Role of Pde2 in the CO_2 Sensing during Fruiting in *Schizophyllum commune*

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Content

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Laymen's summary

Mushrooms are gaining popularity worldwide due to their nutritional value and health benefits. Only several edible mushrooms are currently commercially cultivated such as the white button mushroom, *Agaricus bisporus*. To be able to meet the increasing demand, supply of edible mushrooms needs to increase. Thus, the mechanism behind mushroom formation needs to be investigated. Therefore, the model fungus *Schizophyllum commune* is studied. It is already known that several environmental factors are able to induce mushroom formation, including temperature, light and CO_2 conditions. While high CO_2 concentrations prevent mushroom formation, low CO_2 concentrations allow mushroom formation to occur. How this signal is relayed and results in mushroom formation is not yet known. It is thought that a conserved pathway is used that uses signaling molecule cyclic AMP to sense CO_2. Therefore, the current research focuses on how cyclic AMP levels can be influenced by focusing on phosphodiesterase, the enzyme that degrades cyclic AMP. It was found that by exogenous addition of cyclic AMP and by inhibiting phosphodiesterase, mushroom formation can be inhibited. Also, that the fungus cannot transport exogenously added cyclic AMP. Furthermore, constitutive and conditional overexpression mutants have been generated with a new technique, of which the latter allows for turning gene expression on and off. The results indicate that CO_2 is sensed by intermediation of cyclic AMP. If mushroom formation in *Schizophyllum commune* can be increased and controlled, it could potentially be applied to commercially cultivated mushrooms since the pathway is very likely conserved.

Abstract

Environmental CO_2 is an important cue for initiating fruiting body development in the basidiomycetous fungus *Schizophyllum commune*. While the pathway through which light is sensed is well-studied, CO_2 sensing is mostly studied in the context of pathogenic fungi. Here, a possible CO_2 sensing pathway is proposed for *S. commune* in which the CO_2 signal is mediated by cyclic AMP. cAMP is produced by adenylyl cyclase (AC) upon stimulation by bicarbonate and is broken down by phophodiesterase (Pde). As cAMP plays such an intricate role in the pathway, attempts were made to manipulate the intracellular cAMP levels by 1) addition of exogenous cAMP and Pde inhibitor IBMX; 2) generating constitutive and conditional *pde2* overexpression strains; and 3) generating *pde2* deletion strains. It was found that wild-type dikaryon *S. commune* was not able to fructify in the presence of 2.5 mM IBMX or 2.5 mM cAMP. Moreover, with the use of ring plates it was shown that neither IBMX nor cAMP are transported by *S. commune*. Generation of overexpression strains proved successful when using a new method of PEG-mediated transformation using protoplasted wild-type spores. Two selected constitutive *pde2* overexpression strains were not able to fructify under elevated CO_2 conditions (3%) or in the presence of 2.5 mM IBMX or 2.5 mM cAMP at low CO_2 conditions. A quantitative PCR could show whether other transformant strains overexpress *pde2* and thereby determine functionality of the construct. When using protoplasted ΔKU80 spores, no *pde2* deletion transformants were generated during several attempts. Furthermore, the Tet-on system was introduced in *S. commune* for the first time by PEG-mediated transformation using protoplasted wild-type spores. Quantitative PCR results show that the construct is functional. Further research needs to be performed for phenotyping the constitutive and conditional overexpression strains.

Introduction

Fungi have a wide application in modern society. For example, various fungal species are used in applications within agriculture, biotechnology and biofuel production. Besides the many applications of fungi, they themselves are also a suitable source of food production, such as bread and cheeses (Jouzani et al., 2020; Money, 2016; Thirkell et al., 2017). Thereby, the mushrooms which several fungi form are also an important source of food. Multiple studies have shown that different mushrooms are high in fibre, vitamins and antioxidants, but low in fat and calorie intake (Ferreira et al., 2009; Heleno et al., 2015; Kalač, 2009). For example, three edible mushrooms originating from Poland were found to contain various bioactive compounds and antioxidants such as phenolic compounds, tocopherols and carotenoids. As a result, the ingestion of mushrooms contributes to, among other things, radical scavenging activity and lipid peroxidation inhibition, processes that counteract oxidative damage. The extraction of these substances could play an important role for public health and chronic disease related to oxidative stress (Heleno et al., 2015).

Mushrooms have long been consumed for their refined taste, but their popularity is increasing as the health benefits of mushrooms are becoming more widely known (Kalač, 2009; Royse et al., 2017). The most well-known mushroom-forming fungi belong to the genera *Lentinula*, *Pleurotus* and *Agaricus*, such as the white-button mushroom *Agaricus bisporus*. In 2013, the value of the total mushroom industry was estimated at 63 billion USD. While 38% is covered by medicinal mushroom, 54% is covered by the edible mushrooms (Royse et al., 2017). The value is expected to rise to 100.1 billion USD by 2028 (https://www.zionmarketresearch.com/report/mushroom-market). This can partly be explained by the fact that the vegan diet is becoming a more popular trend in modern society. With the growing world population estimated at 9.15 billion by mid-century (Alexandratos & Bruinsma, 2012), the worldwide demand for high quality food and medicine is expected to increase (Grimm & Wösten, 2018; Royse et al., 2017). This can also be achieved in a sustainable manner. Mushrooms are grown on lignocellulose waste such as straw or wood chips from agriculture, forestry and plant-processing industry (Grimm & Wösten, 2018; Kües & Liu, 2000). Fungi are able to convert these low energy waste products into high quality food for consumption. Also, the media mushroom are grown on can also be repurposed, further lowering the environmental impact (Grimm & Wösten, 2018).

Even though edible mushrooms can be produced at low-cost, little is known about the molecular and genetic processes involved in mushroom development, also known as fruiting body development or fructification. Thus, the biological potential of these organisms is not yet fully exploited. Only a few fungi are used as model organisms for fructification, namely *Coprinus cinereus* and *Schizophyllum commune* (Kües & Liu, 2000). It is important to have a complete picture of the fructification process. By manipulating and optimizing model organism such as *C. cinereus* or *S. commune*, it can subsequently be applied to other fungi that are commercially cultivated.

The basidiomycetous fungus *S. commune* is a wood-degrading fungus. Its genome is known and can therefore be easily manipulated. Several methods have been developed to genetically manipulate *S. commune* (Ohm et al., 2010a; van Peer et al., 2009a; Vonk et al., 2019). Another reason why *S. commune* is often used for research is because it is able to complete its life-cycle in a relative short time of 10 days (Ohm et al., 2010a). Fructification can occur when two sterile monokaryons with different alleles in both mating type loci, *matA* and *matB*, meet. The monokaryons fuse to form a fertile dikaryon which forms basidiospores (Pelkmans et al., 2017). Clamp connections, which can be microscopically observed, indicate that a dikaryon has been established (Niederpruem, 1963). After vegetative growth of the dikaryon, fructification is initiated when aggregates of aerial hyphae form and grow in upward and then inward direction. These aggregates become primordia, structures that continue to grow and swell into fruiting bodies with a typical split-gill structure (Niederpruem, 1963; Wessels, 1992). When grown from point inoculum, fruiting bodies usually develop in a ring at the periphery of the colony after sufficient vegetative growth (Perkins, 1969; Raudaskoski & Yli-Mattila, 1985). Upon fructification, this ring turns brown and the primordia form. Multiple transcription factors and genes under their control are needed to bring about the fructification process. Several environmental cues are required for activation of these transcription factors (Pelkmans et al., 2017).

While *S. commune* requires certain nutrients, humidity, pH and salinity for both vegetative and generative growth (Kües & Liu, 2000; Leonard & Dick, 1968; Niederpruem et al., 1964), changes of

some environmental factors are necessary to initiate fruiting. These include a change in temperature, light conditions and CO_2 concentration (Niederpruem, 1963; Niederpruem & Wessels, 1969). Normally, *S. commune* grows inside a lignocellulose substrate, in dark conditions where the CO_2 concentration builds up due to respiration. When it grows aerial hyphae to grow outside its substrate, there is a drop in temperature, light is perceived and lower atmospheric CO_2 concentration is perceived (Ohm et al., 2010a). Fruiting can occur in *S. commune* at a temperature of about 20-25°C, whereas fruiting is blocked by higher temperatures (30-37°C) or heat shocks (Niederpruem et al., 1964; Niederpruem & Wessels, 1969; Perkins, 1969). Light is essential for fruiting in *S. commune*. From early studies is it known that fruiting in *S. commune* is induced by blue light (320-525 nm) and UV (Perkins, 1969; Perkins & Gordon, 1969). It has to be noted that fruiting can only occur when colonies are photosensitive, thus being of a certain age or size (Leonard & Dick, 1968; Perkins, 1969). Light is perceived through the white collar complex, consisting of blue light receptor WC-1 and WC-2. This mechanism has been well studied in another model fungus, *Neurospora crassa*. If *wc-1* or *wc-2* is deleted in dikaryotic *S. commune* strains, they cannot develop fruiting bodies upon sufficient light exposure, resulting in a blind phenotype (Ohm et al., 2013). Furthermore, WC-2 was found to play an important role in switching from vegetative growth to generative growth, as fruiting *wc-2* mutants did not show fruiting body development (Pelkmans et al., 2017). Less is known about how CO_2 is perceived. It is readily known that fruiting is inhibited by high environmental CO_2 caused for example by respiration (Niederpruem, 1963; Niederpruem et al., 1964). This effect can be undone when alkali such as KOH which binds CO_2 were added (Niederpruem, 1963). Thus, the CO_2 concentration has a significant effect on whether fruiting occurs, yet the molecular sensing pathway remains to be uncovered.

CO_2 is a small non-polar molecule, which could therefore enter fungal cells via diffusion. It is also hypothesized that it could be transported via aquaporins or other transporters (Bahn & Mühlschlegel, 2006). However, disruption of aquaporin Aqy1 in fungal pathogen *Candida albicans*, did not result in diminished CO_2-mediated polymorphism (Klengel et al., 2005). CO_2 sensing is more studied in the context of pathogenic fungi such as *Candida albicans* and *Cryptococcus neoformans*, as they have to adapt to high CO_2 concentrations in their hosts. CO_2 can react with H_2O to form bicarbonate (HCO_3^-) and H^+. This reversible hydration and dissociation reaction can be catalyzed by a metalloenzyme called carbonic anhydrase (CA) (Bahn & Mühlschlegel, 2006; Elleuche & Pöggeler, 2010; Martin et al., 2017). Five classes of CAs have been identified (α, β, γ, δ, and ζ), of which α-CAs and β-CAs have been found in fungi (Elleuche & Pöggeler, 2009; Elleuche & Pöggeler, 2010). In *C. albicans* and *C. neoformans*, deletion of CAs were found to be lethal under low CO_2 conditions, indicating that the speed of the spontaneous reaction to HCO_3^- is not sufficient for the physiology of the organisms (Martin et al., 2017). HCO_3^- directly stimulates fungal adenylyl cyclase (AC) in fungal pathogens *C. albicans* and *C. neoformans*, which thus acts as a chemosensor (Hall et al., 2010; Klengel et al., 2005). It was established that virulence was inhibited when AC was deleted in *C. albicans* (Klengel et al., 2005) and virulence was suppressed using *in vitro* and *in vivo* models when the AC receptor site Lys1373 for CO_2/HCO_3^- was mutated (Hall et al., 2010). Thus, the activation of AC is the next step in the CO_2 sensing pathway. AC next responds by catalyzing the conversion of ATP into adenosine-3',5'-cyclic monophosphate (cAMP), a second messenger involved in various processes (Bahn & Mühlschlegel, 2006).

While cAMP knows many targets, in this specific pathway it activates cAMP-dependent protein kinase A (PKA) (Cao et al., 2017; D'Souza et al., 2001; Hicks et al., 2004). PKA consists of both catalytic and regulatory subunits. The catalytic subunits can be released to phosphorylate their target upon activation by cAMP (Hicks et al., 2004). It was found that disruption of the major catalytic subunit Pka1 in *C. neoformans* serotype A resulted an avirulent strain (D'Souza et al., 2001; Hicks et al., 2004). Likewise the catalytic subunit TPK2 in *C. albicans* is essential for filamentous growth, thus virulence (Cao et al., 2017). PKA enables a negative feedback on the cAMP production by activating phosphodiesterase (Pde). Pde degrades cAMP to adenosine-5'-monophosphate (Uno & Ishikawa, 1973a). In *Saccharomyces cerevisiae* two phosphodiesterases have been identified, namely low-affinity cAMP Pde1 and high affinity cAMP Pde2, of which Pde1 downregulates agonist-induced cAMP and Pde2 regulates the basal cAMP levels (Hu et al., 2010; Ma et al., 1999; Park et al., 2005). A deletion of either phosphodiesterases slightly increased intracellular cAMP levels while deletion of both led to a very high increase of cAMP levels. This was also the case for attenuated PKA, indicating that PKA activity is required for cAMP breakdown by Pde (Ma et al., 1999; Nikawa et al., 1987).

The AC-cAMP-PKA pathway initiated by CO_2 seems to be conserved throughout evolution. CAs have been investigated in many organisms and can be categorized in five classes (Elleuche & Pöggeler, 2009). Of the β-CAs found in fungi, it was shown that CAs from *C. neoformans* could complement for deleted CAs in *C. albicans*, showing that β-CAs are conserved in fungi (Schlicker et al., 2008). ACs appear to be conserved across phyla (Chen et al., 2000). These cAMP producing enzymes know both transmembrane AC (tmAC) activated by G-proteins and soluble AC (sAC) activated directly by HCO_3^- in mammals while fungi know a sAC-like AC activated by both G-proteins and HCO_3^- (Bahn & Mühlschlegel, 2006; Chen et al., 2000; Klengel et al., 2005; Mogensen et al., 2006). It may well be that cAMP signaling initiated by AC may play an important role in fruiting in *S. commune* as it is part of the CO_2 sensing pathway. Studies on fructification in *Coprinus macrorhizus* have shown that cAMP can induce fructification (Uno & Ishikawa, 1973a, 1973b). Similarly, it has been observed in *S. commune* that there is an increase in cAMP levels upon exposure to light (Yli-Mattila, 1987). The latter contrast findings from Schwalb (1974). Here, it was found that addition of cAMP to the agar medium affected morphogenesis in *S. commune* as fruiting body development is arrested at a primordial stage (Schwalb, 1974). More recent studies also showed similar results when intracellular cAMP levels were raised in *S. commune* due to constitutively active AC subunits, called *ScGP-A* and *ScGP-C* (Yamagishi et al., 2002, 2004). The contrasting results can be explained by the fact that cAMP levels peak before the primordial stage in *S. commune* and gradually increases until maturation of the fruiting bodies (Kinoshita et al., 2002). Thus, it appears cAMP signaling plays a significant role in CO_2 sensing and subsequent fruiting.

Altogether, this shows that cAMP is involved in fruiting in S. *commune*. Based on the aforementioned literature, a model for fruiting in *S. commune* based on CO_2 sensing has been proposed (See Fig. 1). It is hypothesized that a high atmospheric CO_2 concentration leads to high AC activity, leading to high intracellular cAMP levels. As a result, the catalytic subunits of PKA will phosphorylate their potential target and fructification will not occur. A low CO_2 concentration, on the other hand, will cause a low AC activity, so that PKA is not activated by cAMP. As a result, the potential target is not phosphorylated, and fructification can take place. It is hypothesized that the potential target of phosphorylation by PKA could be the *hom2* gene, an important gene involved in fruiting in *S. commune*. In a *hom2* deletion strain, fruiting cannot occur (Ohm et al., 2010a; 2011).

In this study, it was attempted to pinpoint the role of Pde2 in the pathway of CO_2 sensing in *S. commune*. It was hypothesized that, apart from light and temperature, the atmospheric CO_2 concentration via cAMP signaling would determine whether fruiting occurs. Pde2 was chosen as the focus point in this study of the CO_2 sensing pathway since it can have a major effect on intracellular cAMP levels and thus on fruiting. Fruiting in dikaryotic *S. commune* was studied by 1) manipulating cAMP levels by adding IBMX and cAMP to the agar medium; 2) generating conditional and constitutive high-affinity *pde2* overexpression strains; 3) generating a *pde2* deletion strain. Among other substances, caffeine is able to inhibit the degradation of cAMP by Pde (Kinoshita et al., 2002; Uno & Ishikawa, 1973a). IBMX, 3-isobutyl-1-methylxanthine, is a known Pde inhibitor 5-7 times more potent than caffeine (Coffin & Spealman, 1989; Wu et al., 1982). IBMX would thereby increase the intracellular cAMP levels. It was found that addition of 2.5 mM IBMX to the agar medium generated clear results (Braat and Lugones, 2020). By adding either IBMX or cAMP to the agar medium, the same effect was hypothesized, namely an increase in intracellular cAMP levels which is expected to inhibit fruiting in the wild-type according to the proposed CO_2 sensing pathway (See Fig. 1). In contrast, an overexpression of *pde2* is expected to fruit regardless of the atmospheric CO_2 concentration or exogenous addition cAMP, while being inhibited by IBMX. Therefore, colonies were grown under different CO_2 concentrations. The deletion strain was hypothesized to not develop fruiting bodies as cAMP is not broken down by Pde2. The fruiting patterns of the *pde2* overexpression and the *pde2* deletion strain together could confirm the CO_2 sensing pathway as proposed in Figure 1.

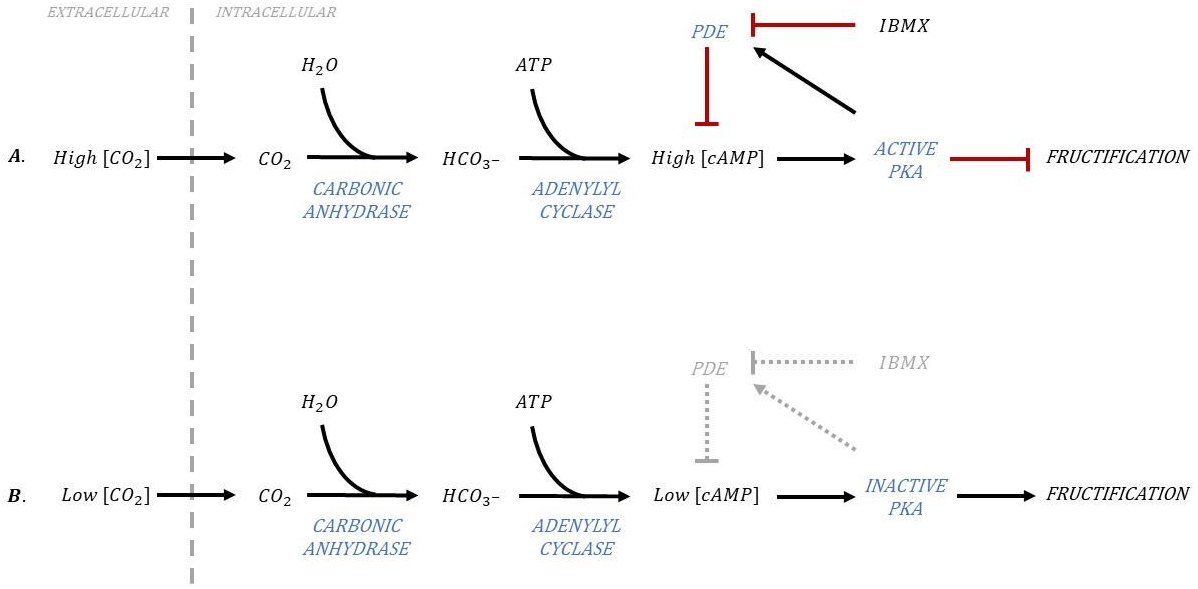

Figure 1. Hypothetical model of CO_2 sensing in *Schizophyllum commune*. Enzymes in this pathway are written in blue, chemical reactions or activation of proteins are depicted with black lines while inhibitory functions are depicted with red lines. A) Hypothetical pathway of CO_2 sensing under high CO_2 conditions. B) Hypothetical pathway of CO_2 sensing under low CO_2 conditions.

Materials and Methods

Culture conditions and strains

Schizophyllum commune strains were grown from point inoculum on minimal medium (MM) with or without 1.5% agar (Dons et al., 1979) for either liquid medium or agar medium. For phenotyping, agar medium either did or did not contain 2.5 mM IBMX (Sigma-Aldrich, St. Louis, USA), 2.5 mM cAMP (Sigma-Aldrich, St. Louis, USA) or a combination of both substances. IBMX and cAMP were added as powder before solidification of the medium and dissolved in the agar by shaking. Along with 2.5 mM IBMX and/or 2.5 mM cAMP, 100 µg ml^{-1} ampicillin and 35 µg ml^{-1} chloramphenicol was added to the medium before solidification. Strains were grown at 30°C in the dark (0 lux) for vegetative growth conditions or at 25°C in the light (~1000 lux) for generative conditions, with cycles of 16 hours of light and 8 hours of darkness. The CO_2 concentration varied for generative conditions. Strains were either exposed to low CO_2 concentrations (~0,04%) or high CO_2 concentrations (3%). The strains of *S. commune* used in this study were H4-8A (MATA43MATB41; FGSC 9210; Ohm et al., 2010a) and H4-8B (MATA41MATB43; Ohm et al., 2010b) as a wild-type (WT). Their derived dikaryon was used as a control when phenotyping compatible monokaryon *pde2* transformant strains 1 and 10, which resulted from the transformation of the *pde2* overexpression construct. Lastly, the ΔKU80 strain (de Jong et al., 2010) was used in order to generate *Δpde2* transformants.

Ring plate assay

Custom made ring plates were sterilized with Halamid for 30-60 minutes and cooked in boiling water for 5 minutes. The plates could not be autoclaved as they were made of Perspex and would deform when stacked in the autoclave. Ring plates were left to dry in the flow cabinet and would be inoculated as described above in 'Culture conditions and strains'. Ring 1 throughout 6 contained increasing volumes of MM (100 µl, 500 µl, 1200 µl; 1800 µl; 2400 µl; 3600 µl) a total volume of approximately 10 ml MM. Inoculated plates were wrapped in wet tissue paper, cling foil and aluminum foil to prevent light to be perceived by the strains. After 4 days of vegetative growth colonies reached the edge of ring 4 and the plates were transferred to a box and placed under generative conditions. The ring plates were placed in the box without the lids for aeration of the colonies to achieve fruiting. Excess CO_2 was extracted from air in the box by tissues wetted with 4% KOH (Niederpruem, 1963). Proper humidity of the box was achieved by tissues wetted with water. Together, these conditions make for optimal fruiting conditions. After 5 to 7 days, fructification of all colonies was assessed. It was assessed whether fruiting occurred and where the fruiting bodies developed. Factors that were able to influence the fruiting of *S. commune* in this assay were 2.5 mM IBMX (Sigma-Aldrich, St. Louis, USA), 2.5 mM cAMP (Sigma-Aldrich, St. Louis, USA), a combination of both substances, and light. While 2.5 mM IBMX and 2.5 mM cAMP were added to the medium as described above, light was assessed as a factor by only letting certain rings of the plate be able to perceive the light source during generative conditions by using aluminum foil. Strains of *S. commune* that were used in this assay were H4-8A (MATA43MATB41; FGSC 9210) and H4-8B (MATA41MATB43), and compatible monokaryon *pde2* transformant strains 1 and 10.

Phosphodiesterase 2 Tet-on constructs

Two construct were fabricated for the Tet-on system in *S. commune*. The constructs were made by Remmers (2022), whom previously worked on this project and is referred to for a more detailed description of the construction of the constructs mentioned in this study. The first construct consisted out of the transcriptional activator sequence (rtTA). The sequence was placed under control of the *β-tubulin* promoter for constant expression of the transcriptional activator. This construct contained a resistance cassette for hygromycin. The second construct consisted out of the promotor sequence (TetO7). This construct contained dTomato and a phleomycin resistance cassette (Remmers, 2022). The constructs had to be transformed separately and brought together via the crossing of two monokaryons bearing each one of the constructs. Also see Table 1.

Phosphodiesterase 2 overexpression construct

The *pde2* overexpression construct was fabricated by Remmers (2022). Here, the PKA binding domain RRXS is replaced by RRXD. The aspartate which substitutes for the serine may be seen as a phosphorylated serine, resulting in an active Pde2 molecule. The *pde2* gene is under constant expression as a tubulin promotor is present in the construct. The selection for this construct is phleomycin (Table 1). Integrity of the construct was checked in this study by digestion using restriction enzyme EcoRI.

Phosphodiesterase 2 deletion construct

The *pde2* deletion construct was fabricated by Remmers (2022). pDelPde2 contained a nourseothricin cassette which was placed between two *pde2* flanks (Table 1). After transformation with this construct, its presence would be confirmed by PCR using primer pairs u1fw/surv, sdfw/u2rv and u1fw/ufrv. While u1fw/surv and sdfw/u2rv together confirm the presence of the construct by overlapping sequences with the nourseothricin resistance cassette, u1fw/ufrv serves as a control.

Table 1. An overview of the constructs used and their resistance cassettes.

Experimental line	Construct	Resistance cassette
Tet-on system	prtTASc prTetO7-dTom	Hygromycin Phleomycin
pde2 overexpression strain	pTub-Pde2*	Phleomycin
Δ*pde2* strain	pDelPde2	Nourseothricin

Spore harvesting and spore swelling

Small 10 ml plates containing MM with 1.5% agar were point inoculated with *S. commune* WT dikaryon or ΔKU80 dikaryon. After 4 days of growth in vegetative conditions, plates were taken out of the incubator and placed on a bench to induce fruiting by the changed conditions. Plates were placed upside down so that fruiting bodies would face downwards and spores would fall onto the inside of the lid. Spores of *S. commune* WT dikaryon or ΔKU80 dikaryon were harvested by taking them up in MQ. A sample of 1 µl of the solution was taken and spores were counted using a Bürker Türk Bright-Line counting chamber. The following formula was used to calculate the number of spores:

Number of spores (16) * dilution * 2,5 * 10E5*

Depending on which grid is used to count, the number between brackets is included in the formula. As this formula gives the amount of spores per milliliter, the number requires recalculation according to the amount of MQ which the spores have been taken up in. Spores were spun down at maximal speed for 3-4 minutes. The spores were allowed to swell by taking them up in liquid MM in a concentration of 1.6E8 spores ml^{-1} and incubating them overnight at 30°C with shaking at 200 rpm. The next day, spore swelling was assessed using a microscope.

Electroporation of S. commune

Swollen spores were used for electroporation. Spores were spun down for 3-4 minutes at maximal speed and the supernatant was discarded. The spores were washed one time in 1 ml of ice cold sorbitol 1 M. Spores were taken up in 45 µl of ice cold sorbitol and 15 µg of DNA was added to an end volume of 50 µl. The solution was gently mixed and kept on ice for 15 minutes. The solution was transferred to an ice cold cuvette which was then placed in the Gene Pulser II and Pulse Controller II (Bio-Rad, 200 Ω resistance, 25 µF capacitance, 2.30 kV to reach a time constant of 5 ms). Immediately after providing the shock, 1 ml of ice cold sorbitol 1 M was added to the cuvette. The content was then transferred to a 50 ml tube and spun down for 5 minutes at 5000 rpm. Spores were embedded using 1% low melting point agarose (LMP) with MM containing 0.05% glucose to an end volume of 30 ml. The content was plated out over three plates. When the medium solidified, a liquid overlay of 5 ml MM without glucose,

100 µg ml^{-1} ampicillin, 35 µg ml^{-1} chloramphenicol and the selective antibiotic was placed over the solidified medium. The plates were incubated at 30°C with shaking at 50 rpm. After 3 days of growth, a second selection could be performed in order to assess and purify the transformant. Cylinders of selected transformant colonies were placed on a dry Petri-dish. A drop of 65 µl LMP agar along with the selective antibiotic was placed on top. Plates were wrapped in wet tissue paper and cling foil to prevent dehydration of the agar and incubated at 30°C for 2-4 days depending on the growth rate. Both the liquid overlay and embedding colonies using LMP agar were done in order to prevent aerial hyphae from forming, which may lead to a false positive selection. In addition, the liquid overlay provides the option to increase or decrease the amount of the selective antibiotic.

Protoplast production from Spores

Swollen spores of WT dikaryon or ΔKU80 dikaryon were used for total protoplasting. An additional step for the protoplasting of ΔKU80 spores, is that spores are firstly incubated for 3 hours in fresh liquid MM with 72 mM hydroxyurea. Swollen spores were spun down at maximal speed for 3-4 minutes. Spores were then taken up in 1 ml of lysing enzyme solution (1 M MgSO4, 25 mM malate buffer pH 5.8, 20 mg lysing enzymes (LE)). The spores were incubated for 3 hours at 30°C. Hereafter, the extension of protoplasting progression was checked under the microscope using a diluted sample. The number of protoplasts could be counted using Bürker Türk Bright-Line counting chamber and calculated via the same calculation as previously mentioned, namely:

Number of protoplasts (16) * dilution * 2,5 * 10E5*

Depending on which grid is used to count, the number between brackets is included in the formula. Protoplasts were spun down at 2000 g for 10 minutes. The protoplasts were washed once in sorbitol 1M with 1/10 CaCl 500 mM. WT protoplast were taken up in 1E7 protoplasts per 100 µl sorbitol 1M with 1/10 CaCl 500 mM, while ΔKU80 protoplasts were taken up in 5-6E7 protoplasts per 100 µl sorbitol 1M with 1/10 CaCl 500 mM. Protoplasts were gradually frozen overnight using a freezing container (Nalgene®, Mr. Frosty) and stored at -80°C.

E. coli transformation and generation of closed plasmid DNA

100 µl with an aliquot of competent *E. coli* cells were thawed on ice for transformation. 1 µl of diluted closed plasmid DNA was added to the cells. The cells were kept on ice for 15 minutes. Hereafter, a heat shock of 42°C for 1 minute was given. 1 ml liquid LB was added and the competent cells were incubated for 1 hour at 37 °C. 50 µl of the competent cells were plated out on an LB agar plate with 100 µg ml^{-1} ampicillin for selection. The plate was incubated overnight at 37°C. One colony was scooped up for DNA extraction and 5 ml of liquid LB with 100 µg ml^{-1} ampicillin was added. This was incubated at 37°C with shaking at 200 rpm. After 7-8 hours, the volume of LB was increased to 200 ml and further incubated at 37°C with shaking at 200 rpm overnight. Midi-prep was performed according to the NucleoBond protocol (https://www.mn-net.com/media/pdf/e4/c4/67/Instruction-NucleoBond-PC-BAC.pdf). The protocol was slightly adapted as up until 'NucleoBond Ax100, Step 5' everything is performed in duplicate to overload the column with the goal to gain more closed plasmid DNA. The quantity of the DNA was checked with the NanoDrop spectrophotometer.

Transformation of S. commune and monokaryon selection

Protoplasts were thawed on ice and 15 µg DNA was added to the protoplasts and incubated on ice for 15 minutes. For ΔKU80 protoplasts, 10-20 U of the restriction enzyme SgsI (Thermo Fisher Scientific) could be added hereafter, followed by 1 hour of incubation at 37°C for optimal functioning of the restriction enzyme. Hereafter, 1 V filter sterilized 40% PEG4000 buffered with 10 mM Tris pH 7 was added. This was gently mixed and kept on ice for 5 minutes. Up to 2,5 ml of regenerative medium (glucose, $MgSO_4.7H_2O$, KH_2PO_4, K_2HPO_4, peptone (mycological), yeast extract) was added to the mixture along with 100 µg ml^{-1} ampicillin and 35 µg ml^{-1} chloramphenicol. When WT protoplasts were used, 25 ug ml^{-1} phleomycin was added to the medium for overnight incubation. Phleomycin was added in the form of zeocin with the goal to create double-strand breaks in the DNA (van Peer et al., 2009a). Protoplasts were incubated overnight at 25°C. Only when the restriction enzyme SgsI (Thermo Fisher

Scientific) was used, protoplasts were incubated overnight at 30°C. The next day the regenerated protoplasts were spun down at 5000 rpm for 5 minutes. Regenerated protoplasts were embedded using LMP agarose with MM containing 0.05% glucose, 100 µg ml^{-1} ampicillin, 35 µg ml^{-1} chloramphenicol and the selective antibiotic to an end volume of 30 ml. The content was poured out over three plates. When the medium solidified, a liquid overlay of 5 ml MM without glucose, 100 µg ml^{-1} ampicillin, 35 µg ml^{-1} chloramphenicol and the selective antibiotic was placed over the solidified medium. Plates were incubated at 30°C with shaking at 50 rpm. After 3 days the liquid overlay would be removed and the growth on the plates would be checked regularly. When sufficient amount of colonies had formed, they would be selected before contact with other colonies. Cylinders of selected transformant colonies were placed on a dry Petri-dish. A drop of 65 µl LMP agar along with the selective antibiotic was placed on top. Plates were wrapped in wet tissue paper and cling foil to prevent dehydration of the agar and incubated at 30°C for 2-4 days depending on the growth rate. Both the liquid overlay and bedding in colonies using LMP agar were done in order to prevent aerial hyphae from forming, which may lead to a false positive selection. Hyphae of each colony were observed using an inverted microscope in order to determine which colonies were dikaryons or monokaryons on the basis of the presence of clamp connections. Individual 10 ml MM agar plates were inoculated with the selected monokaryons and allowed to grow for at least 4 days at 25°C and ~1000 lux to exclude potential dikaryons and common A's from the selection.

Mating type assessment

Transformants that were determined as monokaryons were assessed for their mating type. All selected monokaryons were crossed with monokaryons of known mating types. Drops of 65 µl MM agar were placed in four columns and multiple rows on a large, square Petri-dishes. Cylinders of monokaryons of one out of four known mating types were placed in columns and the to be identified monokaryons were placed in the rows. Plates wrapped in wet tissue paper and cling foil to prevent dehydration of the agar and were incubated at 30°C for 4 days. Hereafter, the mating type was determined by searching for the common A and by using the inverted microscope and searching for clamp connections.

DNA isolation

DNA isolation was accomplished by homogenizing mycelium by freezing and crushing with a pestle. 500 µl extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and the mixture was kept in a water bath of 65°C for 30 minutes. 250 µl of chloroform was added and the vortex was spun down for 5 minutes at maximal speed. The water phase was transferred to a fresh tube where 315 µl isopropanol was added. This was spun down for 5 minutes at maximal speed. The pellet was washed with 70% ethanol and briefly dried at 65°C to remove all the remaining ethanol. The pellet was dissolved in 30 µl TE buffer at 65°C for five minutes. To check the quality of the DNA, 1 µl was used for PCR, with the proper primers. The PCR products were run on agarose gel for 20-25 minutes at 100 V.

RNA isolation

RNA isolation was accomplished by firstly growing the colonies of interest on sterile cellophane membrane on top of MM agar plates. Plates were incubated at 30°C for 3 days. Colonies were quickly removed from the cellophane and frozen in liquid nitrogen in order to prevent changes in gene expression. Mycelium was homogenized by a metal bead at 25 Hz for 1 minute using the TissueLyser II (Qiagen, Hilden, Germany). Samples were kept frozen after homogenization. Subsequently, samples were suspended in 500 µl Invitrogen TRIzol Reagent (Thermo Fisher Scientific). Samples were kept at room temperature (RT) for 5 minutes. Hereafter, 100 µl chloroform was added to each sample and samples were inverted 10 times. Centrifugation for 5 minutes at maximal speed caused the water phase containing the RNA to separate from the chloroform phase. Subsequently, the water phase was carefully transferred to a fresh tube to which 250 µl 100% isopropanol was added and kept at RT for 10 minutes. Centrifugation for 10 minutes at maximal resulted in caused precipitation of the RNA. The RNA pellet was taken up in 100 µl RNase free water and 1 V 4 M LiCl. RNA was precipitated at -18°C for at least 1 hour. Centrifugation at 12000 rpm for 20 minutes at 4°C caused precipitation of the RNA. The RNA pellet was washed twice in 80% ethanol and air dried. RNA was dissolved in 30 µl RNase free water.

The quantity of the RNA was determined using the NanoDrop spectrophotometer. The quality of the RNA samples was checked by running the samples on a denaturing gel with Hepes/triethanolamine buffer (Mansour & Pestov, 2013) for 25 minutes at 100 V. Samples were first denatured by placing 1 µl of the RNA sample in 5 µl of loading buffer (50% formamide, 0.4 M formaldehyde, 1x running buffer (30 mM Hepes, 30 mM triethanolamine), 0.5 mM EDTA, 0.02% bromophenol blue) at 70°C for five minutes. The samples were quenched on ice and loaded onto the gel.

cDNA synthesis and quantitative PCR

cDNA was synthesized from RNA by using the RevertAID RT Reverse Transcription kit (Thermo Fisher Scientific). Here, random primers included in the kit were used. The cDNA was stored at -20°C. The quantitative PCR (qPCR) was performed using the generated cDNA and the Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). The primers used for this qPCR were scrtTAfw/scrtTArv and arf1qfw2/arf1qrv2. Expression levels of *tTa* were calculated relative to the expressions levels of *arf1* (Protein ID 2623093).

Results

IBMX and cyclic AMP inhibit fructification is S. commune

Dikaryotic WT colonies of *S. commune* were exposed to IBMX and cAMP using ring plates to test whether these substances act as inhibitors of fruiting. It was expected that WT dikaryons of *S. commune* are not able to fructify when IBMX or cAMP is present.

Preliminary work showed that ring plates with MM agar as opposed to liquid MM with a cellophane membrane, generated reproducible results. Liquid MM with cellophane membrane resulted in asymmetrical growth of the colonies, making reproducibility difficult (data not shown). In addition, IBMX or cAMP would be dissolved in DMSO which by itself had an effect on the growth and fruiting of the colonies (data not shown), rendering this method useless. Thus, 2.5 mM of IBMX and/or 2.5 mM of cAMP was added as a powder to the agar of certain rings of the ring plates (Braat and Lugones, 2020). The tested set-up included adding IBMX and/or cAMP in the outer two rings (ring 5-6), the outer four rings (ring 3-6), the inner four rings (ring 1-4), and all rings of the plate (ring 1-6).

Several findings were made during this study. First, it has been found that IBMX as well as cAMP can reduce fructification of *S. commune* by pushing back the location of fructification to the center of the colony. This can be seen in the plates where IBMX or cAMP is added to the outer two rings of the plate (See Fig. 2 and Fig 3., first and second column). Relative to the control, the colony cannot fructify on the outer rings. This raised the question of whether the fructification of *S. commune* can be reduced even further, namely to the center where the plate is inoculated. For this, IBMX or cAMP is added to the outer four rings of the plate. However, it can be seen in the third column of both Figure 2 and Figure 3 that no fructification takes place in these plates when compared to the control. Only once was it observed that fructification occurred in the center of the colony namely in two plates in which each ring contained cAMP. Pronounced fruiting bodies formed in the center (data not shown). However, due to a bacterial infection that came with the inoculum, it is not certain what caused this effect. Since fructification in the center of the colony was never observed again hereafter, this observation is disregarded. Thus, in general fructification cannot take place in the center of the colony. Primordia did form in various places. Interestingly, these primordia are often formed on the edges of the rings, where the colony is in contact with the Perspex of the plate, and not with the agar itself containing IBMX or cAMP. It is hypothesized that these primordia are less affected by the added substances here and are therefore somewhat capable of inciting fructification. It may also be a stress response of the colony. The effect of reducing fruiting was more strongly observed in the plates containing IBMX as more primordia form in the plate containing cAMP. This was also observed in the second finding. The plates where IBMX or cAMP is added to all rings, no fructification takes place when compared to the control (See Fig. 2 and Fig. 3, first and last column). Here again, primordia are observed, but again mainly where the colony is in contact with the Perspex of the plate and mainly in the plate where only cAMP is present. Lastly, the effects of the substances on the fructification of *S. commune* are enhanced in the plates where both IBMX and cAMP has been added. First, it can be seen in the third column of Figure 4 that fewer primordia are found compared to the plates where only IBMX or cAMP was added to the agar. This also applies to the plate where there is IBMX and cAMP in all the rings. Furthermore, an interesting finding is made when looking at the growth of the colonies pre-fructification. Although the growth of the colonies pre-fructification does not differ much from the control in the plates where solely IBMX or cAMP is present, this seems to be the case when both substances are present. Colonies growing on a plate containing IBMX and cAMP in all rings from the start show less growth regarding diameter of the colony. It seems that the excess of intracellular cAMP generated from extracellular addition and by blocking Pde from the start can have an effect on growth.

From these results, it is clear that IBMX and cAMP can suppress the fructification of WT dikaryon *S. commune*. Also, the fructification can be pushed back to the center of the colony to some extent. The observed effects on fructification are enhanced when both IBMX and cAMP are added to the medium. However, it is not yet clear why fructification cannot take place in the center of the colony. Perhaps, this can be deduced from further investigation into gene expression, which may be elucidating when comparing gene expression of the hyphae per ring.

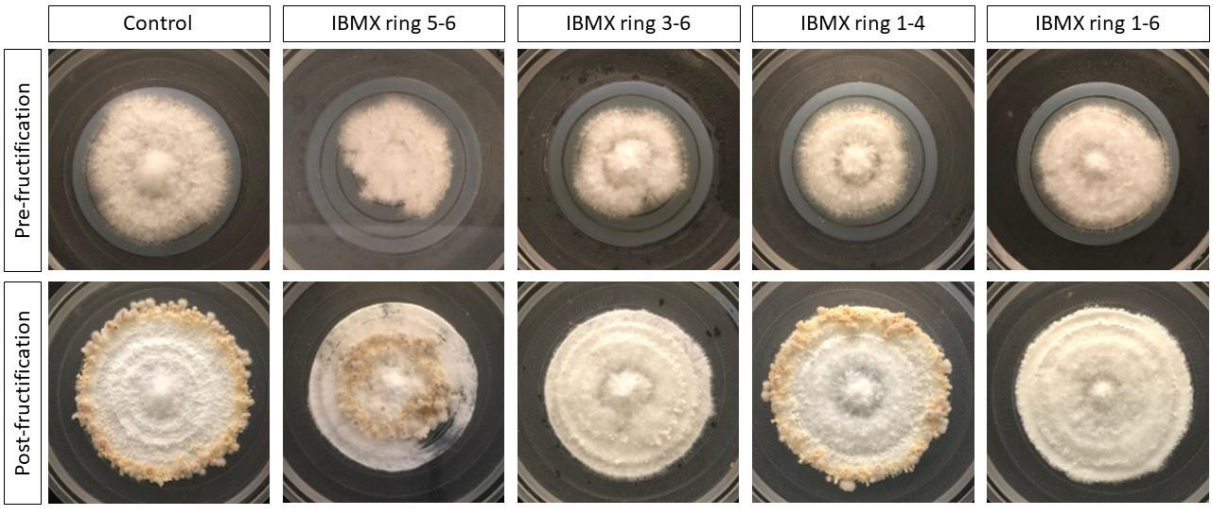

Figure 2. Fructification patterns of WT dikaryon *S. commune* grown with IBMX. 2.5 mM of IBMX was added to certain rings of the ring plates. From left to right, plates contained no IBMX (control); IBMX in the outer two rings (IBMX ring 5-6); IBMX in the outer four rings (IBMX ring 3-6); IBMX the inner four rings (IBMX ring 1-4); and IBMX in all rings of the plate (IBMX ring 1-6). The top row shows the growth after 4 days of vegetative growth (pre-fructification). The bottom row shows the growth and fructification after 5-7 days of generative growth (post-fructification).

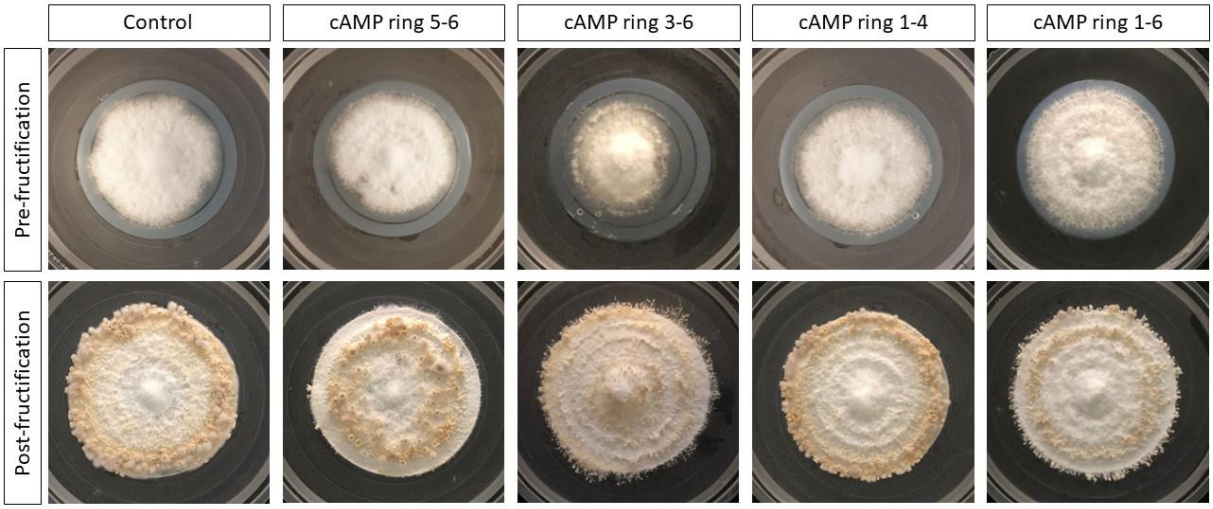

Figure 3. Fructification patterns of WT dikaryon *S. commune* grown with cAMP. 2.5 mM of cAMP was added to certain rings of the ring plates. From left to right, plates contained no cAMP (control); cAMP in the outer two rings (cAMP ring 5-6); cAMP in the outer four rings (cAMP ring 3-6); cAMP in the inner four rings (cAMP ring 1-4); and cAMP in all rings of the plate (cAMP ring 1-6). The top row shows the growth after 4 days of vegetative growth (pre-fructification). The bottom row shows the growth and fructification after 5-7 days of generative growth (post-fructification).

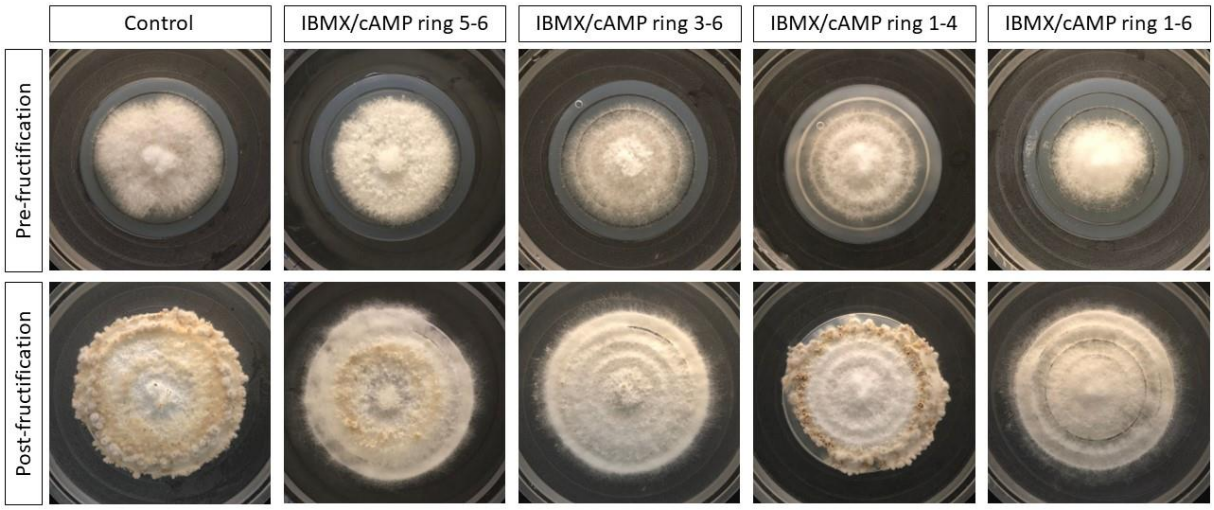

Figure 4. Fructification patterns of WT dikaryon *S. commune* grown with IBMX and cAMP. 2.5 mM of IBMX and cAMP was added to certain rings of the ring plates. From left to right, plates contained no IBMX or cAMP (control); IBMX and cAMP in the outer two rings (IBMX/cAMP ring 5-6); IBMX and cAMP in the outer four rings (IBMX/cAMP ring 3-6); IBMX and cAMP in the inner four rings (IBMX/cAMP ring 1-4); and IBMX and cAMP in all rings of the plate (IBMX/cAMP ring 1-6). The top row shows the growth after 4 days of vegetative growth (pre-fructification). The bottom row shows the growth and fructification after 5-7 days of generative growth (post-fructification).

IBMX and cyclic AMP are not transported by hyphae of S. commune

Hyphal transport by cytoplasmic streaming in *S. commune* relies on the dynamic opening and closing of septal pores, which appears to depend on environmental conditions (van Peer et al., 2009b). In other species, it is known that transport of certain substances, for example glucose, only occurs from the center of the colony to the periphery and not vice versa due to certain transporters present in the septal wall (Bleichrodt et al., 2015). Dikaryotic WT colonies of *S. commune* were exposed to 2.5 mM IBMX and 2.5 mM cAMP using ring plates to test whether these substances can be transported and subsequently act as inhibitor of fruiting. Therefore, IBMX and cAMP were added to certain rings as aforementioned. It was hypothesized that in the presence of IBMX or cAMP, no fructification occurs. This implies that the expectations were that IBMX and cAMP could be transported through hyphae of WT dikaryon *S. commune* and that no fructification would occur despite the location of IBMX and cAMP.

When solely IBMX or cAMP is added to the agar of the inner four rings of a plate, fructification is observed to occur in the outer two rings of the plate. These plates are similar to their corresponding control (See Fig. 2 and Fig. 3, first and fourth column). Fruiting can still occur on the outer rings were no IBMX or cAMP is present, even though vegetative growth occurred on agar with one of said substances. The same results are found on a plate where both IBMX and cAMP are present in the inner four rings (See Fig. 4, first and fourth column). The fact that fructification can still take place on the outer two rings in all of the above cases indicates that IBMX and cAMP are not transported from the center of the colony to the periphery or are degraded in the process of transport. The opposite is also true. When IBMX and/or cAMP is added to the outer two rings of a plate, the colony can still fructify where there is no IBMX and cAMP in the agar (See Fig. 2-4, first and second column), namely on the fourth ring. These results suggest that transport of IBMX and cAMP does not occur in WT dikaryon *S. commune* from either the center of the colony to the periphery or vice versa.

In addition to IBMX and cAMP, light has also been tested as a factor. An attempt was made to test whether the light signal is indeed transported and where fructification would take place. After 4 days of vegetative growth, lidded plates were placed under generative conditions. The lid was covered with aluminum foil so that the light could only reach ring 1-2, ring 3-4 and ring 5-6 compared to the complete light or complete dark controls. However, this experimental set-up had its limitations. First, light could still slightly reach the sides of the plates, seen as in the dark control primordia were observed (data not shown). Light could also reach several rings via diffraction, since the lid is several millimeters above the colony and as the Perspex of the plate could refract some light. Finally, the set-up is different from that mentioned before, since the ring plates are not placed in a box containing 4% KOH. Since both the

lids remained on the plates and no 4% KOH was used in this set-up, the CO_2 concentration in the plates could build up which is not ideal for fructification. For these reasons, the results of this experiment have not been taken into consideration.

Pde2 transformants do not fructify under high CO_2

Constitutive dikaryotic *pde2* overexpression transformant strains and dikaryotic WT *S. commune* were exposed to high atmospheric CO_2 concentrations (3%) during generative growth to test if the transformants are proper *pde2* overexpressors. It was expected that the *pde2* overexpression transformant strains can fructify under high CO_2 conditions while the WT dikaryon cannot.

Transformation with the pTub-Pde2* construct generated 22 transformants, which were determined monokaryons. Therefore, their mating types were assessed after which all monokaryons were crossed with their potential partners. These dikaryotic colonies were grown under generative conditions from the start to induce fruiting. The colonies were viewed several times to assess the timing and the extent of mushroom formation relative to the control. Of all the colonies, two looked promising, namely strain 7x1 and 10x1 as they showed early fructification and the formation of lots of fruiting bodies compared to their relative control. Both strains were generated from monokaryons of the same mating types. Thus, WT dikaryon, strain 7x1 and strain 10x1 were grown on MM agar plates under vegetative growth conditions for 4 days. Hereafter, plates were transferred to an incubator (25°C, ~1000 lux) in which the CO_2 concentration could be controlled. The CO_2 concentration was set to 3%. After 5 to 7 days, plates were assessed for fruiting. No fruiting had occurred in the WT, strain 7x1 or strain 10x1 (See Fig. 5, first column). The experiment was performed several times due to infections which occurred within the incubator. Strain 10x1 did seem to fructify in one attempt (data not shown) but these data were discarded due to the large amount of infections within the plates. To further test the extent of *pde2* overexpression of both strains, they were exposed to 2.5 mM IBMX or 2.5 mM cAMP under high atmospheric CO_2 concentrations (3%) during generative growth. This experiment was performed parallel to testing solely the effect of elevated atmospheric CO_2. Neither the WT or *pde2* overexpression strains 7x1 or 10x1 were able to form fruiting bodies under elevated CO_2 conditions combined with the presence of either 2.5 mM IBMX or 2.5 mM cAMP (See Fig. 5, second and third column).

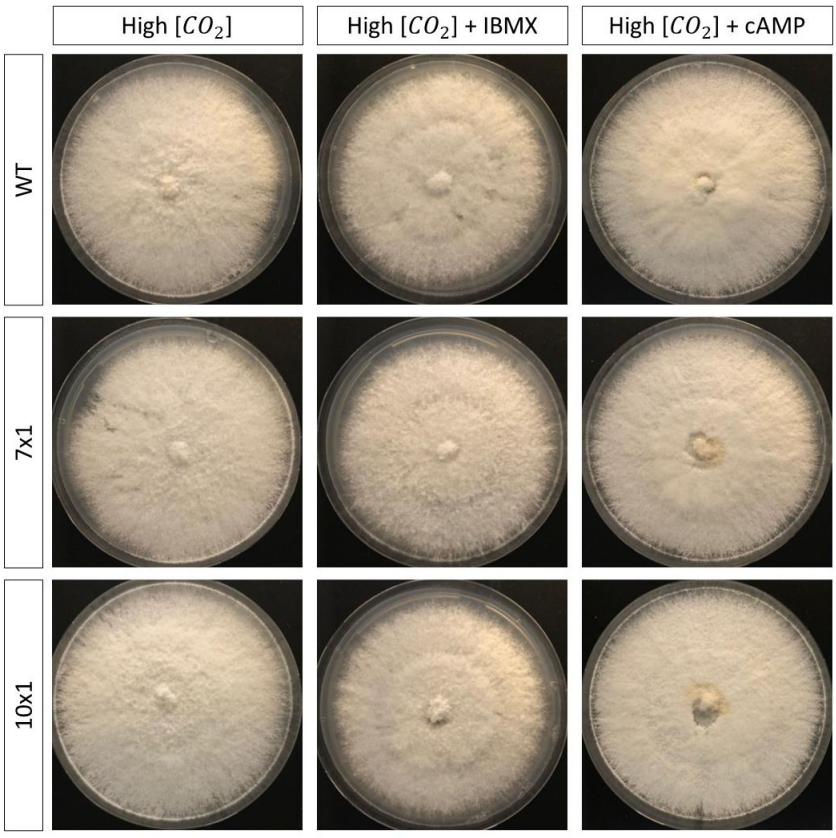

Figure 5. Fructification pattern of WT dikaryon *S. commune* and *pde2* overexpression strain 7x1 and strain 10x1 under several conditions. From left to right, plates were incubated 5-7 days under high CO_2 conditions (3%); high CO_2 conditions (3%) and 2.5 mM of IBMX; or high CO_2 conditions (3%) and 2.5 mM of cAMP. The first row shows the generative growth of WT dikaryon *S. commune*; the second row shows the generative growth of dikaryon *pde2* overexpression strain 7x1; and the last row shows the generative growth of dikaryon *pde2* overexpression strain 10x1 after 4 days of vegetative growth.

IBMX and cyclic AMP inhibit fructification for Pde2 transformants at low CO_2

To further test the extent of the *pde2* overexpression of strain 10x1, both strain 10x1 and WT dikaryon were exposed to 2.5 mM IBMX, 2.5 mM cAMP or to a combination of 2.5 mM IBMX and 2.5 mM cAMP using ring plates. Strain 7x1 was not included in this assay as it was observed that strain 10x1 seemed to fructify in a first attempt under high CO_2 conditions. However, this was refuted later on. Another explanation could be that reversions occurred, which occurred often when overexpressing *pde2* in Braat and Lugones (2020). It was expected that the *pde2* overexpression strain 10x1 was able to fructify on agar containing 2.5 mM cAMP while the WT dikaryon is not. The fructification of both strain 10x1 and the WT would be inhibited by addition of 2.5 mM IBMX as Pde2 molecules would be inhibited with disregard to the expression level. It was hypothesized for the WT to resemble the phenotypes seen in Figure 2 and 3. For strain 10x1, phenotypes resembling the 10x1 control were expected for addition of cAMP, while phenotypes resembling those seen in Figure 2 were expected for addition of IBMX.

Pde2 overexpression strain 10x1 is not able to fructify in the presence of 2.5 mM IBMX at low CO_2 conditions. Strain 10x1 resembles its relative WT control for all treatments (See Fig. 6). The expected fruiting pattern on the outer rings occurs for the controls and the plates were IBMX is added in the inner four rings only. Fruiting does not occur on the outer rings for the other three treatments where IBMX is present in the outer rings. Therefore, these results suggest that fructification of strain 10x1 is inhibited by IBMX. While it may seem as if strain 10x1 is able to form fruiting bodies on the plate where IBMX is present in all rings (See Fig. 6, last column), the WT is also able to form fruiting bodies to some extent while it was not able to do so in previous experiment (See Fig. 2, last column).

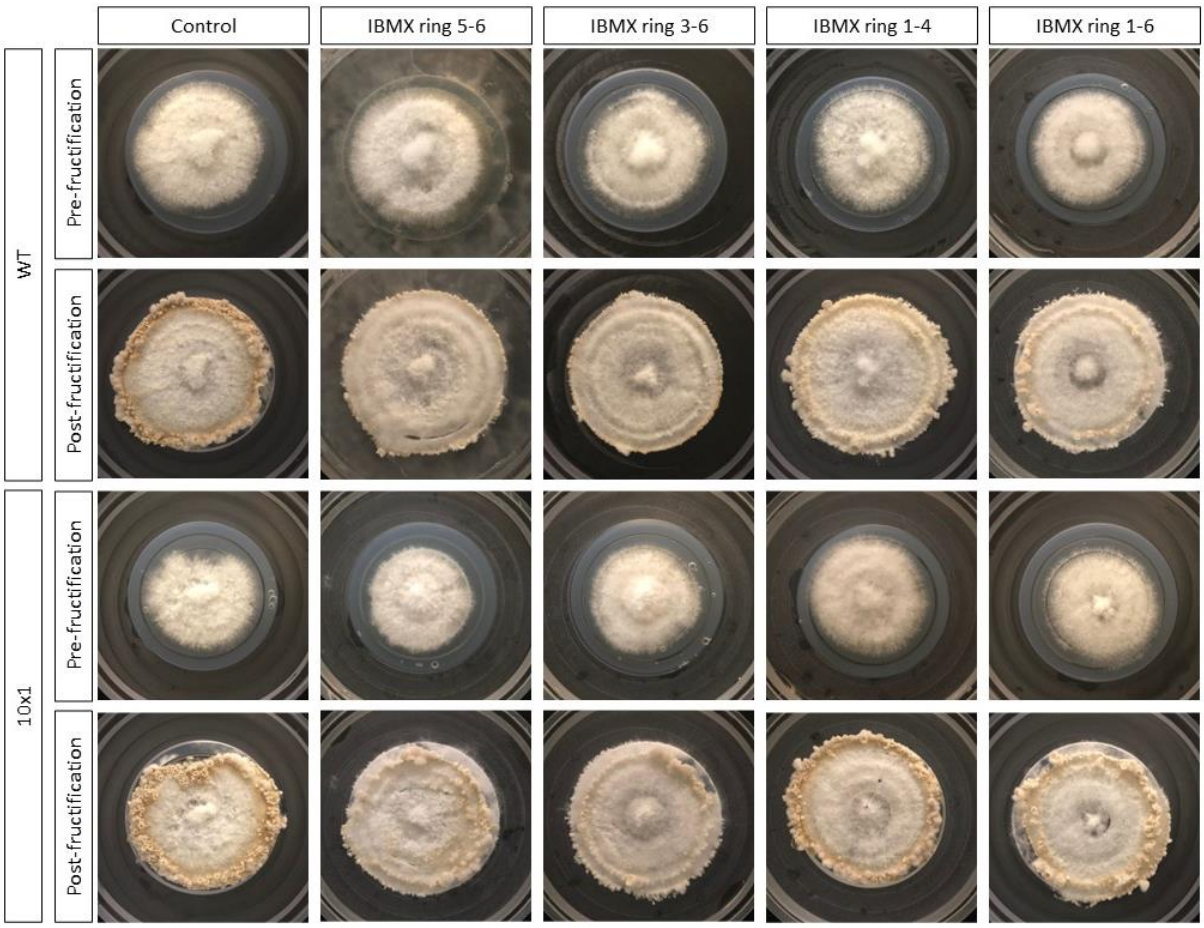

Figure 6. Fructification patterns of WT dikaryon *S. commune* and *pde2* overexpression strain 10x1 grown with IBMX. 2.5 mM of IBMX was added to certain rings of the ring plates. From left to right, plates contained no IBMX (control); IBMX in the outer two rings (IBMX ring 5-6); IBMX in the outer four rings (IBMX ring 3-6); IBMX in the inner four rings (IBMX ring 1-4); and IBMX in all rings of the plate (IBMX ring 1-6). The top two rows show the WT dikaryon and the bottom two rows show the *pde2* overexpression strain 10x1 after 4 days of vegetative growth (pre-fructification) and after 5-7 days of generative growth (post-fructification).

Another striking result are phenotypes of the WT and strain 10x1 when IBMX is added to the outer two rings. While fructification was pushed back to the center of the colony in previous experiments (See Fig. 2), this effect is not observed now. Recalculations have shown that the correct amount of IBMX has been used. Furthermore, it was investigated whether the temperature of the agar medium had affected the stability of IBMX. By repeating this part of the experiment, this also turned out not to be the case. The reason why fructification no longer pushed back to the center of the colony is therefore not clear. One note to take into account is that the 25°C incubator was in need of reparations as temperatures increased to 29°C during the course of this experiment. This may have affected the observed fructification patterns of the colonies in this experiment.

Pde2 overexpression strain 10x1 is not able to fructify in the presence of 2.5 mM cAMP at low CO_2 conditions. Strain 10x1 resembles its relative WT control for all treatments (See Fig. 7). While expected, fruiting bodies do not develop on the outer rings for the treatments where cAMP is present in the outer rings. Thus, fructification of *pde2* overexpression strain 10x1 is also inhibited by cAMP. This indicates that 10x1 is not a true overexpressor. This is also indicated by fructification being pushed back to the center of the colony. Yet, this pushed back fructification pattern is not as strongly observed as in previous experiments (See Fig. 3). Recalculations have shown the correct amount of cAMP has been used. Furthermore, primordia are observed for both the WT and strain 10x1 in the plates were cAMP is present in the outer four rings and in all rings (See Fig. 7, third and fifth column). The inhibitory effect of cAMP thus seems to disappear somewhat. Yet, primordia were also observed in previous experiments (See Fig. 3). Perhaps this has to do with the fact that cAMP is metabolized intracellularly. Therefore, it

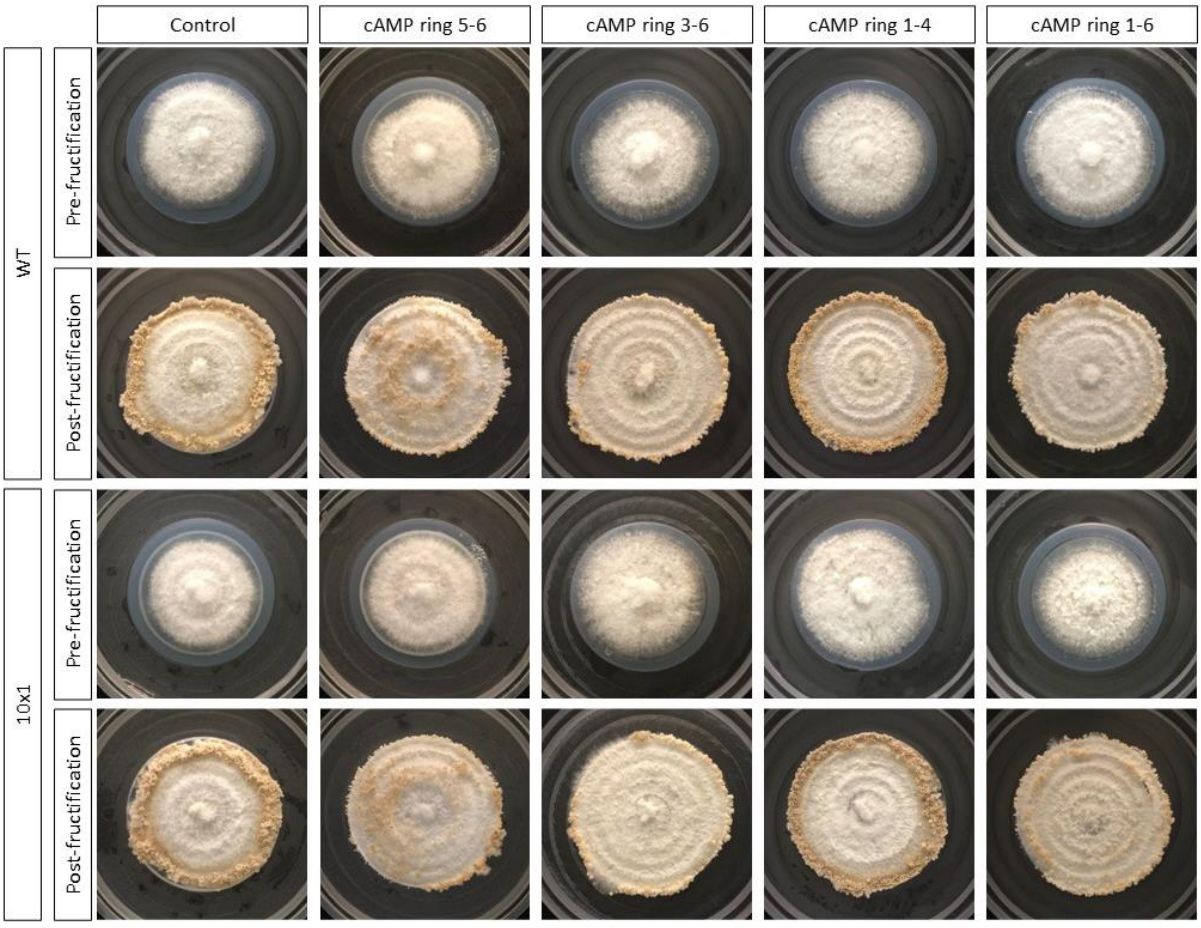

Figure 7. Fructification patterns of WT dikaryon *S. commune* and *pde2* overexpression strain 10x1 grown with cAMP. 2.5 mM of cAMP was added to certain rings of the ring plates. From left to right, plates contained no cAMP (control); cAMP in the outer two rings (cAMP ring 5-6); cAMP in the outer four rings (cAMP ring 3-6); cAMP in the inner four rings (cAMP ring 1-4); and cAMP in all rings of the plate (cAMP ring 1-6). The top two rows show the WT dikaryon and the bottom two rows show the *pde2* overexpression strain 10x1 after 4 days of vegetative growth (pre-fructification) and after 5-7 days of generative growth (post-fructification).

was previously suggested that IBMX had a stronger inhibiting effect as these primordia were not found here (See Fig 2). However, this is again contradicted by results found here with IBMX.

Pde2 overexpression strain 10x1 is not able to fructify in the presence of 2.5 mM IBMX and 2.5 mM cAMP at low CO_2 conditions. Strain 10x1 resembles its relative WT control for all conditions (See Fig. 8). Fruiting is inhibited more clearly in as primordia do not form on these plates compared to when solely IBMX or cAMP are added. This resembled earlier findings (See Fig. 4). Fructification which is pushed back to the center of the colony is strongly observed here, both in the WT and strain 10x1. Another interesting finding is the growth of the WT and 10x1 colonies of pre-fructification on the plates where IBMX and cAMP are in the inner four rings and in all rings (See Fig. 8, fourth and fifth column). The colonies grew in the presence of these substances from the start and this seems to affect their growth pre-fructification. These findings are consistent with results from previous experiments (See Fig. 4, fourth and fifth column).

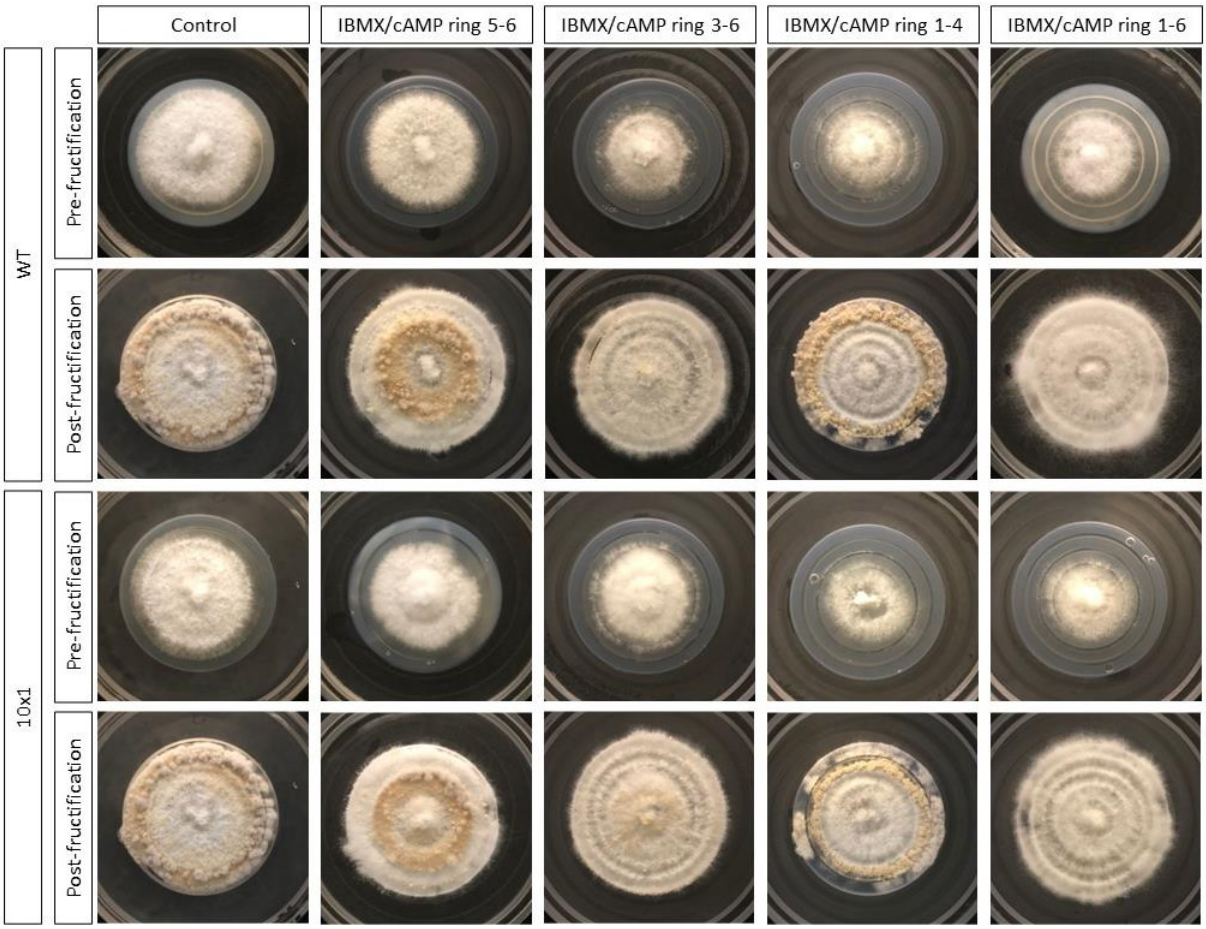

Figure 8. Fructification patterns of WT dikaryon *S. commune* and *pde2* overexpression strain 10x1 grown with IBMX and cAMP. 2.5 mM of IBMX and cAMP was added to certain rings of the ring plates. From left to right, plates contained no IBMX and cAMP (control); IBMX and cAMP in the outer two rings (IBMX/cAMP ring 5-6); IBMX and cAMP in the outer four rings (IBMX/cAMP ring 3-6); IBMX and cAMP in the inner four rings (IBMX/cAMP ring 1-4); and IBMX and cAMP in all rings of the plate (IBMX/cAMP ring 1-6). The top two rows show the WT dikaryon and the bottom two rows show the *pde2* overexpression strain 10x1 after 4 days of vegetative growth (pre-fructification) and after 5-7 days of generative growth (post-fructification).

Deletion of Pde2

The goal was to obtain transformants containing the pDelPde2 construct. The hypothesis was that PEG-mediated transformation using spores protoplasts would work equally well for ΔKU80 protoplasts as for WT protoplasts.

ΔKU80 protoplasts were generated and transformed in the same way as WT protoplasts. The only difference for transformation using ΔKU80 protoplasts is that no 25 µg ml^{-1} phleomycin in the

form of zeocin is added for overnight incubation. Multiple attempts were made to generate transformants containing the pDelPde2 construct in order to obtain a deletion strain. No transformation led to a successful finding. Three attempts were made to transform to ΔKU80 protoplasts. This resulted in 0 transformants. After these attempts, steps in the protocol were adapted. First, attempts were made to add the restriction enzyme SgsI (AscI, Thermo Fisher Scientific) for overnight incubation of the protoplasts. SgsI is a restriction enzyme that recognizes and cuts GG^CGCGCC sites, which could lead to integration of the construct. However, after two attempts using 20 U SgsI or 10 U SgsI, 0 transformants were generated. Next, overnight incubation was attempted with the addition of 1 µg ml^{-1} phleomycin with the intention of also making some cuts in the genome (van Peer et al., 2009a). One attempt resulted in 0 transformants. Hereafter, new protoplasts were generated with *S. commune* ΔKU80 spores. Before protoplasting, these spores were incubated for 3 hours with 72 mM hydroxyurea. Hydroxyurea inhibits ribonucleotide reductase and thereby lowers the amount of dNTPs available for DNA synthesis. This synchronizes cells to arrest in S-phase when there high homologous recombination activity, thus increasing gene targeting rates (Tsakraklides et al., 2015). Transformation was carried out without addition of SgsI or phleomycin. One transformation attempt with these protoplasts resulted in 0 transformants. These results indicate that transformation of the pDelPde2 construct does not work using protoplasted ΔKU80 *S. commune* spores. However, after overnight incubation of the protoplasts, samples were taken and plated on MM agar without selection. These plates showed regeneration of the protoplasts, indicating both protoplasts generated with and without hydroxyurea (data not shown). All plated protoplasts regenerated on the MM agar without selection.

Establishment of an inducible expression system in S. commune

The Tet-on system, and its counterpart Tet-off system, are genetic strategies that can be used to create conditional mutants. The systems have often been successfully applied in *Aspergillus* species. Using this system, genes and also essential genes can be examined by putting the desired gene under the control of the Tet-on/Tet-off system. This system can be turned on or off by a transcriptional activator that binds tetracycline, or its derivative doxycycline (Dümig & Krappmann, 2015; Das et al., 2016; Wanka et al., 2016). The goal of this experiment was to generate a *S. commune* strain containing the overexpression construct of Pde2 under the control of the Tet-on system. Two separate transformations were performed to gain transformants that contained either of two constructs that together formed the Tet-on system for conditional *pde2* overexpression. While the prTetO7-dTom construct contained the promotor sequence with a dTomato coding region, the prtTASc construct contained the transcriptional activator. The aim was to find a strain containing the prtTASc construct with high activity and cross this with its potential partner. It was expected that a dikaryon strain with a highly active transcriptional activator could be found based on dTomato fluorescence cassette of the prTetO7-dTom construct.

PEG-mediated transformations using protoplasted *S. commune* spores resulted in a high number of transformants. The first transformation with the prTetO7-dTom construct resulted in a total of 528 transformants. Of these, 36,67% transformants were monokaryons. The second transformation with the prtTASc construct resulted in a total of 192 transformants of which 56,67% were monokaryons. 11 monokaryon strains containing the prTetO7-dTom construct and 17 monokaryon strains containing the prtTASc construct were selected. The mating type of these monokaryons was assessed after which the monokaryons were crossed with every potential partner. To determine which dikaryon strain had a highly active transcriptional activator, a drop of MM agar containing 10 µg ml^{-1} doxycycline was added adjacent to the drop of MM agar on which the dikaryon strain was growing. After 2-4 days of growth after the addition of the doxycycline, the colonies were screened for dTomato fluorescence. No fluorescence was observed in the dikaryon strains upon addition of doxycycline. A higher concentration of doxycycline, namely 50 µg ml^{-1} as described by Remmers (2022), also did not result in any fluorescence. Thus, no proper expressor of the transcriptional activator could be selected based on phenotyping.

In order to select a strain containing a highly active transcriptional activator, RNA was isolated from 12 of the 17 monokaryon strains containing the prtTASc construct, namely strain 6 throughout 17. cDNA was generated and a quantitative PCR was performed. Analysis of the qPCR data shows that relative gene expression levels differ between samples (See Fig. 9). The relative expression of the construct is highest for strain 13, followed by strain 15 and strain 17. From the results of the qPCR it is clear that the construct functions in *S. commune* as there is a difference in gene expression of the prtTASc

construct. Other monokaryons not included in this qPCR analysis could be analyzed next to determine their relative expression levels of transcriptional activator.

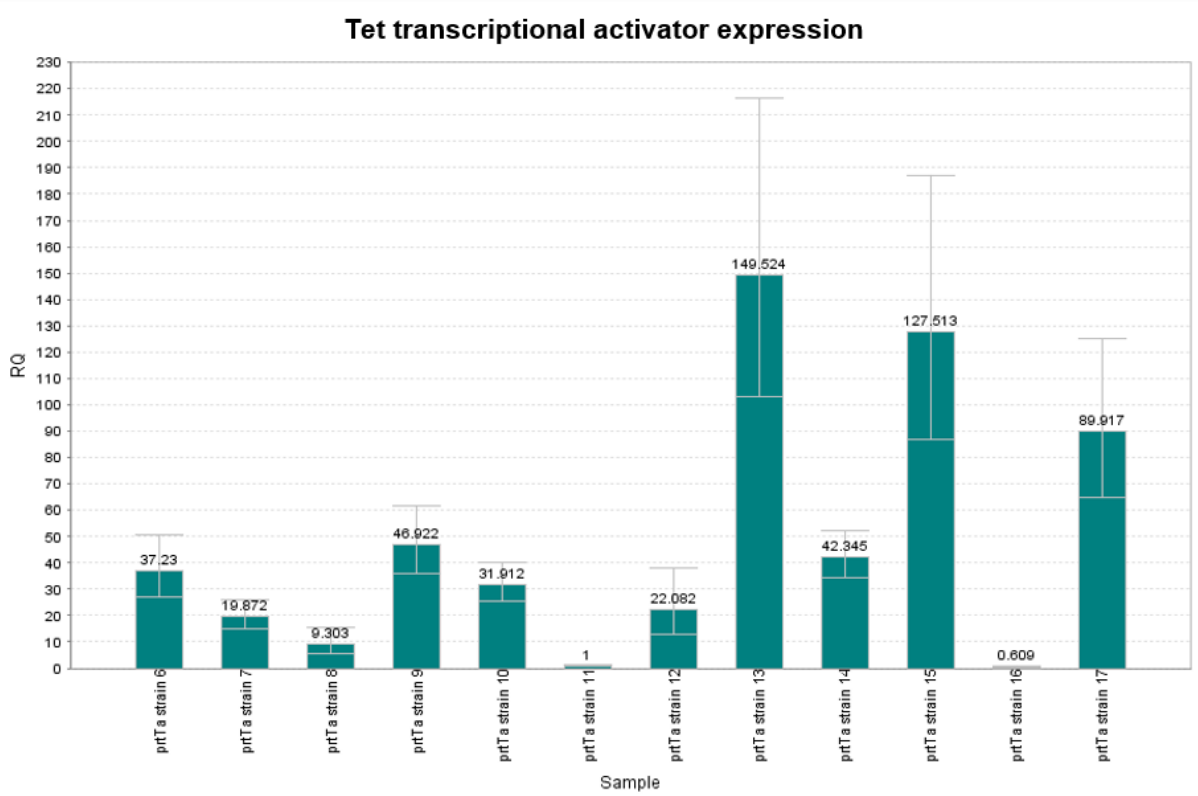

Figure 9. Bar graph displaying the relative expression of the Tet transcriptional activator of transformant strains 6 throughout 17. The x-axis shows the sample names and the y-axis shows the relative quantification (RQ) of the expression levels of the transcriptional activator of each sample relative to the expression levels of strain 11. Numbers above the bars indicate the mean relative expression level. The error bars show the 95% confidence interval.

Transformation using spores

The purpose of transformation using *S. commune* spores was to try to create an easier method of transformation with high efficiency. Multiple methods have been investigated using spores of *S. commune*. Spores have been used because they have two nuclei in contrast to mycelial cells or their derivative protoplasts that bear only one. Protoplasts of mycelium often do not contain a nucleus since more protoplasts arise from a single cell compartment in which only one nucleus is present. Thus, only one protoplast arising from the cell compartment contains this nucleus. As a result, the transformation efficiency is much lower. Therefore, it was expected to achieve a higher efficiency by electroporation of WT spores or by PEG-mediated transformation using protoplasted WT spores.

Preliminary work showed that electroporation using WT spores did not result in a high transformation efficiency. Swollen spores were used for electroporation with the idea that their cell walls were more permeable. As electroporation with simply swollen spores in a concentration of 1.6E8 spores ml^{-1} resulted in 1 colony out of 20 million used spores (0,000005%), it was secondly tested how spore swelling could be increased the most. 1 M histidine, 1 M proline, 1 M cysteine or yeast extract was added to liquid MM with a concentration 1.6E8 spores ml^{-1}. It was found that the addition of histidine resulted in most swelling (data not shown). However, when these spores were used for electroporation, only 25 colonies regenerated out of 100 million spores (0.000025%) that were used for two electroporation attempts. A survival assay of spores swollen with histidine showed that 17 out of 50.000 spores (0.034%) germinated after overnight incubation with histidine, and only 2 spores (0.004%) germinated after electroporation performed without the addition of a DNA construct and selection. Thus, it was concluded that the combination of both histidine and electroporation put too much strain on the spores and could not be used for electroporation. Hereafter, spores were swollen in liquid MM in a

concentration of 1.6E8 ml^{-1} and treated with a LE solution for 1 hour before electroporation. This resulted in a transformation efficiency of 0.000046% as 23 transformants were found. This was the highest efficiency so far. However, when repeated to test whether 1 or 2 hours of LE incubation before electroporation worked best, no colonies were found for both electroporation attempts. Perhaps this could be due to the age of the spores. However, this seems unlikely as fresh plates were made for spore harvesting three weeks prior to these electroporation attempts. Thereby, it was seen that the PEG-mediated transformation performed parallel to the electroporation had a higher transformation efficiency. Thus, after the last two electroporation attempts, the method was disregarded and PEG-mediated transformation was continued.

For PEG-mediated transformations, WT spores were swollen overnight in a concentration of 1.6E8 ml^{-1} and incubated in a LE solution for 3 hours to generate protoplasts. The first transformation was performed with a dTomato construct to firstly see if the method worked and secondly to generate a positive fluorescent control. This transformation resulted in a transformation efficiency of 0.00786%. Next, two transformations with two different constructs belonging to the Tet-on system brought about a transformation efficiency of 0.00528% and 0.00192%. Two other transformations performed with the same *pde2* overexpression construct resulted in much lower transformation efficiencies, namely 0.00002% and 0.0002%. It has to be noted that the latter two transformations were performed with protoplasts from another batch than the first three transformations. The protoplasts from the second batch were stored in the wrong concentration. When a sample was subsequently taken to obtain the correct number of protoplasts for transformation, CaCl was not added to the protoplasts for the penultimate transformation. It was seen that in the last transformation when CaCl was added, the number of colonies had increased tenfold. However, the number of transformants still does not come close to the numbers of the previous transformations. Protoplast quality did not differ between batches as protoplasts from both batches regenerated on MM agar plates without selection (data not shown). However, it was observed that an infection was present in the protoplast mixture of the second batch. Since the same kind of infection was observed in the last two transformations that were performed independently of one another, it was concluded that the infection originated from the protoplast mixture. No other type of colonies, including *S. commune* transformants, could grow on the plates where the infection grew (data not shown). This infection could have had an effect on the regeneration of *S. commune*. Furthermore, it was observed that there was less regeneration after overnight incubation. This could possibly be explained by the infection being present. To see if the reduced transformation efficiency was dependent on this, a new batch of protoplasts should be made to perform the transformations again to see if higher transformation efficiencies can be reached.

From these results it is clear that electroporation results in low transformations efficiency. PEG-mediated transformation results in high transformation efficiency if protoplast quality is good and no infections are present in the protoplast mixture.

Discussion and Conclusion

With the increase in the global demand for edible fungi (Grimm & Wösten, 2018; Kalač, 2009; Royse et al., 2017), there is a need to understand the mechanisms behind the initiation of fruiting. While it is known how light is perceived (Ohm et al., 2013; Pelkmans et al., 2017; Perkins, 1969; Perkins & Gordon, 1969), the mechanism through which CO_2 is sensed remains to be elucidated. It is known from early studies that CO_2 has an inhibitory effect on fruiting body development in *S. commune* (Niederpruem, 1963; Niederpruem & Wessels, 1969). Moreover, elevated CO_2 levels can arrest fruiting body formation at every stage of development (Pelkmans, 2016). Here, a preliminary attempt was made in order clarify the CO_2 sensing pathway in *S. commune*. CO_2 sensing is mostly studied in the context of pathogenic fungi (D'Souza et al., 2001; Hall et al., 2010; Hicks et al., 2004; Klengel et al., 2005; Mogensen et al., 2006). CO_2 would be sensed through cAMP as bicarbonate made by carbonic anhydrase from CO_2 would stimulate adenylyl cyclase, which would in turn produce cAMP. cAMP would in turn activate PKA, which also activates Pde. Pde can affect the cAMP levels as it breaks it down (Hu et al., 2010; Ma et al., 1999; Park et al., 2005). It was found that *S. commune* has analogous for all genes involved in the cAMP-PKA pathway (Pelkmans, 2016). Moreover, some genes involved in the CO_2 sensing pathway have been identified in *S. commune*, such as PKA catalytic subunits genes *ScPKAC1* and *ScPKAC2* (Yamagishi et al., 2014). While overexpression does not lead to increased cAMP levels, it does result in suppression of aerial hyphae formation similar to *ScGP-A*, an AC subunit found in *S. commune*. *ScGP-A* overexpression does increase cAMP levels, indicating *ScPKAC1* and *ScPKAC2* act downstream of *ScGP-A* (Yamagishi et al., 2002, 2004, 2014). Thus, CO_2 is likely sensed through cAMP. Therefore, a CO_2 sensing pathway through cAMP signaling was proposed (See Fig. 1). In line with the hypothesis, it was observed that WT dikaryon *S. commune* was unable to form mature fruiting bodies in the presence of 2.5 mM cAMP or 2.5 mM IBMX at low atmospheric CO_2. This is in accordance with several other studies (Knabe et al., 2013; Yamagishi et al., 2002, 2004), but contradicts findings by Schwalb (1974). Differences in results found by Schwalb (1974), can be explained by the used concentration of cAMP. Here, the amount was higher, 2.5 mM cAMP instead of 1 mM, thus explaining the greater inhibition. Inhibition by IBMX was stronger than inhibition by cAMP as some primordia formed on the plates with cAMP. The reason that primordia were able to form on various places can be explained by local metabolization of cAMP (Uno & Ishikawa, 1973a). While cAMP is metabolized by Pde, IBMX inhibits Pde which could result in higher intracellular cAMP levels, thus inhibiting fruiting more. Hence, primordia were not found when IBMX was present. As cAMP is probably broken down by Pde at a fixed rate, addition of more cAMP could result in more inhibition of fruiting as intracellular cAMP levels further increase. The effect of inhibition was strongest when both IBMX and cAMP were added to the agar, likely due to an even higher increase in cAMP levels. While it is assumed that intracellular cAMP levels increase, it has to be noted that empirical evidence for cAMP concentrations lacks from this study. When combined, IBMX and cAMP reduced colony growth during vegetative growth (See Fig. 4, fourth and fifth column). In addition, it was found by Remmers (2022) that exogenous addition of 2 mM cAMP to the agar medium resulted in lower mycelial density compared to the control. Higher concentrations of cAMP resulted in different morphology and higher concentrations of HCO_3^-, which activate AC to produce cAMP, were seen to reduce growth (Remmers, 2022). While it is not yet known how raised cAMP levels in fungi cause growth inhibition, it is known that there is a link between HCO_3^- and cAMP, which inhibit growth and biofilm formation in bacteria (Dobay et al., 2018).

Furthermore, with the use of ring plates, it is concluded that cAMP and IBMX are not transported by the hyphae upon exogenous addition. Fructification of WT *S. commune* dikaryon could still occur on the outer two rings without IBMX or cAMP when vegetative growth occurred in the presence of IBMX or cAMP. Vice versa, fructification is pushed back when IBMX or cAMP is present in the outer two rings. Thus, transport does not occur as fructification does not fail. It could be that both substances are metabolized locally. It is known that Pde2 metabolizes cAMP (Uno & Ishikawa, 1973a). It would be interesting to see if the activity of Pde2 actually increases in the rings where cAMP is present through gene expression to verify this hypothesis. Another explanation could be that the substances do not enter the cell. cAMP is a negatively charged molecule which is actually not able to pass the cell membrane. Yet, in this study and previous studies (Braat and Lugones, 2020; Pelkmans, 2016;

Remmers, 2022; Schwalb, 1974), it was found that cAMP inhibits fruiting. Moreover, one study found that WT *S. commune* could mimic the phenotype of a mutant strain where cAMP levels raised intracellular by exogenous addition (Schwalb, 1978). The mechanism through which cAMP is able to enter the cell remains unidentified. Although it has not been investigated why transport of IBMX and cAMP does not occur, it can be theorized why transport does not occur. In natural conditions, *S. commune* grows inside a substrate under vegetative growth conditions. Aerial hyphae growing outside the substrate under generative growth conditions have the ability to incite fruiting. The cAMP concentration in the aerial hyphae must therefore be low. However, the cAMP concentration of the hyphae still growing in the substrate is high. If cAMP was transported through the hyphae, the concentration would be equally high in all hyphae and thus the aerial hyphae would not be able to fructify. In this regard, it makes sense that cAMP is not transported.

It was observed that fructification can be reduced as it was pushed back to the center of the colony by addition of IBMX and/or cAMP to the outer two rings of a ring plate (See Fig. 2-4, second column). This was not observed in later experiments with solely IBMX or cAMP but was restored when IBMX and cAMP were added together (See Fig. 6-8, second column). Two explanations can be provided for these varying results. Firstly, different boxes were used for the incubation time under generative conditions for the plates containing solely IBMX or cAMP, which may have led to different environmental conditions per type of box. Secondly, temperatures within the 25°C incubator increased to 29°C during the experiment. Repeating the experiment with solely IBMX or cAMP may point out which factor caused differences in fruiting patterns between the experiments. Fructification could not occur in the center of the colony itself (See Fig. 2-4, third column). One explanation could be that colonies have to be of certain size before they become photosensitive and can incite fruiting (Leonard & Dick, 1968; Perkins, 1969). Yet, once was it observed that fruiting occurred in the center of the colony. However, due to a bacterial infection that came with the inoculum, these results were disregarded. The bacterial infection may be the reason why fruiting could occur. As cAMP was present in the agar of the entire plate, fruiting was inhibited. Perhaps the bacteria were able to breakdown cAMP, enabling fruiting for *S. commune* in the center of the colony.

A first attempt was made in order to determine whether light as a signal is transported in *S. commune* as opposed to IBMX and cAMP. The question of whether fruiting bodies would develop at the edge of the colony despite where the light signal is sensed, was investigated. While it appeared as if a signal provoked by light perception was transported and fructification occurred on the outer rings (data not shown), the used experimental set-up was not optimal for investigating this query. Firstly, a better alternative to aluminum covered lids is to use an opaque graffiti spray that can be sprayed on top of the colony. Certain rings could not be sprayed to get more accurate results to assess light as a factor in fructification. In addition, dark ring plates should be used that do not transmit light or refract it to other parts of the plate. When plates are sprayed with the graffiti, a box containing tissue paper wetted with 4% KOH can also be used once more. This will keep the atmospheric CO_2 concentration low as the CO_2 concentration could have accumulated in lidded plates used previously. In this way, the aforementioned limitations have been overcome. Investigating this query would be interesting as it was seen that cAMP levels increase in *S. commune* upon exposure to light and gradually increase until mature fruiting bodies have formed (Kinoshita et al., 2002; Yli-Mattila, 1987). Based on these findings, Remmers (2022) proposed a model for cAMP fluctuations during fruiting body development. It was found by Braat and Lugones (2020) that *pde2* overexpression strains were able to fructify in the dark. Thus, the light signal was no longer needed. In addition, Westhoff and Lugones (2014) observed that CA activity was reduced during light exposition. Thus, light also has an effect on the cAMP levels. By repeating the experiment attempted in this study, the interplay between the influence of light and CO_2 on cAMP levels could be elucidated.

During the course of this study, *pde2* transformants were gained with constitutive overexpression and conditional overexpression (Tet-on system). Only the constitutive *pde2* overexpression strains were tested for their ability to develop fruiting bodies compared to the WT control. Strains 10x1 and 7x1 were exposed to elevated CO_2 concentrations (3%), but did not fructify. Therefore, it can be concluded that strain 7x1 and strain 10x1 are not proper overexpressors of the pTub-Pde2* construct. Other studies which generated *pde2* overexpression strains did observe such finding (Braat and Lugones, 2020; Pelkmans, 2016). However, it has to be noted that different constructs were transformed. Two explanations can be proposed for the findings in this study. Firstly, it could be that

proper *pde2* overexpression strains were not correctly selected based on phenotyping. Square plates were point inoculated with either nine colonies or four colonies of different *pde2* transformant dikaryons based on their determined mating type. Control plates were point inoculated with four dikaryotic colonies including all mating types. This unproper control for plates with nine colonies could have caused biased phenotyping for multiple reasons. The plates with nine colonies were closer to the sides of the plate, which allowed for more aeration than the control colonies leading to more fructification. In addition, the plates with nine transformant dikaryon colonies resulted in more respiration, causing more CO_2 to build up inside the middle of the plate. Thus, the dikaryon transformant colonies which grew in the middle of the square plates were likely more inhibited in fruiting than other colonies. This problem could have been solved by choosing a proper control or simply by using small individual plates to create more consistent conditions for fruiting for each strain. A more conclusive method to identify proper *pde2* overexpression strains is to perform a qPCR with the generated transformants in order to determine which transformant has a high expression of the pTub-Pde2* construct. No qPCR was performed during this study due to time constraints. If strains with high *pde2* gene expression are identified, they can be exposed to high CO_2 conditions to investigate the extent of *pde2* overexpression. In addition, repeating the experiment with 2.5 mM IBMX and/or 2.5 mM cAMP with a true *pde2* overexpression strain at low CO_2 conditions will provide even more insight. A second explanation why the investigated *pde2* overexpression strains do not fructify under high CO_2 conditions could be the construct made by Remmers (2022) used in this study. The substitution of serine by aspartate in the PKA binding domain (RRXS to RRXD) of Pde2 may not function as desired. It was expected that aspartate with its negative charge would function as a phosphorylated serine, thus activating Pde2. However, it could be that phosphorylation of serine that can occur in WT Pde2 when cAMP levels are high is crucial for proper functioning of Pde2. Thus, by substitution with aspartate, the function of Pde2 may be destroyed or its sensor cannot function anymore. Performing a quantitative PCR could confirm this hypothesis.

To our knowledge the Tet-on system has not been introduced in *S. commune* before. The constructs made by Remmers (2022) were transformed separately and introduced into *S. commune*. Many transformants were gained using PEG-mediated transformation of protoplasted spores. While it was expected that phenotyping by dTomato fluorescence would result in finding a strain with a highly active transcriptional activator, no colonies showed such fluorescence. Remmers (2022) described to use 50 µg ml^{-1} of doxycycline while 10 µg ml^{-1} is often used. However, neither concentration resulted in any fluorescence when tested in this study. Two explanations can be proposed for this. First, the transcriptional activator may not be functional in *S. commune*. Secondly, it could be that the transcriptional activator cannot bind to the promoter. Therefore, a qPCR was performed to check whether the transcriptional activator is functional in *S. commune*. Results of the qPCR have shown that there is difference in gene expression of the construct for the twelve strains tested (See Fig. 9). Strain 13 shows the highest relative expression of the transcriptional activator. A possible next step would be to cross strain 13 with potential partners containing the prTetO7-dTom construct. An alternative would be to transform strain 13 itself with the prTetO7-dTom construct. The functionality of the Tet-on system and fructification pattern of the strain could be assessed by using ring plates with subsequent the addition of 10 µg ml^{-1} doxycycline.

Several attempts were made to generate *pde2* deletion strains. No *pde2* deletion strains were generated despite changes in the transformation protocol. It was observed that all protoplasts regenerated when plated out without the selection. Since the protoplasts regenerate after the addition of PEG and overnight incubation, this shows that there is nothing wrong with the protoplasts quality and that PEG does not damage the protoplasts too much. Therefore, the question remains why no colonies are able to grow once nourseothricin is added. It could be that the construct may not function as desired. Perhaps the nourseothricin resistance cassette is not functional, making it impossible for regenerated protoplasts to survive the selection conditions. A possible next step would be to sequence the construct to assess its functionality. An alternative would be to generate *pde2* deletion strains using a different method, for example a proposed in Vonk et al. (2019), using pre-assembled Cas9-sgRNA ribonucleoprotein.

Lastly, a new transformation technique was set up during this study. This technique has proven both easy and efficient. Since spores are used in this technique, only a few lab materials in small quantities are needed. The choice to use spores is based on achieving higher transformation efficiencies. Spores all bear one to two nuclei. When spores are protoplasted, they will all bear one or two nuclei. This is in contrast to mycelial protoplasts, where only one of the protoplasts generated from the same

cell compartment will have a nucleus. However, using protoplasted spores poses two problems when using them for transformation. The first problem is the formation of dikaryons and common A's. This issue was addressed by embedding regenerated protoplasts in LMP agar with low glucose (0.05%) and the selection. In addition, a liquid overlay was added with the selection. In this way, false positives are not able to grow through aerial hyphae and the concentration of the selection could be adjusted if necessary. Together, this makes for a strong selection, which allows small colonies to grow making it possible to select monokaryons. For two PEG-mediated transformations, it has been calculated that 36.67% and 56.67% of the transformants were monokaryons from all four sexes of *S. commune*. This percentage could be increased when regenerated protoplasts are plated out over more plates. Three plates were used in the present study. The second problem is recombination and chromosome segregation which may occur during sporulation. As a result, the spores would all be genetically different. This issue was addressed through the use of co-isogenic strains such as H4-8A (MATA43MATB41; FGSC 9210) and H4-8B (MATA41MATB43). These strains have been backcrossed so that the spores are genetically identical. Thereby, the obstacles of using spores have been overcome. By applying this technique to transformation via electroporation and PEG-mediated transformation, high transformation efficiencies have been achieved. Electroporation was firstly investigated as an easier method for transformation using spores. Several substances were tested for their ability to swell *S. commune* spores with the aim to make their cell walls more permeable. While histidine was found to swell spores the most, the combination of histidine and electroporation put too much strain on the spores. LE-mediated electroporation resulted in lower transformation efficiencies than PEG-mediated transformation using protoplasted spores. Therefore, PEG-mediated transformation was continued. High transformations efficiencies were reached with the first three transformation using WT protoplasted spores. The last two transformations with the WT protoplasted spores resulted in lower efficiencies. This was explained by an infection which was present in the protoplast mixture. It seems that the infection repressed the ability of *S. commune* protoplasts to regenerate as no *S. commune* colonies grew in the presence of infection. PEG-mediated transformation using ΔKU80 protoplasted spores did not generate any deletion transformants. However, it is yet to be determined whether this was due to the functionality of the construct used in this study or the transformation technique itself. While PEG-mediated transformation seems a proper method to gain transformants, it has to be noted that this method was not compared to PEG-mediated transformation using protoplasted mycelial cells in this study. Altogether, PEG-mediated transformation combined with using protoplasted spores and a strong selection offers a new and efficient method of transformation of which the full potential has not yet been unlocked.

To conclude, it seems likely that CO_2 is sensed via cAMP through a conserved pathway. The inhibiting effect of Pde inhibitor IBMX and cAMP on fructification is once again established in this study. IBMX and cAMP are not transported. Further research into whether light is transported as a signal may provide more insight into the interplay between CO_2 sensing and light sensing as both rely on cAMP signaling. Further research is needed concerning the Tet-on transformant strains generated in this study to investigate the functionality of the Tet-on system in *S. commune*. Also, further screening is necessary for the constitutive *pde2* overexpression strains generated in this study. When assessed, both the conditional and the constitutive overexpression strains can be compared to the wild-type using the ring plate assay. The transformation and selection technique set up over the course of this study and used to generate the aforementioned transformants, has proven itself easy and efficient as high transformations efficiencies were generated when WT spore protoplasts were used. It remains to be determined whether ΔKU80 protoplasted spores can be transformed using this method. Together, the results of this study provide a little more insight into how CO_2 is sensed by *S. commune* and how the signal is relayed. When the pathway is understood and manipulable in *S. commune*, it can be applied to other commercially cultivated fungi to meet the growing demand for edible mushrooms.

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