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Characterisation of serine proteinase expression in *Agaricus bisporus* and *Coprinopsis cinerea* using GFP and the *A. bisporus SPR1* promoter.

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Running title: Serine proteinase expression in basidiomycetes

Abstract

The *Agaricus bisporus* serine proteinase 1 (SPR1) appears to be significant in both mycelial nutrition and senescence of the fruiting body. We report on the construction of an SPR promoter::GFP fusion cassette (pGreen_hph1_SPR_GFP) for the investigation of temporal and developmental expression of SPR1 in homobasidiomycetes, and to determine how expression is linked to physiological and environmental stimuli. Monitoring of *A. bisporus* pGreen_hph1_SPR_GFP transformants on media rich in ammonia or containing different nitrogen sources, demonstrated that SPR1 is produced in response to available nitrogen. In *A. bisporus* fruiting bodies, GFP activity was localised to the stipe of postharvest senescing sporophores. pGreen_hph1_SPR_GFP was also transformed into the model basidiomycete *Coprinopsis cinerea*. Endogenous *C. cinerea* proteinase activity was profiled during liquid culture and fruiting body development. Maximum activity was observed in the mature cap, while activity dropped during autolysis. Analysis of the *C. cinerea* genome revealed 7 genes showing significant homology to the *A. bisporus* *SPR1* and *SPR2* genes. These genes contain the aspartic acid, histidine and serine residues common to serine proteinases. Analysis of the promoter regions revealed at least one CreA and several AreA regulatory motifs in all sequences. Fruiting was induced in *C. cinerea* dikaryons and fluorescence determined in different developmental stages. GFP expression was observed throughout the life cycle, demonstrating that serine proteinase can be active in all stages of *C. cinerea* fruiting body development. Serine proteinase expression (GFP fluorescence) was most concentrated during development of young tissue, which may be indicative of high protein turnover during cell differentiation.

Index descriptors: Basidiomycetes, *Agaricus bisporus, Coprinopsis cinerea,* serine proteinase, green fluorescent protein (GFP), humic protein.

1. Introduction

Agaricus bisporus, the cultivated mushroom has economic and biotechnological significance. It is the most extensively cultivated mushroom worldwide, with an annual production in the region of 5 million tonnes (33) and is a major protected crop in the UK accounting for 10% of such horticultural production (20). In addition to its value as a food crop there is considerable interest in *A. bisporus* as a host for molecular pharming of heterologous proteins (21, 51, 58, 62), and it also appears to produce a number of compounds of potential biomedical/nutraceutical importance (13). Application of biotechnology to *A. bisporus* has greatly increased due to the development of a transformation system (14, 26) and recently Burns *et al.,* (2006) developed an *A. bisporus* 'molecular toolkit' which tested different promoters for efficient gene expression. Despite these recent advances, developmental studies in Agaricus have been hampered due to the time and containment issues that exist when studying a genetically modified strain.

The ink-cap mushroom, *Coprinopsis cinerea* (formally *Coprinus cinereus*), is a well-studied homobasidiomycete (12, 43, 47) that forms an excellent model system for studies of gene expression at several levels of differentiation, particularly mushroom development and meiotic processes (46, 59). It has been used as an object for studies of development (32) mainly because of its relatively short life cycle, which can be completed in the laboratory within 2 weeks (44). In addition, genetic studies and experimental manipulation of all phases of its life cycle are simple and relatively straightforward (63). The *C. cinerea* genome sequence was released in 2003 (http://www.broad.mit.edu) and recently gene silencing has been demonstrated in the basidiomycete (24, 47). We have exploited these characteristics of *C. cinerea* for the investigation of a serine proteinase from *Agaricus bisporus.*

A serine proteinase (SPR1) has been purified from senescent sporophore tissue of *A. bisporus,* which has a molecular mass of 27 kDa and an isoelectric point of 9.0 (11). The protease has a broad pH optimum, 6.5-11.5, and a narrow substrate specificity, requiring both a hydrophobic amino acid in the P1 position and a minimum peptide chain length (11). The most active proteolysis of *A. bisporus* culture filtrate was observed with Suc-Ala-Ala-Pro-Phe-pNA at neutral pH (10). Serine proteinase was found to be the major proteinase produced by *A. bisporus* in sporophores during senescence (9), and extracellular to mycelium in colonized compost where nitrogen is largely in the form of protein suggesting a nutritional role for this enzyme (10). The serine proteinase extracellular to mycelium was produced to a greater degree in response to protein associated with humic substances than other pure proteins, suggesting factors additional to the protein are involved in its induction. The cDNA for this proteinase has been cloned and sequenced (accession no Y13805), which revealed that this serine proteinase (SPR) belongs to the 'proteinase K family' (31). The *SPR1* gene expression was not detected in freshly harvested mushrooms, while increased transcript levels were observed 1-3 days post harvest. Expression of *SPR1* was strongest in post harvest stipe tissue (31) which correlated well with the increase in enzyme activity and protein level detected in senescent stipe (9). The relatively high transcriptional and translational levels of SPR in the stipe demonstrate that the enzyme is important during the metabolism of senescing mushrooms.

This paper reports on the construction of a promoter::GFP fusion cassette for the investigation of the temporal and developmental expression of *SPR1* in *A. bisporus* and *C. cinerea* and to characterise expression in response to physiological and environmental stimuli. This paper further investigates the utility of *C. cinereus* as a model system for basidiomycete gene expression and fruiting body production, as

development of a model species for basidiomycetes research is vital for future progress.

2. Materials and methods

2.1 Strains and culture maintenance

Escherichia coli strain DH5α was the host strain for recombinant plasmids. *Agrobacterium tumefaciens* AGL1 (34) was used for *A. bisporus* transformations and cultured as previously described (17, 26). The *A. bisporus* commercial strain A15 (18) was used for transformations. Mycelia were routinely maintained at 25°C on MPA (35) agar plates and supplemented with 25μgml^{-1} hygromycinB to select for transformants. A tryptophan auxotroph, LT2 (*A6B6, trp1.1;1.6*) (4) was used for *C. cinerea* transformations. *C. cinerea* strains AT8 (*A43B43, trp-3, ade-8*) and AmutBmut (*A43mutB43mut*, *pab1*) (41, 56) were used for fruiting studies. *C. cinerea* mycelia were routinely maintained at 37°C on YMG agar (4) supplemented when appropriate with 100μgmL^{-1} L-tryptophan.

2.2 Construct design

An 877bp *A. bisporus SPR* putative promoter region (5'UTR) was amplified from a cosmid clone template using primers spr1-fwd (TCCCCGCGGCGGGCTCAGAAGGTTTCTAT) and spr1(rev)m (AAATCCATGGTCGGTGAAGAGATC) that respectively introduced 5' *Sac*II and 3' *Nco*I restriction sites. The resulting amplicon was cloned using pGEM-Teasy (Promega Corp.) and *SPR1* promoter integrity confirmed by dsDNA sequencing of recombinants. The *SPR1* promoter was cloned into a pBluescriptII based GFP expression construct (pBlue-SPR-GFP) following removal of the *A. bisporus GPD*II promoter (*Sac*II-*Nco*I restriction) from an intron-GFP expression vector p004iGM (6).

The 1884bp SPR::GFP expression unit was excised by *Sac*I-*Kpn*I restriction and ligated into the *Cla*I-*Kpn*I restricted binary pGreen_hph1 (18) by addition of a *Cla*I-*Sac*I oligolinker (CGAGCT) to yield pGreen_hph1_SPR_GFP.

2.3 Fungal transformations

Plasmid DNA for fungal transformation was prepared using QIAgen Midi Prep Kits. *C. cinerea* protoplast co-transformations were performed as previously described (4, 6, 22, 24) using *ca.* 1μg of pCc1001 (*trp1*) (54) with 5μg of plasmid pGreen_hph1_SPR_GFP. Trp+ transformants were maintained on *Coprinus* regeneration agar (6, 16, 24). Putative transformants of *C. cinerea* were cultured as described above and genomic DNA extracted as previously described (36). PCR screening of *C. cinerea* transformants was performed using Reddymix components (Abgene) with a general thermal cycling program of 95°C for 3 min, (95°C for 30 sec, 50°C for 1min, 72°C for 30 sec,) 30 cycles, 72°C for 10 min.

A. bisporus was transformed using *Agrobacterium tumefaciens* mediated transfection of gill tissue as previously described (6, 7, 14, 35, 42). Transformants of *A. bisporus* were identified using previously published methods (18, 35) and transcription of both *hph* and GFP transgenes confirmed using rtPCR and/or quantitative rtPCR (24). *A. bisporus* transformants for fruiting were selected from a large sample set by fluorometric quantification of GFP activity in mycelia (24) following induction with humic fraction (10).

2.4 Fruiting studies

Dikaryons of *C. cinerea* were produced on YMGT plates by placing mycelial blocks of AT8 and LT2 trp^+ transformants 5mm apart at 37°C; dikaryotization was

confirmed by the presence of clamp cells. For growth and induction of fruiting bodies, dikaryons on YMGT plates were incubated at 12 hr light/12 hr dark, 25°C, 90% humidity under standard fruiting conditions (22, 37). *C. cinerea* strain AmutBmut was selected as a control strain for fruiting studies as it exhibits clamp formation and fruit body development like a dikaryon and produces uninucleate oidia like a monokaryon (56). GFP expression in fruiting bodies was examined using a Leica MZFL111 microscope with SPOT 2.2.1 (Diagnostic Instruments inc.) imaging software.

A. bisporus sporophores were produced in small-scale compost cultures at the University of Warwick's transgenic mushroom containment facility, harvested and stored as previously described (18). GFP activity was measured in detached mushrooms, 3-days post-harvest, using a portable GFP meter (ADC BioScientific Ltd., UK; excitation 450nm/ emission 530nm/ gain setting 55). Metered readings were recorded for both cap and stipe tissues of whole mushrooms and the freshly cut face of longitudinally bisected sporophores. A minimum of three replicate readings was taken for each sample tissue from two replicate sporophores. Sectioned mushrooms were also viewed using a blue LED floodlight (Inova X5^{TM}, Emissive Energy, RI) with appropriate blue/yellow filter sets (57) and photographed using a Nikon Coolpix 990.

2.5 Proteinase assays

A proteinase plate assay was carried out by inoculating *C. cinerea* LT2 onto ammonium free regeneration agar (RA) plates containing 0.5% (w/v) skimmed milk powder. To assess proteinase activity, colony size was measured, as well as the clearing zone around each colony, produced by degradation of the milk layer by

extracellular proteinase activity. LT2 was inoculated onto standard RA media as a control. Five replicate plates were measured per assay.

Expression of serine proteinase activity in liquid culture was determined by inoculating LT2 into ammonium free RA containing 0.5% (w/v) milk solution and into standard RA. Cultures were grown for 264 h and samples (8 ml) were aseptically removed every 24 h and assayed. Serine proteinase activity was measured in fruiting body developmental stages by homogenising fungal tissue in 50mM Tris buffer, pH 8.0 and centrifuging at 10,000g to remove particulate material.

Serine proteinase activity was assayed spectrophometrically by absorbance at 405nm following the release of p-nitroaniline from the synthetic peptide Suc-Ala-Ala-Pro-Phe-pNA (0.15mM) in 50mM Tris buffer, pH 8.0. Hydrolysis was performed for 30 min at 37^{o}C. Inhibition of serine proteinase was performed by pre-incubation of 0.1M PMSF (Fluka) inhibitor with the enzyme at 37°C for 30 min. Soluble protein concentrations were measured by the dye-binding method of Bradford (5). Bovine serum albumin was used as a standard. Biochemical assays were performed in triplicate.

2.6 Sequence analysis

Sequences were analysed by BLAST (NCBI) (1) and aligned using ClustalW (25). The sequence manipulation suite (55) performed molecular weight and isoelectric point prediction. Prosite was used to identify motifs and signature sequences in the deduced protein sequences (3) and signal sequences were identified using SignalP (48). Structural classification of sequences was based on SCOP (45). Transcription factor binding sites were predicted using MOTIF search on Genome Net (http://motif.genome.jp/).

3. Results

3.1 Analysis of *A. bisporus* pGreen_hph1_SPR_GFP transformants

To investigate the temporal and spatial expression of the *A. bisporus SPR1* gene, a promoter::GFP fusion cassette was constructed. This expression vector was engineered to contain a 5′ intron, which has previously been shown to be necessary for GFP expression in *A. bisporus* and *C. cinerea* (6). Plasmid pGreen_hph1_SPR_GFP was transformed into *A. bisporus* via *A. tumefaciens* and transformants were recovered on hygromycin selection. Nine transformants were selected for further analysis. The presence of the intact expression cassette, pGreen_hph1_SPR_GFP, was confirmed via PCR. Primers SPR1Fwd (5′-CCGCGCAACATATGTATGTGAGAG-3′) and GFPrev (5′-GTGGCGGATCTTGAAGTTCACCTTG-3′), which bind 256bp downstream from the 5′ end of the *SPR1* promoter and 234bp upstream from the 3′ end of the GFP gene respectively, resulted in a 1226bp PCR product. Primers GFPFwd (5′-GGCGTGCAGTGCTTCAGCCGC-3′) and TrpCRev (5′-GCACTCTTTGCTGCTTGGAC-3′) which bind 222bp downstream from the 5′ end of the GFP gene and 146bp upstream from the 3′ end of the TrpC terminator resulted in a 665bp PCR product. Positive amplification of both fragments confirmed the presence of the intact expression cassette. *A. bisporus* pGreen_hph1_SPR_GFP transformants, wild type *A. bisporus* A15, and an *A. bisporus* strain expressing the plasmid pGR4-4GiGM3' (G26) which contains GFP under the *A. bisporus GPD II* promoter (6), were inoculated onto a range of media to investigate if changes in nutrient availability would alter the expression of the proteinase which is know to be involved in nutrient acquisition. GFP expression was monitored on media rich in

ammonia (YMG, MMP, and regeneration agar (RA)), potato dextrose agar (PDA), and ammonia free regeneration agar containing one of the following sole nitrogen sources; 0.094% (w/v) humic fraction, 0.084% (w/v) glutamic acid (GA), or 0.5% (w/v) skimmed milk power. GFP expression was observed in the pGreen_hph1_SPR_GFP transformants grown on humic fraction, milk, GA and PDA media, while no GFP expression was observed on YMG, MMP and RA (Table 1). Fig. 1A shows expression of GFP in an *A. bisporus* SPR::GFP transformant (TP17) on ammonia free regeneration agar containing 0.094% humic fraction, and its repression on standard regeneration media. As expected the GPD::GFP control transformant (G26) exhibited strong GFP expression on all media, whilst GFP fluorescence was not observed on any media with the wild type strain (Table 1).

3.2 Monitoring of *SPR1* expression in *A. bisporus* sporophore development

Fruiting was induced in *A. bisporus* transformants and GFP expression was detected using blue LED illumination of bisected mushrooms (Fig 1B). Transformants for fruiting were selected from a large sample set by fluorometric quantification of GFP activity in mycelia (23) following induction with humic fraction (10). TP196 was selected as a typical phenotypic representative of transformants, which also exhibited excellent culture and fruiting capabilities. GFP expression was clearly observed in both the cap and stipe tissues of freshly harvested (day 0) *A. bisporus* G26 fruiting bodies, expressing GFP under the control of the GPD promoter (Fig. 1B: Panel A). In senescing mushrooms (3d post harvest) no GFP expression was observed in a hygromycin resistant (control) transformant of A15hph (no GFP cassette, Fig. 1B: Panel B: leftmost mushroom), while GFP expression was clearly detected in the stipe tissue of SPR::GFP transformant TP196 (Fig. 1B: Panel B: rightmost mushroom).

Metered readings (relative fluorescence units (RFUs)) for cap and stipe tissues of whole and longitudinally bisected mushrooms of TP196 (*SPR*::GFP), G26 (GPD::GFP) and A15hph (no GFP cassette) were recorded 3 days post harvest (Fig. 2). GFP activity was substantially elevated in the stipes of senescing mushrooms for the SPR::GFP transformant TP196. The tissue (stipe) specific expression of GFP in TP196 is consistent with earlier histochemical observations of SPR activity in senescing mushrooms (9). RFUs recorded for G26 represent background fluorescence of the fruiting body, while A15hph exhibits a slight increase in RFUs compared to G26 due to autofluorescence.

3.3 Expression profiles of serine proteinases in *C. cinerea* LT2

Endogenous proteinase activity was assessed by inoculating LT2 onto ammonium free regeneration agar (RA) with and without a 0.5% (w/v) milk solution. Clearing zones, indicative of proteinase activity, were only produced on media containing the milk overlay (Fig. 3A). A proteinase expression profile was developed for LT2 grown in broth by measuring the hydrolysis of the synthetic peptide Suc-Ala-Ala-Pro-Phe-pNA. Proteinase activity was observed in LT2 cultures grown in ammonium free RA containing 0.5% (w/v) milk solution after 120 h (0.0259 AU/mL) and continued to increase until 240 h (1.283 AU/mL) (Fig. 3B). A small decrease in activity was observed at 264 h but increased again at 288 h. Pre-incubation of the crude enzyme extracts with the serine proteinase inhibitor PMSF resulted in a large decrease in activity (from 1.283 AU/mL to 0.17 AU/mL at 240 h), thus confirming that the majority of proteinase activity detected was the serine mechanistic class. Little or no proteinase activity was observed in LT2 cultures grown in standard RA media, which is rich in ammonia (Fig. 3B).

Serine proteinase activity was measured during the primordium, karyogamy, meiosis, immature, mature and autolysis stages of fruiting body development (Fig. 3C). Activity increased slowly from the primordium (1.29 units/g) to the meiosis stage (1.69 units/g) with a slight dip at immature (1.52 units/g) followed by a large increase in activity during the mature development stage (6.32 units/g). Maximum activity was detected in the mature cap (6.32 units/g) followed by a decrease in activity during autolysis (3.45 units/g). Similarly, pre-incubation of the crude enzyme extracts with the inhibitor PMSF resulted in a large decrease in activity (from 6.32 units/g to 0.34 units/g in the mature cap), demonstrating that the class of proteinase activity detected was serine proteinase (Fig. 3C).

3.4 Identification and sequence analysis of homobasidiomycete serine proteinases

Following confirmation of endogenous serine proteinase activity in *C. cinerea*, identification of the encoding genes was undertaken using the published *C. cinerea* genome sequence. Two *A. bisporus* serine proteinases have been previously identified (*SPR*1, *SPR2*) and their sequence deposited in public databases under accession numbers Y13805 and AJ344211 respectively (30, 31). Predicted molecular weights (Table 2), for full-length *SPR*1 and *SPR*2 are considerably larger than the ca. 27kDA experimental estimates from SDS-PAGE, cDNA and N-terminal amino acid sequencing. Mature proteins for *SPR*1 (286 aa, 28.29kDa) and *SPR*2 (275 aa, 27.70kDa) are much closer to the 27kDA estimate previously observed (11, 31). Blast analysis (1) of the *A. bisporus SPR1* and *SPR2* genes against the *C. cinerea* database revealed 7 genes (04562.1, 10592.1, 10615.1, 07792.1, 10606.1, 0.3122.1 & 04470.1) showing significant homology to the serine proteinases. ClustalW alignments of these *C. cinerea* genes with the *A. bisporus SPR1* revealed amino acid sequence identity

values ranging between 44% and 61% while homology of the *SPR2* with the *C. cinerea* genes ranged between 42% and 55% (Table 3). *SPR1* and *SPR2* have an amino acid identity value of 75% while the *C. cinerea* genes have homology ranging between 31% and 77% (Table 3).

Three motifs were identified within the *C. cinerea* genes that are common to other serine proteinases; the aspartic acid residue (consensus: [STAIV]-X-[LIVMF]-[LIVM]-D-[DSTA]-G-[LIVMFC]-X(2,3)-[DNH], the histidine residue (consensus: H-G-[STM]-X-[VIC]-[STAGC]-[GS]-X-[LIVMA]-[STAGCLV]-[SAGM]) and the serine residue (consensus: G-T-S-X-[SA]-X-P-X(2)-[STAVC]-[AG]) (31). These residues were conserved between the *C. cinerea* genes and the *A. bisporus SPR1* and *SPR2*, with the exception of the *C. cinerea* gene 10606.1 that lacked the serine residue. This suggests that the *C. cinerea* genes are serine proteinases and they appear to belong to the subtilisin family.

The probable *C. cinerea* serine proteinase genes ranged between 346 and 500 amino acids in length (Table 2), and all contained introns. Each intron began with GT and ended with AG, which is a common feature of fungal introns and has been observed in the serine proteinase genes from *Acremonium chrysogenum* (28), *Lecanicillium psalliotae* (60) and *Arthrobotrys conoides* (61). The number of introns varied between 2 and 14 depending on the gene (Table 2), and some conservation of intron position was observed between the *C. cinerea* genes and *SPR2*.

The theoretical molecular weight and isoelectric points for the *C. cinerea SPR* genes range between 35kDa and 53kDa and 5.83 and 9.97 respectively (Table 2), while the theoretical molecular mass and isoelectric points for *SPR1*and *SPR2* are 39.39 kDa and 5.93 and 38.85 kDa and 5.53 respectively (Table 2). A predicted signal peptide was observed in the *C. cinerea* and *A. bisporus* serine proteinases, with cleavage

occurring either between amino acids 19 and 20, 20 and 21 or 21 and 22, suggesting that these enzymes are secreted. Using the highest homology sequences, the predicted secondary structure of these genes is composed of between 20-30% helices, 16-35% strands and 42-61% loops (Table 2) and analysis of the degree of protein globularity suggests that these enzymes exist as compact (globular) domains.

One kb of sequence upstream from the ATG start codon of each gene was analysed for the presence of regulatory motifs. At least one CreA and several Nit2/AreA regulatory elements were identified in the promoter regions of the *C. cinerea* and *A. bisporus* genes (Table 2). No other regions of homology were detected between the promoters.

3.5 Analysis of *C. cinerea* pGreen_hph1_SPR_GFP transformants

From a preliminary screen of one hundred Trp+ co-transformants on RA media (rich in ammonia), and on ammonia free regeneration agar containing 0.094% (w/v) humic fraction as the sole nitrogen source, 32% of transformants were found to express GFP on humic fraction, which correlates well with the reported rate of co-transformation (30-49% (6)). However, GFP expression was not observed on RA media. Four GFP+ transformants, non-transformed LT2, and a *C. cinerea* strain (PG78Gr) expressing GFP under the regulation of the *A. bisporus GPDII* promoter (24), were selected for further studies. GFP expression was monitored on media rich in ammonia (YMG, and regeneration agar (RA)), potato dextrose agar (PDA), and ammonia free regeneration agar containing either 0.094% (w/v) humic fraction, 0.084% (w/v) glutamic acid (GA), or 0.5% (w/v) milk as the sole nitrogen source. LT2 exhibited no fluorescence on any media while PG78Gr expressed GFP on all the media. GFP fluorescence was observed in transformants grown on humic fraction, milk, GA and potato dextrose

media while no GFP fluorescence was observed on RA media (Table 1). The only transformant to exhibit fluorescence on YMG media was T47.

C. cinerea transformants were mated with AT8 and the dikaryons inoculated onto a range of media and screened for GFP expression. Similar expression profiles were observed for both the monokaryons and dikaryons (Table 1). Fig. 4A shows *C. cinerea* transformant T47 monokaryon and dikaryon expression of GFP on ammonia free regeneration agar containing 0.094% (w/v) humic fraction, and repression of GFP on standard regeneration media.

3.6 Monitoring of *SPR1* expression in *C. cinerea* fruiting body development

Fruiting was induced in the *C. cinerea* dikaryon strains and the control strain AmutBmut. Different developmental stages of the fruiting body were examined microscopically for fluorescence. Low levels of fluorescence were observed in the hyphal knot (Fig 4B: Panel A). Fluorescence was also observed in the primordium stage but was not localised (Fig 4B: Panel B). A similar observation was made for karogamy stage but GFP localisation began to occur at the edge of the forming gill tissue (Fig 4B: Panel C). GFP appeared more localised at the forming gill tissue during meiosis (Fig 4B: Panel D), while at the immature stage GFP was observed high up in the stipe close to the cap (Fig 4B: Panel E). In mature sporophores, fluorescence was observed in the cap but was most concentrated at the junction of the stipe and cap (Fig 4B: Panel F), while fluorescence was reduced in the stipe (Fig 4B: Panel G). During autolysis fluorescence was greatly reduced in the cap (Fig 4B: Panel H) but was concentrated in the stipe tissue (Fig 4B: Panel I). In the control strain AmutBmut some autofluorescence was observed throughout the different

developmental stages. Fig 4C depicts a schematic of GFP fluorescence under the control of the *A. bisporus SPR1* promoter through out the *C. cinerea* life cycle.

4. Discussion

A. bisporus SPR1 has previously been shown to be significant in both mycelial nutrition and senescence of the mushroom fruit body (8-10). This study used an SPR::GFP fusion construct to investigate temporal and developmental expression of *SPR1* in *A. bisporus* and a heterologous host *C. cinerea* in response to physiological and environmental stimuli. Developmental studies in *A. bisporus* are still hindered due to the time and containment issues that exist when studying a genetically modified strain. *C. cinerea* provides a model system for the studies of gene expression throughout mushroom development (47, 59) and heterologous expression of the *A. bisporus SPR1* promoter fusion is a further demonstration of the inkcap host utility as a model species.

GFP has been widely used as a reporter molecule or as a fluorescent tag for fusion proteins (53) and is now a valuable tool in the molecular analysis of filamentous fungi (38). The use of GFP in ascomycete fungi has been widely reported (2, 27, 49), and recently expression in homobasidiomycetes has also been achieved (6, 39, 40).

The aim of this study was to carry out a comparative molecular analysis of serine proteases in both *C. cinereus* and *A. bisporus*. To this end, identification of *C. cinereus* SPR genomic sequences was performed to establish the homology between Agaricus and Coprinus SPR genes. Bioinformatics was employed to help predict if the genes would be regulated in a similar fashion, thus providing evidence for the suitability of *C. cinereus* as a heterologous host for *A. bisporus* SPR1. Blast analysis of the *A. bisporus SPR1* cDNA and *SPR2* genomic sequence against the *C. cinerea*

database revealed 7 genes showing significant homology. Conservation of the aspartic acid, histidine and serine residues within the genes suggested that they are serine proteinases belonging to the subtilisin family. However, lack of a serine residue at the active site in 10606.1 suggests that some of these are 'pseudogenes' that would be unable to code for active enzymes. Signal peptide analyses were indicative of extracellular activity and protein globularity infers that the enzymes would exist as compact globular domains. Sequencing of the *A. bisporus* genome is currently underway (http://www2.warwick.ac.uk/fac/sci/whri/research/agaricusgenome/) which may reveal further SPR homologues.

All the *C. cinerea* genes contained introns with numbers varying between 2 and 14; only two genes had less than 10 introns, six of the seven analysed contained between 11-14 introns. Short exons and high intron density in basidiomycetes and the comparatively poor conservation of intron splice sequences compared with other fungi can result in some inaccuracies when using intron predictive software. This may account for the low number of introns identified in CC1G_10615.1 and CC1G_04470.1.

In *A. bisporus*, two serine proteinases (*SPR1* and *SPR2*) were isolated from the same cosmid clone, within 30kb of each other (30). Similarly, three *C. cinerea* serine proteinases (CC1G_10592.1, CC1G_10606.1 and CC1G_10615.1) lay within 50kb of each other on the genome suggesting either local duplication or a common ancestor. Conservation of intron positions observed in these genes is indicative of local duplications.

Endogenous protease activity was investigated in *C. cinereus*. A preliminarily plate based assay resulted in a clearing zone around the fungal colonies thus confirming the presence of proteases in the basidiomycete. As previously demonstrated in *A. bisporus*

(10), little or no serine proteinase activity was detected in *C. cinerea* cultures grown in ammonia rich media. Activity was observed in cultures grown on ammonia free RA containing milk as the sole nitrogen source after 120 h and continued to increase until 240 h with a slight decrease at 264 h before increasing again at 288 h which may be indicative of the onset of autolysis.

Expression of SPR1 in response to physiological and environmental stimuli was examined by inoculating the *A. bisporus* and *C. cinerea* pGreen_hph1_SPR_GFP transformants onto a range of media to investigate if changes in nutrient availability would alter the expression of the proteinase. At least one CreA and several Nit2/AreA transcription factor-binding sites were identified in both the *A. bisporus* and *C. cinereus* SPR promoter sequences, signifying regulation by factors such as carbon and nitrogen sources. Conservation of these sites was not observed across the promoters. Experimental evidence for the regulation of serine proteinases in response to nitrogen sources is provided from *C. cinerea* biochemical profiles in broth culture; serine proteinase was not detected on ammonia rich RA media but was observed on ammonia free RA supplemented with milk. GFP expression was observed in *A. bisporus* and *C. cinereus* transformants grown on PDB and on ammonia free RA containing humic fraction, milk, or glutamate as the sole nitrogen source. GFP expression was not observed on YMG, MMP or regeneration media (rich in ammonium), with the exception of *C. cinereus* transformant TP47. GFP expression was observed in TP47 grown on YMG media, which may result from multiple insertion events, however this expression profile was atypical of the population of *C. cinereus* transformants analysed. Expression profiles were similar for both monokaryons and dikaryons. Collectively these results suggest that both *C. cinerea* and *A. bisporus* produce serine proteinases in response to available nitrogen.

Developmental regulation of serine proteinase expression was investigated. Serine proteinase activity has previously been reported during fruiting body development of *A. bisporus* (9). From stages 2 to 6 of development (23), activity was relatively low and cap and stipe activities were similar. *A. bisporus* developmental stages 2-6 roughly correspond to the primordium, karyogamy, meiosis, immature and mature stages of *C. cinereus* development. In our SPR biochemical assays, activity was relatively low in the first four stages of *C. cinerea* development but increased rapidly at the mature stage. GFP expression was ubiquitous in the primordium stage, which may be the result of a higher density of cytoplasm in the developing primordium. GFP expression was observed throughout the karogamy and meiosis stages though localization of fluorescence began to occur at the edge of the forming gill tissue at the karyogamy stage and became more pronounced at the meiosis stage. *C. cinerea* is described as having a rupthymenial mode of hymenophore development, where the gill is envisaged as widening towards the periphery of the cap as a differentiating front moves into, and differentiates from, the basidiocarp (50). As the widest part of the gills is those at the cap margin, the differentiating front is also moving upwards towards the apex of the cap (52). GFP fluorescence was most concentrated at the base of the gills in the karogamy stage and moved upwards towards the apex of the cap in meiosis suggesting that *SPR1* promoter activity was enhanced during the development of young tissue, which may be indicative of high protein turnover during cell differentiation. This could also result from autolysis of connective tissue as the gills begin to separate from the stipe i.e. creating an abscission zone. At the immature stage GFP was observed high up in the stipe close to the cap. Studies of *C. cinerea* stipe elongation have revealed that it is variable along its length and that elongation is

greatest at the mid-upper portion (the stipe that is enclosed by the developing cap); the apex and base of the stipe shows little elongation (15, 29). The rapid increase in length is chiefly due to cellular elongation (29) but divisions also contribute, with cells doubling in number and increasing six to eight fold in length (19). The fluorescence observed in the mid-upper stipe demonstrates that the *SPR1* promoter is activity upregulated during elongation, and is likely to support the elongating stipe by providing free amino acids via protein degradation. Highest activity was recorded in the mature cap with slightly less activity in the mature stipe, contrasting to the levels recorded for *A. bisporus*. In the mature fruiting body GFP fluorescence was observed in the cap but was most concentrated at junction of the stipe and cap. This may result from a high density of cells where younger tissue is still developing resulting in elevated protein turnover. With *A. bisporus* developmental stage 7, a large increase in activity in the stipe and a small increase in the cap occurs (9), and further increases are observed as stage 7 mushrooms progress to senescence. During *C. cinerea* autolysis serine proteinase activity decreased and fluorescence was greatly reduced in the cap but was highly concentrated in stipe tissue. Accumulation of serine proteinase in the stipe during autolysis would suggest a role in the export of nutrients from the stipe to the cap tissue during senescence. Similarly in *A. bisporus* sporophores, highest SPR::GFP activity was observed in senescing stipe tissues, suggesting that the stipe may act as an 'active source' during the export of nutrients to reproductive spore-bearing tissues.

The results reported here confirm that the *A. bisporus* (*SPR1*) promoter is able to regulate mycelial serine proteinase production in response to specific nitrogen sources and have demonstrated tissue specific (stipe localised) expression in detached

sporophores. Use of the SPR::GFP fusion construct, coupled with genome data-mining, suggests that serine proteinases also play an integral part in the development of *C. cinerea* sporophores. The approaches developed in this study should underpin further promoter analysis in these homobasidiomycete mushrooms and may permit characterisation of promoter elements that regulate differential expression and nutritional regulation of serine proteinases. Furthermore *C. cinereus* has been validated as a potential model for expression and regulation studies of *A. bisporus* genes.

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Table 1. Evaluation of GFP fluorescence in *A. bisporus* and *C. cinerea* monokaryon and dikaryon pGreen_hph1_SPR_GFP transformants on a range of different media. Control strains included non transformed *A. bisporus* (A15) and *C. cinereus* (LT2) and *A. bisporus* (G26) and *C. cinereus* (PG78Gr) strains expressing GFP under the *A. bisporus GPD II* promoter. GFP expression was monitored on media rich in ammonia (YMG, MMP, RA), potato dextrose agar (PDA), and ammonia free regeneration agar containing 0.094% (w/v) humic fraction, 0.084% (w/v) glutamic acid (GA), or 0.5% (w/v) milk as sole sources of nitrogen.

Table 2. Sequence analysis of the *A. bisporus* and the predicted *C. cinerea* serine proteinases and promoter regions. *A. bisporus* genes: *SPR1* & *SPR2*. *C. cinerea* genes: 04562.1, 10592.1, 10615.1, 07792.1, 10606.1, 0.3122.1 & 04470.1

Table 3. Percentages of amino acid sequence identity between the *A. bisporus* and the predicted *C. cinerea* serine proteinases. *A. bisporus* genes: *SPR1* & *SPR2*. *C. cinerea* genes: 04562.1, 10592.1, 10615.1, 07792.1, 10606.1, 0.3122.1 & 04470.1

Fig 1. A: GFP expression in the *A. bisporus* SPR::GFP transformant TP17 when grown on regeneration agar with or without 0.094% humic fraction under phase contrast microscopy and UV light. Actively growing mycelia were examined using 40× objective on a Leitz Dialux 20 research microscope with excitation filters at 450–490 nm, dichroic filter at 510 nm, and emission filter at 515 nm. Images clearly show GFP fluorescence in TP17 grown on humic fraction while no fluorescence was observed when grown on RA. **B:** Stipe localised GFP fluorescence in *A. bisporus* transformant TP196. Fruiting was induced in *A. bisporus* transformants and bisected mushrooms viewed under white light (WL) and blue LED illumination (BL). Panel A: Images clearly show fluorescence in both the cap and stipe tissues of freshly harvested (day 0) *A. bisporus* G26 fruiting bodies, expressing GFP under the control of the GPD promoter. Panel B: In senescing mushrooms (3d post harvest) no GFP expression was observed in a hygromycin resistant transformant of A15 (no GFP cassette, leftmost mushroom), while GFP expression was clearly detected in the stipe tissue of SPR::GFP transformant TP196 (rightmost mushroom).

Fig 2. GFP activity in senescing *A. bisporus* sporophores. Metered readings (relative fluorescence units) are presented for cap and stipe tissues of whole and longitudinally bisected mushrooms 3 days post harvest. GFP activity was substantially elevated in the stipes of senescing mushrooms for the SPR::GFP transformant TP196 compared with the control transformants, GPD::GFP (G26) and A15hph (no GFP cassette).

Fig 3. Proteinase profiles in *C. cinerea*. A: Proteinase plate assay of *C. cinerea*. 7mm plugs of *C. cinerea* LT2 were inoculated onto RA and ammonium free RA plates containing 0.5% (w/v) milk solution. Proteinase production was measured by the

clearing zone produced around the colony. B: Expression of serine proteinase activity in culture filtrates during growth of *C. cinerea* LT2 in RA and ammonium free RA containing 0.5% (w/v) milk solution. Cultures were grown for 264 h and samples (8 ml) were aseptically removed every 24 h and assayed using the synthetic peptide Suc-Ala-Ala-Pro-Phe-pNA. C: Proteinase activity during *C. cinerea* AmutBmut sporophore development as determined using the Suc-Ala-Ala-Pro-Phe-pNA substrate in the presence or absence of inhibitor.

Fig 4. A: Expression of GFP in *C. cinerea* T47 monokaryon and dikaryon on ammonia free regeneration agar containing 0.094% (w/v) humic fraction and on standard regeneration media (RA) viewed under phase contrast (PC) microscopy and UV light. Mycelia on actively growing plates were examined microscopically using 40× objective on a Leitz Dialux 20 research microscope with excitation filters at 450–490 nm, dichroic filter at 510 nm, and emission filter at 515 nm. Images clearly show GFP fluorescence in both TP47 monokaryon and dikaryon grown on humic fraction while no fluorescence was observed in transformants grown on RA. **B:** Expression of GFP in the *C. cinerea* developing fruiting body. Fruiting was induced in the dikaryon *C. cinerea* TP24 mated with AT8 and GFP fluorescence was monitored in the hyphal knot, primordium, karyogamy, meiosis, immature, mature and autolysis stages of development. Fruiting was induced in *C. cinerea* AmutBmut and fruiting body stages were also screened for GFP expression as a control. Samples were viewed under phase contrast (PC) microscopy and UV light. **C:** Schematic illustration of GFP fluorescence under the control of the *A. bisporus SPR1* promoter through out the *C. cinerea* life cycle.

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Table 1.

Strain		Media						
		YMG	RA	MMP	PDA	Humic fraction	GA	Milk
A. bisporus	17	-	-	-	+	+	-	-
	18	-	-	-	-	+	-	-
	19	-	-	-	+	+	+	-
	21	-	-	-	+	+	+	+
	22	-	-	-	+	+	-	+
	23	-	-	-	+	+	+	+
	119	-	-	-	+	+	-	+
	120	-	-	-	+	+	+	+
	121	-	-	-	+	+	+	+
	A15	-	-	-	-	-	-	-
	G26	+	+	+	+	+	+	+
C. cinereus monokaryon	1	-	-	ND	+	+	+	+
	24	-	-	ND	+	+	+	+
	37	-	-	ND	+	+	+	+
	47	+	-	ND	+	+	+	+
	LT2	-	-	ND	-	-	-	-
	PG78Gr	+	+	ND	+	+	+	+
C. cinereus dikaryon	1	-	-	ND	+	+	+	+
	24	-	-	ND	+	+	+	+
	37	-	-	ND	-	+	+	+
	47	+	-	ND	+	+	+	+

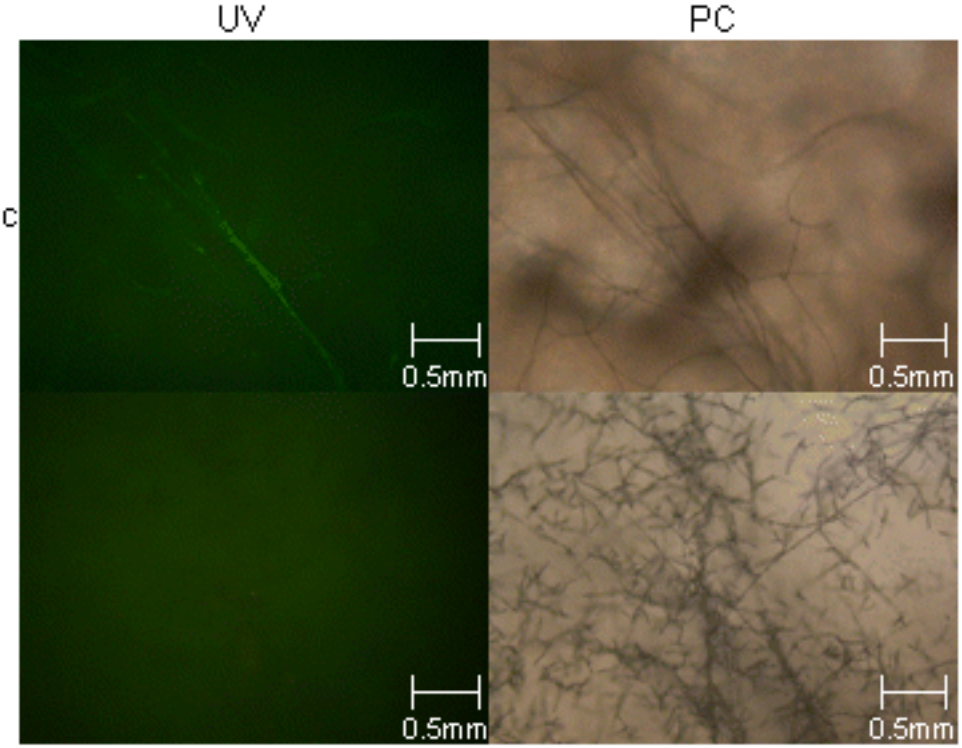
Table 2.

Gene	Length (aa)	Predicted Mol Wt kDa	Predicted PI	Signal peptide	Cleavage point	Introns	Secondary structure			Regulatory sequences in promoter	
							% helices	% strands	% Loops	Nit 2/AreA	CreA
Spr1	387	39.39	5.93	+	19/20	nd	20.41	24.81	54.78	9	1
Spr2	377	38.85	5.53	+	19/20	11	29.44	24.93	45.62	9	1
CC1G_04562.1	394	40.093	7.75	+	20/21	14	27.99	24.94	47.07	6	2
CC1G_10592.1	387	39.332	9.69	+	21/22	12	36.53	21.24	42.23	10	1
CC1G_10615.1	394	40.193	9.97	+	21/22	6	30.53	20.61	48.85	13	3
CC1G_07792.1	395	40.683	6.73	+	20/21	13	28.43	22.84	48.73	7	3
CC1G_10606.1	346	35.359	5.83	+	19/20	13	21.16	35.07	43.77	7	4
CC1G_0.3122.1	421	44.354	7.55	+	20/21	12	20.71	24.29	55.00	4	1
CC1G_04470.1	500	53.18	6.84	+	21/22	2	22.24	16.63	61.12	6	8

Table 3.

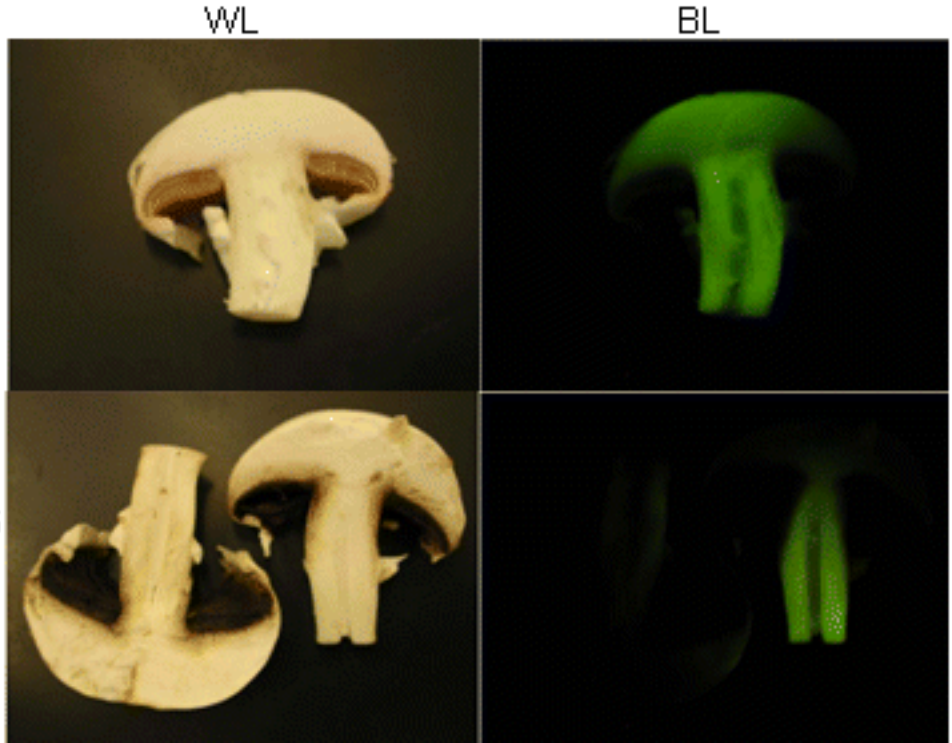
Gene	***Spr1***	***Spr2***	**04562.1**	**10592.1**	**10615.1**	**07792.1**	**10606.1**	**0.3122.1**	**04470.1**
Spr1	100								
Spr2	75	100							
04562.1	61	55	100						
10592.1	57	54	66	100					
10615.1	55	53	62	77	100				
07792.1	55	50	70	58	56	100			
10606.1	47	48	50	54	51	47	100		
0.3122.1	46	45	43	45	41	40	40	100	
04470.1	44	42	41	39	40	39	35	31	100

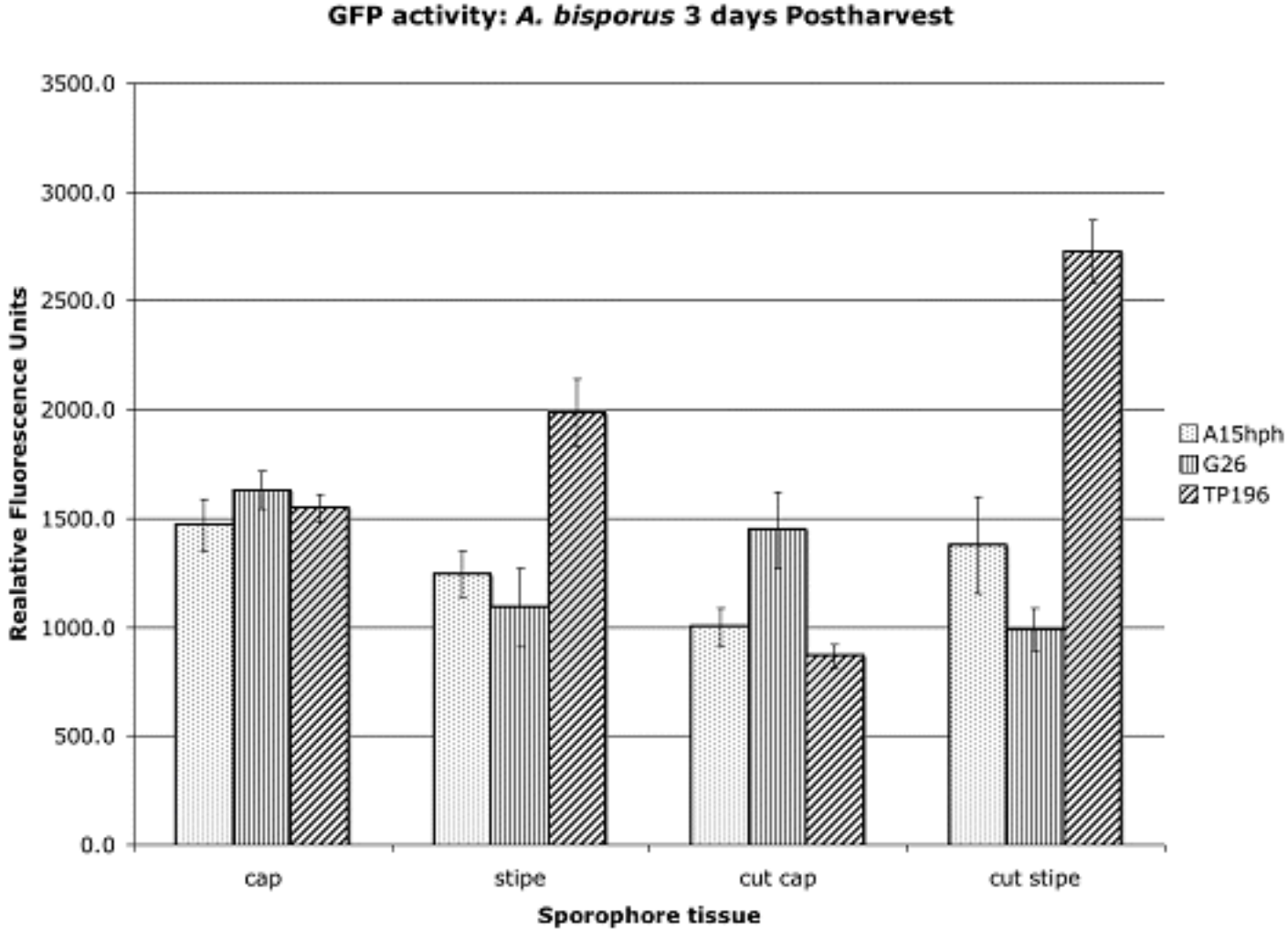
A:

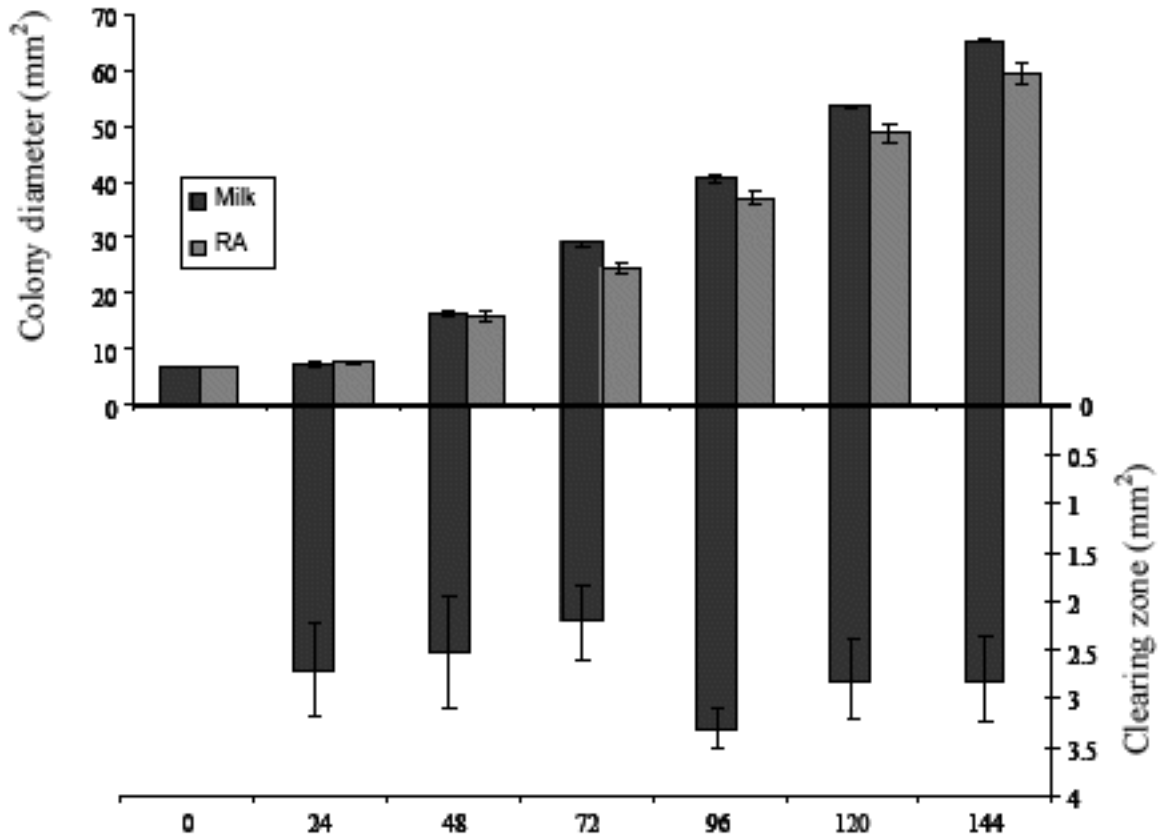

UV
PC
A. bisporus TP17 on humic fraction
A. bisporus TP17 on RA
0.5mm
0.5mm
0.5mm
0.5mm

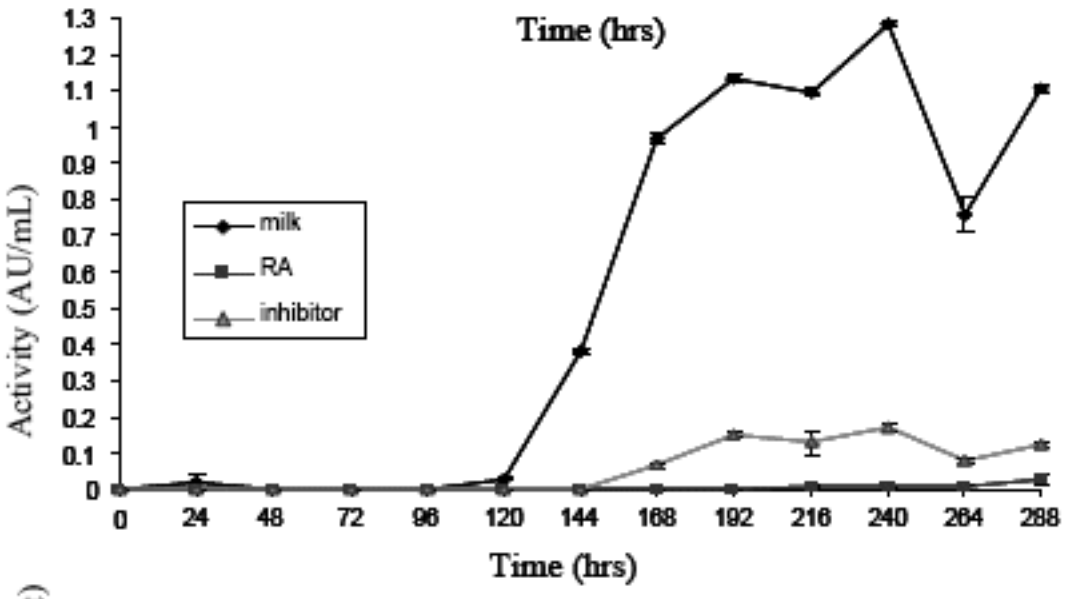
B:

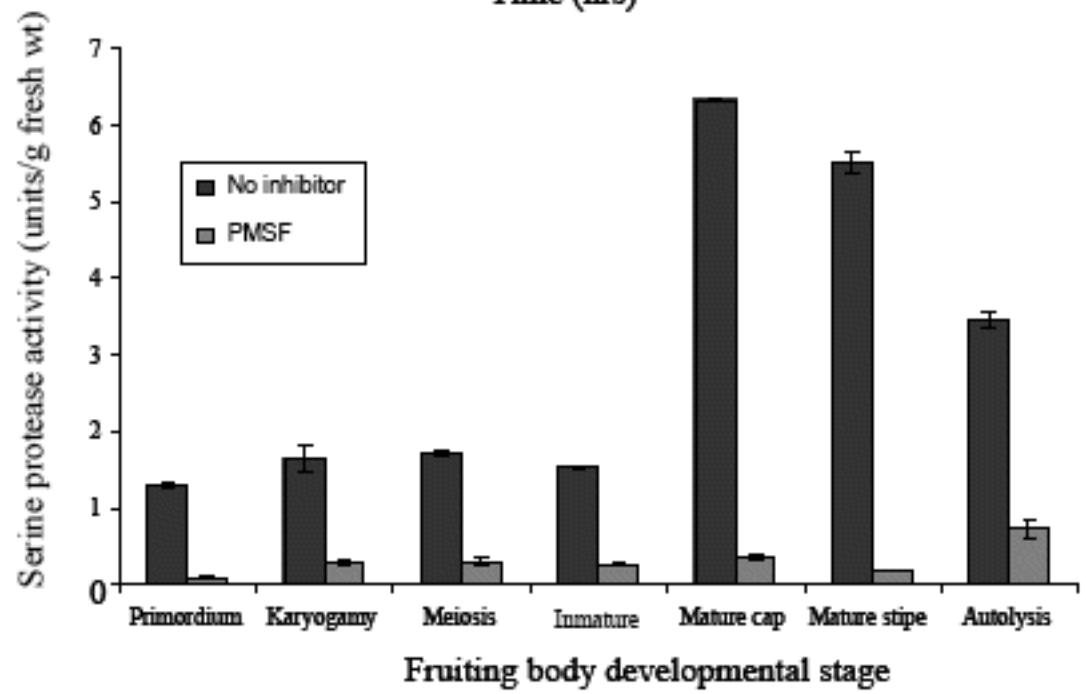
Panel

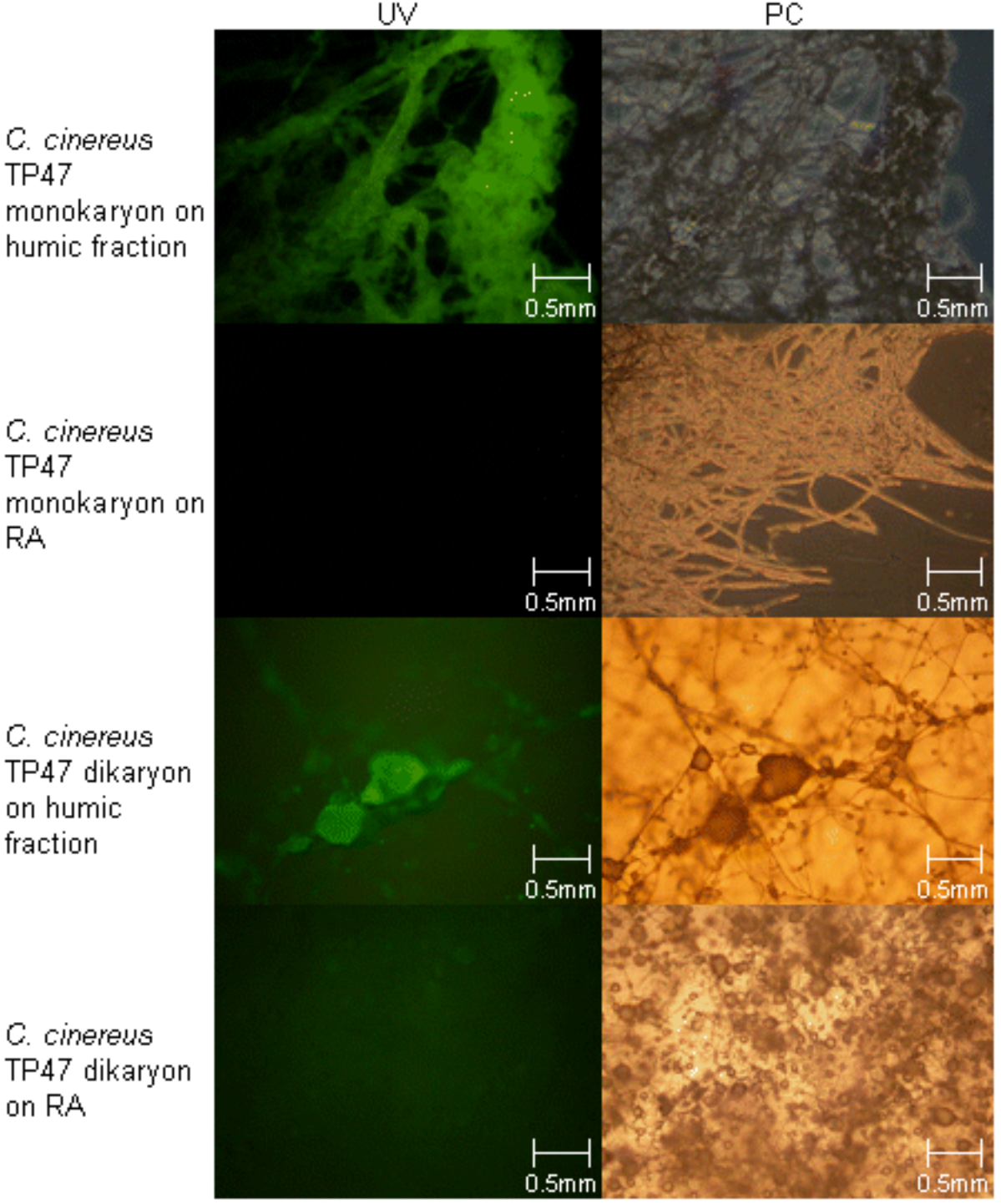

WL
BL
A : A. bisporus G26
B: A. bisporus G26 (left) and TP196 (right)

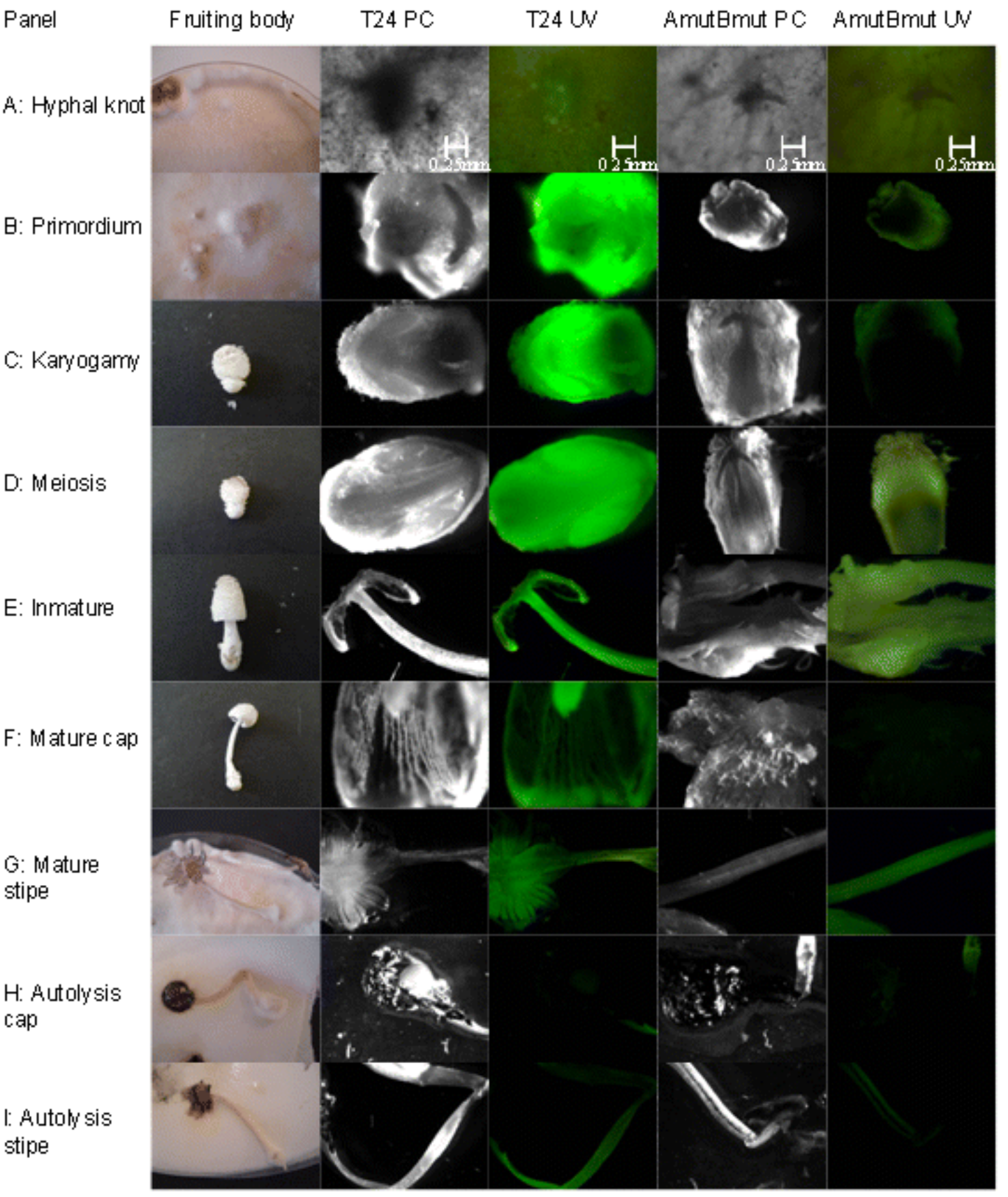

GFP activity: A. bisporus 3 days Postharvest
3500.0
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
Realative Fluorescence Units
A15hph
G26
TP196
cap
stipe
cut cap
cut stipe
Sporophore tissue

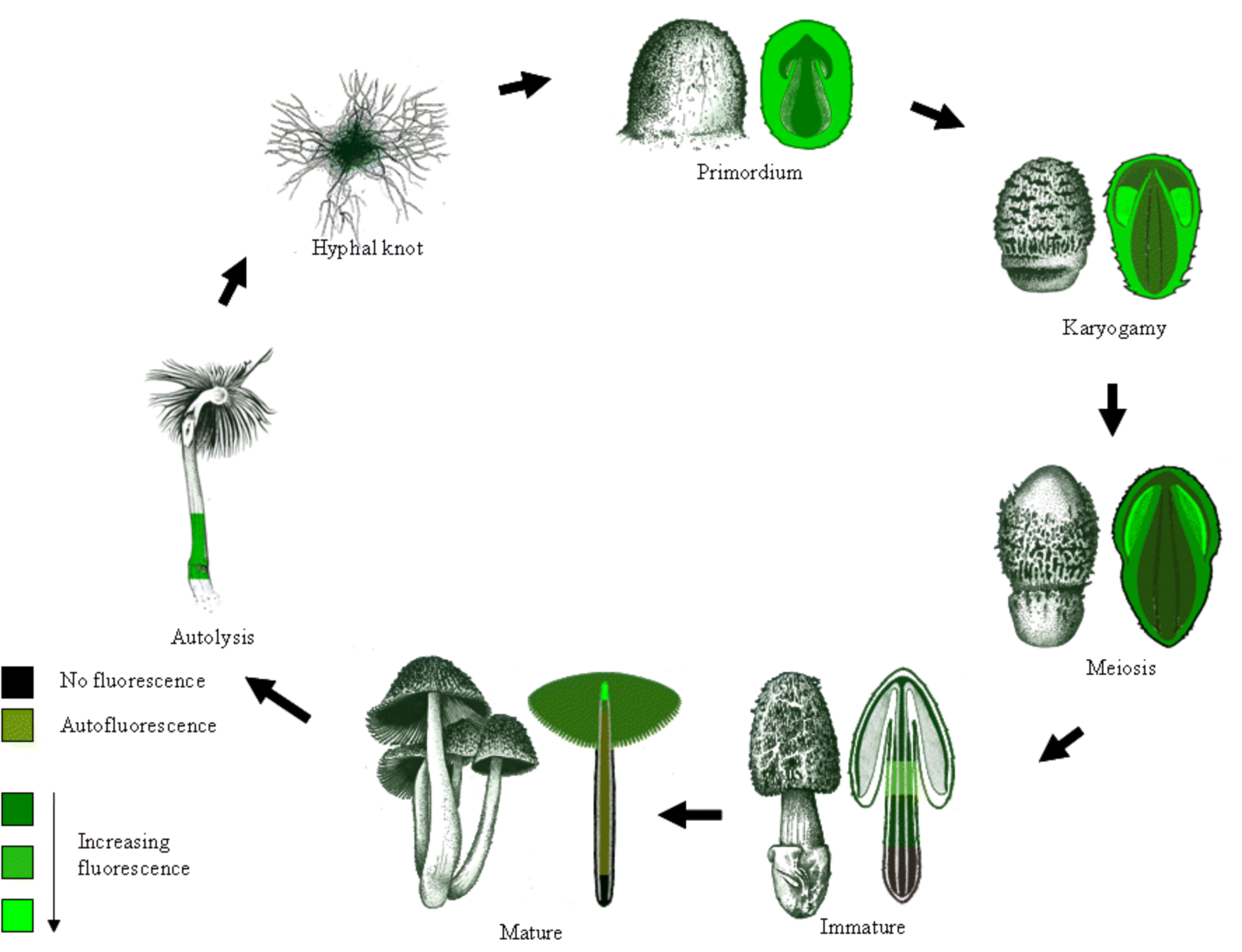

Colony diameter (mm²)
Milk
RA
Clearing zone (mm²)
0
24
48
72
96
120
144
Time (hrs)


Activity (AU/mL)
milk
RA
inhibitor
0
24
48
72
96
120
144
168
192
216
240
264
288
Time (hrs)


Serine protease activity (units/g fresh wt)
No inhibitor
PMSF
Primordium
Karyogamy
Meiosis
Immature
Mature cap
Mature stipe
Autolysis
Fruiting body developmental stage


UV
PC
C. cinereus TP47 monokaryon on humic fraction
C. cinereus TP47 monokaryon on RA
C. cinereus TP47 dikaryon on humic fraction
C. cinereus TP47 dikaryon on RA
0.5mm
0.5mm
0.5mm
0.5mm
0.5mm
0.5mm
0.5mm
0.5mm


Panel
Fruiting body
T24 PC
T24 UV
AmutBmut PC
AmutBmut UV
A: Hyphal knot
B: Primordium
C: Karyogamy
D: Meiosis
E: Inmature
F: Mature cap
G: Mature stipe
H: Autolysis cap
I: Autolysis stipe
0.25mm


Hyphal knot
Primordium
Karyogamy
Meiosis
Immature
Mature
Autolysis
No fluorescence
Autofluorescence
Increasing fluorescence