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Transcription factors of *Schizophyllum commune* involved in mushroom formation and modulation of vegetative growth

Jordi F. Pelkmans¹, Mohini B. Patil¹, Thies Gehrmann², Marcel J. T. Reinders², Han A. B. Wösten¹ & Luis G. Lugones¹

Mushrooms are the most conspicuous fungal structures. Transcription factors (TFs) Bri1 and Hom1 of the model fungus *Schizophyllum commune* are involved in late stages of mushroom development, while Wc-2, Hom2, and Fst4 function early in development. Here, it is shown that Bri1 and Hom1 also stimulate vegetative growth, while biomass formation is repressed by Wc-2, Hom2, and Fst4. The $\Delta bri1\Delta bri1$ and the $\Delta hom1\Delta hom1$ strains formed up to 0.6 fold less biomass when compared to wild-type, while $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$, and $\Delta fst4\Delta fst4$ strains formed up to 2.8 fold more biomass. Inactivation of TF gene *tea1*, which was downregulated in the $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$, and $\Delta fst4\Delta fst4$ strains, resulted in a strain that was severely affected in mushroom development and that produced 1.3 fold more biomass than the wild-type. In contrast, introducing a constitutive active version of *hom2* that had 4 predicted phosphorylation motifs eliminated resulted in radial growth inhibition and prompt fructification in both $\Delta hom2$ and wild-type strains, even in sterile monokaryons. Together, it is concluded that TFs involved in mushroom formation also modulate vegetative growth. Among these TFs is the homeodomain protein Hom2, being the first time that this class of regulatory proteins is implicated in repression of vegetative growth in a eukaryote.

Mushroom forming fungi degrade plant waste, establish mutual beneficial interactions with plants (mycorrhiza), and can be pathogens. Moreover, they are an important food source and produce bioactive components. Mushrooms are the most conspicuous fungal structures. Their formation involves a complex developmental program that has been studied in the model systems *Coprinopsis cinerea* and *Schizophyllum commune*^{1,2}. Germination of a basidiospore of *S. commune* results in a monokaryotic mycelium that can fuse with another monokaryon with compatible mating types. Blue light is required to initiate fruiting in the resulting dikaryotic mycelium³, whereas high CO₂ levels repress this developmental program^{4,5}. Initiation of mushroom formation starts with asymmetrical colony growth, followed by aggregation of aerial hyphae, and subsequent formation of primordia². Primordia develop into fruiting bodies that form basidia within the hymenium. Karyogamy, meiosis, and one round of mitosis in the basidia results in haploid, binucleate basidiospores.

The blue light receptor complex of *S. commune* 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 phenotype⁶. 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 *bri1* shows a similar phenotype⁷. Inactivation of the zinc finger transcription factor gene *fst4* results in dikaryons that still grow irregular in the light under low CO₂ conditions but aggregates, primordia, and fruiting bodies are not produced⁸. Strains in which the Cys2His2 zinc finger protein gene *c2h2* has been inactivated are arrested at the aggregate stage⁷, while deletion strains of *fst3*, *gat1* and *hom1* form smaller fruiting bodies but in higher numbers^{7,8}. The zinc finger protein Fst3 was proposed to play a role in repression of outgrowth of fruiting bodies from primordia, while the GATA type zinc finger protein Gat1 and the

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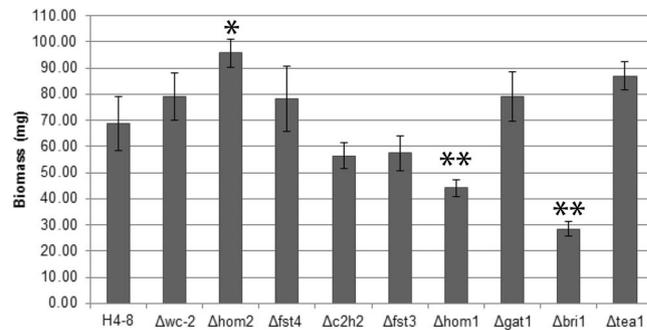


Figure 1. Biomass of biological triplicates of 6-day-old dark-grown agar cultures of the wild-type dikaryon and transcription factor deletion strains using glucose as carbon source. An independent sample t-test (p -value ≤ 0.05) was used to identify strains forming more (*) or less (**) biomass when compared to wild-type H4-8 (lines 506-507).

homeodomain protein Hom1 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 these and other basidiomycetes⁸⁻¹⁰, which is supported by the finding that over-expression of the *c2h2* homologue of *A. bisporus* results in faster fruiting body development¹¹.

In this study, it is shown that transcription factors that are involved in mushroom formation in *S. commune* also modulate vegetative growth. Bri1 and Hom1 were shown to stimulate biomass formation, while Wc-1, Wc-2, Hom2, Fst4, as well as the newly identified transcription factor Tea1 repress vegetative growth. The interplay between vegetative growth and mushroom formation is illustrated by a constitutive version of Hom2 that strongly reduces colony formation and immediately induces mushroom formation under ambient CO₂ conditions, even in a sterile strain.

Results

Genes involved in mushroom development also control vegetative growth. Dikaryotic colonies of wild-type H4-8, and the transcription deletion strains $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$, $\Delta bri1\Delta bri1\Delta fst4\Delta fst4$, $\Delta c2h2\Delta c2h2$, $\Delta fst3\Delta fst3$, $\Delta hom1\Delta hom1$, and $\Delta gat1\Delta gat1$ were grown for 6 days on glucose MM in the dark. The $\Delta hom2\Delta hom2$ strain formed 1.4-fold more biomass when compared to H4-8 (Fig. 1), which was accompanied by increased radial growth (not shown). In contrast, $\Delta bri1\Delta bri1$ and $\Delta hom1\Delta hom1$ formed 0.4 and 0.6-fold less biomass, respectively, while biomass formation of the other strains was not significantly different from H4-8. Strain $\Delta hom2\Delta hom2\Delta fst4\Delta fst4$ also formed more biomass than the wild-type. There was a trend that it also formed more biomass when compared to $\Delta hom2\Delta hom2$ (Supplementary Fig. 1), which was confirmed in liquid shaken cultures where it formed 1.7 fold more biomass when compared to the *hom2* deletion strain (Supplementary Fig. 1). 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. Microscopical analysis revealed that wild-type formed two types of hyphae at the colony periphery. Vacuole-rich hyphae with a diameter of $2.68 \mu m \pm 0.14$ were 5.7-fold less abundant than hyphae with few vacuoles and a diameter of $1.72 \mu m \pm 0.12$. Strain $\Delta hom1\Delta hom1$ formed 5-fold less thick hyphae when compared to the wild-type, while $\Delta bri1\Delta bri1$, $\Delta fst4\Delta fst4\Delta hom2\Delta hom2$ formed 1.3-, 2.0-, and 3.3-fold more vacuole-rich, wide hyphae (Supplementary Fig. 2).

Biomass formation of $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$ and $\Delta fst4\Delta fst4$ was assessed on xylose, pectin, and sucrose (Supplementary Fig. 3). The $\Delta fst4\Delta fst4$ strain formed 2.8-fold more biomass on xylose when compared to H4-8, while $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$ and $\Delta fst4\Delta fst4$ 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) (Supplementary Fig. 3). Together, these data show that Wc-2, Hom2, and Fst4 repress vegetative growth on different carbon sources.

Reduced vegetative growth of the $\Delta bri1\Delta bri1$ and $\Delta hom1\Delta hom1$ strains may slow down fruiting body development. Therefore, fruiting was monitored after 7 days and 15 days of culturing. The $\Delta hom1\Delta hom1$ strain had formed more but smaller mushrooms both after 7 and 15 days (Fig. 2). In contrast, $\Delta bri1\Delta bri1$ had not formed fruiting bodies after 7 days⁷ but did so after 15 days showing that fruiting in this strain is delayed and not abolished as reported previously⁷.

Genome-wide expression analysis. RNA composition of the wild-type H4-8 dikaryon 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* in whole cultures increased or decreased ≤ 2 -fold during development when compared to the vegetative mycelium grown in the dark (Table 1). 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, $\Delta wc-1\Delta wc-1$, $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$, $\Delta fst4\Delta fst4$, $\Delta c2h2\Delta c2h2$, $\Delta fst3\Delta fst3$, $\Delta gat1\Delta gat1$, $\Delta hom1\Delta hom1$, and $\Delta bri1\Delta bri1$ 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

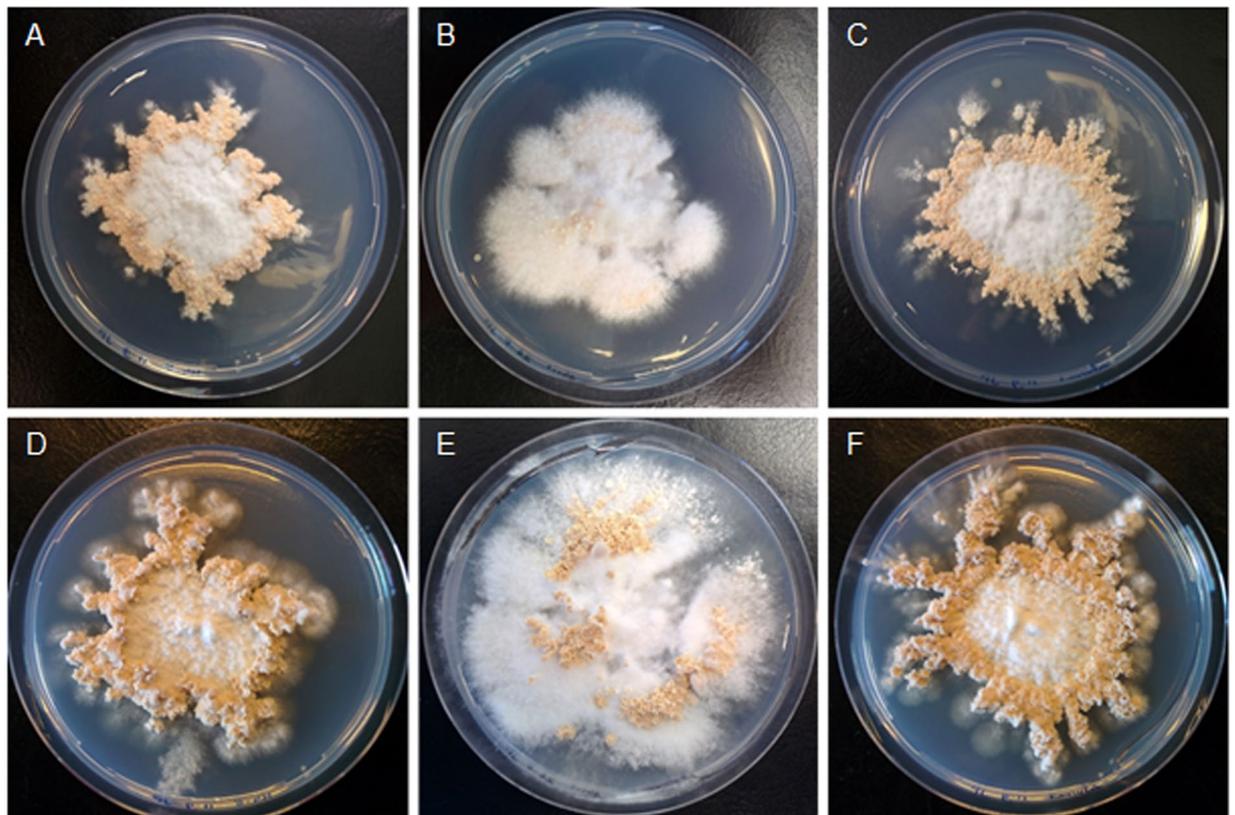


Figure 2. Fruiting body development of the wild-type dikaryon (A,D), $\Delta bri1\Delta bri1$ (B,E), and $\Delta hom1\Delta hom1$ (C,F) after 7 (A–C) and 15 (D–F) days of growth.

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

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

8-day-old colonies revealed a first and second component explaining 38% and 29% of the variation, respectively. Two distinct clusters were observed (Supplementary Fig. 4). The first cluster consisted of $\Delta wc-1\Delta wc-1$, $\Delta wc-2\Delta wc-2$, and $\Delta hom2\Delta hom2$ that are all affected in early stages of fruiting body development. The second cluster consisted of $\Delta gat1\Delta gat1$ and $\Delta fst3\Delta fst3$ 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, $\Delta wc-1\Delta wc-1$, $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$ and $\Delta fst4\Delta fst4$ clustered, whereas the other strains clustered with the wild-type (Supplementary Fig. 4).

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 wild type (Supplementary

Table 1). Enriched functional categories in the upregulated genes of 8-day-old colonies of $\Delta wc-1\Delta wc-1$, $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$ and $\Delta fst4\Delta fst4$ were mainly linked to carbohydrate metabolism, when compared to the 8-day-old wild-type colonies (Supplementary Tables 2 & 3). Downregulated functional groups were linked to energy transfer in these strains. Functional groups involved in energy transfer and carbohydrate metabolism were upregulated in 12-day-old colonies of $\Delta wc-1\Delta wc-1$ and $\Delta wc-2\Delta wc-2$ (Supplementary Tables 3 & 4), while downregulated genes were enriched for nucleosome, catalytic and oxidoreductase activity. The functional group ATPase activity was also overrepresented within the downregulated genes of $\Delta wc-2\Delta wc-2$. In 12-day-old colonies of $\Delta hom2\Delta hom2$ 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 $\Delta fst4\Delta fst4$ 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 $\Delta c2h2\Delta c2h2$, while processes involved in cytoplasm, ATP binding, nucleosome and peptidase activity were enriched in the downregulated genes. In 12-day-old colonies of $\Delta c2h2\Delta c2h2$ 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 $\Delta hom1\Delta hom1$ but functional groups related to chitinase activity, carbohydrate metabolism and energy transfer were enriched in the downregulated genes. 12-day-old $\Delta hom1\Delta hom1$ 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 $\Delta fst3\Delta fst3$ and $\Delta gat1\Delta gat1$ 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 $\Delta fst3\Delta fst3$. 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 $\Delta gat1\Delta gat1$. 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 $\Delta bri1\Delta bri1$ 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 $\Delta bri1\Delta bri1$ colonies were mainly enriched for carbohydrate metabolism, hydrolase activity and transcription repressor activity.

Between 1 and 43 transcription factor genes were ≥ 2 -fold up- or down-regulated in one of the deletion strains when compared to the wild-type (Fig. 3; Supplementary Table 5). Expression of *hom1* and *c2h2* had decreased in 12-day-old $\Delta wc-1\Delta wc-1$ colonies. In 12-day-old colonies of $\Delta wc-2\Delta wc-2$ *hom2* expression was increased, while *c2h2* was downregulated. Expression of *c2h2*, *gat1*, and *hom1* was downregulated in 12-day-old $\Delta hom2\Delta hom2$ colonies. In 8-day-old $\Delta fst4\Delta fst4$ colonies expression of *hom1* was downregulated and in 12-day-old colonies *hom2* expression increased, while *c2h2* and *hom1* expression had decreased. In contrast, *hom1* levels were upregulated in 8-day-old $\Delta fst3\Delta fst3$ colonies. Similarly, expression of *hom1* but also *c2h2* was increased in 8-day-old colonies of $\Delta gat1\Delta gat1$. In 8-day-old $\Delta bri1\Delta bri1$ colonies *gat1* expression was increased, while *hom2* was increased in 12-day-old colonies (Supplementary Fig. 5). Together, these data show that transcription factors involved in fruiting control other regulatory genes involved in this process.

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 8-day-old colonies of $\Delta wc-1\Delta wc-1$, $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$, $\Delta bri1\Delta bri1$ and $\Delta fst4\Delta fst4$ when compared to the 8-day-old aggregate forming colonies of wild-type, while it was upregulated ≥ 2 -fold in 8-day-old colonies of $\Delta fst3\Delta fst3$, $\Delta hom1\Delta hom1$ and $\Delta gat1\Delta gat1$. A ≥ 2 -fold decreased or increased expression of *tea1* was not observed in the deletion strains at day 12 when the wild-type had formed fruiting bodies. This is explained by reduced expression of *tea1* in the wild-type colonies when compared to day 8. Gene *c2h2d* (proteinID 2703923) was also downregulated in $\Delta wc-1\Delta wc-1$, $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$ and $\Delta fst4\Delta fst4$ during aggregation, while it was upregulated ≥ 2 -fold in $\Delta gat1\Delta gat1$ (Supplementary Fig. 5). Furthermore, expression of *c2h2d* was decreased > 2 -fold in 12-day-old colonies of $\Delta wc-1\Delta wc-1$, $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$, $\Delta fst4\Delta fst4$, $\Delta fst3\Delta fst3$ and $\Delta gat1\Delta gat1$. Gene *c2h2d* has a predicted C2H2 DNA binding domain, while *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 1).

Deletion constructs of *c2h2d* and *tea1* were introduced in H4-8 $\Delta ku80$. PCR analysis confirmed that $\Delta c2h2d$ and $\Delta tea1$ strains were obtained. These transformants were crossed with the compatible H4-8 wild-type strain and siblings were selected with an intact *ku80* gene and a deleted *tea1* or *c2h2d* gene. Compatible monokaryons were identified and then crossed to obtain the $\Delta tea1\Delta tea1$ and $\Delta c2h2d\Delta c2h2d$ strains. Growth and mushroom formation of the $\Delta c2h2d\Delta c2h2d$ strain were not affected (data not shown). In contrast, the $\Delta tea1\Delta tea1$ strain showed a 1.3-fold increase in biomass when compared to the wild-type. This increase was similar to that of $\Delta hom2\Delta hom2$ (Fig. 1). Transfer to the light did not induce irregular vegetative growth as observed in the wild-type. Moreover, newly formed light-exposed mycelium did not produce aerial hyphae (Fig. 4B). This resulted in a distinct border between the dark-grown mycelium and mycelium grown in the light. Fruiting body formation was almost completely abolished. The few spore-forming flask-like mushrooms were formed in clusters at random positions in the $\Delta tea1\Delta tea1$ colony, while a typical ring of fruiting bodies is formed in the wild-type (Fig. 4C,D). This and the fact that fruiting body and biomass formation was restored to wild-type levels in 2

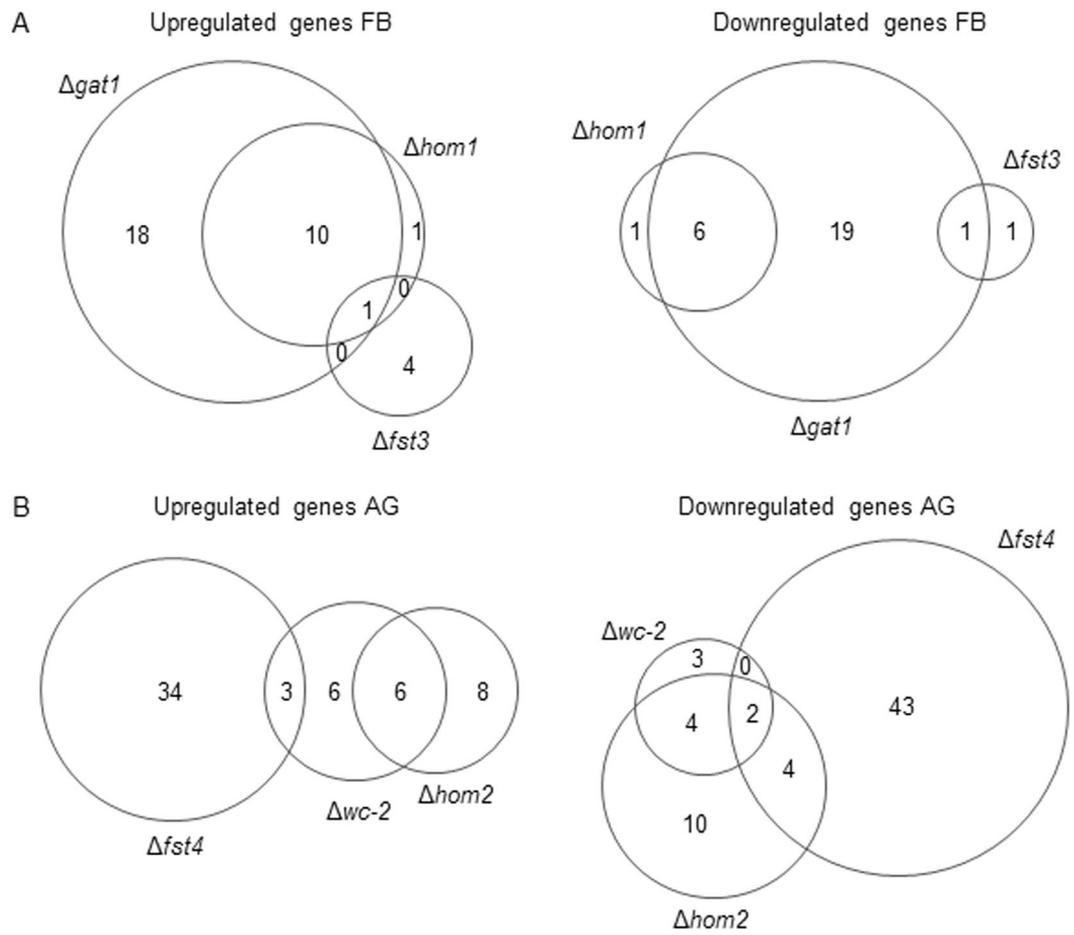


Figure 3. Venn diagrams showing overlapping differentially expressed transcription factor genes in $\Delta fst3\Delta fst3\Delta hom1\Delta hom1$ and $\Delta gat1\Delta gat1$ (A) and $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$, and $\Delta fst4\Delta fst4$ (B) when compared to the wild-type aggregate and fruiting body stage, respectively.

independent complemented strains (data not shown) shows that *tea1* represses vegetative growth and is involved in fruiting body formation.

Hom2^{con} represses vegetative growth and induces fruiting body formation. Hom2 is a key gene involved in mushroom formation but it does not show differential expression during development (see above). Therefore, we searched for motifs that could mediate post-translational modification of this homeodomain protein. Hom2 was found to contain 4 predicted RRXS phosphorylation motifs. The serine codons within these motifs were replaced for alanine codons, resulting in *hom2^{con}* (name based on its phenotype; see below) and introduced in *S. commune* H4-8a and H4-8b. Two types of transformants were observed. 90% of the colonies had a growth speed similar to regenerating wild-type protoplasts, while 10% grew very slowly. Restriction PCR using RNA from colonies of the wild-type, normal growing transformants, and slow growing transformants showed that *hom2^{con}* was much higher expressed than the endogenous copy in the case of slow growing transformants (Fig. 5F). These transformants were selected for further experiments. Monokaryotic *hom2^{con}* transformants grew slower than wild type at 30 °C in the dark at high CO₂. Clamp connections as observed in the wild-type dikaryon were absent (Fig. 5A,B). When colonies were transferred to fruiting conditions (low CO₂, light, 25 °C) the monokaryotic *hom2^{con}* transformants started to form flask like fruiting bodies that did not expose their spore forming gills (Fig. 5D,E). Similar results were obtained when *hom2^{con}* was introduced in a $\Delta hom2$ monokaryon. These data show that Hom2^{con} functions independently of the endogenous Hom2 and that it can induce fruiting in a sterile monokaryon. Monokaryotic *hom2^{con}* transformants crossed with the compatible wild type strain showed less severe phenotypes. However, they all grew slower than the wild type dikaryon and developed spore forming fruiting bodies immediately after transfer to the light at low CO₂ conditions. In contrast, the wild-type continued vegetative growth for 1-2 days and then started to form fruiting bodies (Data not shown).

Discussion

The transcription factor genes *wc-2*, *hom2*, *fst4*, *bri1c2h2*, *fst3*, *gat1*, and *hom1* have been reported to be involved in fruiting body formation in *S. commune*⁶⁻⁸. We here showed that the homeodomain domain protein Hom2, the zinc finger transcription factor Fst4, and the blue light complex transcription factor Wc-2 also repress vegetative growth, while the DNA binding BRIGHT domain protein Bri1 and the homeodomain protein Hom1 stimulate

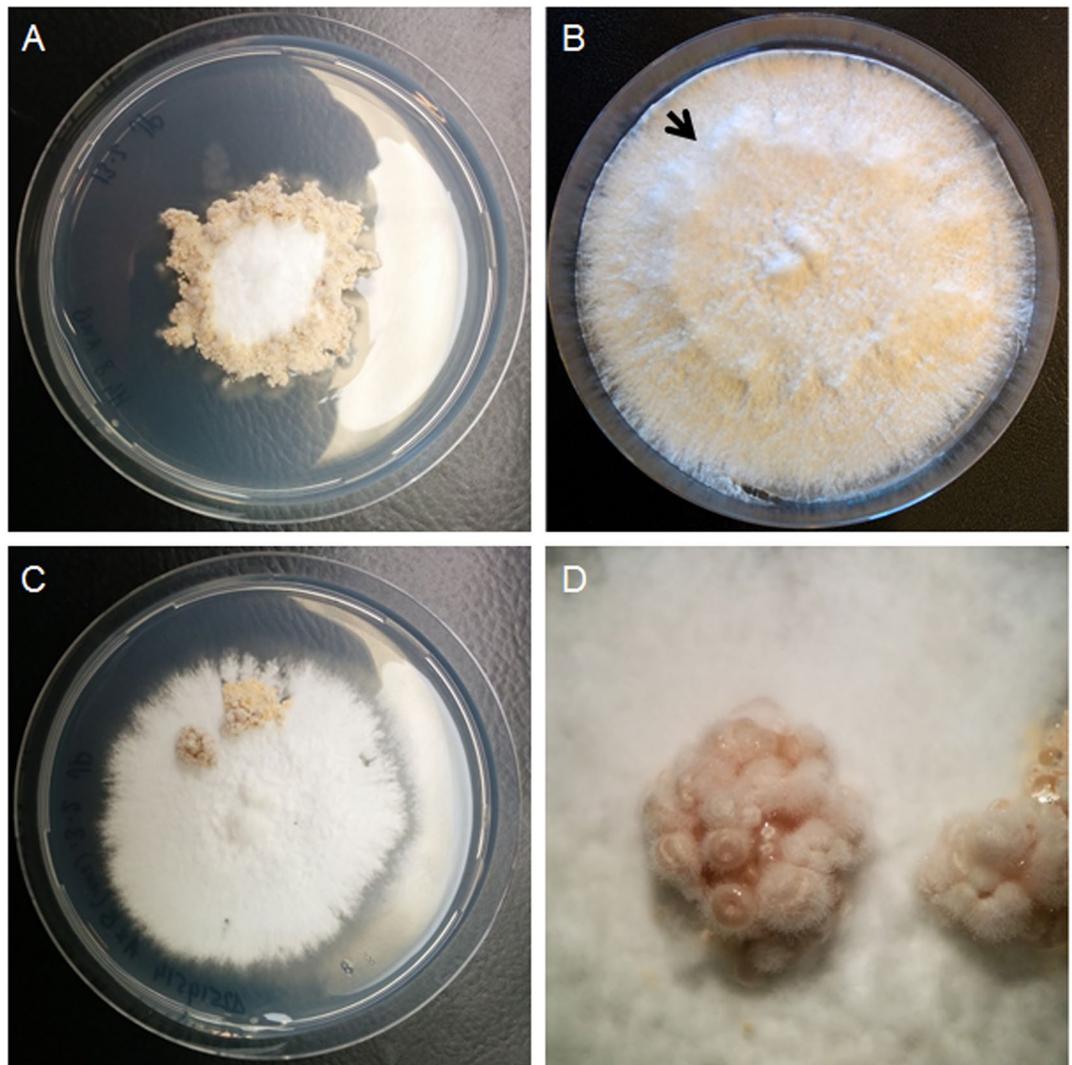


Figure 4. Aerial growth of dikaryotic colonies of H4-8 (**A**) and $\Delta tea1$ (**B,C,D**). Strain $\Delta tea1\Delta tea1$ produced less dense and high aerial hyphae when transferred to light (**B**) and only forms small clusters of fruiting bodies (**C** and **D**). Arrow indicates the transition from dense aerial hyphae production to thinner aerial hyphae production upon light induction.

vegetative growth. We also showed that a newly identified transcription factor, called Tea1, stimulates mushroom formation, while repressing vegetative growth.

The $\Delta bri1\Delta bri1$ strain does not fruit after the standard growth period of 10 days⁷. However, we here showed that fully developed mushrooms had formed after 4 weeks. This shows that Bri1 is not required for fruiting. Delayed mushroom development may be the result of the reduced growth speed of $\Delta bri1\Delta bri1$. Lower biomass formation may well be explained by the fact that functional categories metabolic process, carbohydrate metabolism, catalytic activity, transcription, and cell wall were down-regulated in 8-day-old colonies of the deletion strain. 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 formation¹². Notably, Bri1 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, Bri1 stimulates vegetative growth and functions in mushroom formation by its effect on *tea1* and *gat1* expression (Fig. 6).

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^{6,7} but also increased the vegetative growth rate. Strain $\Delta wc-2\Delta wc-2$ formed more biomass on sucrose and pectin when compared to the wild-type, while $\Delta hom2\Delta hom2$ formed more biomass on glucose, sucrose, and pectin. Increased biomass formation was associated with an increased hyphal diameter and with a higher radial extension rate. In addition, upregulated genes were enriched in the functional class 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,

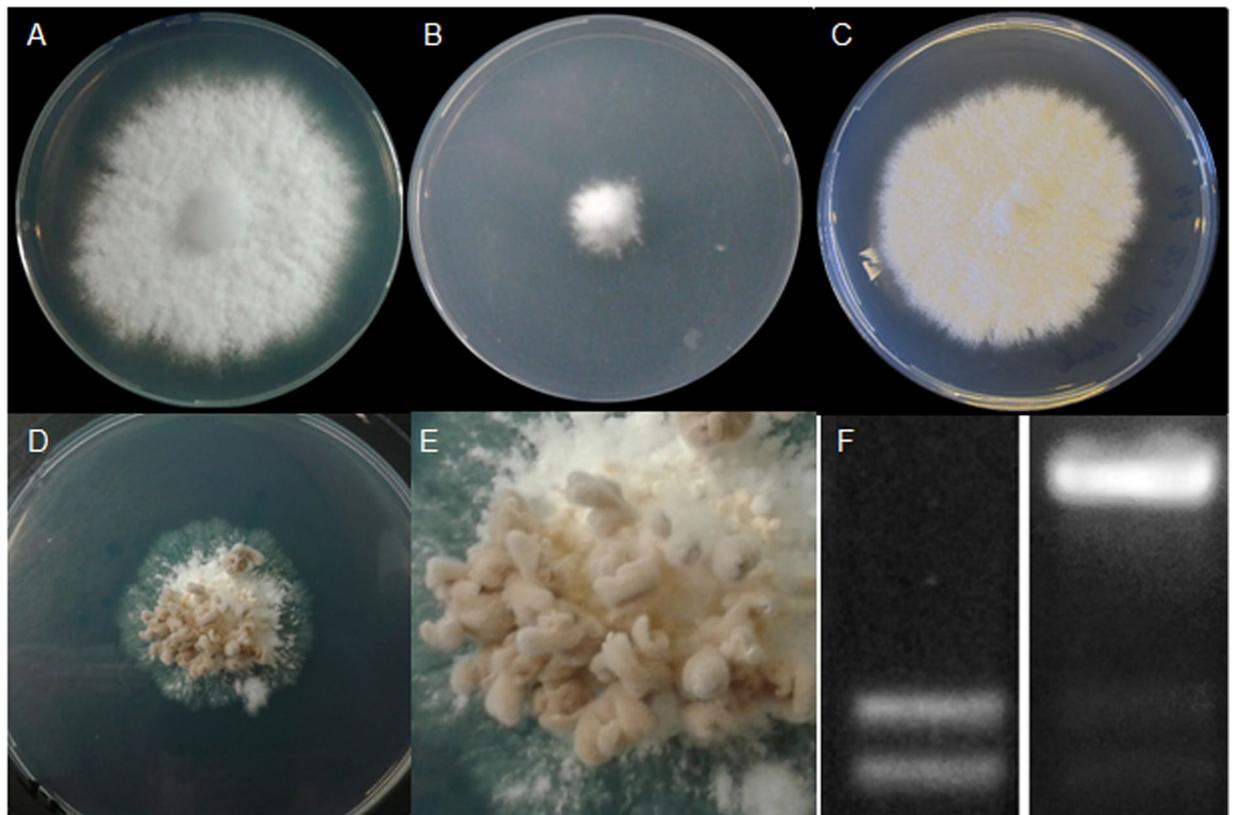


Figure 5. Six-day-old colonies of the wild type monokaryon (A,C) and a *hom2^{con}* strain (B,D) grown continuously at 30 °C in the dark at high CO₂ (A,B) or transferred to 25 °C, low CO₂, and light (C,D). (E) represents a close up of fruiting bodies of the *hom2^{con}* strain, while (F) shows relative *hom2* and *hom2^{con}* expression in wild-type (left) and the *hom2^{con}* strain T4 (right).

although the effect was less pronounced. Together, this confirms that Wc-2 and Hom2 stimulate *c2h2* expression^{6,7} (Fig. 6).

Expression of *hom2* and *wc-2* is rather constant during development, suggesting post-translational regulation of these genes. In the case of Wc-2, this may be accomplished by the interaction with the blue light sensor Wc-1⁶, while post-translational modification of Hom2 was shown to be mediated via its 4 predicted protein kinase motifs. Introduction of *hom2^{con}* that has its 4 protein kinase motifs eliminated resulted in severe phenotypes in sterile wild-type and $\Delta hom2$ monokaryons. The *hom2^{con}* strains grew very slowly when compared to their parental strains, indicating that *hom2* in its non-phosphorylated state represses vegetative growth. Transformants even fruited when exposed to light and low CO₂, thus resembling a dikaryon. This suggests that Hom2 is maintained in a phosphorylated state in the monokaryon resulting in fast vegetative growth and repression of fruiting. The fact that the $\Delta hom2$ monokaryon grows faster than the wild-type implies that a fraction of Hom2 in the monokaryon is not phosphorylated thus impacting vegetative growth to some extent. Data also indicate that the phosphorylation state of Hom2 is the only inhibitory factor that suppresses fruiting in monokaryons in conditions favorable for mushroom formation in the dikaryon. Moreover, data imply that fruiting body formation can be independent of the mating type genes. Indeed, *frt1* of *S. commune* also initiates fruiting in a homokaryon when copies of the gene are introduced in homokaryons with a different *frt1* allele¹³. The role of the mating type genes could be restricted to the establishment and maintenance of the dikaryotic state. Alternatively, different pathways exist that each can lead to fruiting body formation and that may at some point interconnect.

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 $\Delta fst4 \Delta fst4$ 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. Hyphae of $\Delta fst4 \Delta fst4$ were more wide compared to wild-type but not as wide as $\Delta hom2 \Delta hom2$. Together, these data indicate 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 $\Delta hom2 \Delta hom2 \Delta fst4 \Delta fst4$ formed more biomass than $\Delta hom2 \Delta hom2$. Strain $\Delta fst4 \Delta fst4$ showed enrichment of carbohydrate metabolism in the upregulated genes of 8-day-old and 12-day-old-colonies similar to that observed in $\Delta wc-2 \Delta wc-2$ and $\Delta hom2 \Delta hom2$. The fact that *fst4* expression is not affected in $\Delta wc-2 \Delta wc-2$ and $\Delta hom2 \Delta hom2$ 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* (Fig. 6).

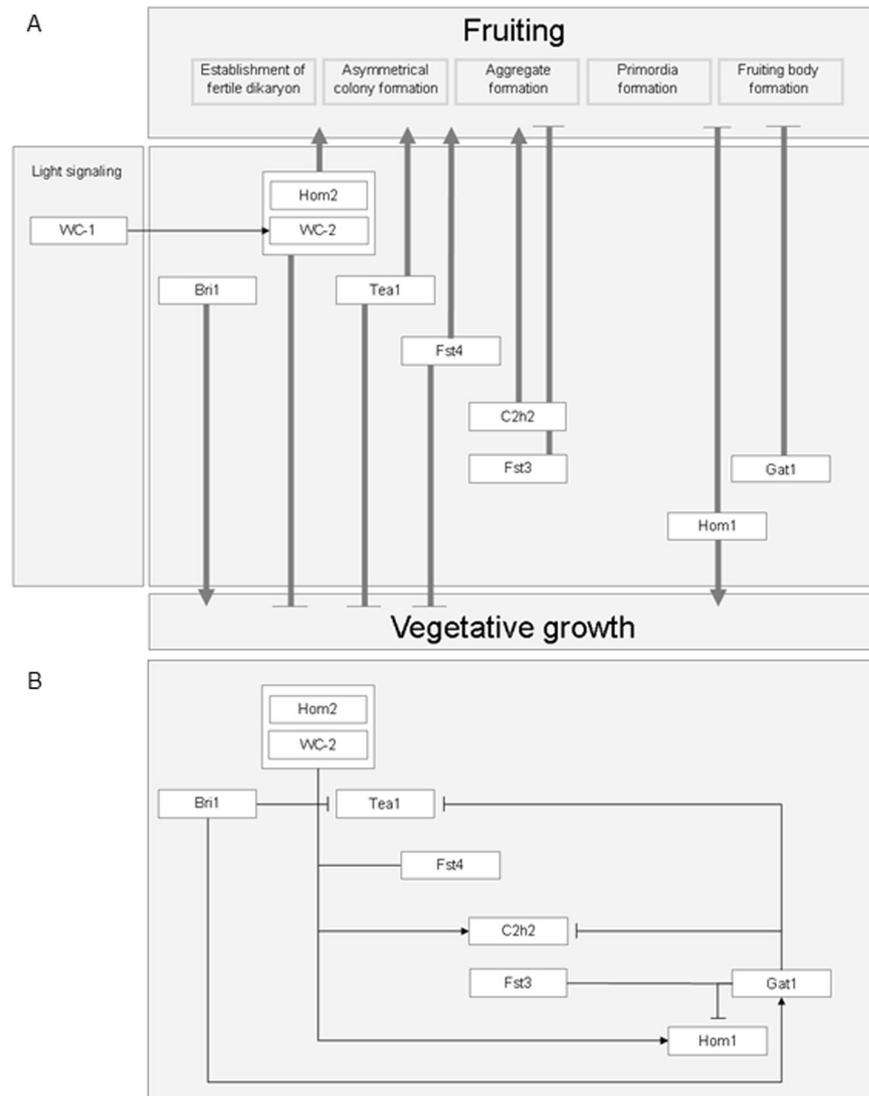


Figure 6. Model of regulation of vegetative growth and fruiting body formation in *S. commune*. Transcription factor genes control both vegetative growth and fruiting body development (**A**) and influence each other's expression levels (**B**).

Transcription factor gene *tea1* was downregulated in $\Delta wc-1\Delta wc-1$, $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$, $\Delta bri1\Delta bri1$ and $\Delta fst4\Delta fst4$ when compared to the aggregating wild-type (8-day-old colonies), while it was upregulated in $\Delta fst3\Delta fst3$, $\Delta hom1\Delta hom1$ and $\Delta gat1\Delta gat1$. 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 $\Delta tea1\Delta tea1$ strain formed more biomass on glucose when compared to the wild-type. Moreover, it was severely affected in mushroom formation. Local clusters of fully developed mushrooms were only occasionally 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 the further increased expression in primordia and fruiting bodies agrees with the phenotype of $\Delta c2h2\Delta c2h2$ forming aggregates but not primordia and fruiting bodies⁷ (Fig. 6). 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 mushrooms⁷. In addition, Hom1 and Gat1 are involved in mushroom tissue formation⁷. 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. Expression of *hom1* gradually increases during progression of fruiting. Wc-1, Hom2, Fst4, and probably Wc-2, stimulate expression of *hom1*, while Fst3 and Gat1 have the opposite effect. The fact that Hom1 has both an effect on vegetative growth and on tissue formation in mushrooms suggests that Hom1 operates at two distinct stages of development (Fig. 6). Alternatively, the reduced size of the $\Delta hom1\Delta hom1$ fruiting bodies and the effect of Hom1 on tissue formation in mushrooms may be explained solely by the reduced size of the vegetative feeding mycelium.

The model of development of *S. commune* may apply to other mushroom forming basidiomycetes as well. Hom2, Hom1, Fst3, Fst4, C2H2, and Gat1 are basidiomycete-specific regulatory proteins¹⁴. Homologues of these genes were identified in *L. bicolor* and *A. bisporus*^{8,9}. The homologues for *fst4*, *fst3*, *c2h2*, and *hom1* were upregulated during sexual development in two *A. bisporus* varieties⁹, while homologues of *hom2*, *fst4*, *c2h2*, *fst3*, *gat1* and *hom1* showed similar expression expression profiles in *L. bicolor* showed similarity to *S. commune*⁹. Expression patterns of *c2h2*, *fst3*, *hom1* and *gat1* were also found to be similar in *C. cinerea*¹⁰. Moreover, it was recently shown that over-expression of the *c2h2* homologue of *A. bisporus* accelerated mushroom development in this mushroom forming fungus¹¹, which agrees with the phenotype of the $\Delta c2h2\Delta c2h2$ phenotype of *S. commune*.

This is the first time a direct link has been shown between repression of vegetative growth and initiation of sexual reproduction. Previously, a link has been shown between vegetative growth and asexual development in *Aspergillus*¹⁵. This link involves trimeric G-protein signaling. The activity of the G α -subunit of *Aspergillus* is regulated by the FlbA protein¹⁶. 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 bodies¹⁷. This suggests that similar signaling pathways are involved in the decision to stop vegetative growth and to invest in reproduction in ascomycetes and basidiomycetes. A link between vegetative growth and development may also exist in *Podospora anserina*. Inactivation of the homeodomain gene *pah1* resulted in increased production of male gametes and increased branching of vegetative hyphae, resulting in smaller but more dense colonies^{18,19}.

Methods

Culture conditions and strains. The compatible *S. commune* strains H4-8 (*matA43matB41*; FGSC 9210)⁸ and H4-8b (*matA41matB43*)²⁰, their derived wild-type dikaryotic strain, as well as the deletion strains $\Delta wc-1\Delta wc-1$, $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$, $\Delta fst4\Delta fst4$, $\Delta c2h2\Delta c2h2$, $\Delta fst3\Delta fst3$, $\Delta hom1\Delta hom1\Delta bri1\Delta bri1$, $\Delta gat1\Delta gat1$ ⁶⁻⁸, $\Delta hom2\Delta hom2\Delta fst4\Delta fst4$ and $\Delta hom2\Delta hom2\Delta fst3\Delta fst3$ were used in this study. The $\Delta ku80$ H4-8 strain²¹ 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 1.5% agar and 2% glucose²². Cultures were inoculated with a point inoculum taken from the periphery of a 7-day-old colony. Liquid shaken cultures were inoculated with a mycelial homogenate²³ and grown at 250 rpm in 250 ml Erlenmeyers containing 100 ml MM. To assess growth on other carbon sources, glucose was replaced by 4% xylose, 3.4% sucrose, or 1% pectin.

Gene inactivation and complementation. 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 cassette²⁰. 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 $\Delta 2519514ufw/\Delta 2519514urv$ and $\Delta 2519514dfw/\Delta 2519514drv$, respectively (Supplementary Table 6). Primer pair combinations $\Delta 2703923ufw/\Delta 2703923urv$ and $\Delta 2703923dfw/\Delta 2703923drv$ were used to amplify the 897 bp upstream and 975 bp downstream flank of *c2h2d*, respectively (Supplementary Table 6). 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.

To complement strain $\Delta tea1$ the coding region of *tea1* was amplified by PCR using primer pair *tea1fw1/tea1rv1* (Supplementary Table 6) that introduce AarI and BamHI sites, respectively. The AarI/BamHI fragment was introduced in NcoI/BamHI cut vector pRO151²⁴ that has a pUC20 backbone containing 1 kb HindIII/NcoI promoter sequence of *gpd1* gene from *S. commune*, a 350 bp BamHI/EcoRI terminator sequence from the *S. commune sc3* gene, and a EcoRI fragment containing a phleomycin resistance cassette. This resulted in pTEA1^{comp}.

Expression vector for Hom2^{con} of *S. commune*. Plasmid pHom2 is a pGMTphleoB derivative containing the *S. commune* gene *hom2*⁷. The 1832 bp HindIII/SbfI fragment of pHom2 containing *hom2* coding and flanking sequences was cloned in pSP72 using the same sites, resulting in pHom2HF. In the next step, a 692 *hom2* coding fragment was synthesized (Genscript, Piscataway, NJ, USA), in which the serine codons in the 4 RRXS motifs were replaced for alanine (*hom2^{con}*). As a consequence of the substitution in the second motif a unique PvuI site was removed in *hom2^{con}*. A fusion PCR was performed with the 692 bp fragment and pHom2HF using T7 and SP6 primers annealing at both sides of the multiple cloning site of pHom2HF. The fusion fragment resulted in a mixture of 1922 bp *hom2* and *hom2^{con}* fragments. The wild-type fragment was digested with PvuI. The remaining 1922 bp band was cut with HindIII/SbfI and used to replace the corresponding fragment in pHom2. This resulted in plasmid pHom2* containing the *hom2^{con}* gene.

Transformation of *S. commune*. Constructs were introduced in H4-8 or H4-8 $\Delta ku80$ as described²² using $1 \cdot 10^7$ protoplasts. For gene deletions, protoplasts were incubated with 20 μg vector DNA and regenerated overnight without antibiotic. Selection of transformants took place for 4 days at 30 °C on MM plates containing 8 $\mu\text{g ml}^{-1}$ nourseothricin. Transformants were transferred to a second selection plate containing 5 $\mu\text{g ml}^{-1}$ 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 2519514ufcfw/nourdelrev and 2519514dfcrv/sc3tersqf were used to screen for *tea1* deletion (Supplementary Table 6), while primer pairs c2h2dufcfw/nourdelrev and c2h2ddfcfv/sc3tersqf were used to confirm *c2h2d* deletion (Supplementary Table 6).

For complementation or expression constructs, protoplasts were incubated with 5 μg vector DNA and regenerated overnight in the presence of 25 $\mu\text{g ml}^{-1}$ phleomycin. Selection of transformants took place for 4 days at 30 °C on MM plates containing 25 $\mu\text{g ml}^{-1}$ phleomycin.

Whole genome expression analysis. The wild-type dikaryon and strains $\Delta wc-1\Delta wc-1$, $\Delta wc-2\Delta wc-2$, $\Delta hom2\Delta hom2$, $\Delta fst4\Delta fst4$, $\Delta c2h2\Delta c2h2$, $\Delta fst3\Delta fst3$, $\Delta hom1\Delta hom1$, $\Delta bri1\Delta bri1$, and $\Delta gat1\Delta gat1$ were grown for 5 days in the dark at 22 °C, after which they were transferred to the light²⁵. Biological duplicate whole cultures 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. RNAseq data have been deposited at NCBI under BioProject PRJNA323434.

RNA-Seq Analysis Pipeline. STAR aligner²⁶ was used 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 analysed by Cufflinks version 2.1.1²⁷, and Cuffdiff using a Benjamini Hochberg false discovery rate of 0.05²⁸ excluding genes that had a number of fragments per kilobase of exon per million < 1 in one or both of the strains or conditions. 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 factors⁸.

Restriction PCR. RNA was extracted from 10-day-old colonies grown on MM plates on top of a polycarbonate membrane using Trizol (Thermo Scientific, Waltham, MA, USA). cDNA was made using the QuantiTect Reverse Transcription Kit (Qiagen, Düsseldorf, Germany). In the next step, cDNA was amplified by PCR with the intron spanning primers hom2rtfw and hom2rtvr (Supplementary Table 6). The amplified wild-type 1117 bp fragment was cut with PvuI resulting in fragments of 609 and 508 bp. The *hom2^{con}* fragment could not be cut by this enzyme, leaving the 1117 bp band intact.

Bioinformatics. RRRS domains were identified in the protein sequences of *S. commune* using a custom Python script. Enrichment analyses were performed with Python and R to analyze over- and under-representation of functional annotation terms in sets of genes using the Fisher Exact test. The Benjamini-Hochberg correction was used to correct for multiple testing using a *p*-value ≤ 0.05 . Orthologues of *S. commune hom2* in other basidiomycetes were identified by the presence of a homeobox PFAM domain (PF00046)²⁹ and a reciprocal best blastp hit to *hom2*. These hits were manually curated using MycoCosm³⁰ based on the available expression and homology data for that genome. The protein sequences were aligned using MAFFT version 7.123³¹ with the E-INS-i method. The alignment was visualized and manually curated using Jalview 2.8.2³². Conserved RRRS sites were identified in the alignment. A phylogenetic tree was reconstructed using RaxML version 8.1.16³³ with the PROTGAMMAWAG model with 100 rapid bootstrap partitions. The tree and domain structures were visualized using the ETE Toolkit version 2.2.

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) using biological triplicates. Mycelium of liquid cultures was separated from the medium using Miracloth filter (Merck Millipore, Billerica, USA). Mycelium was freeze-dried and weighed. Statistical analysis was done with an independent sample *t*-test (*p*-value ≤ 0.05) using IBM SPSS 20.

Microscopy. Strains were grown in Petri dishes with a diameter of 4.5 cm within a 125 μm layer of MM agar for 2 days at 30 °C. Petri dishes were covered with wet tissue, 2 layers of plastic foil, and 1 layer of aluminum foil. Morphology of hyphae was studied with an inverted microscope using a 400-fold magnification. Statistical analysis was done with a χ^2 test (*p*-value ≤ 0.05).

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Author Contributions

J.P., M.H., T.G. and L.L. performed, analysed, interpreted, and designed the experiments; H.W., L.L. and M.R. supervised the project; J.P., L.L., and H.W. wrote the manuscript.

Additional Information

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