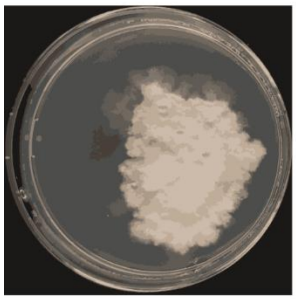

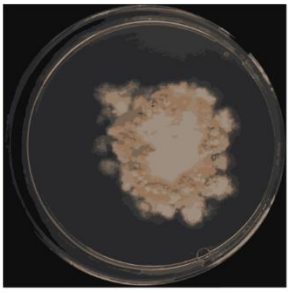

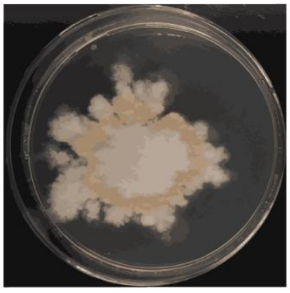

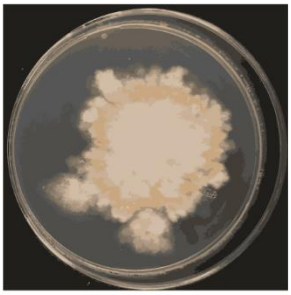

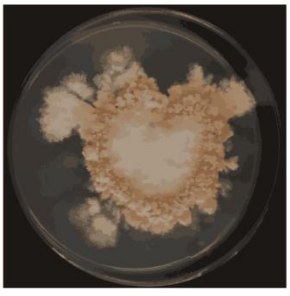

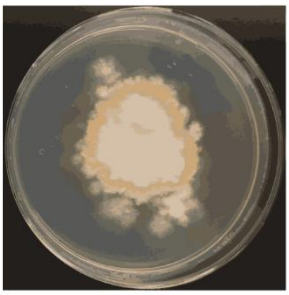

Mushroom Formation in *Schizophyllum commune*: The Central Role of cAMP in CO_2 Sensing

Major Research Project Report

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Laymen's Summary

Mushrooms, the fruiting bodies of fungi, are increasingly grown for their nutritional, economical and medicinal value. To this end, a limited number of species are cultivated intensively. To sustain the future growth of the mushroom industry, in regard of both capacity and variety, mushroom cultivation must be thoroughly researched and optimised. For mushroom formation, the involvement of environmental triggers, including temperature, CO_2, and light, is deemed essential. High environmental CO_2 levels inhibit mushroom formation in most mushroom-forming fungi, including *Schizophyllum commune*, a model fungus for mushroom formation. It produces its split-gill mushroom only when CO_2 levels are low. Little is known about how the sensing of this environmental change and the subsequent signalling cascade that coordinates mushroom formation. The signalling molecule cyclic AMP appears to have a central role in its regulation. Therefore, this study looked further into the functioning of a protein involved in the breakdown of cyclic AMP. However, no conclusions could be drawn from the work performed in this project regarding this protein. Consequently, different strategies may be applied to further research its involvement. Moreover, a first attempt was made at conditional gene expression in *S. commune*, which would allow for accessible interference in gene expression. Both upregulation and downregulation of genes may further elucidate the mechanisms at play in mushroom formation. Additionally, this tool may be applied as a strategy for also the controlled regulation of the cyclic AMP breakdown protein. Altogether, these developments may add to the understanding of mushroom formation, thus facilitating expansion of mushroom cultivation.

Mushroom Formation in *Schizophyllum commune*: The Central Role of cAMP in CO_2 Sensing

Abstract

Environmental CO_2 is recognized to be one of the main factors involved in mushroom formation in many fungi, including the model fungus *Schizophyllum commune*. It has been established that cyclic AMP (cAMP) plays a central role in regulation of fructification, however, it remains unclear how the intracellular cAMP concentration is regulated and how this affects fructification throughout the process. cAMP is produced by adenylyl cyclase, whereas breakdown is achieved by phosphodiesterases (PDEs). An attempt has been made at the deletion of the high-affinity *PDE* (*Pde2*) of *S. commune*, though no transformants were obtained. Moreover, efforts were made towards a titratable conditional gene expression in *S. commune*, by developing the required constructs for the Tet-on system. In this initial setup, the reporter gene *dTomato* is used to show the viability of the system in *S. commune*. The Tet-on system may prove useful as a novel strategy in interference with genes, *Pde2* being a prime candidate. Additionally, the use ring plates has been introduced for phenotyping of colonies. Altogether, valuable tools have been prepared which will improve future research methods. Finally, some colonies were mutated into the streak morphology during transformation by accident. Evidently, streak colonies show red auto-fluorescence, whereas WT colonies do not.

Introduction

Mushrooms have a high potential for becoming the food source of the future. Most edible mushrooms are rich in fibre, minerals, protein, and vitamins, resulting in a highly nutritious product. Many contain antimicrobial, antitumor, anti-inflammatory, and anti-diabetic compounds, which are increasingly studied for application (Chang 2007a; Rathore, Prasad, and Sharma 2017). In addition, biotechnological applications are increasingly researched, though only in limited species (Suzuki 2021). Mushroom-forming fungi are involved in ecological recycling of nutrients. Saprophytes live off lignocellulosic rest-products, such as wood and straw, which contain complex, high-energy compounds. These are released and broken down by fungal enzyme secretions and subsequently remodelled into a high-quality food. Industries such as forestry, agriculture, and their second handlers provide plenty of substrate for these saprophytes, since more than 70% of their materials are non-productive (Chang and Wasser 2017). The cultivation of mushrooms is labour-intensive, thus providing jobs and income (Chang 2007b). Moreover, mushroom-forming species thrive in diverse substrates, allowing for cultivation in many areas on the globe. Beyond the high demand of mushrooms as foodstuff, the medicinal, technological, ecological, and economic benefits add to the increasing popularity of mushroom-forming fungi. Between 1960 and 2012 mushroom production increased annually with 12.9% on average (Chang and Wasser 2017), reaching up to about 35 billion kg of produced mushrooms in 2013 (Royse, Baars, and Tan 2017). As the world population is further growing to 9.7 billion people in 2050, food production must be expanded to serve our population. In this prospect, the many-faceted mushroom is ideal to exploit further.

The expansion of mushroom production is dual. On one hand, there is the intensifying of the yield of mushroom production, by researching and applying new cultivation methods. On the other hand, there is the widening of the palette of species available for commercial cultivation. Historically,

around two hundred species have been experimentally grown around the globe, though more than three thousand species are regarded as prime edible mushrooms (Chang and Wasser 2017). Notably, only a mere twenty species are cultivated commercially, which include *Agaricus*, *Lentinus*, *Pleurotus*, *Auricularia*, *Volvariella* and *Flammunila* (Kues and Liu 2000). For many other Agaricomycetes, attempts at cultivation have not yet led to fruitful results. Though the mushroom is the most conspicuous fungal structure, how it comes to existence is still unknown. Research into the induction and process of fruiting may help us understand the barriers that block fruiting in these species, as well as find ways to increase the yield in already cultivated species.

Schizophyllum commune is a white-rot mushroom-forming fungus that is used as a model for fruiting body formation. Its genome is relatively easy tampered with, due to availability of genetic tools for this species (Ohm, de Jong, Berends, et al. 2010; Ohm, de Jong, Lugones, et al. 2010; Vonk et al. 2019). *S. commune* has a rather convenient life cycle that spans 10 days from spore germination to spore production (Ohm, de Jong, Lugones, et al. 2010). Once a spore has germinated, a monokaryon starts to develop through vegetative growth. When it meets another monokaryon with different alleles for the mating-type loci MatA and MatB, they may fuse together to form a dikaryon (Pelkmans et al. 2017). Environmental conditions may now trigger the process of fruiting. In *S. commune*, the initiation is recognized with the aggregation of aerial hyphae. These turn into primordia, that finally mature into fruiting bodies (Ohm, de Jong, Lugones, et al. 2010). When plate-grown from point inoculum, fruiting generally takes place in the periphery (Raudaskoski and Yli-Mattila 1985). Here, a ring of macroscopic size forms and turns brown during fruiting body formation (Perkins 1969). Humidity, nutrient availability (Manachere 1980) and salinity (Castillo and Demoulin 1997) are crucial for both vegetative and generative growth (fruiting). Environmental factors that lead to fructification include temperature, light, and CO_2 levels (Niederpruem 1963; Niederpruem and Wessels 1969), all of which must change drastically from vegetative growth conditions to induce fruiting (Kues and Liu 2000). A 5 °C drop from 30 °C is optimal for fruiting, whereas the switch from dark to light conditions is obligatory (Yli-Mattila et al. 1989). For *S. commune*, light is photoinductive between 320 and 525 nm and most stimulating around 450 nm (Perkins 1969; Perkins and Gordon 1969; Raudaskoski and Yli-Mattila 1985; Yli-Mattila 1985). It has been established that the proteins White Collar (WC) 1 and 2 are responsible for the sensing of blue light specifically and the subsequent transcription involved in fructification (Ohm et al. 2013). Deletion of the *wc-1* and *wc-2* results in a blind phenotype, which grows vegetatively in both dark and light.

In contrast with the sensing of light in *S. commune*, little is known about the sensing of environmental CO_2 and the pathway that connects it to mushroom formation. Transport of CO_2 across fungal membranes does not require transporters or aquaporins in *C. albicans* (Bahn and Mühlschlegel 2006; Klengel et al. 2005). When CO_2 is dissolved in water, a natural equilibrium arises between bicarbonate and CO_2, which is greatly pushed towards the former by carbonic anhydrases (CAs). CA deletion mutants of *C. albicans* and *C. neoformans* must be compensated for their low CO_2/bicarbonate equilibrium by increasing environmental CO_2, to activate virulence factors (Bahn and Mühlschlegel 2006; Klengel et al. 2005). This shows that CAs are required for subsequent activation of pathways that are indirectly dependent on environmental CO_2 levels. In both *C. albicans* and *C. neoformans*, it has been established that adenylyl cyclase (AC) has a conserved binding site for HCO_3^-, which connects environmental CO_2 levels to the AC – cyclic AMP (cAMP) – protein kinase A (PKA) pathway. Mutation of this site resulted in CO_2 blind colonies in *C. albicans* (Hall et al. 2010). AC responds to bicarbonate by converting ATP into cAMP, a ubiquitous second messenger involved in responses to extracellular signals and nutritional conditions (Buck et al. 1999). One of its confirmed targets in yeasts is PKA, a well-conserved kinase (Wilson et al. 2007). Notably, high cAMP levels are inhibited by PKA, an effect that becomes stronger in PKA overexpression mutants (Nikawa, Sass, and Wigler 1987). This effect may be well explained through its direct activation of phosphodiesterases

(PDEs) that break down cAMP into AMP (Ma et al. 1999). PKA is also renowned for its role in many pathways, through which it may also affect cellular or subcellular cAMP levels (Nikawa, Sass, et al. 1987; Vandamme, Castermans, and Thevelein 2012). Notably, virulence levels in *C. neoformans* are affected by each of the components in the pathway, i.e., AC, cAMP, PKA and PDE (D'Souza et al. 2001).

This principle may also apply to fructification in *S. commune* and other Basidiomycetes, as it is most likely that CO_2-sensing in *S. commune* is also regulated through the same pathway (figure 1). By placing chemicals or substances in the medium or near the fungus the AC-cAMP-PKA pathway may be interfered with, resulting in differences in fructification (Leonard and Dick 1968; Schwalb 1974; Uno and Ishikawa 1971). Notably, cAMP has been found to stimulate fructification in *C. macrorhizus* (Uno and Ishikawa 1971, 1973a, 1973b). This finding is consistent with a later finding in *S. commune*, that exposure to light leads to an intracellular cAMP increase (Yli-Mattila 1987). However, exposure to light was only short and measurements took place over a relatively short period. These results are at conflict with work from (Schwalb 1974), who shows that mushroom formation ceased at a certain stage in *S. commune* when supplemented with cAMP or analogues. This conforms to the supposed positive correlation between environmental CO_2 and cAMP. These differences may all well be explained through a broader narrative of cAMP fluctuations. Kinoshita *et al.* (2002) show that cAMP peaks just before primordia formation and is followed up with a gradual increase until fructification has succeeded. Also, a sudden decrease in intracellular cAMP levels was seen when the culture was moved to generative conditions.

Presumably many proteins and pathways are involved in the fluctuation of cAMP levels, however only AC is responsible for its production (Takagi, Katayose, and Shishido 1988). Fungal ACs are most different from mammalian ACs, which are divided into two categories but together provide a full explanation of the fungal variant. Transmembrane ACs (tmACs) respond to activated heterotrimeric G proteins and are involved in the response to extracellular signals (Bahn and Mühlschlegel 2006). Soluble ACs (sACs) are activated directly by bicarbonate in a concentration dependent manner (Chen et al. 2000; Klengel et al. 2005). Fungal ACs are different as they comprise only one type of AC, which is soluble and carries out the functions of both mammalian ACs (Klengel et al. 2005; Mogensen et al. 2006). Notably, this double function has not been recognized in all fungi. In *S. cerevisiae*, *S. pombe*, *U. maydis*, *C. albicans* and *C. neoformans*, adenylyl cyclase is stimulated by a G_α subunit (D'Souza and Heitman 2001; Maidan et al. 2005). However, only for the latter two species adenylyl cyclase also functions as a bicarbonate sensor (Hall et al. 2010; Klengel et al. 2005). In *S. commune*, AC is sensitive to bicarbonate, apparent from its response to environmental CO_2. Moreover, constitutively active heterotrimeric G_α and G_γ subunits in *S. commune* result in suppression of fruiting body formation, like high levels of environmental CO_2 (Yamagishi et al. 2002). A follow-up study showed that these transformants resulted in elevated intracellular cAMP levels up to 200% (Yamagishi et al. 2004). In conclusion, AC from *S. commune* is thus responsive to both signals.

Furthermore, AC in *C. albicans* is also activated by Ras enzymes through a highly conserved binding domain (Rocha et al. 2017). It has been recognized that each of these three inputs for AC are involved in virulence (Hogan and Sundstrom 2009). Notably, JGI Mycocosm (https://mycocosm.jgi.doe.gov/) shows two main predictions for *AC* in *S. commune*, one of which contains a Ras binding domain (proteinID's 2633355 and 2633357). Constitutively activated Ras results in similar phenotypes compared to constitutively active G_α subunits (Yamagishi et al. 2004). Its phenotype fits earlier research involving addition of exogenous cAMP, which suggests that Ras asserts it effects through the AC – PKA pathway (Palmer and Horton 2006; Schwalb 1974). Notably, both constitutively active Ras and deletion of its inhibitor *Gap1* showed no increase in intracellular cAMP (Schubert et al. 2006; Yamagishi et al. 2004). In a follow-up study, however, it was shown that

PKA levels were significantly higher compared to WT, thus reinforcing the hypothesis that Ras is involved in above-mentioned pathway (Knabe et al. 2013). The idea of multiple AC activators may conform to the fluctuating cAMP levels during subsequent stages of fructification, as shown by Kinoshita *et al.* (2002). Once the mycelium reaches environmental CO_2 levels, intracellular bicarbonate concentrations would lower and give room to initiation of mushroom body formation. After a short period, G subunits and/or Ras may activate AC once more, resulting in higher concentrations of cAMP. Alternative splicing is not uncommon in *S. commune* and possibly, AC needs to be spliced differently for it to be responsive to Ras (Gehrmann et al. 2016). Most clearly, Ras-responsive AC would elevate the intracellular cAMP concentration once more. It remains unclear what role this takes on during fructification.

Fungal genomes generally contain two cAMP phosphodiesterases, *PDE1* and *PDE2*, which respectively encode a low-affinity and a high-affinity variant (Ma et al. 1999). As such, Pde2 regulates the basal cAMP level, which reflects the cell's reaction to stresses like temperature, salts, and heavy metals (Park, Grant, and Dawes 2005). In *S. cerevisiae*, both Pde1 and Pde2 are crucial for regulating cAMP concentrations and both are positively correlated to PKA activity (Hu et al. 2010; Ma et al. 1999; Nikawa, Cameron, et al. 1987). However, deletion of *PDE1* results in much higher cAMP accumulation upon stimulation, compared to deletion of *PDE2* (Ma et al. 1999). Adding to the complexity, a double *PDE1/PDE2* mutant resulted in merely a threefold cAMP elevation (Nikawa, Sass, et al. 1987). This hints at other factors at play in the AC – PKA pathway that have not yet been recognized, or strong feedback inhibition through AC and/or PKA (Vandamme et al. 2012). In *C. albicans* virulence factors are dependent on Pde2; its deletion results in both lack of virulence and higher basal cAMP levels (Bahn, Staab, and Sundstrom 2003; Wilson et al. 2007). This is most different for *C. neoformans*, in which Pde2 appears to play no role in virulence whereas Pde1 does (Hicks, Bahn, and Heitman 2005). These differences may be due to species specific traits, an issue that may extend itself to Pde1 and Pde2 levels in mushroom-forming fungi (Wilson et al. 2007).

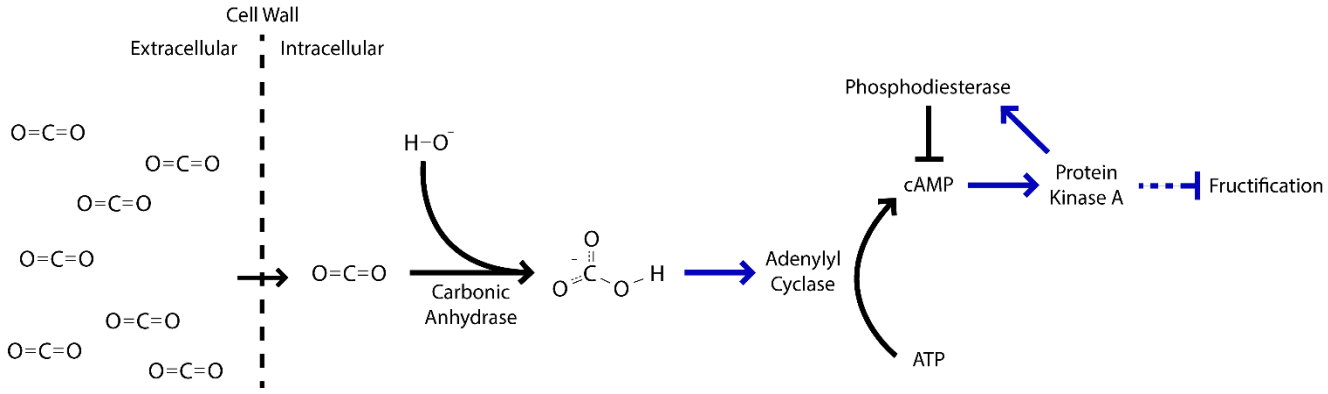

Figure 1. A proposed pathway for the sensing of CO_2 and inhibition of fructification in *S. commune*. The black lines depict chemical reactions, the blue lines depict activation or inhibition of proteins.

During this research project a first attempt has been made at conditional gene expression in *S. commune*. The Tet-on and Tet-off systems, both derived from *E. coli* species, are well-known for their use in *Aspergillus* species (Das, Tenenbaum, and Berkhout 2016; Wanka et al. 2016). Both systems include a constitutively expressed transcriptional activator that binds the titratable doxycycline, a stable derivative of tetracycline. In the Tet-on system, this transcriptional activator is a reverse tetracycline-controlled transactivator, that becomes active upon doxycycline stimulation. The opposite is true for Tet-off systems, which involves a tetracycline-controlled transactivator that shuts down upon stimulation. Both bind specifically to its responsive operator (TetO7), which entails seven copies of a DNA binding sequence (Meyer et al. 2011). The system allows for user-determined gene

expression, from knock-out to overexpression. Notably, the Tet-off system is most useful for characterization of essential genes, for which deletion is not an option but quantitative regulation of expression is. This may be the case with genes involved in mushroom formation (N. Braat and Lugones 2020; Wanka et al. 2016). Currently, this system has not been used in *S. commune* to our knowledge but would prove a useful tool for previously failed gene overexpression and deletion attempts.

Finally, protoplasting and transformation has proven itself hazardous during this project. Failed attempts have inspired opportunities for improvement of these processes. Regarding protoplasting, differently grown mycelia have been exploited, next to the use of spores and a variety of lysing enzymes. Transformation to *S. commune* requires relatively high doses of antimycotics, specifically phleomycin D1 (zeocin), hygromycin, and nourseothricin. Experience learns that 0.5 µg ml^{-1} phleomycin is sufficient for selection against *S. commune*, though most selections happen at 25 µg ml^{-1} (Ohm, de Jong, Berends, et al. 2010; van Peer et al. 2009). Nourseothricin selection is done using 8 or 10 µg ml^{-1} (Ohm, de Jong, Berends, et al. 2010; van Peer et al. 2009), though concentrations up 15 (Vonk et al. 2019) µg ml^{-1} or even 20 µg ml^{-1} have been utilized (Ohm et al. 2011). Selection on nourseothricin is generally reserved for deletion of genes, which is characterized by a low efficiency in *S. commune* due to low incidence of homologous integration (Ohm, de Jong, Berends, et al. 2010). One Cas9 RNP deletion requires around ten plates and consequently large amounts of antibiotic (Vonk et al. 2019). Optimization of transformation would involve the minimization of antibiotic use while at the same time improving, or at least not diminishing the transformation efficiency.

Materials and Methods

Culture Conditions and Strains

S. commune strains were grown from point inoculum on minimal medium (MM) for liquid cultures or supplemented with 1.5% agar for plate cultures (Dons, de Vries, and Wessels 1979). For phenotyping, MM with and without up to 4 mM cAMP (Sigma-Aldrich, St. Louis, USA) or up to 64 mM $NaHCO_3$ (Sigma-Aldrich, St. Louis, USA) was used. NaOH was added to the cAMP stock, until the cAMP was dissolved. Vegetative growth of the mycelium was generally done for 3 to 5 days at 30 °C, whereas generative growth was achieved in 3 to 4 days at 25 °C. For vegetative growth, plates were kept in stoves with and without CO_2 supplementation, up to 7% CO_2 in an incubator (CO28IR-10, New Brunswick Scientific). When not supplemented with CO_2, plates were wrapped together in plastic foil resulting in the accumulation of metabolically produced CO_2. Always, the mycelia were kept in the dark during vegetative growth. Fruiting was achieved by placing the colonies in generative conditions, for which plates were disposed of their lid and kept in a box that allowed for aeration. During fruiting, the mycelia saw 16 hours of white light alternated by 8 hours of darkness. The box was kept humid with tissues wetted with either water or 4% (w/v) KOH for binding of environmental CO_2 (Schwalb 1971). On ring plates and for weighing experiments, the mycelium was grown on a membrane, either cellophane or polycarbonate (PC). For the perspex ring plates, liquid medium was used. The strains *S. commune* H4-8A (MATA43MATB41; FGSC 9210) and H4-8B (MATA41MATB43) were used as wild-type (WT). For phenotypic experiments, their derived dikaryon was created by inoculating point inocula together with a distance less than 0.5 cm between them. Alternatively, the Pde2 overexpression was used for phenotypic comparison (N. Braat and Lugones 2020). For the development of the *ΔPde2* transformant, the ΔKU80 strain was used.

Phosphodiesterase 2 deletion construct

From the *pde2* gene (ProteinID 2636760, https://mycocosm.jgi.doe.gov/Schco3/Schco3.home.html) the upstream and downstream flank, resp. 1200 and 1318 bp, were amplified using Taq polymerase and 3 µL DMSO (SigmaAldrich, St. Louis, USA) per 50 µl PCR reaction mix, and primer pairs pde2uf/pde2df (table 1). pGmtNour was isolated from its *E. coli* transformant and cut with EcoRI, resulting in a 1252 bp fragment containing a nourseothricin resistance cassette. Vector pUC20 was cut with HindIII and EcoRI, creating a linear backbone containing ampicillin resistance. The three fragments were cloned into pUC20 using circular polymerase extension cloning (CPEC), with all fragments present within one order of magnitude of each other. In 20 µl of CPEC reaction mix 8 ng/µL DNA was used, supplemented with 0.6 µL DMSO. Following a PCR-reaction without primers, a plasmid of 6405 bp was obtained in which nourseothricin was placed between the two *Pde2* flanks (pDelPde2). The integrity of the plasmid was checked using restriction enzymes, followed by sequencing, both of which confirmed the expected configuration (table 2).

Transcriptional Transactivator construct

The sequence of the reverse transcriptional activator (rtTA) was codon optimized for S. commune and synthetically produced (Integrated DNA Technologies, Coralville, IA). pTubDst2 was cut using BamHI and EcoRI, resulting in the loss of both the *SC3* terminator and the phleomycin resistance cassette. The plasmid was given a *β-tubulin* terminator (ProteinID 2629605) instead, which was also cut with BamHI and EcoRI. Dst2 was cut out using NcoI and BamHI, which allowed for the insertion of rtTA, which was cut using BsaI and BamHI. Consequently, the rtTA was placed under control of the *β-tubulin* promoter, which also contains an intron before the start codon. Finally, a resistance cassette for hygromycin was added, after both the vector and the resistance cassette were cut with EcoRI

(prtTASc). For additional amplification of the rtTA, primer pair scrtTAfw/scrtTArv was developed (table 1). The Tet-promoter, comprising of 7 Tet boxes (TetO7) and a minimal GPD promoter, was amplified from an *Aspergillus niger* construct using Taq polymerase and primer pair teto7fw/teto7rv (table 1). These primers were designed to contain HindIII and NcoI sites outside the sequence. The *GPD* promotor (ProteinID 2673958) of pRO25 was cut out and replaced by the TetO7 with HindIII and NcoI. dTomato, already present in the plasmid, was now under control of the TetO7 promoter (pTetO7-dTom). For the TetO7-Pde2 construct, this gene was taken out using restriction enzymes NcoI and BamHI. From chromosomal DNA *Pde2* was amplified using the primer pair teto7pde2fv/sc3terpde2rv thus creating overlap with the open plasmid (table 1). Using CPEC the gene was then amplified into the TetO7 backbone, resulting in a TetO7-Pde2 construct (pTetO7-Pde2). The integrity of the plasmids was checked using restriction enzymes which confirmed the expected configuration (table 2).

*Phosphodiesterase 2 * construct*

The sequence of Pde2*, in which the PKA binding domain RRXS was replaced by RRXDS, was synthetically produced (Integrated DNA Technologies, Coralville, IA) and amplified using primer pair pde2cpecfw/pde2cpecrv (table 1). pTubDst2 was cut with NcoI and BamHI resulting in the loss of Dst2. Using in vitro assembly as described below, the Pde2* sequence was cloned into the pTub vector (pTubPde2*). The integrity of the plasmids was checked using restriction enzymes which confirmed the expected configuration (table 2).

Protoplast Production from Mycelium

Protoplasts were prepared by growing ten point inocula of *S. commune* H4-8 A, B, or ΔKU80-A for 3 to 4 days in 25 mL minimal medium. Afterwards, the culture was diluted and macerated twice. The two maceration steps were followed by incubation periods of respectively 24 and 16 hours at 200 rpm at 30 °C. The mycelium was spun down at 4700 rpm for 15 minutes, before washing with 1 M MgSO4·7H2O and resuspended in *Trichoderma harzianum* lysing enzyme mix. The latter was prepared with 7,5 mL 2X YT, 300 mg *T. harzianum* lysing enzymes, 0.9 mL 0.5 M malic acid, 2.5 mL MilliQ. 3.96 g MgSO4·7H2O, followed by centrifugation at >10.000 rcf. The supernatant was filter sterilized (0.2 µm, Sarstedt) before being added to the culture, which was subsequently incubated for three to six hours at 30 °C. During incubation, protoplasts were occasionally counted using a Bürker Türk counting chamber. When protoplasts had sufficiently formed, one volume of MilliQ was added before another incubation period of 30 minutes at 30 °C. This allowed protoplasts to be released from the mycelium. The protoplasts were spun down at 1000 g for 1 minute to remove larger pieces of undigested material and subsequently passed through nylon mesh (50 µm). The cells were then incubated with one volume of sorbitol for ten minutes at 30 °C. Next, they were spun down at 2500 g and washed with sorbitol twice before resuspending them in transformation buffer (1 M sorbitol, 0.05 M CaCl2), in aliquots of 200 µl or 1000 µl for H4-8 WT or ΔKU80-A respectively, at a concentration of 10^8 protoplasts mL^{-1}. Finally, they were frozen at -80 °C in a freezing container (Mr. Frosty, Nalgene).

Transformation of S. commune

For transformation, protoplast portions were thawed on ice and 5-10 µg of circular plasmid was added dissolved in a maximum volume of 20 µl TE (10 mM Tris pH 8, 3 mM EDTA]. After an initial incubation period of 15 min on ice, one volume of fresh 40% (w/v) PEG4000 buffered with 10 mM Tris was carefully added. After slow stirring, the mixture was kept at room temperature for 5 min. Regeneration medium (10 g L^{-1} yeast extract, 20 g L^{-1} pepton, 0.5 M $MgSO_4$) was added to a volume

of 2.5 mL. For selection on phleomycin, 25 µg mL^{-1} zeocin was added, whereas for other selections 10 µg mL^{-1} zeocin was added. After overnight regeneration at 25 °C, regenerated protoplasts were spun down and resuspended in MM. SCMM supplemented with low-melting point agarose (LMPA) at 37 °C was added, resulting in a fourfold dilution of protoplasts and a final concentration of 1% LMPA. After mixing, the cells were spread on square plates containing 40 mL of solid MM, supplemented with up to 25 µg mL^{-1} zeocin or up to 20 µg mL^{-1} hygromycin. Selection plates were incubated at 30 °C for at least 5 days. Per plate 10 ml of resuspended protoplasts is needed. For integration transformations up to 3 plates are used.

For knockout attempts using Cas9, the transformation was carried out in with accordance to Vonk *et al.* (2019) with few modifications. The sgRNAs were prepared using primer pairs p1_sgrna_uf/p2_ sgrna_uf and p1_ sgrna_df/p2_sgrna_df (table 1). In preparation of the transformation, 20 µg of one sgRNA for 100 µg Cas9 was used, followed by 100 µg of repair template. Moreover, a volume of 25 mL instead of 2.5 mL of regeneration medium was applied. Selection was done using nourseothricin, up to 15 µg mL^{-1}. A total of 10 square plates were used for plating out.

E. coli cloning

In all cases, the DH5α *E. coli* strain was used for cloning. The Pde2 deletion construct was cloned to *E. coli* using electroporation. Electrocompetent cells were prepared by growing E. coli to 0,8 OD. Next, they were centrifuged at 220 rpm, and washed twice using MilliQ. Finally, the cells were solved in 1 ml 10% glycerol. When not being handled, the cells were kept on ice. Prior to electroporation, the DNA were cleaned of salts. Electroporation was performed using 1 µL plasmid DNA with 40 µL electrocompetent cells. The cells were electroporated at 2.5 kV, 25 µF capacitance and 200 Ω resistance (Gene Pulser II and Pulse Controller II, Bio-Rad). Immediately after electroporation, the cells were resuspended in 1 ml 2x L-broth for an hour. Finally, they were plated on L-broth with 1.5% agar and ampicillin.

For the in-vitro assembly of the two fragments for the Pde2* overexpression construct, their fragments were cloned in PCR in accordance with Jacobus and Gross (2015). For vector amplification, 0.5 ng of DNA per 50 µl was used, whereas 2 µl was used for the insert. Both fragments were cycled 30 times for amplification, their products corrected to a 1:2 vector:insert molar ratio afterwards. For all plasmids and fragments, except for the Pde2 deletion construct, transformation was performed using heat shock on *E. coli* cells prepared using the RbCl method. Competent cells and the DNA were kept on ice for 15 minutes, before applying heat shock at 42 °C. After 1 minute, the cells were supplemented with 1 ml 2x LB for outgrowth. For IVA, the cells were put in ice for 5 minutes before adding LB. Afterwards, the cells were regenerated for 1 hour before being plated on agar / L-broth with ampicillin.

Table 1. Primers used in this study

Name	
pde2uffw	GGA AAC AGC TAT GAC CAT GAT TAC GGG GTG GAC GAG CGA CGA ACA G
pde2ufrv	TCC CAG ACC ACC ATG CCG GGA ATT CAC GCG ACC GGC AGT GCC A
pde2dffw	GTC CCC CTC GAG GCG CGC CGA ATT CCC CAG CTT TCC CGT ATG TTT ATC
pde2dfrv	CGT TGT AAA ACG ACG GCC AGT GCC AGT ACG TCC CGA GGG CCT AG
scrtTAfw	TAT ATA TGG TCT CTC ATG TC
scrtTArv	ATA TAG GAT CCT CAG CC
teto7fw	TAT AAA GCT TCG TAT CAC GAG GCC CTT TC
teto7rv	TAT ACC ATG GAA ACG GTG ATG TCT GCT CAA G
teto7pde2fv	TCG TCT GTA GTG GCA AAG GTA CCA TGT GCC CCC TCG GGA CCG GTG C
sc3terpde2rv	AGA CTG ACG TGC ACT CAC AGT CAT TGG CGC GAG CCC GC
pde2cpecfw	CGC TCA CGC CCG CCC GCA GCA TGT GCC CCC TCG GGA CC
pde2cpecrv	AGA CTG ACG TGC ACT CAC AGT CAT TGG CGC GAG CCC GC
p1_sgrna_uf	TAA TAC GAC TCA CTA TAG GTC AAG ACC TAT GTG CCC CCT
p2_ sgrna_uf	TTC TAG CTC TAA AAC AGG GGG CAC ATA GGT CTT GA
p1_ sgrna_df	TAA TAC GAC TCA CTA TAG GCA AGG CTG CAG GTG CAG TAA
p2_sgrna_df	TTC TAG CTC TAA AAC TTA CTG CAC CTG CAG CCT TG

* Colours indicate overlap with sequences. Red depicts overlap with *Pde2* upward flank, yellow depicts overlap with the nourseothricin cassette, blue depicts overlap with *Pde2* downward flank, orange depicts overlap with *Pde2* coding region, green depicts overlap with the T7 promoter and tracrRNA promoter used for sgRNA production.

Table 2. An overview of constructs cut using various restriction enzymes and their resulting bands

Experimental line	Construct	Restriction enzymes	Expected bands (kb)	Bands (kb)
ΔPde2 strain	Pde2 (CPEC)	NdeI / XhoI	4,9; 1,5	5,1; 1,5
		BglII / XhoI	4,9; 1,5	5,1; 1,5
		MluI	5,1 ;1,3	5,0; 1,4
		BglII / SspI	3,4; 3,0	3,4; 3,0
Tet-on system	pTetO7-dTom	NcoI		6,0
		HindIII / NcoI		5,0; 0,8
		NcoI / BamHI		4,8; 0,7
		EcoRI		4,8; 1,4
		XhoI	3,7; 1,9	4,8; 1,3
		ScaI	4,4; 1,3	5,5; 2,4
	prtTA -Hyg	HindIII / BamHI		3,2; 1,5
	prtTA +Hyg	EcoRI	4,6; 1,3	4,6; 1,3
		BamHI		4,9; 1,8
	pTetO7-Pde2	HindIII / NcoI		7,0; 1,3
		EcoRI		7,0; 0,5
		XhoI		4,0; 2,5; 0,9
*Pde2*Pde2** *strain*	pTub-Pde2*	NdeI		9,0
		XbaI / BglII		7,5; 1,6

Results and Discussion

cAMP decreases the density of the mycelium in S. commune

It was previously shown that 2.5 mM cAMP inhibits mushroom formation in *S. commune* when added to the medium (N. Braat and Lugones 2020). Subsequently, increasing concentrations of cAMP were applied to determine the minimal effective concentration for inhibition of mushroom formation and how higher concentrations affected fructification. It was found that 0.5 mM cAMP still allowed for some pigmentation, but none was found at 1 mM cAMP (figure 2). The colony eventually formed small mushroom bodies at the lowest concentration, though not in the allocated time of 4 days. Therefore, it seems likely that 0.5 mM cAMP impedes fructification, but does not fully prevent it. Moreover, it seemed that addition of cAMP up to 2 mM led to larger surfaces of mycelium and thus faster growth rates, even before switching to generative conditions. Evidently, the surface peaked around 2 mM, since 4 mM cAMP results in colonies with sizes between control and 2 mM cAMP. Therefore, it was hypothesized that cAMP strengthened vegetative growth up to 2 mM. Consequently, follow-up experiments were performed using cellophane or PC-membranes allowing for biomass determination. Cellophane membranes resulted in irreproducible values with no correlation found. Moreover, fructification occurred in the centre of the colonies where the mycelium was furthest away from the medium, instead of at the periphery (data not shown).

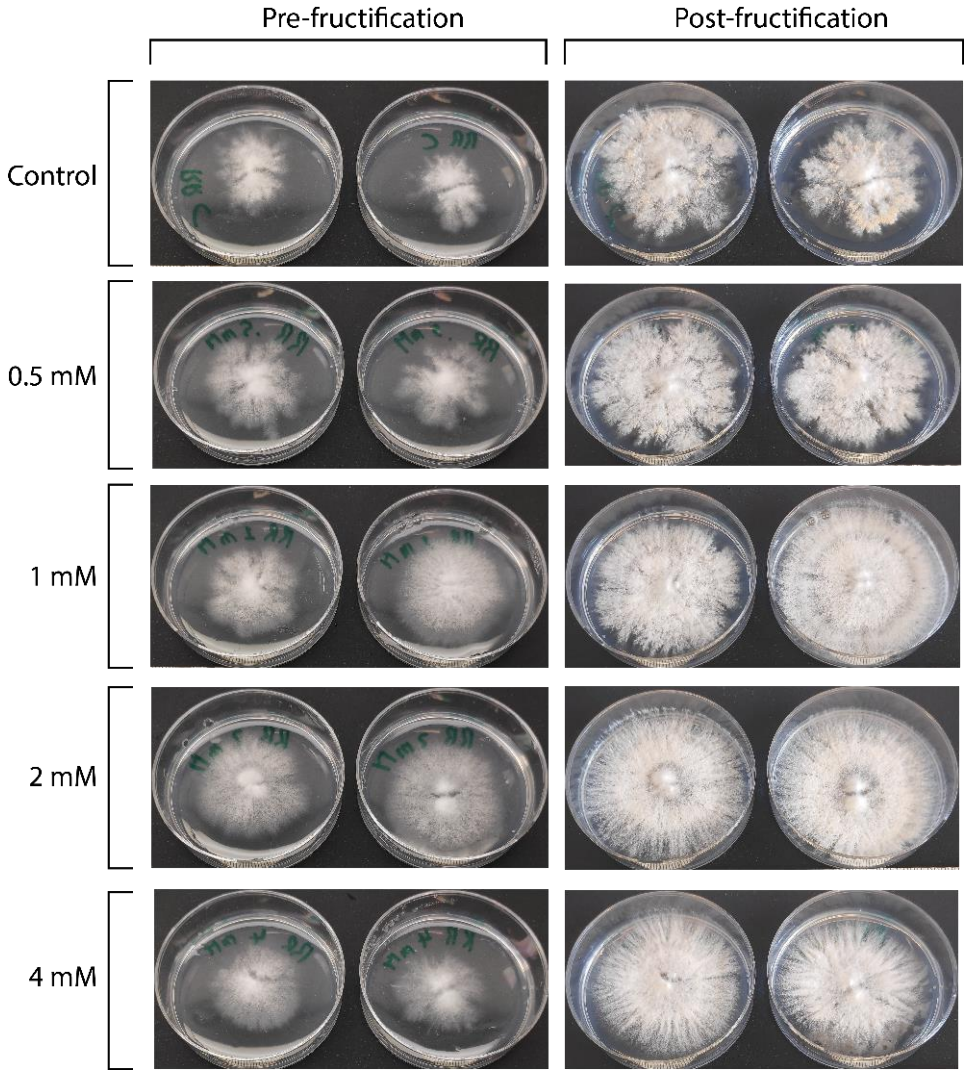

Figure 2. Colony growth and fructification after respectively three days of vegetative growth and three additional days of generative growth, on increasing concentrations of cAMP in the medium.

When grown on PC-membranes, comparisons were made between control conditions and 2 mM cAMP for both dikaryons and monokaryons. For both groups, 5 days of vegetative conditions and 3 days of vegetative conditions followed by 2 days of generative conditions were also studied in their contrast. Monokaryons were tested since they are unable to produce fruiting bodies, limiting their growth output to vegetative growth. Moreover, monokaryons are generally applied for the development of mycelium materials. Vegetative and generative conditions were compared to see the effect of fructification on biomass production. Interestingly, no fruiting bodies were formed, most likely due to the membranes. After growth completion, the mycelia were scraped of their membranes and dried for 24 hours in a 65 °C stove. The biomass was then measured of each colony.

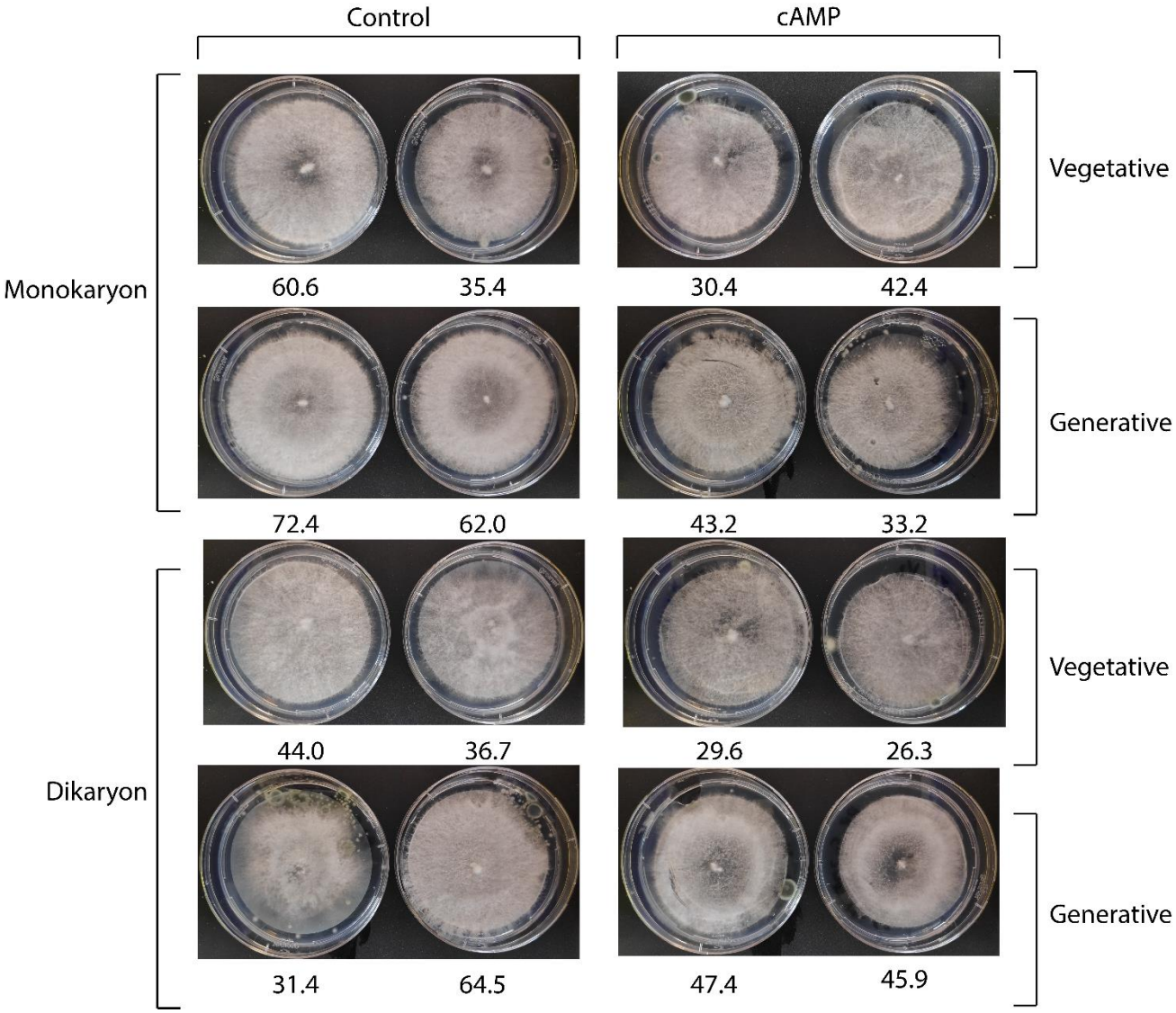

Figure 3. Growth of monokaryons and dikaryons with and without 2 mM cAMP. *Vegetative* involves 5 days of vegetative conditions; *Generative* consisted of 3 days of vegetative conditions followed by 2 days of generative conditions. No mushrooms were formed on the membranes. The weight of the colony is depicted in mg underneath each picture.

The control conditions showed weights between 31.4 and 72.4 mg, whereas cAMP resulted in weights between 26.3 and 47.4 mg (figure 3). Infections were seen on 5 plates, which appeared to have lowered their weight substantially compared to uninfected plates. When infected plates are left out, control and cAMP conditions showed respective averages of 55.1 and 36.8 mg. The two plates in generative conditions with dikaryons were both infected, rendering them invaluable as a control. Except for this condition, the control variants were heavier than the experimental variants in all cases. Interestingly, there are no significant differences between generative and vegetative conditions in monokaryons on cAMP, regarding biomass production. In contrast, the generative condition of dikaryons on cAMP is much heavier compared to solely vegetative conditions.

Altogether, these results proved the abovementioned hypothesis wrong. Despite an increase in radial growth, the produced biomass diminishes under the influence of 2 mM cAMP. It can therefore be concluded that 2 mM cAMP reduces the density of the mycelium. Consequently, cAMP or analogues would not be advantageous in the development of mycelium materials or as a stimulant of growth. Simultaneously, cAMP inhibits fructification despite generative conditions. The fructification of colonies on 0.5 mM cAMP suggests that the cAMP only impedes fructification. On higher concentrations of cAMP, fructification is fully abolished. Most likely, PDEs cannot break down intracellular cAMP at the rate it enters the cell. Little is known about both rate of uptake and breakdown of cAMP. 2- and 4-mM cAMP in the medium result in different morphologies, suggesting that uptake is not limited at 2 mM cAMP. Since cAMP is evenly distributed throughout the medium, uptake of cAMP is assumed to be constant. At a constant influx of cAMP, PDEs are expected to hydrolyse cAMP at a fixed rate, resulting in a dynamic equilibrium of cAMP.

Apparently, 0.5 mM cAMP is not enough for *S. commune* to fully abolish fructification, but its delay shows that cAMP affects fructification nonetheless. These results suggest either one of two options applies to intracellular cAMP and fructification. The first option is that cAMP is not an intracellular switch that, once below a threshold concentration, commits the cell to fructification. Rather, cAMP would act on a spectrum. Small decreases in cAMP would already cease PKA activation sufficiently for fructification signs to appear macroscopically. The intracellular cAMP concentration is crucial for the rate at which fructification occurs. When environmental cues minimize intracellular cAMP, fructification would be achieved quickly. Alternatively, environmental cues (i.e., light, temperature, and CO_2) are perceived through diverse signalling pathways, some of which would circumvent the cAMP concentration. N. Braat and Lugones (2020) found that *Pde2* overexpression strains could fructify in the dark, indicating light also affects intracellular cAMP. Similarly, Westhoff and Lugones (2014) show that expression of CA is reduced under light conditions, effectively also lowering intracellular cAMP levels. Taken together, there are strong indications that light is, at least partly, sensed through intracellular cAMP. Notably, the White-Collar Complex, made up by WC-1 and WC-2, is a transcription factor that is necessary for fructification and appears not to rely on intracellular cAMP (Ohm et al. 2013). In conclusion, there appears to be crosstalk between the two pathways, but it is not clear at what level this occurs. More research into these various pathways needs to be done to see understand how they are connected. An overexpression strain of AC may highlight additional entry points for environmental cues to initiate fruiting.

Increasing cAMP concentrations delay fructification in WT and Pde2Pde2

In line with previous experiments, *Pde2Pde2* and WT were compared on increasing cAMP concentrations, namely 2 mM and 4 mM. The Pde2 overexpression strain was produced in previous work (N. Braat and Lugones 2020). In all cases, the transformant was able to produce small but numerous mushrooms (figure 4). However, the extent to which mushrooms were formed decreased with increasing concentrations of cAMP. Similarly, WT was able to produce few but large mushroom bodies in control conditions but produced only large aggregates on 2 mM. Aggregates of the smallest

size were seen on 4 mM cAMP. At all concentrations, the *Pde2Pde2* strain produces more mushrooms compared to WT, presumably the result from lower intracellular cAMP levels. In both strains, it was clear that that cAMP addition slows the fructification process but does not fully stop it. Notably, even in the *Pde2Pde2* strain cAMP can impede fructification, indicating that the increased expression of *Pde2* is insufficient for invulnerability against exogenous cAMP. It appears that *Pde2Pde2* on 4 mM cAMP produces similar degrees of fructification as WT in control conditions. Possibly, this amount of fructification is the result of comparable intracellular concentrations of cAMP. These results support the hypothesis that cAMP does not act as a molecular switch for fructification, as discussed above. Instead, higher exogenous cAMP concentrations appear to induce higher intracellular cAMP levels, which inhibits fructification proportionally.

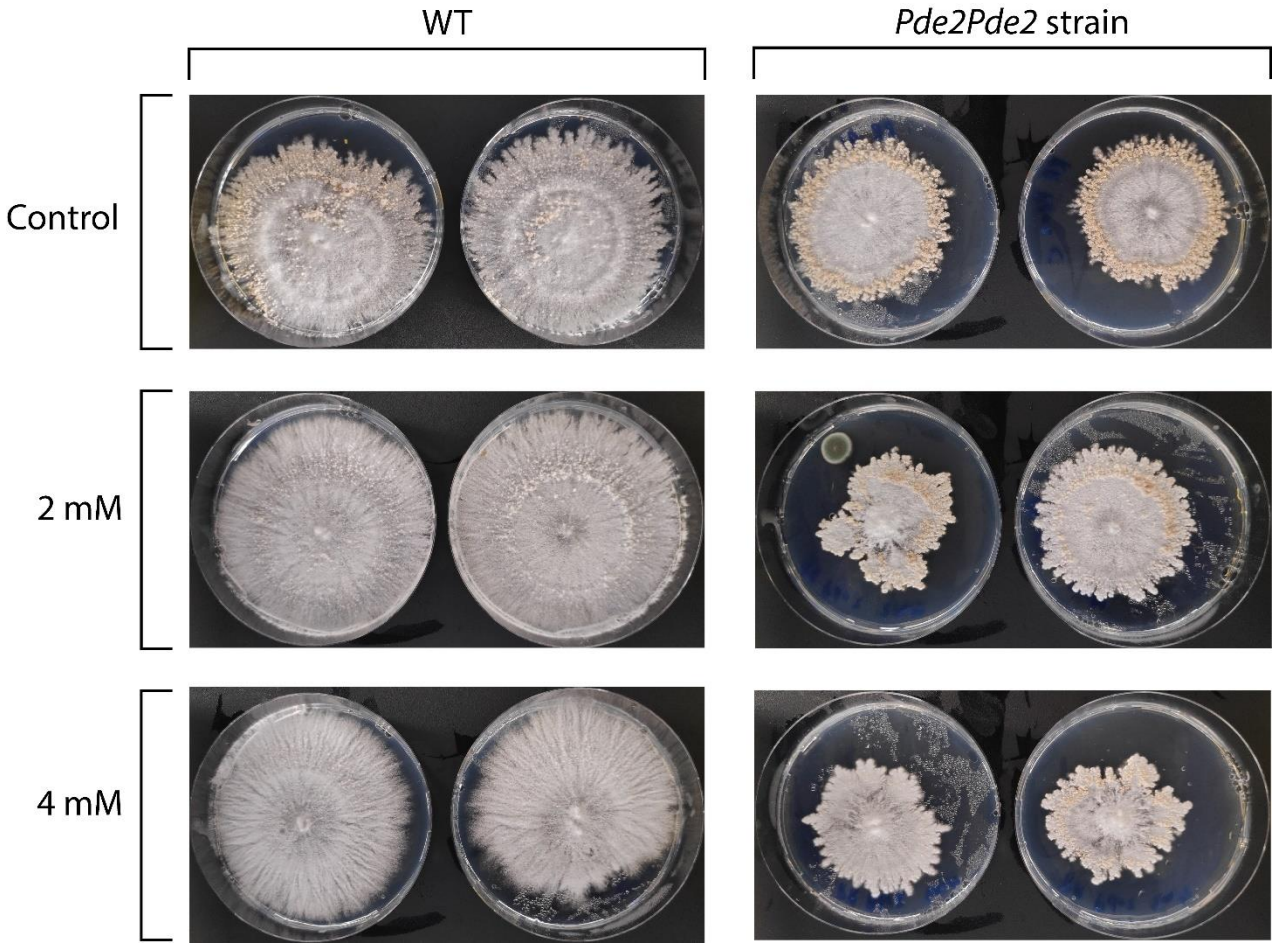

Figure 4. Fructification of Wild-Type and a *Pde2Pde2* strain on increasing concentrations cAMP in the medium.

HCO_3^- strongly inhibits growth but barely affects fructification

To further test the involvement of the AC – PKA pathway in fructification, it was assessed whether exogenous HCO_3^- could activate AC in the cell, thus inhibiting mushroom formation. To this end, colonies were grown on solid media containing $NaHCO_3$ concentrations of 1 mM, 4 mM, 16 mM, or 64 mM. Additionally, during previous experiments, it was found that *S. commune* would not always produce mushrooms in the allocated time under controlled conditions. It was hypothesized that the atmospheric CO_2 concentration in the stove for generative conditions was too high. Therefore, this experiment was carried out in two separate conditions, i.e., one box contained tissues wetted with water and another box contained tissues wetted with 4% KOH (w/v).

No differences were seen between control and 1 mM $NaHCO_3$ (Figure 5). However, at 4 and 16 mM, the colonies showed less pigmentation and became smaller compared to control in presence of 4% KOH (w/v). At 64 mM, the colony grows only scarcely and shows no signs of fructification. Fructification could only be seen in colonies that were placed in an environment where KOH was present, except for the colony grown on 64 mM $NaHCO_3^-$ which showed no fructification in either condition.

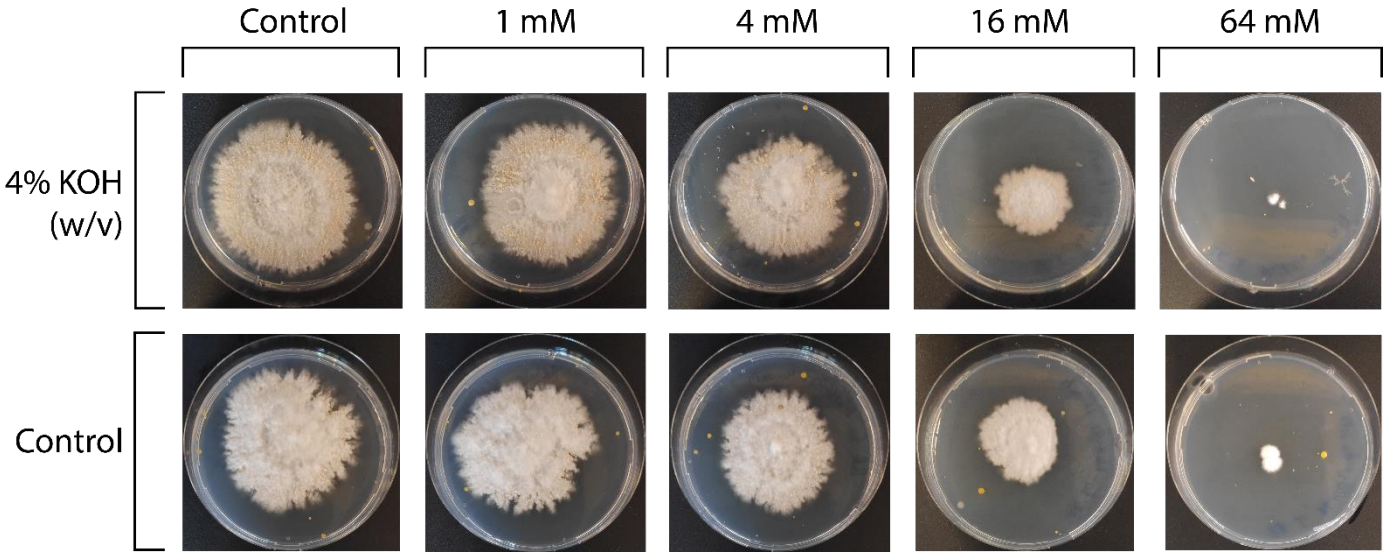

Figure 5. Fructification of *S. commune* on increasing concentrations of $NaHCO_3$. Mushroom bodies were only seen when 4% KOH (w/v) was used to wet the tissues underneath the plates.

The growth inhibition is most clearly an effect from the $NaHCO_3$, though it is not evident what sensing mechanism is involved here. Bicarbonate is known to block mycelial growth in Ascomycetes, but there is no mention in literature of such blockage in Basidiomycetes (Hang and Woodams 2003; Spadaro, Garibaldi, and Gullino 2004). Its mechanism of action has not been fully deciphered in fungi (Letscher-Bru et al. 2013). Interestingly, sodium bicarbonate can raise intracellular cAMP levels in bacteria, resulting in inhibited growth and biofilm formation (Dobay et al. 2018; Ruksakiet et al. 2021). In fungi no link has yet been established between raised cAMP levels and growth inhibition. The exception, demonstrated first in this project, is the lowered density under influence of exogenous cAMP. Moreover, the link between bicarbonate and growth inhibition is clearly demonstrated here. The comparison of higher concentrations of both chemicals suggest that bicarbonate inhibits fungal growth to a much greater extent than fructification. Contrarily, cAMP greatly affects fructification and morphology, but is the inferior growth inhibitor. Taken together, it appears that that *S. commune* responds differently to these signals, despite their apparent link in bacteria. Still, this does not exclude the possibility that there is overlap in their modes of action.

In *C. albicans*, addition of 20 mM $NaHCO_3$ resulted in similar degrees of germ tube formation compared to 5-10% CO_2 (Matare et al. 2017). This concentration compares to human serum levels, which are between 20 and 26 mM, that enable filamentation. Possibly, HCO_3^- is converted to OH^- and CO_2, the latter of which could then leave the medium and enter the cell. Intracellularly, it would be converted to HCO_3^- once more, initiating the AC – PKA pathway. In a closed system, $NaHCO_3$ would lead to accumulation of CO_2, which would explain the overlap between $NaHCO_3$ and 5-10% for *C. albicans* (Matare et al. 2017). During the vegetative growth of *S. commune*, plates are stacked and wrapped with plastic foil. Though the lid is not fixed on the plate, the stacks are wrapped tightly, possibly creating small closed systems in which HCO_3^- may result in high accumulation of CO_2. No mention of such adverse effects is found in literature. Notably, when the culprit is indeed CO_2, the

colonies must have resided in their own closed system during vegetative conditions. If this is the case, it is unknown how high local CO_2 concentrations may have become per plate.

The notion that bicarbonate barely affects fructification in comparison with cAMP, contradicts the hypothesis that CO_2 would be responsible for high intracellular cAMP levels. Possibly, the growth inhibition stems from direct interaction between the cell wall and bicarbonate. Alternatively, it has been suggested that AC performs as a hub in its pathway (Huang et al. 2019). Microdomains may arise, resulting in only locally elevated concentrations of both bicarbonate and cAMP when also CA is kept in proximity (Westhoff and Lugones 2014). This way, it is guaranteed that the growth inhibitory effects of bicarbonate are kept to a minimum. This latter option assumes that bicarbonate does enter the cell and can stimulate AC to produce cAMP. Evidently, cAMP levels were not raised enough, if at all, for fructification to be visibly impaired. In this case, a concentration of bicarbonate (e.g., 16 mM) would be a strong growth inhibitor but a poor fructification inhibitor.

Notably, both exogenous cAMP and exogenous bicarbonate appear to be poor inhibitors of fructification due to their side-effects. Addition of these chemicals exogenously would affect cellular components not relevant to CO_2 sensing and fructification. This underlines the need for methods that allow for interference of the pathway without using these compounds, such as altered gene expression.

The stove that is used for generative conditions has no opening to atmospheric conditions except for the doors. It is customarily filled with many boxes and stacks containing mycelia that, together, produce high amounts of CO_2. The plastic boxes that are used for fructification are around 10 L and contain three holes of each 1 cm^2 in the lid. In these experiments, the holes are covered with folded dry tissues and tape. Consequently, it is assumed there is aeration, but contaminants are kept out. Fructification has only occurred in the box supplemented with tissues wetted with 4% KOH (w/v). This suggests that the CO_2 concentration in the control box was too high for fructification to occur. In an open system, these differences could not exist, since the two boxes would share their CO_2. Consequently, the degree of fructification would be the same for both boxes. Therefore, the systems must be either partly closed or fully closed. In a closed system, it is merely logical that the 4% KOH (w/v) box allows for fructification and the control box does not. In a partly closed system CO_2 may dissipate only at a low rate, allowing for limited mixture. Consequently, the control box may have slightly higher CO_2 concentrations, sufficient for blocking fructification. Similarly, the presence of 4% KOH (w/v) results very low local CO_2 levels, but external CO_2 enters the system only at a very slow rate. This rate would be too low for it to accumulate; hence the colonies can form mushrooms.

Supposedly, the lid of the box allows for little aeration and the system is therefore partly closed. The 4% KOH (w/v) box allows for production of mushrooms, whereas the control box does not. In both boxes colonies produce CO_2 but only in the latter, the CO_2 cannot be removed from the system. This means that the CO_2 levels in the stove is close to equilibrium with the CO_2 levels of the box. During other experiments, also without $NaHCO_3^-$, colonies would not always produce mushrooms simultaneously. Here, it is shown that addition of KOH may solve this issue. Therefore, it can be concluded that the CO_2 concentration in the stove is too high to allow for fructification.

Ring plates allow for accessible spatial and temporal fructification experiments

As an alternative to the experiment described above, ring plates can also be used to test temporal factors. Instead of taking the colony and the membrane off the solid agar, the membrane is lifted and cAMP can be added into the liquid medium. Notably, there are a few downsides to ring plates, i.e., oscillating growth speed (Herman, personal communication), sensitivity to infections and air bubbles under the membrane. Since liquid medium is used, the environment of the plate is preferably kept humid and closed off. Regardless, the ring plates allow for more accurate experimentation as the

plate is divided into 6 volumes by the rings present in the plate. The middle circle is considered the first and smallest volume, the most outer trench is considered the sixth volume.

The idea of using ring plates in fructification experiments was only put forward towards the end of this project. In addition, the Perspex ring plates are scarce and only two plates were available for experimentation. Consequently, only two proof-of-concept experiments have been carried out. For the first experiment, colonies were grown in vegetative conditions before adding 2 mM cAMP to the fifth volume. Next, the colonies were incubated with 4% KOH (w/v). Whereas the control plate resulted in numerous small mushrooms on the outside of the sixth volume, the experimental condition gave only few slightly larger mushrooms clustered together on the sixth volume. Moreover, the cAMP gave rise an unevenly dense mycelium, an effect that becomes visible from the fourth volume.

In the second experiment, the control colony showed only little growth and was therefore inoperative. 2 mM cAMP was added to the outer two rings for the experimental condition. Just before the mycelium reached the fifth ring, the plate was shifted from vegetative to generative incubation for 10 days. The colony developed mushrooms in half a circle, on the fourth volume. Outside this volume, the membrane was fully overgrown, though also cordlike. The circle of mushrooms is incomplete, possibly due to leakage cAMP over the volumes.

cAMP intervention overrules fructification process

Based on the cAMP fluctuations shown by Kinoshita *et al.* (2002), it was tested whether cAMP addition would affect fructification differently when added at a later moment in the system. To this end, WT colonies were grown on PC membrane. It was noted that these colonies went through the fructification process at different rates, despite being in the same generative growth conditions. By wetting the tissues with 4% KOH, this compound was present in the system. Five days of generative growth resulted in colonies in both the stage of primordia formation (group 1) and the stage of maturing fruiting bodies (group 2) (figure 6). It was unclear why this heterogeneity was found and if KOH was involved. From each group, one or two colonies were selected as control and placed on fresh medium. The other two colonies were placed on media containing 2 mM cAMP. Another three days of growth resulted in small setbacks for colonies in experimental conditions compared to control. Colonies in group 1 could not produce mature mushrooms on 2 mM as much as the control condition allowed. Mushrooms are either closed still or have not fully opened. For colonies of group 2 that were placed on fresh media, three days was sufficient to fully mature all mushrooms. Though many primordia of the colonies on 2 mM developed onto mushrooms, there are still some undeveloped fruiting bodies left. Together, these results show that 2 mM is still able to influence the fructification process at later stages.

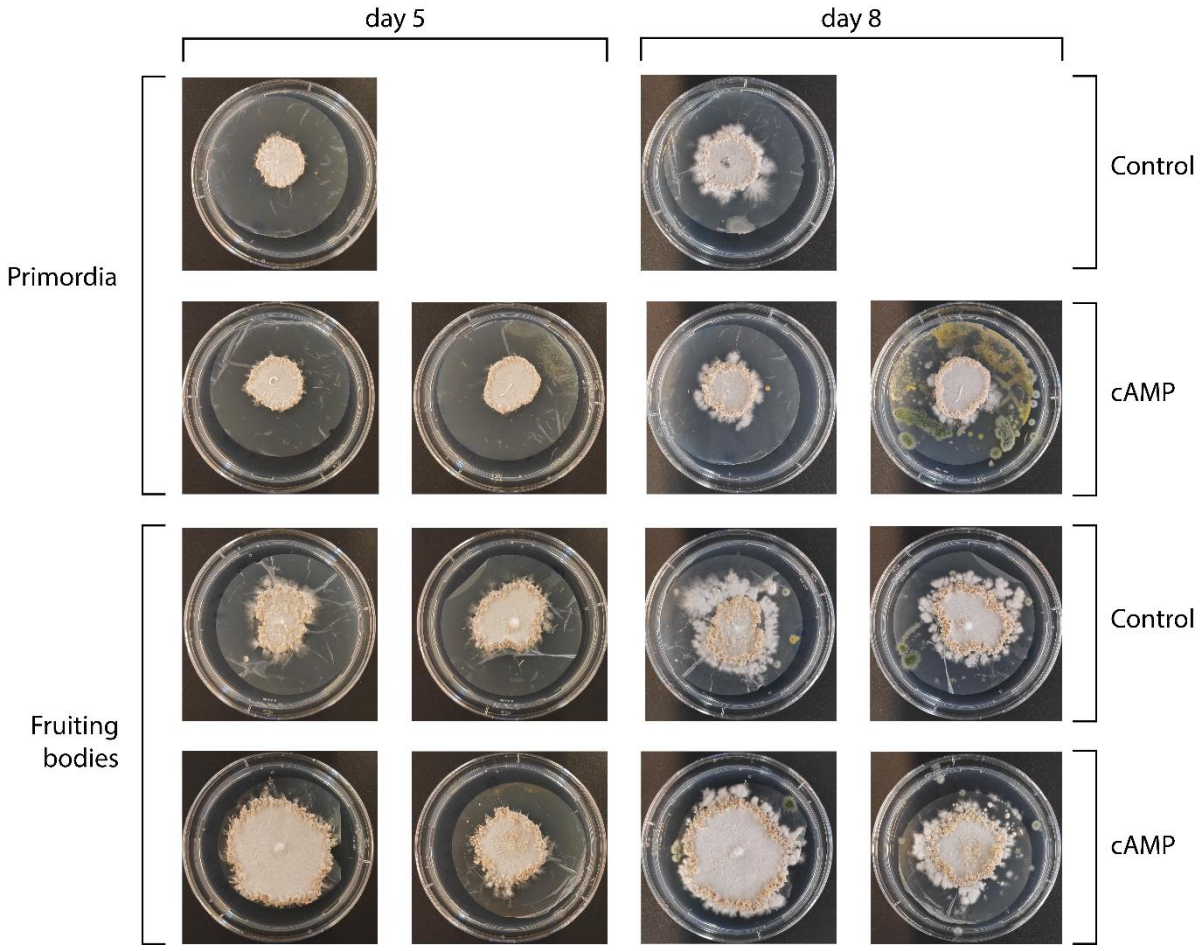

Figure 6. *Fructification of colonies at the stage of primordia and fruiting bodies, on the fifth and eighth day of growth. After day 5, 4 colonies were placed on new media containing 2 mM cAMP. 3 control colonies were placed on new media.*

Notably, cAMP levels were measured in the aerial hyphae of the mycelium by Kinoshita *et al.* (2002). The previous experiment indicated that cAMP effects are experienced relatively locally. Still, it is reasonably assumed that the whole colony, including the aerial hyphae, senses the effects of cAMP in the medium in this experimental setup. Consequently, it is expected that there is peak in intracellular cAMP after switching media. Kinoshita *et al.* (2002) showed that cAMP peaked just before primordia formation and during the maturation process of the fruiting bodies. However, the addition of cAMP in the medium has not facilitated the fructification process, but sooner restricted it. Assuming the cAMP fluctuations are significant and involved in the fructification process, they are of insignificant size compared to the cAMP levels in the medium. Using both a radio immunoassay and an enzyme immunoassay, values between 50 and 400 fmol/OD_{260} were found in the aerial hyphae. In contrast, the experiments in this work involved cAMP concentrations up to 4 mM. Next to the distinct setups that render these values incomparable, it is unknown what the rate of uptake of cAMP is for *S. commune*. However, the altered morphology shows that the fungus is sensitive to values up to at least 4 mM cAMP.

Therefore, it is most likely that cAMP fluctuations are involved in the fructification signalling, which happens only around the small boundaries displayed by Kinoshita *et al.* (2002) (figure 7). The relatively high cAMP values, between 300 and 400 fmol/OD_{260}, are expected to maintain vegetative growth. Once the mycelium reaches conditions that resemble atmospheric CO_2 levels and light cycles, the cAMP levels drop. The subsequent rise in cAMP, both just before primordia formation and during fruiting body maturation, cannot be the result from bicarbonate-stimulated adenylyl cyclase.

Rather, a Ras- or heterotrimeric G protein-sensitive adenylyl cyclase must be present to achieve these effects.

Moreover, the morphology resulting from 2 mM cAMP addition to the medium may be a consequence from phosphodiesterase-mediated cAMP breakdown. Continuous hydrolyzation of cAMP results in accumulation of AMP. This may alter the ratio between ATP, ADP, and AMP, thus affecting the perceived energy state of the cell (Hardie, Ross, and Hawley 2012; de La Fuente et al. 2014). In control conditions, AMP is probably mostly produced by ATP hydrolyzation and *de novo* synthetization, whereas cAMP hydrolyzation is not even considered (de La Fuente et al. 2014). Consequently, an intracellular signal initiated by AMP may override the cAMP fluctuations involved in fructification.

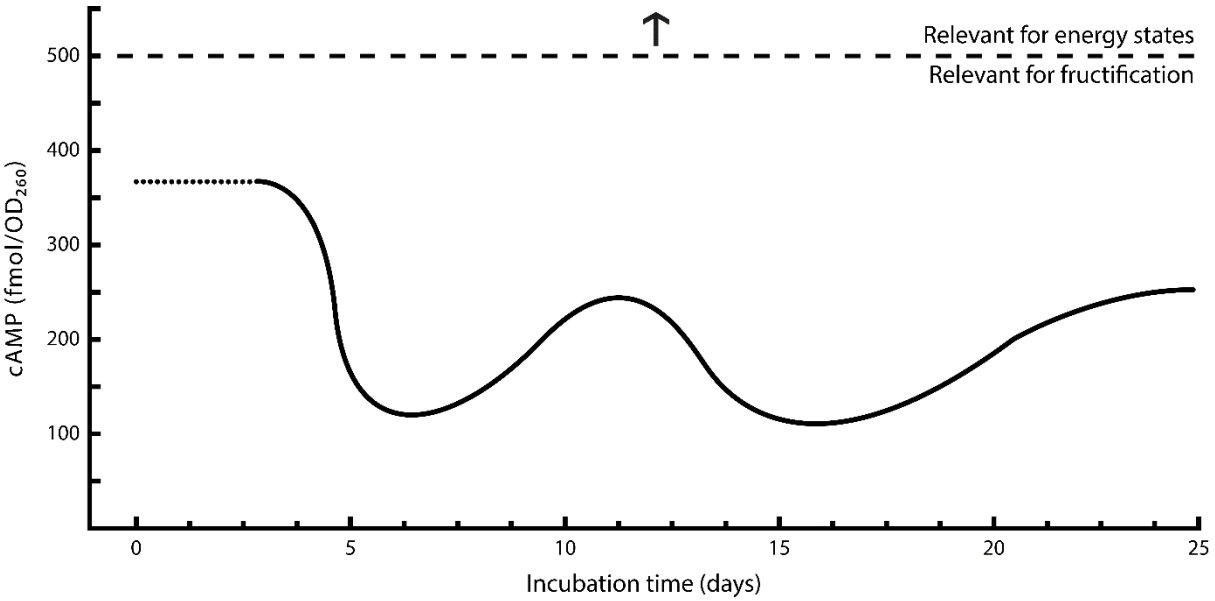

Figure 7. Hypothesis for cAMP fluctuations based on Kinoshita *et al.* (2002). The dashed line concerns vegetative growth, whereas the filled line concerns generative growth. After the first cAMP peak, around day 14, primordia were first seen. During the final increase of cAMP, fruiting bodies were first seen. Most likely, absence of bicarbonate is responsible for the first drop initiated at day 4. The subsequent peaks are hypothesised to be under influence of Ras and/or heterotrimeric G proteins. The straight dashed line denotes a broad border which distinguishes cAMP in its role for fructification and cAMP as a substrate for AMP production.

Note that there are three possible inputs for AC, namely, bicarbonate, Ras, and heterotrimeric G proteins (specifically G_α and G_γ). It is hypothesized that *S. commune* relies on these signals during subsequent time intervals, as envisioned in figure 7. Mutating *AC* into losing its sensitivity to each of these separate inputs would highlight how fructification is dependent on both extracellular and intracellular signals. Specifically, the roles of both Ras and heterotrimeric G proteins are interesting to compare, because either one of the two is generally considered to be the prime signalling protein (Knabe et al. 2013; Palmer and Horton 2006; Schubert et al. 2006; Yamagishi et al. 2004).

No ΔPde2 strains were obtained

As a means to interfere with the AC – PKA pathway, a construct was developed for the deletion of the *Pde2* gene. pDelPde2, which contains the flanks of the *Pde2* coding region and the nourseothricin cassette in between. It was developed using CPEC including 3% DMSO in the reaction, though later experimentation learnt that 5% DMSO was optimal for constructs of this size. pDelPDe2 was

transformed to the ΔKU80 strain, suitable for knock-out transformations in *S. commune*, using preassembled Cas9 ribonucleoproteins (Vonk et al. 2019). No sgRNA was obtained for the downward flank, most likely due to unresponsiveness of the reverse transcriptase to the cDNA.

Because the materials for the desired protocols are hard to get by, the construct was first tested as an integration transformation. Despite numerous attempts and variations in glucose levels and nourseothricin concentration, no colonies survived a second round of selection. Consequently, these colonies were determined to be false positives due to their initial growth phase. It has not become clear during this project what had been the underlying reason for these drawbacks. A previously qualified construct containing a resistance cassette for nourseothricin could also not be transformed, suggesting that the problem lies with the transformation materials or procedure. Possibly, a *ΔPde2* strain may also be inherently inviable, meaning that *Pde2* is an essential gene. In this regard, *S. commune* would stand apart from yeasts *S. cerevisiae*, *C. albicans* and *C. neoformans* (Hicks et al. 2005; Hu et al. 2010; Jung et al. 2005). Bicarbonate stimulation of *S. commune* has resulted in strong growth inhibition, perhaps through cAMP accumulation. Deletion of *Pde2* may realize the same effect, heavily impairing growth of *S. commune*. Notably, deletion of *AC* has also not been accomplished in *S. commune* during earlier work, in line with this hypothesis (N. Braat and Lugones 2020). Accumulation of cAMP in *C. albicans* following hyperactivity of AC resulted in broad defects, including cell sensitivity and cell arrest (Bai et al. 2011).

The viability of *PDE* deletions in other species may well be explained through the existence of multiple feedback loops. Deletion of *Pde1* (considered the prime PDE) in *C. neoformans* results in negligible increases of cAMP (Hicks et al. 2005). In contrast, intracellular cAMP increases fiftyfold when *Pka1* is deleted, due to its role in a negative feedback loop (D'Souza et al. 2001). Crossing of these two strains reinforces these effects, resulting in cAMP values 500 times higher than WT (Hicks et al. 2005). Note that Pde2 alone barely affects the cAMP concentration, which suggests that PKA is responsible for additional negative feedback. Similarly, both PKA and Pde2 are involved in negative feedback on intracellular cAMP in *S. cerevisiae* (Nikawa, Cameron, et al. 1987). Here, deletion of PDEs results in a mere threefold increase of cAMP. Further disruption of the regulatory and catalytic subunits of PKA increases cAMP up to 10.000 times. Possibly, such additional feedback loops are not present in *S. commune*, leaving solely Pde2 responsible for the negative control of intracellular cAMP.

To collect evidence on the interplay between PKA and Pde2, a *Pde2* overexpression construct was built. The RRXS sequence within the *Pde2* sequence, responsible for PKA binding and activation, has been replaced by RRXD (Hicks et al. 2005). The switch from serine, an amino acid that can be phosphorylated, to aspartic acid, an amino acid that contains a negatively charged COO^- group, is expected to result in constitutive activation of Pde2. However, this construct has not yet been transformed to *S. commune*.

Development of a Tet-on system in Schizophyllum commune

As a new means to control gene expression in *S. commune*, the reverse transcription transactivator (rtTA) of the Tet-on was codon-optimized for the codon usage of *S. commune*. The synthesized sequence was placed in pRO25, under control of a *β-tubulin* promoter. The transcriptional unit of rtTA is expected to contain one intron in the untranslated 5' region of this promoter, essential for proper processing of the transcript (Lugones et al. 1999). Originally, this plasmid contained a SC3-terminator, which was replaced by a *β-tubulin* terminator. Consequently, the promoter and terminator sequence originate from the same gene and possible complementarity between the 5' and 3' UTR's is conserved. To check the viability of the system in this species, the TetO7 promoter was placed upstream of *dTomato*, which produces red fluorescence (Ohm, Wösten, and Lugones 2010). In *Aspergillus* ssp., in which the Tet-on and Tet-off system are extensively applied, one construct carries both the rtTA and the TetO7 promoter. Consequently, the *rtTA* is expressed in the

nucleus, in which it also binds the TetO7 promoter. Since the rtTA is folded and activated outside the nucleus, it is reasonably expected that it does not matter whether *rtTA* and *TetO7* are transformed to the same nucleus. For *S. commune*, two constructs (prtTASc and prTetO7-dTom) have been designed for each distinct function.

Several transformations to *S. commune* were performed for both constructs. At first, the rtTA construct was transformed to H4-8A and the TetO7 construct to H4-8B. Only one rtTA construct appeared resistant to hygromycin after two selection rounds and was therefore crossed with all TetO7 constructs. The resulting dikaryons were grown both with and without 50 µg mL^{-1} doxycycline. Out of the 14 tested dikaryons, 2 showed red fluorescence under the influence of doxycycline (data not shown). Further phenotyping of this colony made clear that it was not responsive to doxycycline, but in fact fitted the morphology of the Streak mutation (Papazian 1950). After this dazzling finding, old Streak colonies and WT colonies were also tested for red auto-fluorescence. Whereas WT colonies showed no red auto-fluorescence, all Streak-colonies did. As such, Streak emerged most likely because of the mutagenic zeocin, most coincidentally in a colony that was expected to show red fluorescence.

Due to the scarce availability of lysing enzymes, ultimate rtTA transformations were done with spore protoplasts. Though many transformants were obtained, it was not possible to determine their quality due to time constraints. The next step in the process would involve the assessment of the mRNA of the expressed product. The codon-optimized sequence may contain cryptic introns that result in incomplete proteins following translation, giving an inoperative product. Running a PCR with the cDNA of the mRNA product may show whether this is the case. The presence of cryptic introns would mean that the sequence must be further optimized until no cryptic introns are present. Next, the expression level of the product can be assessed using quantitative PCR. The colony with the highest expression level without any adverse effects will be picked for further development. Crossing the colony with a *TetO7-dTom* transformant will reveal whether the Tet-on system is viable in *S. commune*. Naturally, this concerns the production of red fluorescence solely under influence of doxycycline. Positive results are considered a breakthrough, as controlled gene expression allow for accessible interference of an extensive body of genes in *S. commune*.

To this end, the TetO7-Pde2 construct has already been developed. rtTA/TetO7-Pde2 transformants would express *Pde2* in response to addition of doxycycline, next to basal expression of *Pde2*. Growing this colony on ring plates would allow for both temporal and spatial experimentation with *Pde2* expression. One example experiment would involve doxycycline in the inner three rings of the plate, resulting in high Pde2 activity. Consequently, the middle of the colony would be pushed towards fructification. N. Braat and Lugones (2020) showed that overexpression of *Pde2* prompted mushroom formation in the dark, which occurred in the periphery. This experiment would demonstrate whether the fructification signal can be relayed throughout the colony.

The *TetO7-Pde2* transformant contains the flaw that is does not consider basal *Pde2* expression. Preferably, all expression of *Pde2* would be under control of the Tet-system. However, integration of the TetO7-Pde2 construct only allows for control of *Pde2* outside basal levels. To develop a system less leaky, the promoter of the native *Pde2* would have to be replaced by the TetO7 promoter. However, this would require targeted deletion, a process more demanding than gene insertion (Vonk et al. 2019). For foreign genes such as dTomato, this is not an issue and integration of the gene suffices. Notably, without doxycycline added to the system, no *Pde2* would be expressed and the transformant can be considered a knockout. For essential genes, it is therefore vital that doxycycline is invariably present before experimentation. Alternatively, the Tet-off system may be applied using the same promoter. The counterpart of rtTA, called the transcriptional transactivator (tTA) is constitutively activated until it is bound by doxycycline (Wanka et al. 2016). Essential genes under control of tTA and TetO7 would be active after crossing or after co-transformation. The Tet-off

system allows colonies to grow independently before the gene expression is diminished. Therefore, the Tet-off system may also prove a suitable alternative for the *ΔPde2* strain. Notably, the TetO7-Pde2 construct has not yet been transformed to *S. commune*.

Production of protoplasts

Within the protocol that is used for protoplast production the quality of the material and that of the enzymes play a major role at a chance of success. Regarding the former, it is assumed most productive when a small portion of material contains as many growing tips as possible. The mycelium is macerated two to three times in the protocol, allowing for many branching points. Consequently, the fungus is pushed towards many cell divisions. However, the intensive handling of the mycelium has led to many infections, after which the process had to start anew. During this project, it had occurred during numerous attempts that the material was either infected or that it would not produce protoplasts. For this reason, two different manners of producing material have also been applied. The first concerns the maceration of ⅛th of a fully grown plate and growing it in liquid culture for up to 7 days. The result is a dense stock culture which likely contains fewer branching points but is probably uninfected. Part of the material, around 5 mL when spun down, is macerated, and washed with MilliQ until it is rid of polysaccharides, which takes three to four rounds. In the final round, the material is resuspended in minimal medium and allowed to grow for a period of 2 to 6 hours at 30 °C before resuspending the mycelium in the lysing enzyme (LE) mix. It was found that growing the mycelium for 2 hours resulted in 3.0×10^8 protoplasts mL^{-1}, which went up to 3.7×10^8 when growing for 4 hours. Additional 2 hours of growth did not improve the protoplast production. Growing the material in YT negatively affected the protoplast production. The stock can be kept in the fridge for up to two weeks, before the quality of the material starts to deteriorate.

Alternatively, spores have been used to produce protoplasts. First, up to four 4.8 dikaryons are grown on plates for 3 to 5 days, before being placed in generative conditions. After fruiting bodies have been formed, the plates are kept upside down for at least a week. During this period, spores fall from the fruiting bodies onto the lid, from which they can be harvested afterwards. The spores are allowed to swell in YT supplemented with antibiotics, which takes around 2 days. Afterwards, the spores are resuspended in the LE mix.

The lysing enzymes that are used for protoplast production have long been taken from a large collection that was produced by a previous student in 2017. Halfway the project, this batch was exhausted. Therefore, alternative batches of unknown quality were used, until a reliable batch was found. At the same time, a new project was started in which a student would produce a reliable way of producing effective lysing enzymes. His enzymes, like other batches, were also used for protoplasts in this project. However, their unpredictable effectivity sometimes resulted in insignificant amounts of protoplasts. This uncertainty troubled the determination of the quality of the material itself, when a novel mycelium method and new LE batch were combined and did not yield protoplasts.

Spores are ideal for protoplast production when enzymes are scarce, due to a high ratio between nuclei and total surface. Mycelium contains only one nucleus per compartment, whereas spores contain two. Moreover, lysing enzymes act on growing mycelium, creating protoplasts when new tips are formed. Notably, this process occurs all over the surface, instead of solely where the nucleus resides. Consequently, up to 9 in every 10 protoplasts is empty and thus incompetent. However, spores are compact and therefore would only produce viable protoplasts. The use of spores for protoplasting is therefore especially useful when only lysing enzymes are scarce. In addition, smaller portions should therefore suffice for transformation, though no extensive experimentation has been carried out to test this. Notably, there are also disadvantages to spore protoplasting. Naturally, spores are sexual and therefore mate with each other, which hinders

selection. In contrast, protoplasts of one mating type remain separate after transformation and are therefore easily isolated and tested. Protoplasts of spores were therefore regenerated in schizophyllan, thus immobilizing the protoplasts and limiting their interactions. It is not possible to prevent these interactions once plated out, though colonies can be inoculated on new plates before they occur.

Glucose concentration in transformation

Knock-out transformations are renowned for their low efficiency and high number of false positives (background) (Ohm, de Jong, Berends, et al. 2010). To compensate for low efficiency, five times the number of protoplasts is used compared to integration transformations. Consequently, around ten plates need to be used per knock-out transformation. Naturally, untransformed protoplasts experience more difficulty regenerating. Still, the high degree of background indicates that there is room for improvement. Therefore, transformations have been carried out using varying concentrations of glucose. It was hypothesized that all regenerants would undergo less cell divisions on low glucose, before being selected for on antibiotic. Consequently, untransformed colonies have less cell wall acting as buffer against said antibiotic, effectively raising their sensitivity. Standard SCMM contains 2% glucose, which was lowered down to 0.002% to create more stringent selection. Preliminary results showed empty plates after transformation, indicating that the antibiotic concentrations were too demanding. Therefore, the antibiotic levels were also decreased, down to ten times lower minimally. For nourseothricin, used for the *ΔPde2* knock-out, no transformants were obtained. Consequently, no results were obtained for lower glucose concentration in combination with this antibiotic. Similarly, no binding results have been obtained for the TetO7 transformation and use of lower zeocin concentrations.

For the integration of the rtTA construct, 0.002% glucose was used in combination with 5 µg mL^{-1} hygromycin, compared to 15 µg mL^{-1} for 2% glucose. Both resulted in correct transformants, though the latter resulted in faster growing colonies due to the abundance of glucose. Transference to fresh selection media, all at high glucose and antibiotic concentrations, learnt that the high concentration of glucose resulted in a higher percentage of correct transformants. This discrepancy may be the result from lower expression of the resistance cassette in transformants selected with lower antibiotic concentrations. The assumption that lower glucose levels also result in a more stringent selection can therefore not be fully supported. Consequently, this must mean that different factors were involved in the empty plates following transformation.

Altogether, more transformations need to be carried out to check the viability of low glucose transformation. However, these early indications do not show any advantages compared to conventional transforming, except for the reduced need for antibiotics. The main disadvantages concern the reduced growth speed and the possibility at reduced gene expression for both the resistance cassette and the gene of interest.

Conclusion

During this work, many experiments have been carried out to interfere with the AC – PKA pathway, to further the knowledge of CO_2 sensing and fructification in *S. commune*. It appears that intracellular cAMP does not act as a molecular switch for fructification, but rather that cAMP acts on spectrum. In addition, both exogenous bicarbonate and cAMP affect fructification, though side effects of these compounds heavily affect the growth of the colony. Future work must circumvent these issues, possibly by means of gene alteration and conditional gene expression systems. Despite the many developments that further drive the transformation process of *S. commune*, it remains a difficult process to carry out. Deletion of *Pde2*, if possible, helps aid in the recognition of novel proteins involved cAMP regulation. Moreover, the construct for *Pde2* containing a mutated RRXS sequence, under control of a *β-tubulin* promoter, has been developed but must be transformed still. In this work, it has been suggested to mutate *AC* into losing its sensitivity to either Ras, heterotrimeric G proteins, or bicarbonate.

Furthermore, the Tet-on system may be used to control gene expression, specifically useful for expression of genes that failed during previous attempts, e.g., *AC* (N. Braat and Lugones 2020). Moreover, the Tet-on system may prove useful when applied for genes that are specifically up- or downregulated during the process of fructification. In this work, a first attempt has been made at developing the Tet-on system for *S. commune*. The constructs are ready for transformation and subsequent testing. *Pde2* is a suitable candidate for a first application of the Tet-on system since its expression is vital for initiation of fructification. Specifically in combination with ring plates, the Tet-on system is most viable for research into fructification.

In conclusion, this work adds to the growing body of research performed in the field of CO_2 sensing in *S. commune*. Future work can well be built on results and considerations presented in this work, but even more so, make use of the tools that have been set up here. As such, the field progresses towards a clear understanding of sensing and fructification mechanisms. Finally, these may be applied to further mushroom cultivation of both commercialized and more experimental mushroom-forming fungi.

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