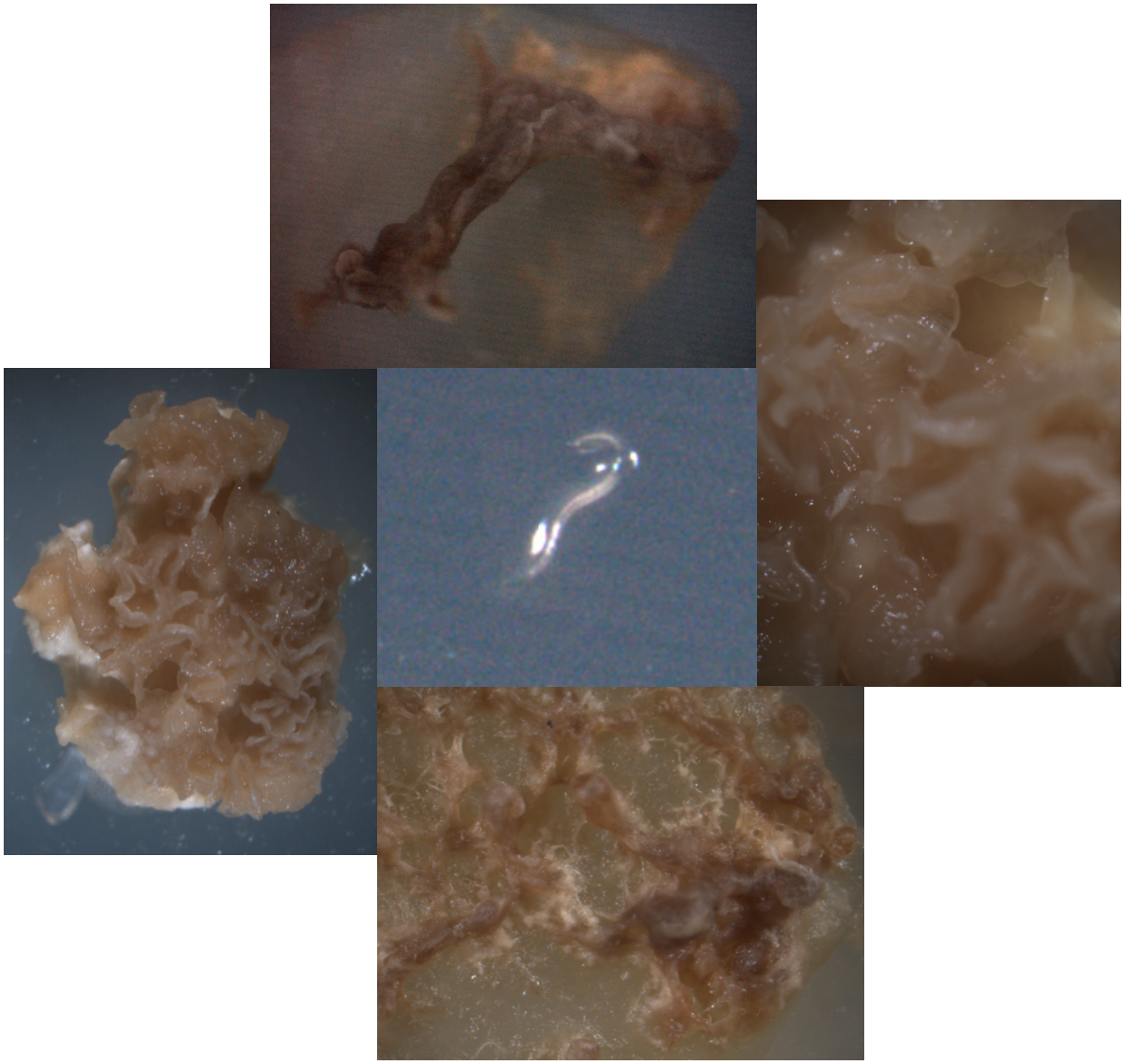
The function of *Schizophyllum commune* agglutinin 1 in the defense against fungivorous nematodes *Ditylenchus myceliophagus*



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ABSTRACT

Recent studies suggest that many mushroom lectins are up-regulated during fruiting body formation. This led to the hypothesis that these lectins could be involved by linking hyphae to each other or as effector molecules in the defense of fungi against predators and parasites. Previous research shows that Agglutinin 1 (*Agl1*) mRNA steady state levels increase to very high values in fruiting bodies of *Schizophyllum commune.* The expression of *Agl1* could be induced by fungivorous organisms as nematodes, specifically *Ditylenchus myceliophagus*. The main issue of this report is to determine if *Agl1* has a function in the fungal defense system of *S.commune*. A gene disruption is done to produce Agglutinin 1 knock-outs (*ΔAgl1)*. *D. myceliophagus* is used to test the fungal defence system of wild type *S.commune* fruiting bodies(4-8Ax4-8B*ΔKu80)* and knock-out fruiting bodies. Three knock-outs (*ΔAgl1)* were made. No morphologic differences were seen between wild type and the *Δ Agl1.* In the fungal defense system of *S.commune*, a significant difference between the wildtype fruiting bodies and the knock-outs is detected.

INTRODUCTION

Many mushroom-forming fungi cannot be cultured in the lab nor genetically modified, an exception is the split gill mushroom *Schizophyllum commune*. This basidiomycete is used as model system to study the mechanisms behind mushroom development (Wösten & Wessels 2006).
The life cycle of *S. commune* consist of approximately 10 days. Monokaryons and dikaryons form aerial hyphae after vegetative substrate mycelium has been formed. In stage I of the dikaryon, these aerial hyphae develop into aggregates. The aggregates develop further into primordia (stage II) and subsequently into mature fruiting bodies. (Ohm, De Jong & Lugones 2010)
The mushrooms of *Schizophyllum commune* are used as traditional food source in Africa and Asia. Apart from being a food source, fruiting bodies produce anti-tumor and immuno-stimulatory molecules (Liu & Kües 2000, Kothe 2001) and enzymes that can be used for bioconversions (Lomascolo et al. 1999).
Several types of lectins have been identified in mushrooms (Wang 1998). Lectins are defined as non-immunoglobin, carbohydratebinding proteins without catalytic activity towards the recognized carbohydrate (Peumans & Van Damme 1995). Many mushroom lectins are up-regulated during fruiting body formation, which led to the hypothesis that they are involved in this process, for example by linking hyphae to each other (Boulianne et al. 2000). However, recent studies suggest that fruiting body lectins play a role as effector molecules in the defense of fungi against predators and parasites (Wohlschlager et al. 2011). Moreover, it was shown that the β-galactoside binding lectin CGl2 is toxic for the nematode *Caenorhabditis elegans* (Butschi et al. 2010).
Fruiting body lectins are characterized by their spatial and temporal expression pattern and their subcellular localization in the fruiting bodies. Fruiting body lectins are synthesized on free ribosomes in the cytoplasm (Kuenzler et al. 2010).
The *Schizophyllum commune* agglutinin 1 gene (*Agl1*) contains a N-terminal, ricin B-type domain. Ricin is a legume lectin from seeds of the castor bean plant, *Ricinus communis*. The presence of a similar domain in *Schizophyllum commune*, the ricin B lectin domain, could bind simple sugars, such as galactose or lactose. A chimeric lectin homologous to *Agl1* is present in the fairy ring mushroom *Marasmius oreades* (Appendix C). *Marasmius oreades* agglutinin (MOA) is a Galα1,3Gal/GalNAc-specific lectin that also contains a ricin B-type lectin domain (Wohlschlager et al. 2011).
It is suggested by previous data that the expression of fruiting body lectins can be induced by fungivory (Kuenzler et al. 2010). An example of a fungivorous organisms are nematodes, specifically *Ditylenchus myceliophagus* (Goodey 1958). This parasitic nematode severely damages mushroom mycelium (Goodey 1960).

It is proposed that predators such as fungivorous nematodes ingest the cytoplasmic lectin when feeding on the content of the fungal cell and that binding of the lectin to specific glycans in the intestine of the worm induces toxicity by an unknown mechanism (Wohlschlager et al. 2011). In the case of MOA (*Marasmius oreades* agglutinin), this toxicity depends on binding of MOA to glycosphingolipids of the worm via its lectin domain. Furthermore, MOA has cysteine protease activity and this has a catalytic function in MOA nematotoxicity (Wohlschlager et al. 2011).
However, less research has been done in the case of *Schizophyllum commune* lectins. Recent studies suggest that Agglutinin 1 (*Agl1*) mRNA steady state levels increase to very high values in fruiting bodies of *S.commune* (figure 1)(Appendix D). Agglutinin 1 is even the most abundant mRNA in fruiting bodies according the MPSS expression analyse 1 (Ohm, De Jong & Lugones 2010). When placing the promoter of agglutinin in front of the red fluorescent protein dTomato, it resulted in very strong fluorescence in mushrooms (data not shown). Therefore, it is thought that *Agl1* could have a function in defense of fruiting bodies, like MOA. Furthermore, *Agl1* could also be involved in ensuring cohesion between hyphae (Khan & Khan 2011), thus have a function in hyphal aggregation. It is hypothesized that *Agl1* is an effector of a fungal defense system against predators and parasites.

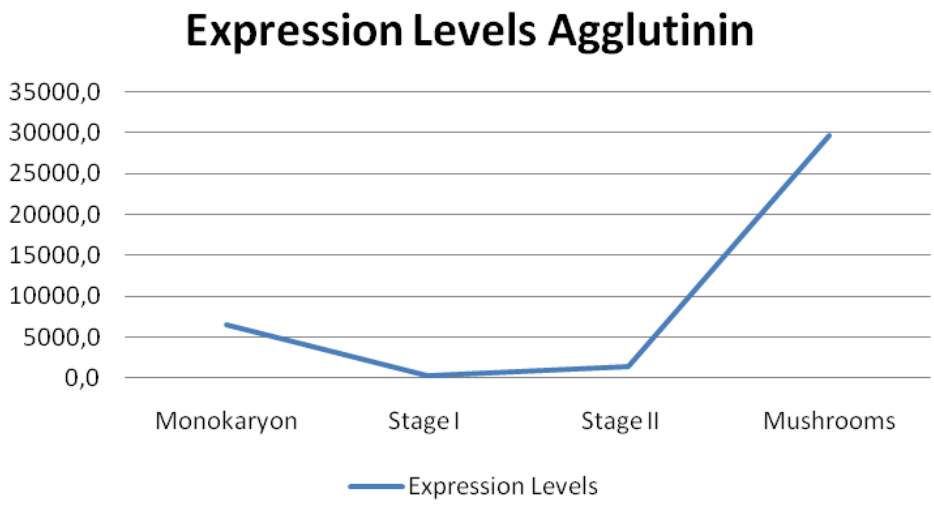


Figure 1. Expression levels in four different stages of growth of Agglutinin (protein ID 109852). Expression was determined by MPSS in tags per million (tpm) (Ohm, De Jong & Lugones 2010).

To determine if *Agl1* has a function in the fungal defense system of *S. commune*, gene disruption was done. An efficient gene deletion procedure for *S. commune* was produced by Ohm (2010). Moreover, by deletion of the ku80 gene, increase of the relative number of transformants with a homologous integration of a knock-out construct was reached. Therefore, 4-8Ax4-8B*ΔKu80* was used (De Jong et al. 2010).
After knock-outs were produced, wild type 4-8Ax4-8B*ΔKu80* fruiting bodies and knock-out fruiting bodies were tested in the fungal defence system. This was done by the nematodes *Ditylenchus myceliophagus*.

MATERIAL AND METHODS

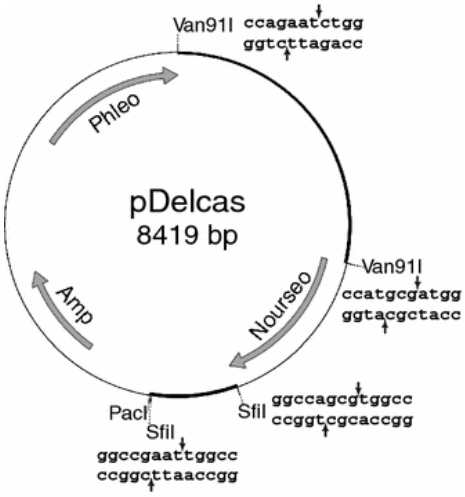
Strains and cultures

Strains used in this project were the *S. commune* monokaryotic strain 4-8A and 4-39, and the dikaryotic strain 4.8A x 4-8B. For creating the *Agl1* knock-out, 4-8Ax4-8B*ΔKu80* spores were used because of their defect on the heterologous recombination machinery (De Jong et al. 2010). Monokaryotic and dikaryotic strains were grown on solid minimal medium (MM) (Dons, de Vries & and Wessels 1979) at 25˚C. Monokaryons are grown for 3 days at 130 Lux. Dikaryons are grown for three days at 130 Lux, followed by one day at 175 Lux and three days at 130 Lux. Liquid shaken cultures were produced by macerating one fourth of a colony in 50 ml MM and mixing the macerate with 50 ml MM in an Erlenmeyer flask (250 rpm, 25˚C).

Deletion construct

The deletion construct for *Agl1* (Protein ID 109852), was based on pDelcas (figure 2)(Ohm et al. 2010). The up- and downflank of the targeted gene were ligated in plasmid pDelCas Aglut up- and downstream of a nourseothricin resistance cassette, respectively (figure 3).

The resistance cassette and flanks allows selection for a homologous crossover between the flanks of the gene and the flanks in the plasmid via screening for nourseothricin resistance. Standard transformations were performed as described previously (Ohm et al. 2010). However, adjustments are made. To generate protoplasts of 4-8Ax4-8B*ΔKu80* spores that are all the same size before the protoplast state, a Hydroxyurea (Hu) experiment is done. Liquid shaken cultures are produced by macerating one fourth of a colony in 50 ml MM and mixing the macerate with 100 ml MM in an Erlenmeyer flask (250 rpm, 25˚). After one day, the Erlenmeyer flask is taken and 6ml is transferred to a 12ml tube (Tube A). From tube A, 3ml is transferred to another 12ml tube (Tube B). 0,0152g Hu (70mM) is then added to Tube A, Tube B functions as a control without Hu. Tube A and B are then placed at 25˚C for two hours. After two hours Dapi is added to visualize the cells. Therefore, 300µl of each Tube is transferred to an Eppendorf tube (1.5ml) wrapped in aluminium foil, and 3µl Dapi (100µg/ml) is added. After 5 minutes incubation with Dapi, the Eppendorf tubes are washed three times with PBS. After washing, 200µl PBS is added. Then a sample of each (A and B) is viewed under the Microscope (Leica MZ16FA, PlanaPo 2.0x).



Flank	Primer	Sequence
Upstream	forward	GGCCNAATNGGCCN$_{18}$
	reverse	GGCCNCGCNGGCCN$_{18}$
Downstream	forward	GGCCNGCGNGGCCN$_{18}$
	reverse	GGCCNATTNGGCCN$_{18}$

Figure 2. Vector pDelcas (De Jong et *al.*, 2010) contains a phleomycin and a nourseothricin resistance cassette. Van91I and SfiI restriction sites flank fragments from bacteriophage λ of 2.4 and 6.4 kb, respectively (indicated in bold).

Transformation and selection

The deletion construct was linearlized with the restriction enzyme PacI before transformation. To ensure homologous recombination, strain 4-8A4-8B*ΔKu80* is used (De Jong et al. 2010). Thirty µl of pDelcas Aglut (350 ng/µl) is incubated with $7{,}5 \cdot 10^6$ protoplasts. Selection was done on minimal medium with 10µg/ml nourseothricin. Transformants were transferred to a second selection plate with nourseothricin in order to eliminate false positives.

PCR screening

From the transformants that grew on the second selection nourseothricin plate, a small piece of mycelium (2–25 mm2) was taken from the colony on the neurseothricin selection plate.

The fragment was placed in a 2 ml Eppendorf tube together with a new metal ball (Marabu, 3/1600 or approximately 5 mm in diameter). The mycelium was frozen in liquid nitrogen and homogenized for 1 min at 25 Hz using a Retsch Tissue Lyser II. One ml of CTAB-buffer (2% CTAB, 0.1 M Tris–HCl pH 8.0, 1.4 M NaCl) was added to the powered mycelium. The tubes were placed at 65 °C for one hour. After 5 minutes the metal ball was removed with a magnet. Cell debris was pelleted by centrifugation for 5 min at 14.680 rpm and 500 µl chloroform was added to the supernatant. After mixing, the samples were centrifuged for 5 min at 14.680 rpm. The water (upper) phase was transferred to a clean tube and DNA was precipitated by adding 0.7x the volume of the upper phase of isopropanol (for example 800 µl upper phase = 640 µl isopropanol). After centrifugation for 5 min at 14.680 rpm, the pellet was washed with 1 ml 70% ethanol for two hours. Then the tube was centrifuged for 5 min at 14.680 rpm, after which the ethanol was removed. The pellet was then dissolved in 50 µl TE buffer. For PCR, 1 µl of the DNA was mixed with primers, Taq polymerase, Taq buffer (as supplied by the manufacturer), 1.5% DMSO, 1.5 mM MgCl2 and 200 µM of each nucleotide(Ohm et al. 2010).

For screening, primers were used that only render a product in case of homologous integration of the flanking sequences of the deletion construct (figure 3). The forward primer pr1 (Agglutscfw1) anneals to the genomic DNA immediately outside the upstream flank. Its sequence depends on the gene that is inactivated. The reverse primer pr1' (GPDdscrv) anneals in the nourseothricin resistance cassette and is used for screening of all gene deletions. In the same way primers pr2 (sc3tersqf) anneals in the nourseothricin resistance cassette and pr2' (Agglut_outrev) are used to check the downstream flank. As a negative control, a PCR is performed with primers pr3 (Agglut_contfw) and pr2' (Agglut_outrev). Primer pr3 anneals in the deleted fragment of the gene (Agglut1). Generally, 35 PCR cycles were sufficient to amplify a specific band. In this case, 40 PCR cycles were used. Agar from the medium did not inhibit the PCR.

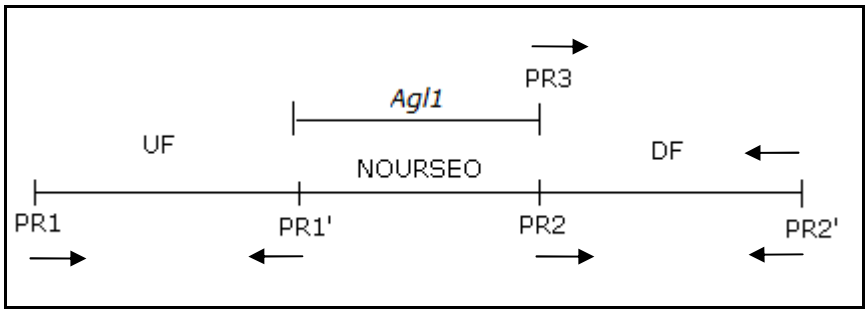


Figure 3. Deletion construct. PR1 = Agglutscfw1 ; PR1' = GPDdscrv ; PR2 = sc3tersqf PR2' = Agglut_outrev ; PR 3 = Agglut_contfw.

Analyses proteins

In order to measure the protease activity, fruiting bodies of 4-8Ax4-8B*ΔKu80* and the three putative *Agl1* knockouts 1.1/ 2.1/ 3.1 were collected into an Eppendorf tube with a metal ball. The tubes were directly placed in liquid nitrogen and then placed in the Tissuelyser (1min. 25 Hz). After the fruiting bodies were pulverized, caps with holes were placed on the tubes. Then, the tubes were placed in freeze drying equipment as described before (Simione & Brown 1991). After 24 hours of freeze drying, the dry samples were stored at room temperature. These samples were then used for the proteolytic assays.

SDS-PAGE and Coomassie blue staining

The proteolytic assay was done by SDS-PAGE and Coomassie blue staining. First, 150g of each sample is collected in an Eppendorf tube. The proteins are then mixed 4:1 with 5x Sample Buffer (10% (w/v) SDS, 10mM Beta-metacapto-ethanol, 20% (v/v) Glycerol, 0.2 M Tris-HCL pH 6.8, 0.05% (w/v) Bromophenolblue). The samples were then heated to 95 °C for 2-3 minutes.

Electrophoresis was done in 12.5% (w/v) polyacrylamide gels according to (Laemmli 1970). Gels were stained with Coomassie Blue (Neuhoff et al. 1988) R250 in 10% (v/v) acetic acid, 25% (v/v) methanol for 30 minutes, and de-stained for two times 15 minutes in the same solvent. After this staining, gels were further stained with Colloidal Coomassie Blue (0.08% CBB G250/ 1.6% ortho-phosphoric acid/ 8% ammonium sulfate/ 20% methanol) overnight. De-staining is done in demi-water for 2 times 12 hours.

Grazing by Nematodes

Nematodes (*Ditylenchus myceliophagus*) were obtained from a mushroom grower. *D. myceliophagus* is a nematode of 0.6-1.4 mm long. Embryonic development of *D. myceliophagus* is completed in 75 hrs or 279 hrs at 25°C or 13°C, respectively under laboratory conditions. It occurs in temperate regions as France and Britain; in mushroom-growing areas. *D. myceliophagus* is a pest in commercial mushroom production. It feeds on a wide range of fungal hyphae, swarms and aggregates on mushroom beds. *D. myceliophagus* becomes cryptobiotic if drying is slow and it remains viable for 3,5 years. Furthermore, it has a sticky surface that sticks to insect or human vectors. *D. myceliophagus* withdraws contents of fungal cells, mycelium disappears: mushroom beds become soggy and foul smelling[1].

To prevent the cryptobiotic stage of *D. myceliophagus* and mite-infections (House dust mites, *Dermatophagoides pteronyssinus)* every two weeks nematodes were transferred to a new plate with monokaryotic *S. commune* mycelium. The plates are taped, first with parafilm and then with yellow tape to ensure that *D. pteronyssinus* wouldn't contaminate the petridishes with the nematodes. A one week photo is made to follow the process of grazing of *D. myceliophagus* on monokaryotic mycelium (Appendix E.3).

For the experiments of grazing by nematodes on fruiting bodies of S.commune, water agar plates are used. Fruiting bodies of S. commune are placed on water agar plates, so the nematodes only would be able to eat fruiting bodies (vegetative mycelium does not contain the protein).

Restoring *Ku80* in *ΔAgl1* 1.1

Spores of S4 knockout, 2 and 3, are crossed with strain 4.8A and 4-8B to find the compatibilities.

S4-2 x 4.8A and S4-3 x 4.8B produced fruiting bodies. Spores of these crossings were first selected on nourseothricin presence. Selection was done on minimal medium with 10µg/ml nourseothricin, two days at 25°. In order to eliminate the false positives, transformants were plated on second nourseothricin (10µg/ml) plates. These nourseothricin-resistant spores are then placed on hygromycin (20µg/ml), two days at 25°. The spores where the *Ku80* is restored are the ones that are nourseothricin resistant and hygromycine sensitive.

[1] Ferris, H. NEMATODE-PLANT EXPERT INFORMATION SYSTEM. A Virtual Encyclopedia on Soil and Plant Nematodes - *Ditylenchus myceliophagus.* http://plpnemweb.ucdavis.edu/NEMAPLEX/index.htm Last viewed on 6.8.2011

RESULTS

Construction of the *Agl1* knock-outs

Using double homologous recombination, it was attempted to create *Agl1* knock-outs. To generate protoplasts of 4-8Ax4-8B*ΔKu80* spores that are all the same size before the protoplast state, a Hydroxyurea (Hu) experiment was done. Sample A (with Hu) exhibit more equal protoplasts compared to sample B (data not shown). In Transformation, after regeneration in sorbitol (2.5h), the protoplasts were in the pellet as well as in the supernatant. Therefore, it is decided to continue with SUPERNATANT-protoplasts and PELLET-protoplasts. Among the PELLET transformants screened for a knock-out, five *Agl1* knock-out candidates were found to exhibit strong resistance against nourseothricin. Furthermore, among the SUPERNATANT transformants screened for a knock-out, two *Agl1* knock-out candidates were found. All transformants became dikaryons, which is normal because we worked with spore protoplasts. The 7 knock-out candidates were screened via PCR (for method see Materials and methods section). Amplification with the primers for the downstream flank gave bands for the strains S4, P2, P5, P6 and P8 at 1400 bp (figure 4, left panel). However, when the amplification was done with the primer combination for the upstream flank (figure 4, right panel) it was found that only S4, P2 and P5 had the second insertion at 1700 bp.

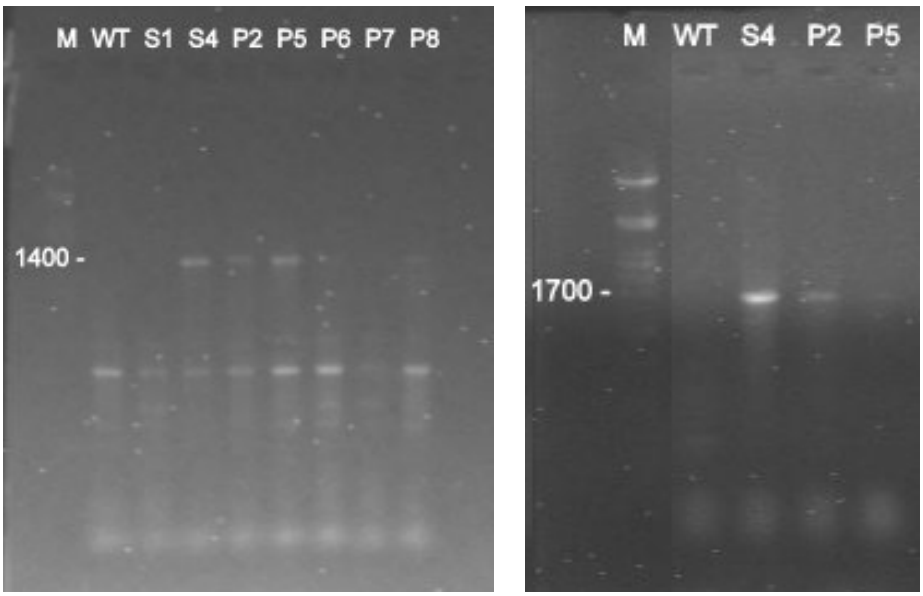


Figure 4. PCR analysis of the Agl1 deletion candidates. *Left panel, DOWNSTREAM: lane 1; λ-PstI ladder, lane 2; wild type gDNA*, lane 3; SUP1, lane *4; SUP4, lane 5; PELLET2, lane 6; PELLET5, lane 7; PELLET6, lane 8; PELLET7, lane 9; PELLET8. PCR was performed with primer pair 1 and 5 (Appendix A). In lane 4,5,6,7 and 9, a band at 1400 bp can be seen. In lane 1,2,3 and 8 this band is absent.*
Right panel, UPSTREAM: lane 1; λ-PstI ladder, lane 2; wild type gDNA, lane 3; S4, lane 4; P2 and lane 5; P5. PCR was performed with primer pair 2 and 3 (Appendix A). In lane 3-5 a band at 1700 bp can be seen. In lane 2 this band is absent.

Spores of the strains (S4, P2 and P5) were harvested and plated to obtain monokaryotic *Agl1* knock-out strains, which were selected on nourseothricin. P2 and P5 had a 100% grow on nourseothricin, S4 had a 50% grow. Amplification with the primers 2 and 3 for the upstream flank (figure 3) gave bands at 1700 bp (figure 5). As can be seen: S4-2, S4-3, S4-9, P2-4, P2-8, P2-9, P5-1, P5-7 and P5-8. So the nourseothricin resistance gene has replaced the *Agl1* gene in these cases. Furthermore, the monokaryotic strains of each deletion line that grew on nourseothricin were crossed against each other to see which ones produced a positive dikaryon (table 1).

Table 1. *Positive dikaryons produced by crossing between spores of monokaryotic strains (S4, P2 and P5)*

Monokaryotic strains	Positive dikaryons		
S4	S4-2 x S4-3		
P2	P2-4 x P2-9	P2-4 x P2-8	P2-3 x P2-8
P5	P5-7 x P5-8		

Remarkably, only the spores that formed dikaryons (table 1) turned out to contain the *Agl1* deletion (figure 5) at 1700 bp.

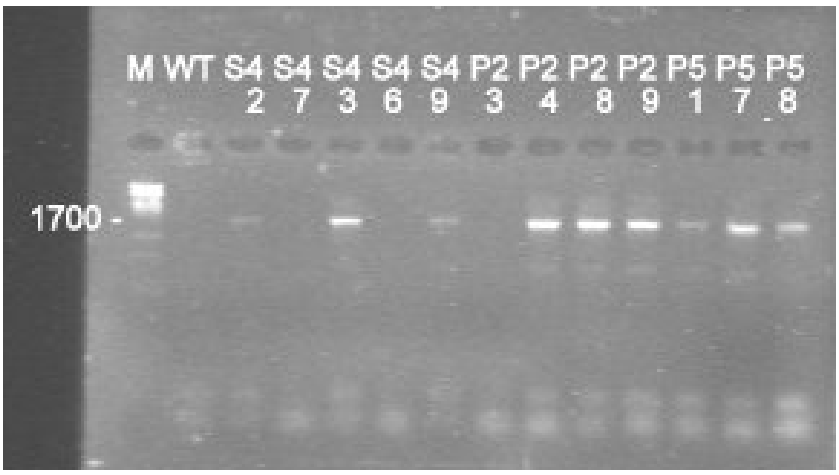


Figure 5. PCR analysis of the Agl1 deletion spores*. UPSTREAM: lane 1; λ-PstI ladder, lane 2; wild type gDNA*, lane 3; SUP4-2, lane *4; SUP4-7, lane 5; SUP4-3, lane 6; SUP4-6, lane 7; SUP4-9, lane 8; PELLET2-3, lane 9; PELLET2-4, lane 10; PELLET 2-8, lane 11; PELLET2-9, lane 12; PELLET5-1, lane 13; PELLET5-7, lane 14; PELLET5-8. PCR was performed with primer pair 2 and 3 (Appendix A). In lane 3,5,7,9-14 a band running at 1700 bp can be seen. In lane 2,4,6, and 8 this band is absent.*

Considering the compatibilities, one dikaryotic strain, homocygous for the *Agl1* deletion, could be synthesized for each of the deletion lines (S4, P2 and P5). Table 2 provides the designation of the positive dikaryons (S4, P2 and P5).

Table 2. *Number of positive dikaryons (S4, P2 and P5)*

Designation	Dikaryotic strains
1.1	S4-2 x S4-3
2.1	P2-4 x P2-9
3.1	P5-7 x P5-8

As a negative control, a PCR was performed with primers 4 and 5 (Appendix A) on one knock-out, *Δ Agl1* 1.1. Primer 4 (Agglut_contfw) anneals in the deleted fragment of the gene (Agglut1). Primer 5 (Agglut_outrev) anneals in the downflank and this should give a band in the wild type gDNA. However in the knock-out spores, this band must be absent. As can be seen in figure 6, a band at 1400 bp is visible at the wild type gDNA and in the case of the two knock-out spores this band is absent.

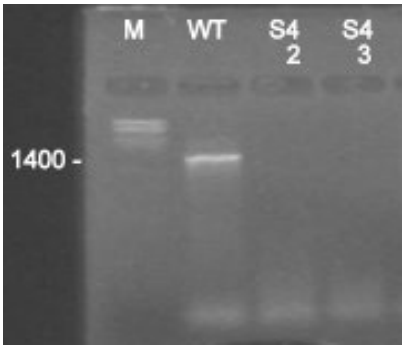


Figure 6. PCR analysis of the Δ Agl1 1.1 spores (negative control)*. DOWNSTREAM: lane 1; λ-PstI ladder, lane 2; wild type gDNA*, lane 3; SUP4-2, lane *4; SUP4-3. PCR was performed with primer pair 4 and 5 (Appendix A). In lane 2 a band running at the 1400 bp can be seen. In lane 3 and 4 this band is absent.*

Identification of the Agglutinin protein band with SDS-PAGE

Using SDS-PAGE it was attempted to analyze the presence of the *Agl1* gene of the wild type (4-8A4-8B*ΔKu80)* and the three putative knock-outs (1.1/ 2.1/ 3.1). As can be seen in figure 7, a band at 28 kDa is visible of the wild type, whereas this band is absent in the three knock-outs. This supports the evidence for the deletion of *Agl1*.

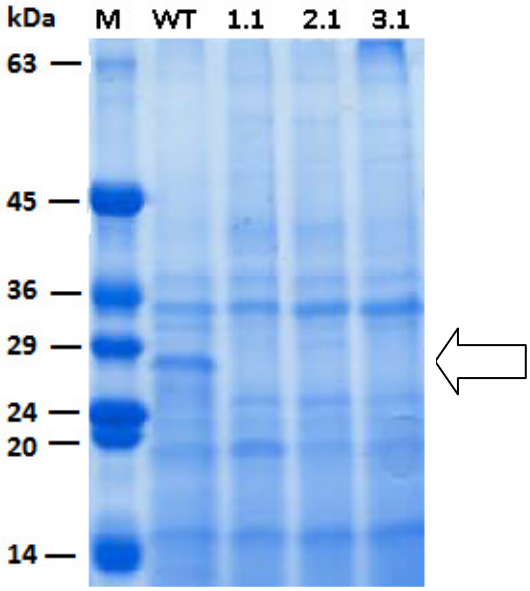


Figure 7. SDS-PAGE of wild type and three knock-outs (1.1/ 2.1/ 3.1). *The arrow indicates the place where the wild type band of 28 kDa is visible. Furthermore, the bands of the three knock-outs (1.1./2.1./3.1) are absent indicating a real Agl1 gene deletion in all three knock-outs. Marker = σM.*

Analysis of Nematodes

Using a four-week experiment, nematodes were observed on wild type (4-8A4-8B*ΔKu80*) fruiting bodies with *Agl1* and knock-out fruiting bodies (*Δ Agl1* 1.1/2.1/3.1). In this experiment, three spots of 10 mm2 were chosen randomly on each plate. On this spot, the nematodes that were alive and dead were counted by making a 30 sec. movie. These results (Appendix E.1) are incorporated into a graphic (Appendix E.2) (figure 8).

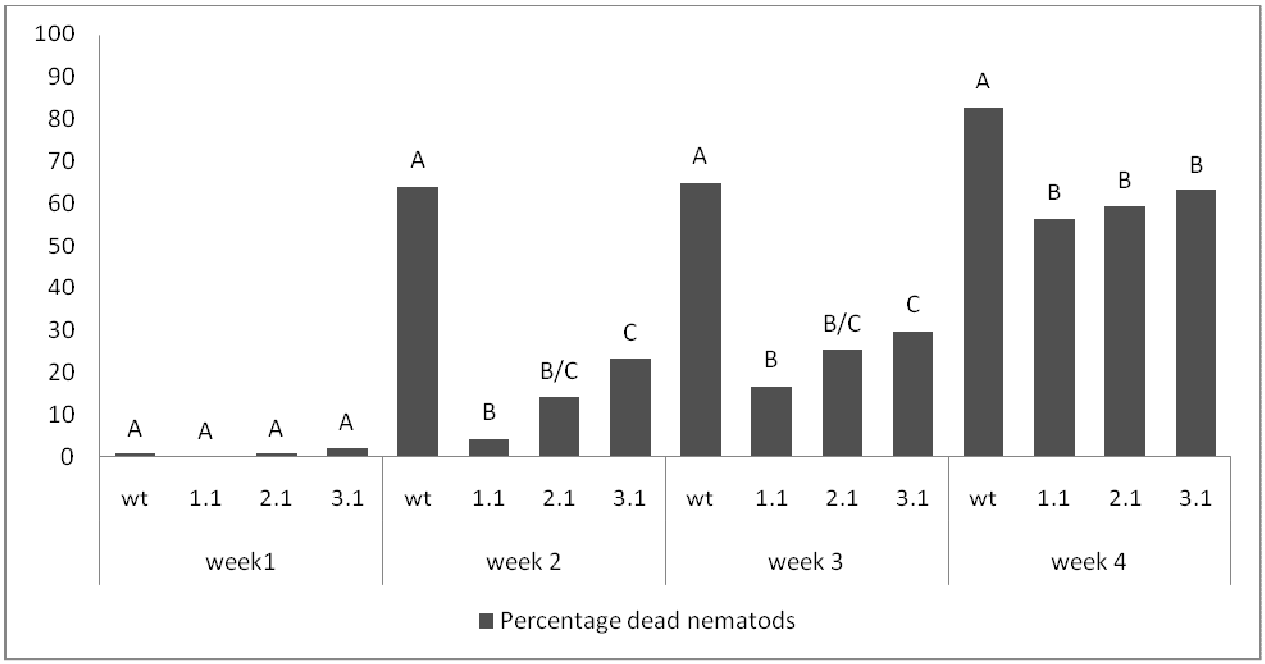


Figure 8. Mean percentage dead nematodes. *Nematodes (dead & alive) were counted on each spot and the percentage of dead nematodes compared to the nematodes that were alive is calculate. The average of all three spots on each plate per week is shown (%). The percentage dead nematodes on the wild type fruiting bodies is larger in week 2 – 4 than on the knock-out fruiting bodies. Letters show the averages which were significantly different.*

In week 2, there were much more dead nematodes on wild type plates than on the knock-out plates. The same pattern was visible in week 3 and 4. Using a T-test ($p<0.05$), these differences turned out to be significant (Appendix E.2). However, in week 1 the number of dead nematodes were not significantly different between the plates. The cause of the higher dead rate could be that the fruiting bodies (the only food source for the nematodes on each plate) were exhausted. At that point they could die because of food shortage.

Characterization of the Agl1knock-outs (*Δ Agl1)*

Monokaryotic and dikaryotic strains were grown on solid minimal medium (MM) (Dons et al., 1979) at 25 or 30˚C as can be seen in figure 9.

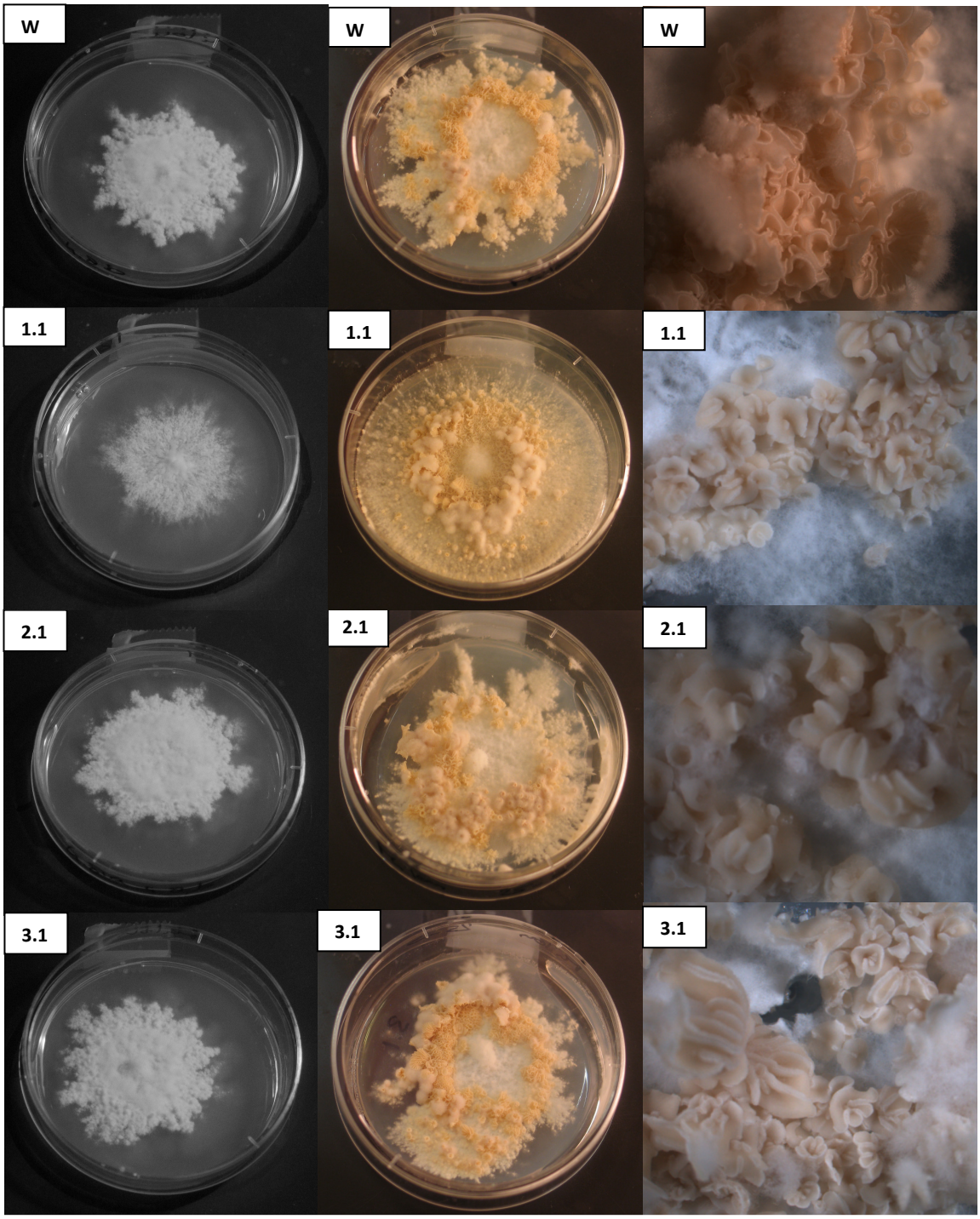


Figure 9. Characterization of the Agl1knock-outs (ΔAgl1). *Phenotype of the mutants as compared to the wild type at two different developmental stages: vegetative (first column) and generative (second column). A detail of the fruiting bodies is shown in the third column. WT = wild type; 1.1./2.1/3.1 = Δ Agl1*

No morphologic differences were seen between wild type and the *Δ Agl1*. However, differences in fructification were seen in one of the knock-outs. Wild type, 4-8Ax4-8B*ΔKu80*, and knock-out strains, *Δ Agl1* 1.1/2.1/3.1 were grown in triplo's and some differences in fruiting body development were observed. As can be seen (figure 9), the wild type dikaryons produce aggregations of fruiting bodies, like the *Agl1*knock-outs 2.1 and 3.1. However, *ΔAgl1* 1.1 produced smaller fruiting bodies and less spores (1:4 compared to *ΔAgl1* 2.1 and *ΔAgl1* 3.1).

Restoring *Ku80* in *ΔAgl1* 1.1

On the second nourseothricin selection plates (10µg/ml), eight of ten colonies grew at S4-2 x 4.8A and S4-3 x 4.8B. The (2x8) colonies that grew on nourseothricin were then placed on hygromycin (20µg/ml). Remarkably, after two days at 25° it turned out that all nourseothricin resistant colonies were hygromycin resistant. The experiment is performed in duplo, though the results were the same.

DISCUSSION

MPSS expression analysis I identified *Schizophyllum commune* agglutinin 1 as an interesting candidate for a role in mushroom development, because it was strongly up-regulated in fruiting bodies (figure 1). *S. commune* agglutinin 1 could also have a function in the defense of fruiting bodies. In previous data, (Kunzler et al. 2010) suggested that the expression of lectins can be induced by fungivores such as nematodes. In order to assess the toxicity of fruiting body lectins and other cytoplasmic proteins toward such organisms, they developed a biotoxicity assay towards the nematode *Caenorhabditis elegans*. This assay is based on the feeding of omnivorous and bacterivorous organisms with recombinant *Escherichia coli* cells expressing the heterologous protein in their cytoplasm. Lectin-mediated toxicity was assessed by scoring larval development. This method is done while *C. elegans* does not feed on fungal mycelium.
However, our research indicates that the fungivorous nematode *Ditylenchus myceliophagus* is a nematode that eats not only monokaryotic mycelium but also the fruiting bodies from *S.commune*. Therefore, no *Escherichia coli* is needed to assess the nematotoxicity of *Schizophyllum commune* agglutinin 1. The results of the four-week experiment indicates a significant difference (T-test; $p<0.05$) in week 2, 3 and 4 between the wild type (4-8A4-8B*ΔKu80*) and the three knock-outs (*ΔAgl1* 1.1/ 2.1/ 3.1). The high percentage of dead nematodes in week 4 can be explained, while 50% of all nematodes in control plates are dead after 3 weeks (data not shown).
A extension of this experiment could be when looking at the amount of spores produced by the strains with and without nematodes. In the end, it should translate in more spores for the wild type than for the mutants when there is infection, if this is a defense system of *S. commune* that concentrates on sparing the reproductive parts of the fungus.

It was tried to visualize the grazing of the nematodes. This was done by agg-dTomato. Monokaryotic mycelium of 4-40, mating type A43B43 was used and a dikaryon was made by crossing with 4.8 D (mating type A41B41). This wild type dikaryon gives red fruiting bodies. It was thought that nematodes eating the fruiting bodies, would turn red. However, the nematodes didn't turn red when the fruiting bodies were eaten. It is assumed that the nematodes can break down agg-dtomato.

House dust mites (*Dermatophagoides pteronyssinus*) were also used for the experiments in the defense of fruiting bodies (Appendix F). Mites were obtained by placing a monokaryotic strain 4-39 on MM for one week on the table in the lab. The mites that contaminated the plate after one week were normal house dust mites, *D. pteronyssinus.* However, because mites can live in very extreme environments it is difficult to see whether or not mites die from eating fruiting bodies with or without *Agl1*. Mites were reproducing fast in wild type and knock-outs, so a difference was difficult to detect. Also, there was a huge difference in eating the fruiting bodies between mites and nematodes. The fruiting bodies were fully eaten by the nematodes whereas the fruiting bodies of wild type and knock-outs formed exudates when there were mites around. This could be a stress responds of the fruiting body against the mites. In the end, the fruiting bodies with mites were less grazed than the fruiting bodies with nematodes (data not shown). In addition, a experiment with agg-dTomato wild type and knock-out fruiting bodies could be done. It is thought that *D. pteronyssinus* would not breakdown agg-dTomato. Mites that eating the fruiting bodies, would turn red.

Schizophyllum commune agglutinin 1 (*Agl1*) could have a function in hyphal aggregation. Previously, another homodimeric lactose-binding lectin is isolated from fresh fruiting bodies of *S. commune* (Han et al. 2005). The N-terminal sequence of this lectin was similar to a part of the sequence of the cell division protein from *Gleobactar violaceus*. Based on these results, it is likely that the splitting of the fruiting body is related to accelerated cell division. In fact, if the sequence of *Agl1* is entered on the website of JGI[2], a signal peptide is predicted meaning that it could be located on the cell surface.

[2] Schizophyllum commune JGI. http://genome.jgi-psf.org/Schco1/Schco1.home.html
Last viewed on 7.2.2011

However, if the sequence is entered on SignalP, *Agl1* is predicted to be a non-secretory protein and the signal probability is 0.0013 (Appendix B.2). According to Appendix C, the best hit is 18476512 agglutinin [Marasmius oreades] (model%: 96, hit%: 96, score: 475, %id: 29). *Marasmius oreades* agglutinin (MOA) lacks a classical secretion signal, like other fruiting body lectins, and is thus predicted to be localized in the cytoplasm (Wohlschlager et al. 2011). Besides that, SDS-PAGE from the extracellular and the intracellular fraction of wild type fruiting bodies showed that *Agl1* was almost exclusively in the late (data not shown). It is therefore unlikely that these lectins (MOA and *Agl1*) are localized in the extracellular space or secreted. Moreover, if *Agl1* is that essential in hyphal aggregation, it is expected that fruiting bodies would not be formed in knock-outs. When looking back to the characterization of the *Agl1*knock-outs (*Δ Agl1),* it seems that two of the three *ΔAgl1* produced normal fruiting bodies. Therefore, a function in hyphal aggregation would be improbable. Only *ΔAgl1* 1.1 produced smaller fruiting bodies and less fructification (1:4 compared with the other two *Δ Agl1*). The reason for this must lay in other events during gene disruption. For instance an extra fragment transforming DNA could have cut into a gene for fructification.

When restoring *Ku80* in *ΔAgl1* 1.1, no hygromycin sensitive colonies were found. The cause of this could be that the scaffolds are at the same chromosome. The *Agl1* is on scaffold 6 and the ku80 on scaffold 15. *Agl1* and ku80 could therefore be linked. Much more spores should be analyzed to eventually let segregate both loci.

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Figure on the front contains one picture in the middle of a nematode (*Ditylenchus myceliophagus*) and four pictures of fruiting bodies of *Schizophyllum commune* grazed by nematodes (*Ditylenchus myceliophagus*)

APPENDIX A: List of Primers

Nr.	Name	Sequence 5' → 3'
1	Agglutscfw1	ACGATGCAAACGAGTTC
2	GPDdscrv	TTGCCCTACTTGTGTAGC
3	Sc3tersqf	GCCTCAGGTCCCGAAGTAAG
4	Agglut_contfw	TTAACAAACAATC
5	Agglut_outrev	ATTACCCTAACAT

APPENDIX B: Genome sequence Agglutinin 1

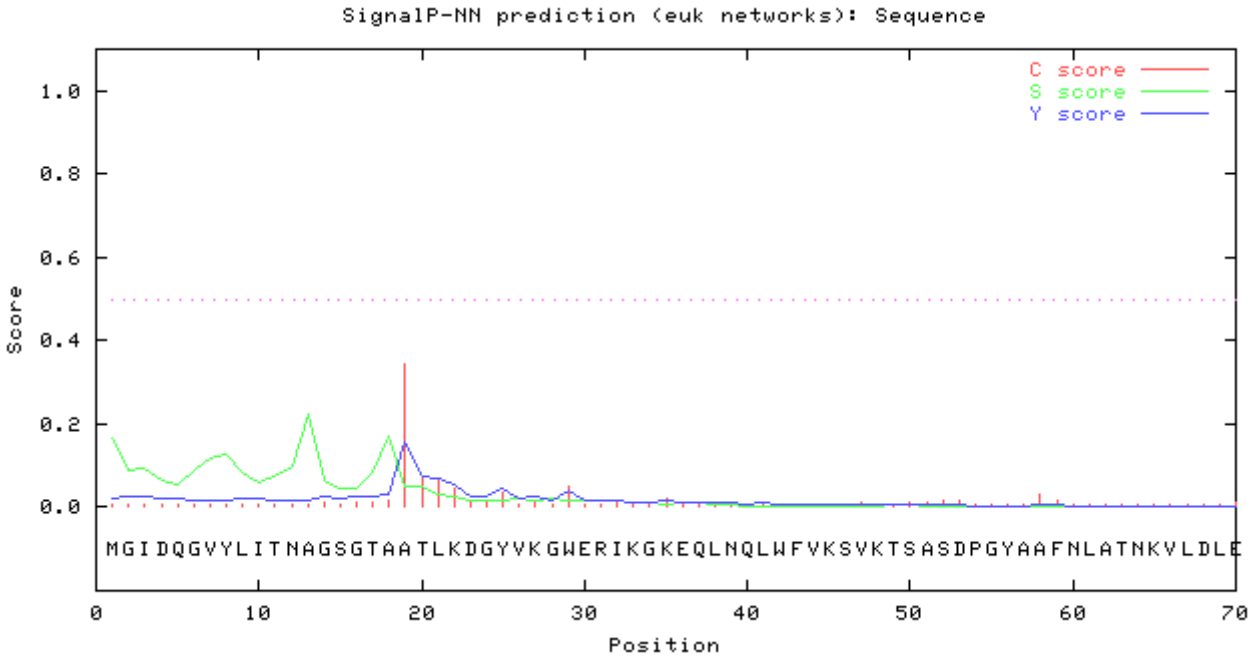
B.1 JGI Sequence

>jgi|Schco1|109852|augustus-scaffold_6.g394
MGIDQGVYLITNAGSGTAATLKDGYVKGWERIKGKEQLNQLWFVKSVKTSASDPGYAAFNLATNK
VLDLE
KGLADRGTPILGWDYHEGDNQHWVIKKQADGKYYKIQSVKTQTFVDLNNGGKDNGTKIQGWVGSW
DETNS
HQRWVFNQFSATGSRIKSILDTHPKIHGKVIVEWPDRIYFTPDQYIFEAIWAKTGLADIKPRDPL
FQSEA
YEKVFKGWVVSRAQEIIKVDGFDILVGSLSLVEKSTQKIKVLDVSVRTSDDKSPDLSQIVFFDPE
SGRTL
VDIPEGYEVNSVII

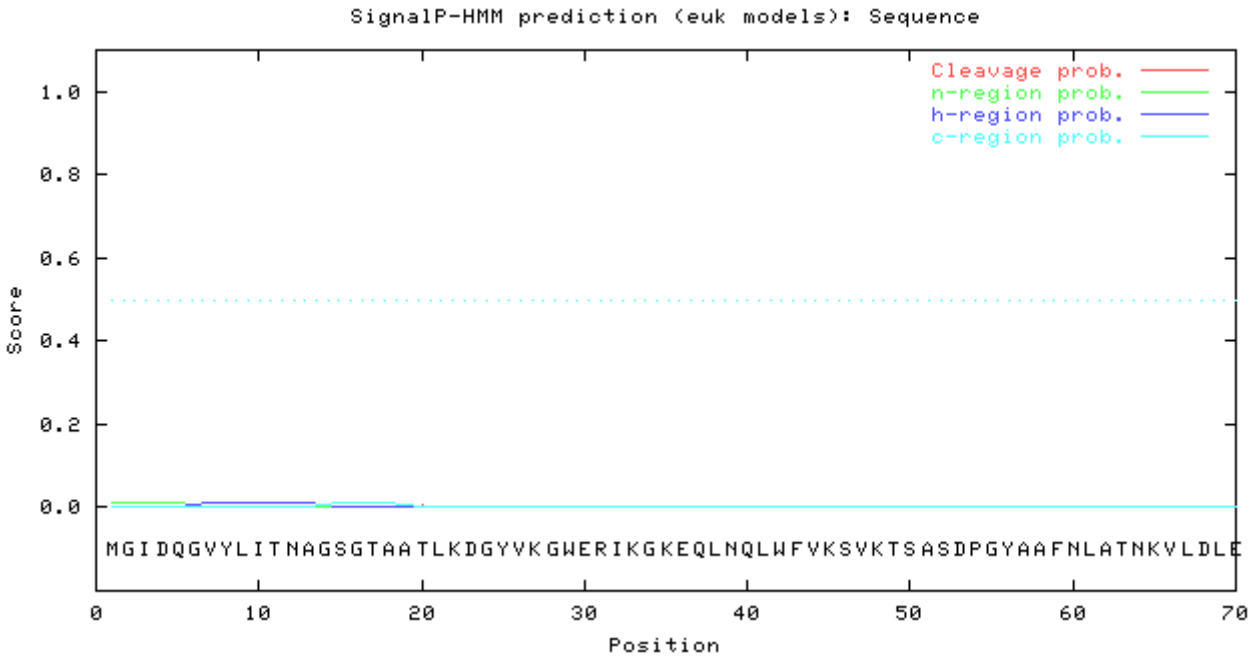
B.2 SignalP prediction

CENTERFORBIOLOGICALSEQUENCEANALYSIS CBS

SignalP 3.0 Server - prediction results
Technical University of Denmark



>Sequence length = 70
Measure Position Value Cutoff signal peptide?
max. C 19 0.344 0.32 YES
max. Y 19 0.158 0.33 NO
max. S 13 0.223 0.87 NO
mean S 1-18 0.098 0.48 NO
D 1-18 0.128 0.43 NO



>Sequence
Prediction: Non-secretory protein
Signal peptide probability: 0.013
Signal anchor probability: 0.000
Max cleavage site probability: 0.007 between pos. 18 and 19

APPENDIX C: Protein search

C.1 Schizophyllum commune v1.0 – Agglutinin 1

C.2 Alignment Marasmius oreades agglutinin

```
QUERY: 109852 (294)
Hit: nr_b_b_18476512 (293)
       defLine: gi|18476512|gb|AAL47680.1| agglutinin [Marasmius
oreades]

SCORE: 475  EVALUE: 1.09987e-32
```

```
Alignment Length: 319   IDENTITIES: 29.15% (93/319) SIMILARITIES:
42.95% (137/319)
GAPS: 0
QUERY START: 1  QUERY END: 283
HIT START: 1  HIT END: 284


    1
MGIDQGVYLITNAGSGTAATLKDG...(1)...VKGWERIKGKEQLNQLWFVKSVKTSA...(8)
...NL    61
      M + +G+Y I NAG  +A  LKDG         + GW+        +QLW  + +   A
NL
    1
MSLRRGIYHIENAGVPSAIDLKDG...(7)...IVGWQFTPDTINWHQLWLAEPIPNVA...(6)
...NL    65


   62 ATNKVLDLEKGLADRGTPILG...(-
1)...GWDYHEGDNQHWVIKKQADGKYYKIQSVKTQTFVDLNNG   120
       +   +DL  G ++ GT + G          G  +    +Q W IKK +DG  YKIQ+
++TFVDL NG
   66 FSGTYMDLYNGSSEAGTAVNG...(
2)...GTAFTTNPHQLWTIKKSSDGTSYKIQNYGSKTFVDLVNG   127


  121
GKDNGTKIQGWVGSWDETNSHQRWVFNQFSATGSRIKSILDTHPKIHG...(7)...DRIYFTPD
QYIFE   188
         +G KI GW G+WDE N HQ+W FN+ S + +  ++ +  +P IHG         D
Y       F
  128
DSSDGAKIAGWTGTWDEGNPHQKWYFNRMSVSSAEAQAAIARNPHIHG...(7)...DGEYLVLP
NATFT   195


  189
AIWAKTGLADIKPRDPLFQSEAYEKVFKGWVVSRAQEIIKVDGFDILVGSLSLVEKS...(19).
..DLS   267
       IW  +GL   K R+ ++  + +    K  V     +  K +GF I  G +  V K+
D +
  196
QIWKDSGLPGSKWREQIYDCDDFAIAMKAAVGKWGADSWKANGFAIFCGVMLGVNKA...(13).
..DHA   268


  268 QIVFFDPESGRTLVDI   283
       IVFF+P++G  L DI
  269 DIVFFEPQNGGYLNDI   284
```

APPENDIX D: Expression Levels Agglutinin 1

Expression levels (in tags per million) of the predicted *Agl1* gene (Protein ID: 109852) of *S. commune* in sense and antisense direction during four developmental stages.

Monokaryon (sense)	Stage I (sense)	Stage II (sense)	Mushroom (sense)	Monokaryon (antisense)	Stage I (antisense)	Stage II (antisense)	Mushrooms (antisense)
647,2	340,8	1423,4	29653,6	203,7	15,5	516,1	821,4

APPENDIX E: Nematodes

E.1 Amount of Nematodes and percentage dead nematodes.

Wild type								
Spot	***Nematods***							
	Week 1		Week 2		Week 3		Week 4	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
A	39	0	26	28	16	30	10	40
B	41	1	9	29	18	31	7	33
C	48	0	10	18	14	28	7	41
% dead A		0		52		65		80
% dead B		2		76		63		83
% dead C		0		64		67		85
Average % dead		0		52		65		80

Knockout 1.1								
Spot	***Nematods***							
	Week 1		Week 2		Week 3		Week 4	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
A	44	0	27	3	22	6	18	12
B	55	0	30	1	28	7	12	20
C	50	0	34	0	29	3	10	19
% dead A		0		10		21		40
% dead B		0		3		20		63
% dead C		0		0		9		66
Average % dead		0		10		21		40

Knockout 2.1								
Spot	***Nematods***							
	Week 1		Week 2		Week 3		Week 4	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
A	34	0	34	6	22	10	13	28
B	22	0	24	5	28	7	18	21
C	44	1	26	3	24	8	18	23
% dead A		0		15		31		68
% dead B		0		17		20		54
% dead C		2		10		25		56
Average % dead		0		15		31		68

Knockout 3.1								
Spot	***Nematods***							
	Week 1		Week 2		Week 3		Week 4	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
A	30	2	24	8	23	10	14	22
B	25	0	27	7	25	9	10	25
C	26	0	19	6	22	11	16	21
% dead A		6		25		30		61
% dead B		0		21		26		71
% dead C		0		24		33		57
Average % dead		6		25		30		61

E.2 T-Test

T-test	WK 1	WK 2	WK3	WK4
wt-1.1	0,3739	**0,0015**	**0,0003**	**0,0314**
wt-2.1	1,0000	**0,0049**	**0,0001**	**0,0128**
wt-3.1	0,6779	**0,0072**	**0,0000**	**0,0053**
1.1-2.1	0,3739	0,0557	0,1145	0,0987
1.1-3.1	0,3739	**0,0058**	**0,0249**	0,1120
2.1-3.1	0,6779	0,0913	0,1890	0,6087

E. 3 Photo's Nematodes

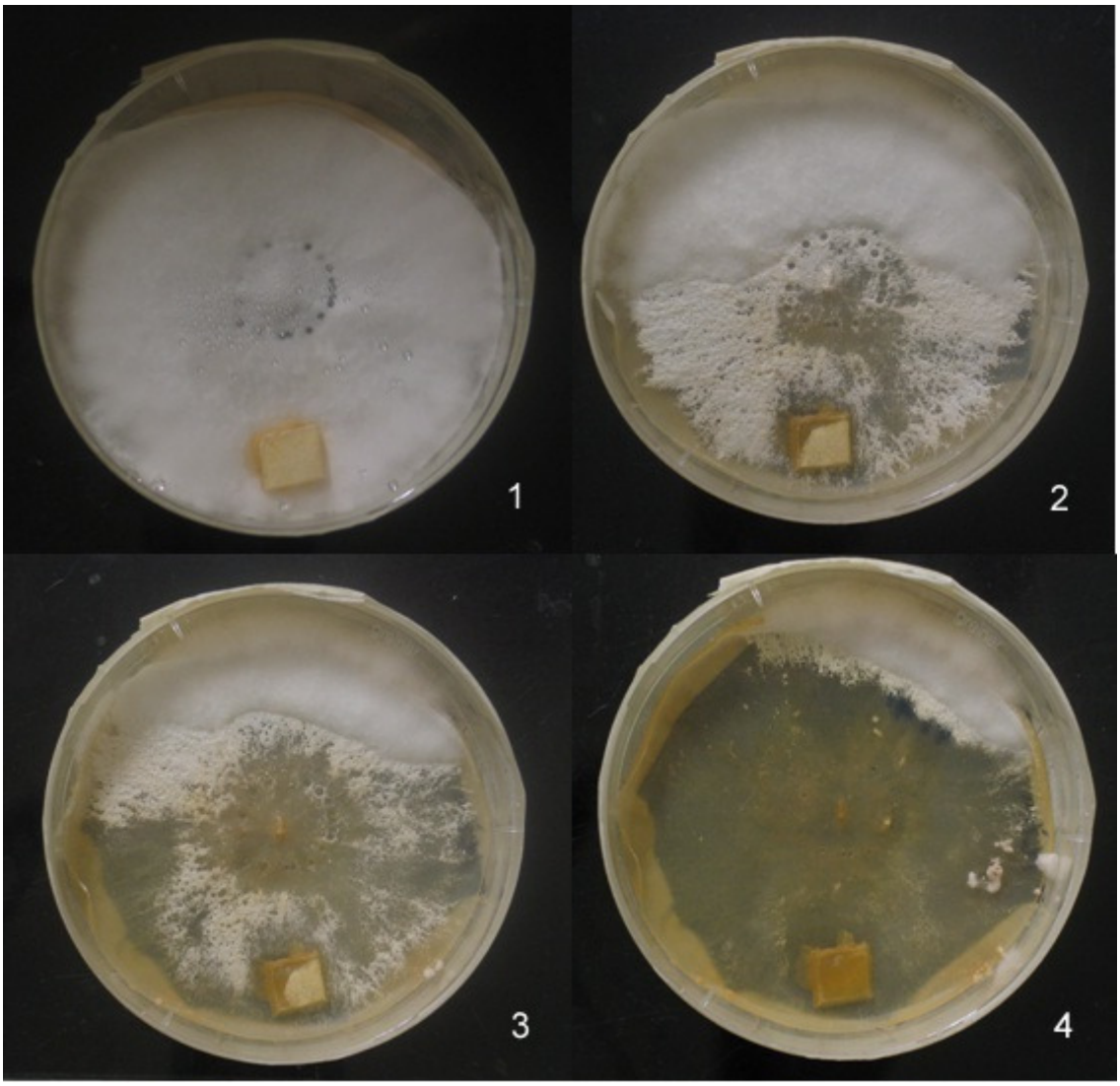


Figure 10. Nematodes (D. myceliophagus) on solid minimal medium (MM) with strain 4-39. *Photo 1 : day 1; Photo 2: day 7; Photo 3: day 14; Photo: 4: day 21*

APPENDIX F: Mites

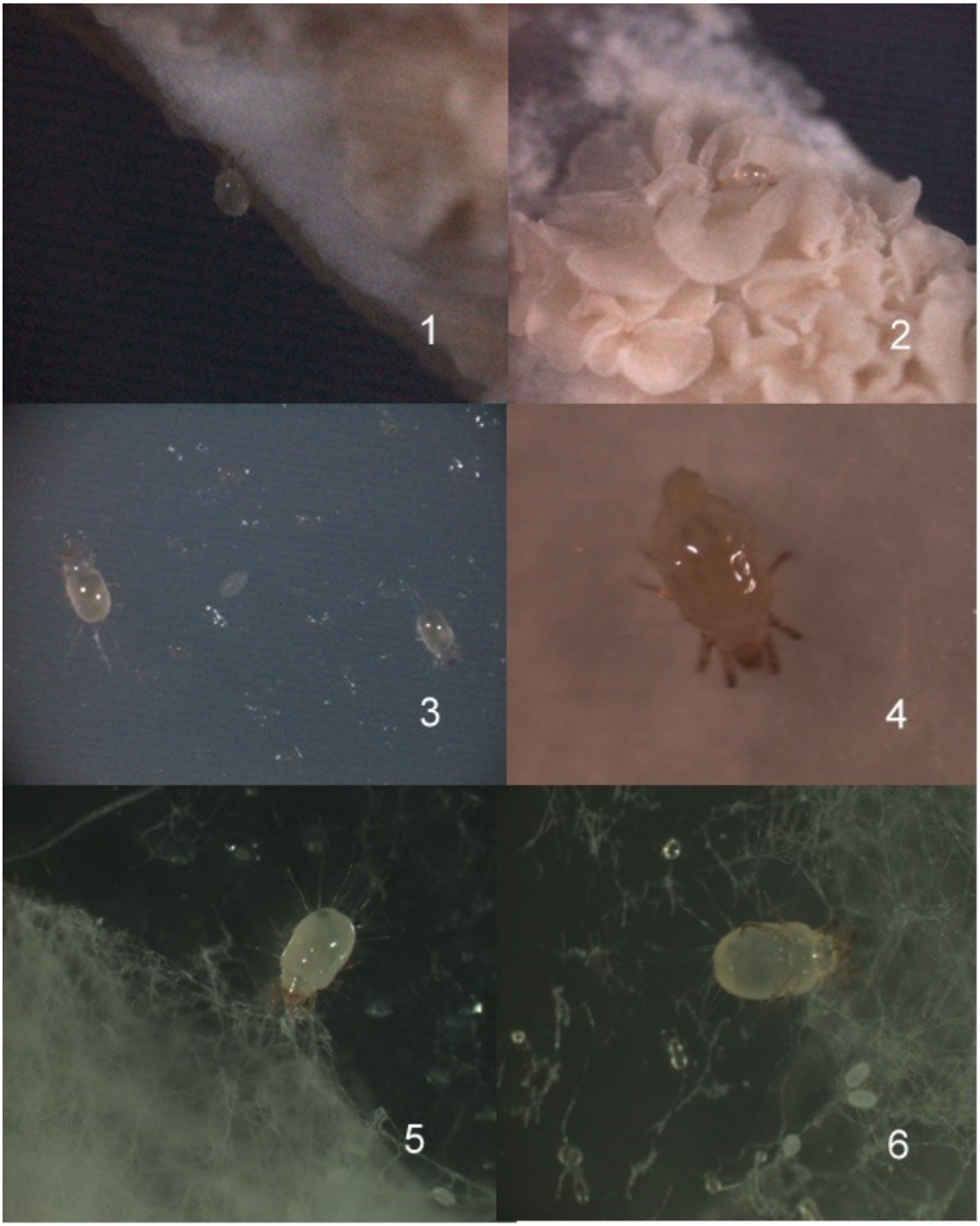


Figure 11. House dust mites (Dermatophagoides pteronyssinus) on MM plates with mycelium (4-39) and fruiting bodies(4-8Ax4-8BΔKu80). *Photo 1: adult mite (8 red legs) on edge of fruiting body; Photo 2: adult mite eating fruiting bodies; Photo 3: larva mites (6 legs); Photo 4: adult mite; Photo 5: adult eating mycelium; Photo 6: adult mite eating mycelium, white eggs visible underneath.*