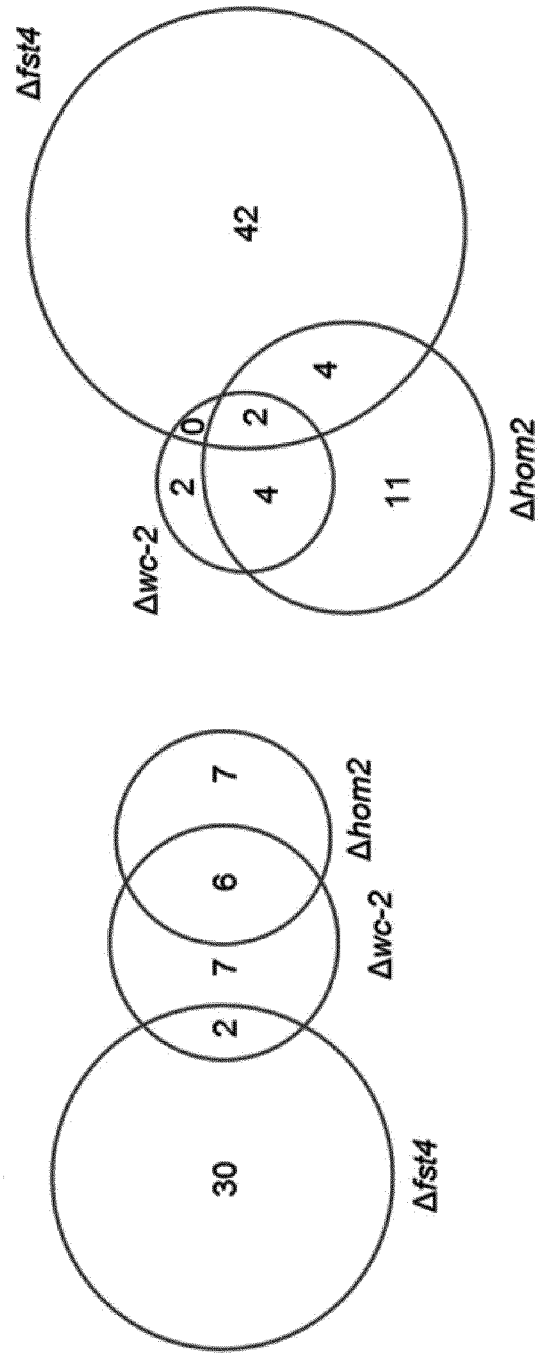
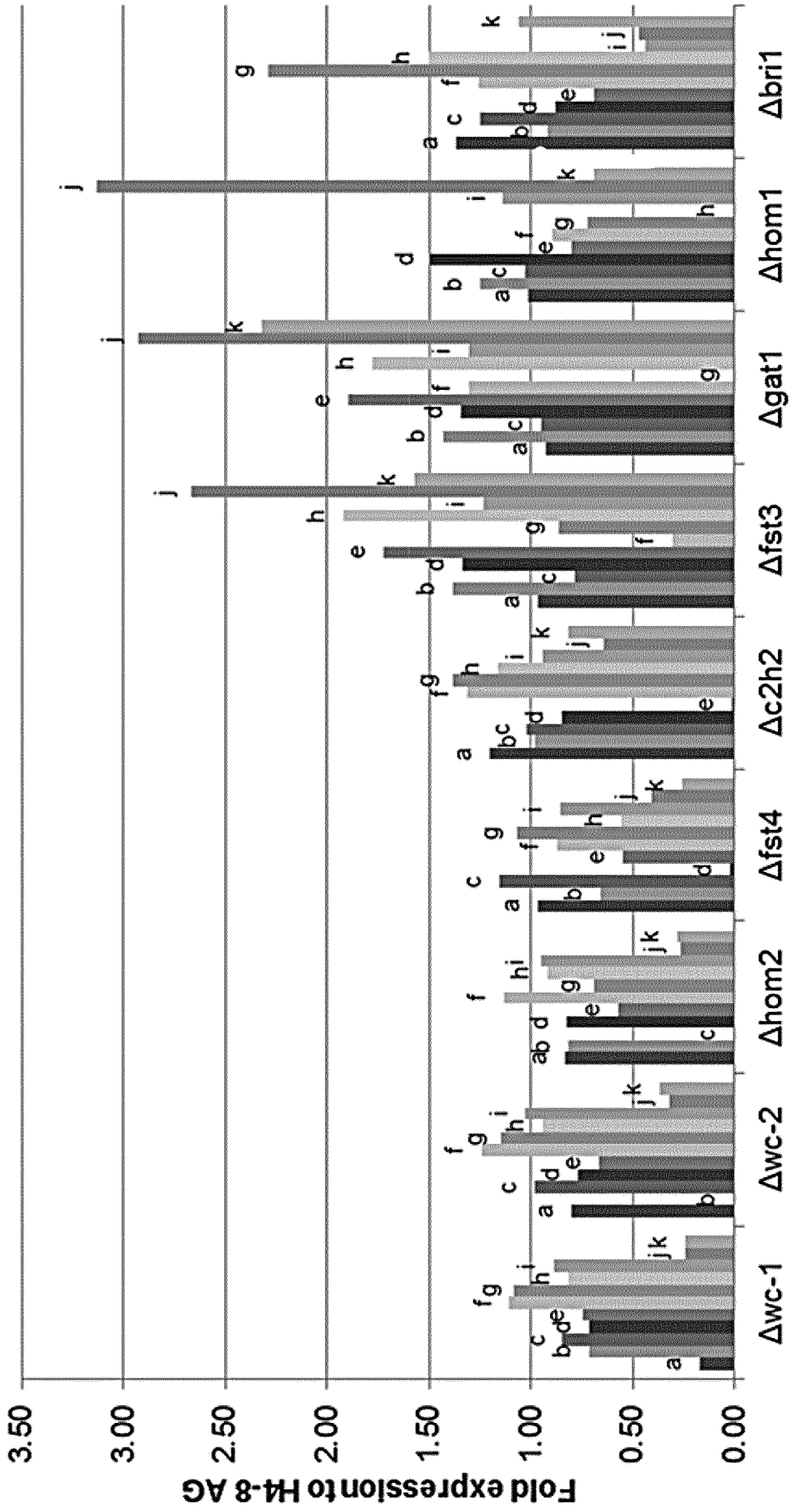

Δfst4
Δwc-2
Δhom2
42
2
0
2
4
4
11
Δfst4
Δwc-2
Δhom2
30
2
7
6
7

Fig. 6B

Fig. 7A


Fold expression to H4-8 AG
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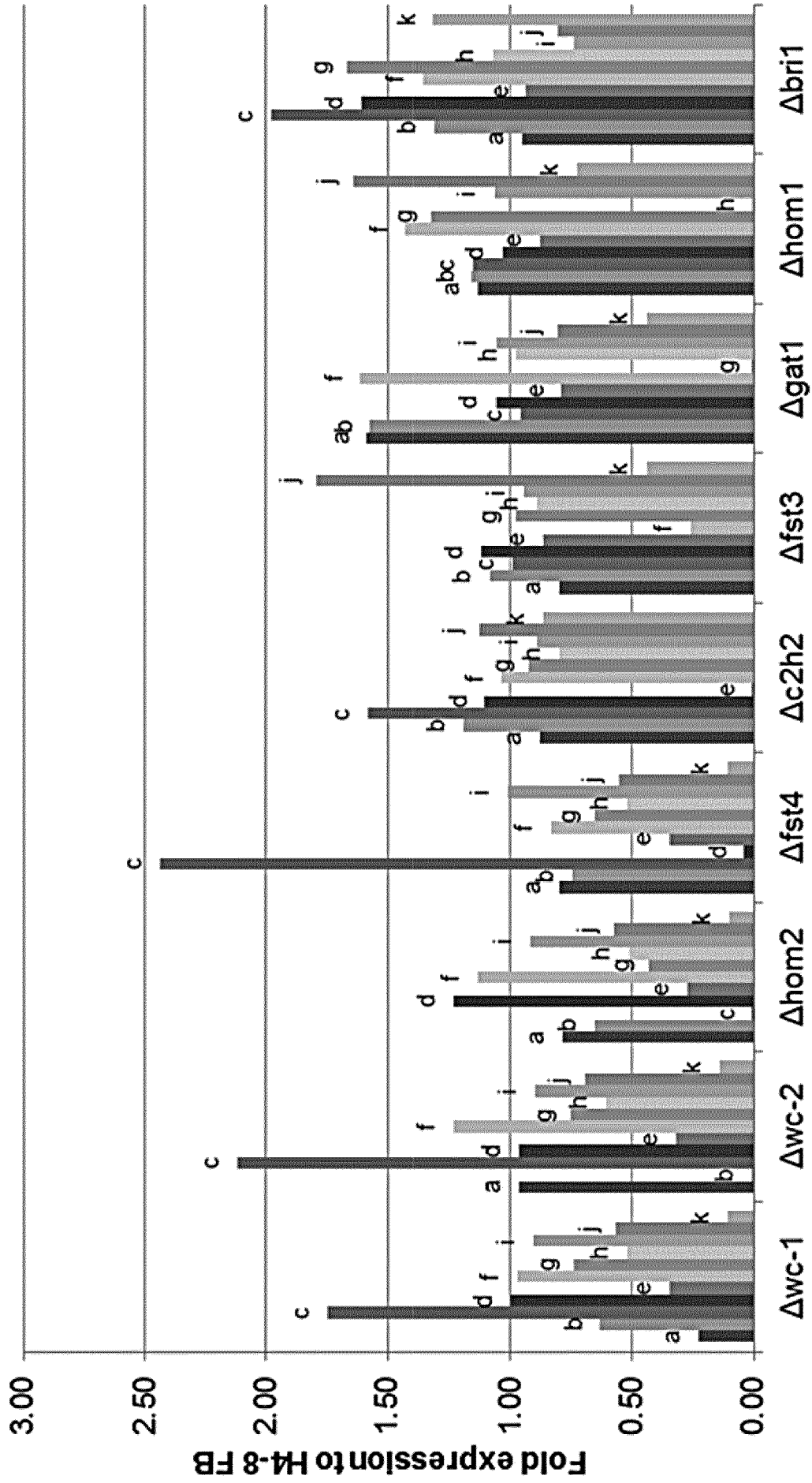
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Δwc-1
Δwc-2
Δhom2
Δfst4
Δc2h2
Δfst3
Δgat1
Δhom1
Δbri1

Fig. 7B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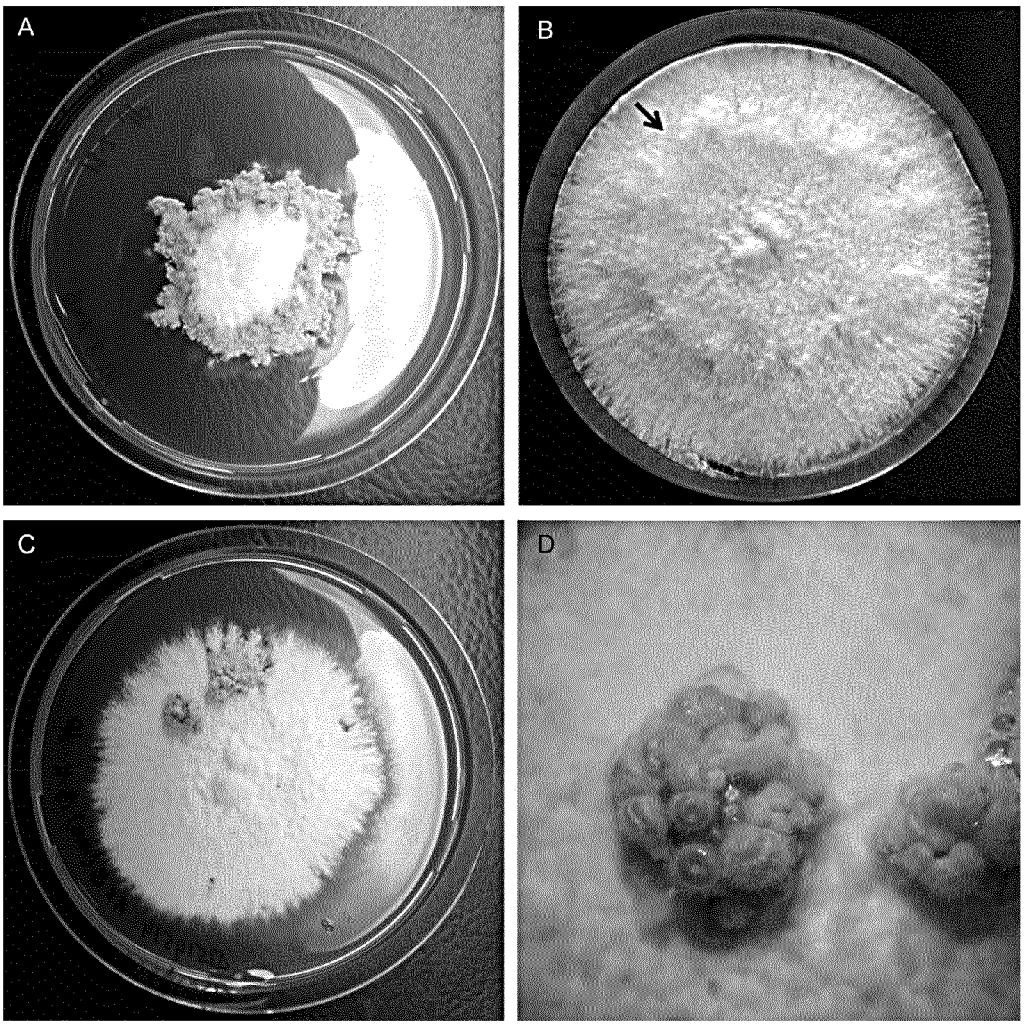
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Δwc-1
Δwc-2
Δhom2
Δfst4
Δc2h2
Δfst3
Δgat1
Δhom1
Δbri1

Fig. 8



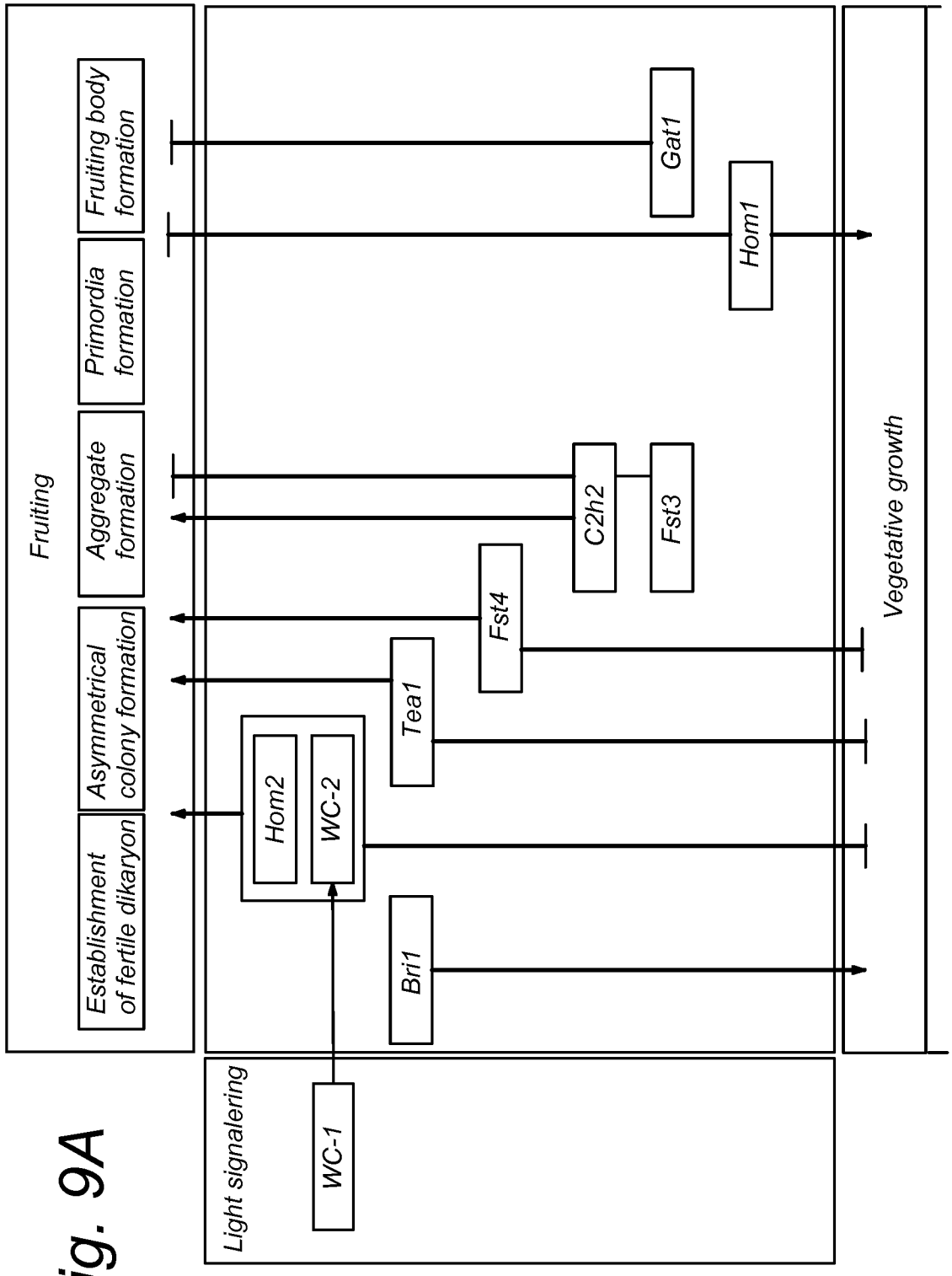

Fruiting
Establishment of fertile dikaryon
Asymmetrical colony formation
Aggregate formation
Primordia formation
Fruiting body formation
Light signalering
WC-1
Hom2
WC-2
Bri1
Tea1
Fst4
C2h2
Fst3
Gat1
Hom1
Vegetative growth

Fig. 9A

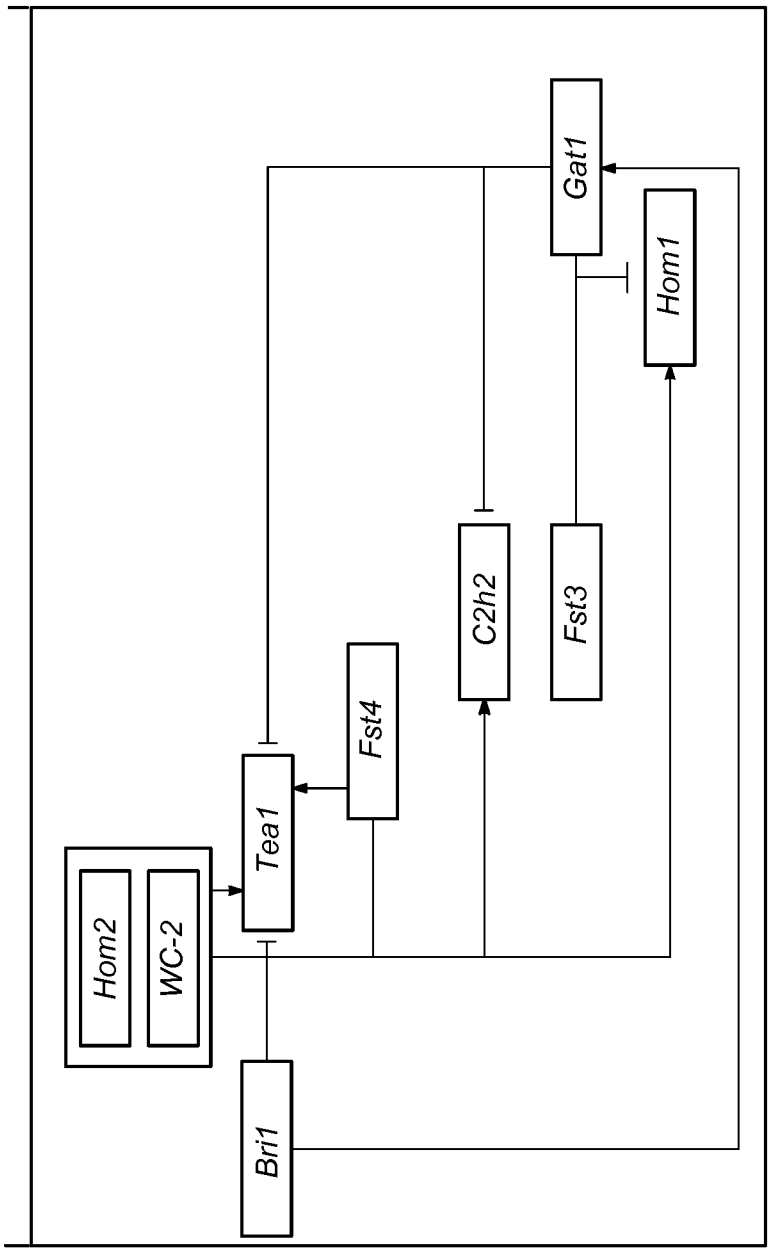
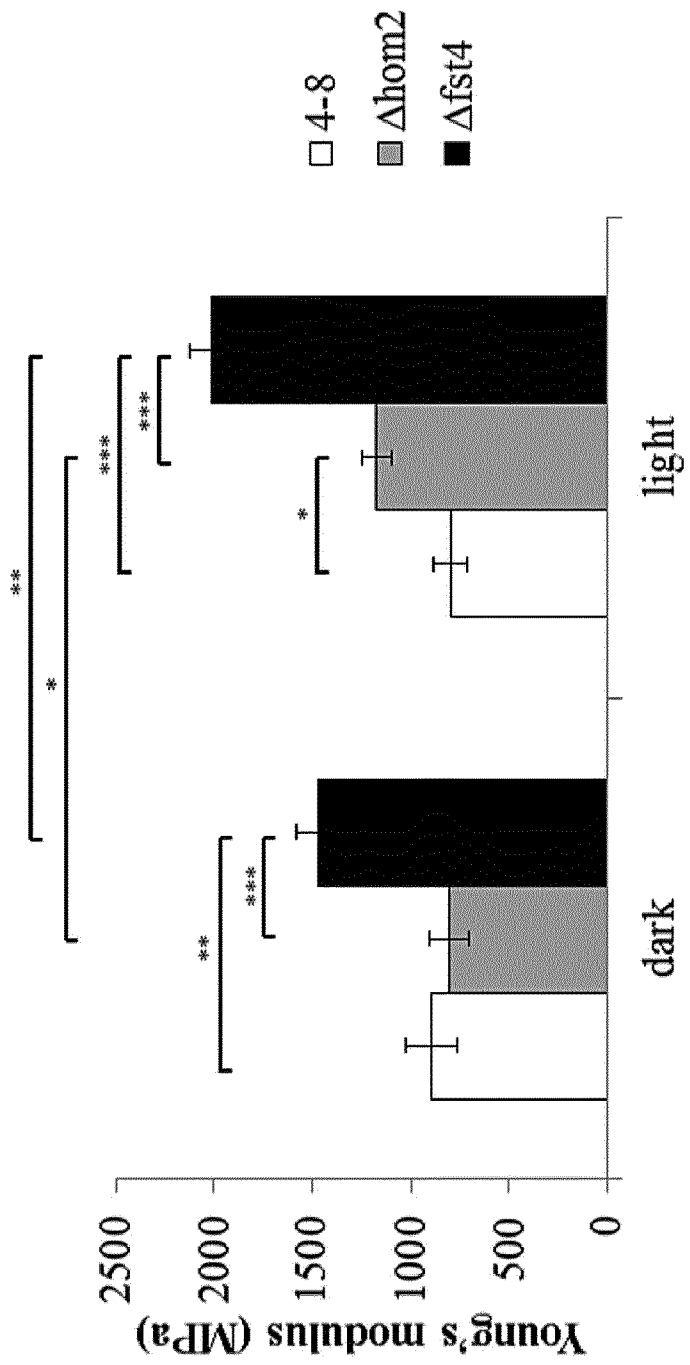


Fig. 9B

Fig. 10A


4-8
Δhom2
Δfst4
Young's modulus (MPa)
2500
2000
1500
1000
500
0
dark
light
**
*
***
**
*
***
***

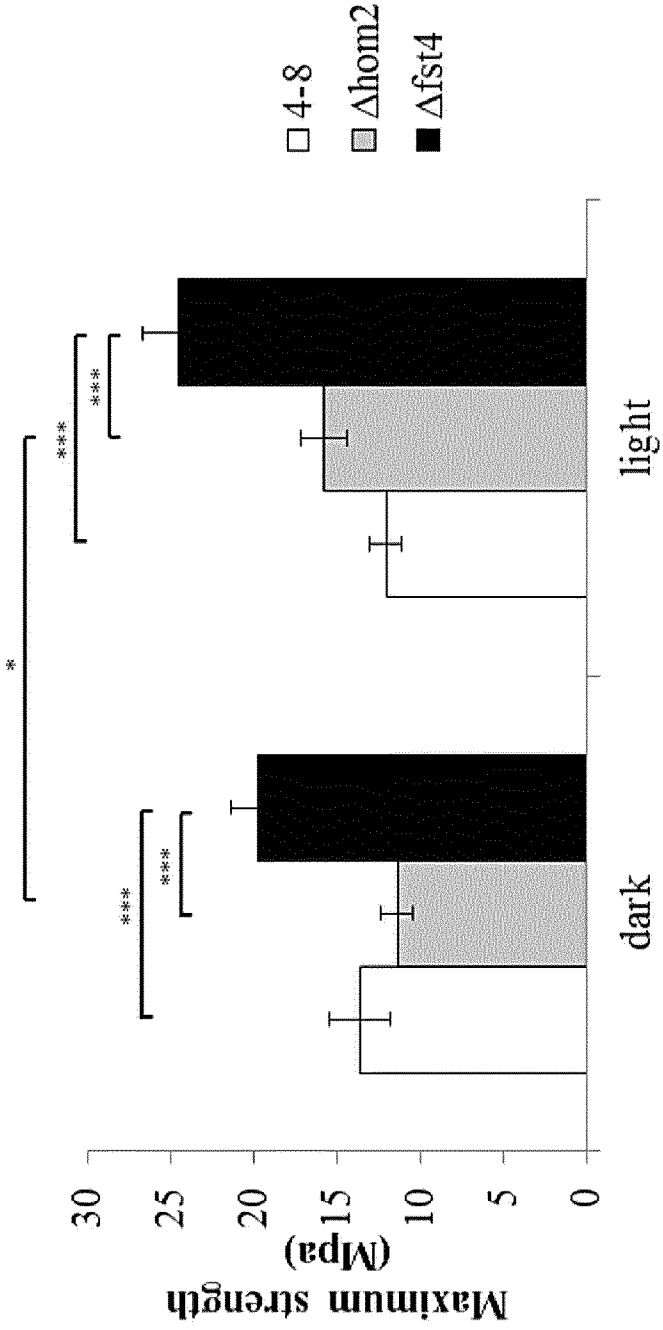

4-8
Δhom2
Δfst4
Maximum strength
(Mpa)
0
5
10
15
20
25
30
dark
light
*
***
***
***
***

Fig. 10B

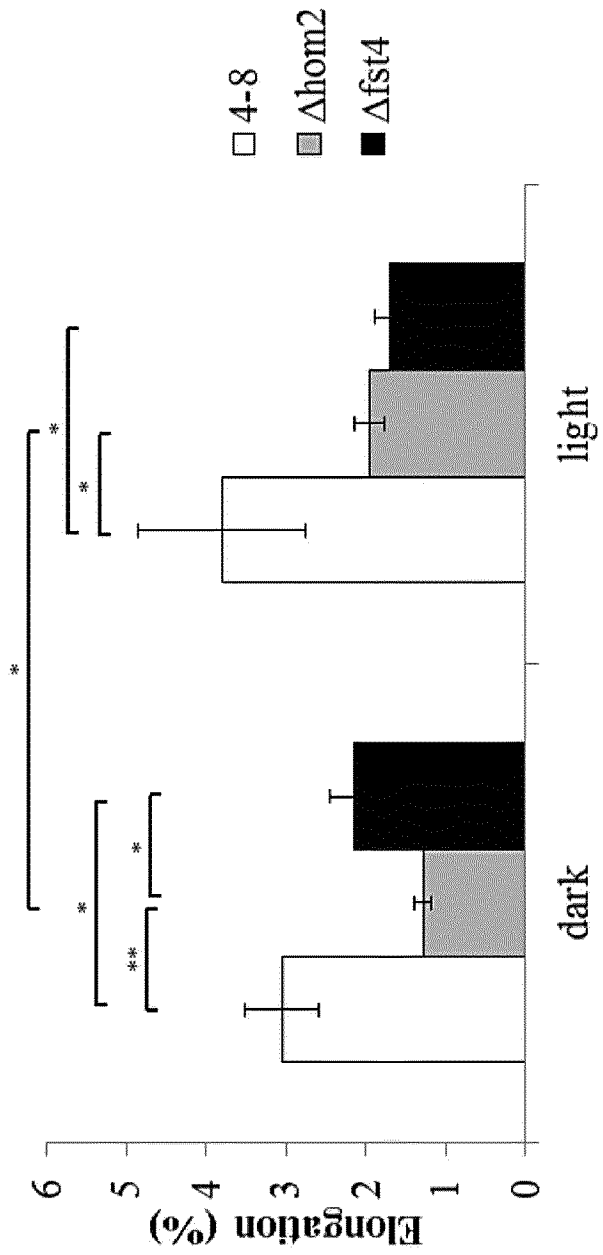


Fig. 10C

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/051310

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/375 C12N1/00
ADD.

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)
C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R.A Ohm: "Regulation of mushroom formation in Schizophyllum commune", , 22 December 2010 (2010-12-22), XP055258852, Retrieved from the Internet: URL:http://dspace.library.uu.nl/bitstream/handle/1874/192084/ohm.pdf?sequence=2 "Chapter 1" "Chapter 5" "Chapter 6" "Chapter 7" page 141; figure 6 ----- -/--	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* 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	Date of mailing of the international search report
9 March 2017	20/03/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Keller, Yves

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROBIN A. OHM ET AL: "Transcription factor genes of Schizophyllum commune involved in regulation of mushroom formation", MOLECULAR MICROBIOLOGY, vol. 81, no. 6, 4 September 2011 (2011-09-04), pages 1433-1445, XP055170230, ISSN: 0950-382X, DOI: 10.1111/j.1365-2958.2011.07776.x page 1434, column 2, paragraph 3 - page 1436 figure 1 figure 4 "Experimental procedures"	1-17
Y	ROBIN A OHM ET AL: "Genome sequence of the model mushroom Schizophyllum commune", NATURE BIOTECHNOLOGY, vol. 28, no. 9, 11 July 2010 (2010-07-11), pages 957-963, XP055078455, ISSN: 1087-0156, DOI: 10.1038/nbt.1643 the whole document	1-17
Y	ROBIN A. OHM ET AL: "The blue light receptor complex WC-1/2 of Schizophyllum commune is involved in mushroom formation and protection against phototoxicity", ENVIRONMENTAL MICROBIOLOGY, vol. 15, no. 3, 1 March 2013 (2013-03-01), pages 943-955, XP055258830, GB ISSN: 1462-2912, DOI: 10.1111/j.1462-2920.2012.02878.x the whole document	1-17
T	: S Travaglini ET AL: "Title: Mycology Matrix Composites Proceedings of the American Society for Composites-Twenty-Eighth Technical Conference", , 13 May 2013 (2013-05-13), XP055259661, Retrieved from the Internet: URL:http://www.mycoworks.com/wp-content/uploads/2014/04/UC-Berkeley-Mycology-Matrix-Composites-ASC-Conference-Paper-13-May-2013-2.pdf [retrieved on 2016-03-18] the whole document	12-17

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