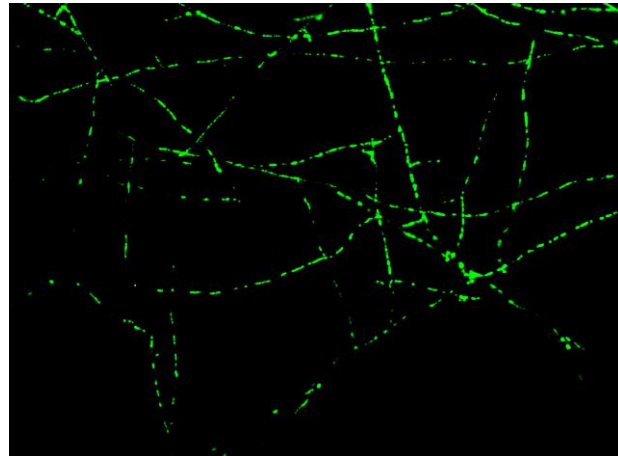


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Mushroom viruses and *Agaricus* genomics



Key external stakeholders:

Mushroom breeders, mushroom scientists, genomics community, mushroom industry,

Practical implications for stakeholders:

New mushroom viruses have been identified in commercial mushroom crops, some of which reduce the quality and saleability of the produce, such as *Agaricus bisporus* Virus 16 (AbV16) of the Mushroom Virus X (MVX) complex. Little is known of how viruses interact with the mushroom to cause detrimental effects or how they are transmitted within and between crops.

- This project has increased our understanding at a genetic level of how mushroom strains respond to each other and to virus infection. This information will enable mushroom breeders to identify interesting incompatibility and anti-viral traits to incorporate into new strains.
- The knowledge that different mushroom viruses can be present in different locations within the mycelium, and at different intensities, sheds light on the complexity of viral dynamics within the mushroom. This may explain the complexity of symptom expression in virus-infected crops.

Main results:

- Unique proteomic responses were detected between co-cultures of different interacting strains of *A. bisporus*, enhancing our understanding of potential barriers to viral transmission through vegetative incompatibility
- MVX was transmitted successfully into the mycelium of five different *A. bisporus* strains, although less successfully into fruitbodies in some strains. Distinct transcriptomic and proteomic responses to infection were recorded for different strains and times of inoculation.
- The AbV16 (see above image) and AbV6 viruses are located in different areas of the mycelial network and can be visualised using a fluorescent *in-situ* hybridisation (FISH) technique.
- The genome of a wild *A. bisporus* strain ARP23 was sequenced, assembled and annotated. It is 33.49 Mb in length and has 13,030 genes, the largest *A. bisporus* strain sequenced to date.

Opportunity / Benefit:

All mushroom breeders have access to the data generated by this project through peer reviewed publications. The availability of a fully annotated genome of a wild *Agaricus bisporus* strain is the starting point for more detailed molecular analyses into the symptomatic responses of *A. bisporus* strains when challenged with MVX.

Collaborating Institutions:

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Mushroom Industry Stakeholder Committee

1. Project background:

The commercial mushroom, *Agaricus bisporus*, is susceptible to a disease caused by a complex of viruses known collectively as mushroom virus X (MVX). Symptoms of MVX include poor quality mushrooms and mushroom cap discolouration (browning), which are correlated with the *A. bisporus* Virus 16 or 'AbV16'. The AbV6 virus is also associated with virus outbreaks but has not been directly correlated with distinct symptoms although it was one of the viruses regularly detected in virus-infected crops in the early 2000's in the UK. Most modern-day commercial varieties of *A. bisporus* are 'mid-range hybrids' and are almost identical genetically, having been derived from a hybrid cross between two strains in the 1980s. One major disadvantage of this lack of genetic diversity is the universal susceptibility of mushroom crops worldwide to the same pathogens, including viruses. Furthermore, the increased internationalisation of mushroom businesses means that the transmission and spread of viruses is facilitated by the transportation of substrates and mushrooms, not only across land borders but also across continents. Mushroom Virus X (MVX) infections continue to cause significant quality and yield losses to the Irish mushroom industry and worldwide. In the past the *Agaricus* species *A. bitorquis* was used as a 'virus breaker' strain as it was incompatible with *A. bisporus* and so virus transmission was halted when this strain was grown. This species is no longer commercially acceptable but an *A. bisporus* strain that is commercially acceptable but incompatible with the current dominant commercial strains could potentially work as a virus breaker. There is a lack of knowledge on how *A. bisporus* strains respond to virus infection at a genomic and proteomic level. The main aim of this project was to characterise, at a proteomic level, the interactions between a number of *A. bisporus* strains with different vegetative compatibilities as well as their antiviral responses. At the same time, we wanted to try and visualize where viruses were located in the mycelium and also to fully sequence a wild *A. bisporus* strain. The results will add to the *A. bisporus* knowledge base for this important crop species, identifying useful traits for future strain development endeavours.

2. Questions addressed by the project:

- What is the proteomic response of interhyphal interactions between different strains of *A. bisporus* in co-cultures in terms of their vegetative incompatibility?
- What is the transcriptomic and proteomic response of different *A. bisporus* strains to infection with MVX in general and to AbV16 in particular?
- Where are the AbV16 and AbV6 viruses located within a living mycelial network and can they be visualised using a fluorescent *in-situ* hybridisation (FISH) technique?
- What is the genome of wild *A. bisporus* strain ARP23 and how does it differ to the genome of other sequenced *A. bisporus* strains?

3. The experimental studies:

Five strains of *A. bisporus* were used in these studies (Strains A, B, C, D & E). Strain D was a modern commercial mid-range hybrid and Strains A, B, C & E were considered to be not very compatible with Strain D. Strain E was a wild strain 'ARP23' from the *Agaricus* Resource Program (ARP) collection. All strains were obtained from Sylvan Inc., France.

Compatibility interaction studies: Three strains of *A. bisporus* were used in this study, Strains C, D & E. Co-cultures of different strains were set up with two agar plugs placed either end of a Petri dish, then grown for two weeks at 25°C to allow enough time for ample hyphal interactions to occur. Proteins were extracted from mycelial tissue and identified using LC-MS/MS. Comparative quantitative proteomics was carried out with particular focus on proteins which were statistically differentially abundant (SSDA; $P < 0.05$, fold change ≥ 1.5) between pairwise comparisons of samples.

Virus-*Agaricus* interaction studies: All five strains of *A. bisporus* were used in this study. The strains ('acceptors') were grown in dual culture with strain MVX 1153 ('donor'), a culture which had been isolated in October 2016 from commercial mushrooms showing brown-cap disease symptoms. Presence or absence of the AbV16 virus in the donor and acceptor cultures was determined by RTPCR once hyphal interactions had taken place between the dual cultures (Day 0), and again 6 and 12 days later. *In vivo* crop inoculation studies were done with the five strains, with each strain inoculated with MVX 1153 at either crop-spawning or crop-casing time. Harvested mushrooms were tested for presence of AbV16 by RTPCR. Mushrooms for strains C, D and E were used for transcriptomic and proteomic analyses.

FISH studies. Cultures of *A. bisporus* strains, with and without MVX viruses AbV6 and AbV16, were grown on thin layers of agar on sterile microscope slides in a humid chamber. Hyphae were soaked in permeabilisation buffer to facilitate the entry of the virus-specific probes, designed to target AbV16 and AbV6, and labelled at the 5'-end with fluorescein isothiocyanate (FITC). After hybridisation, slides were prepared for fluorescence light microscopy and photographed.

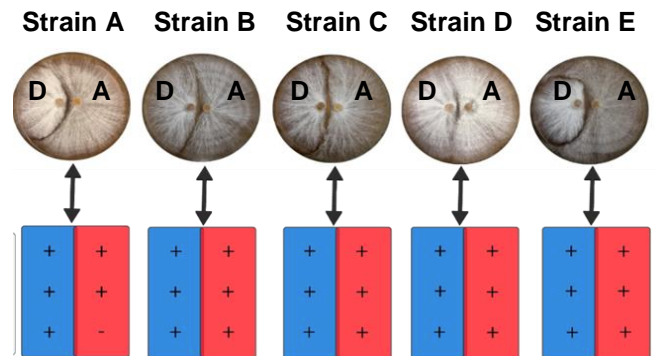
Genome sequencing. DNA was isolated from fungal mycelium of ARP23 homokaryons with the Wizard Genomic DNA Purification Kit (Promega). DNA library construction and sequencing on the Pacbio (RSII) and Illumina (HiSeq 4000) platforms was carried out by BGI Tech Solutions Co., Ltd. (Hong Kong, China). Synteny between the genome assemblies of ARP23, and those for *A. bisporus* JB137-S8 and H97, was assessed and visualized. Similarities and gross genomic differences were recorded.

4. Main results:

The work presented here provides novel genomic, transcriptomic, proteomic and cellular resources to help address the gaps in knowledge concerning MVX infection of *A. bisporus*.

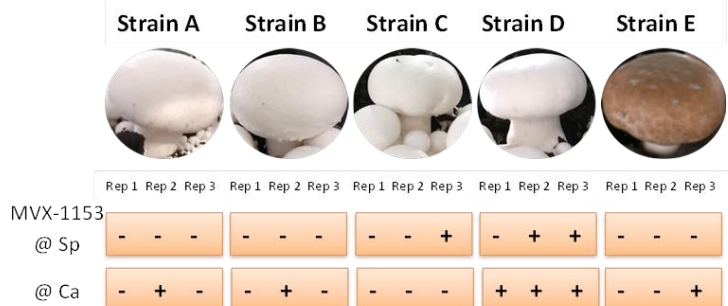
Anastomosis, compatibility and virus transmission: Our results show that anastomosis (hyphal fusion) occurs readily *in-vitro* between a standard commercial strain and all *A. bisporus* strains tested, despite different levels of vegetative compatibility. This allowed transmission of the AbV16 virus into the mycelium of all strains quite readily (Fig 1). However, under cropping conditions AbV16 was less effectively transmitted to Strains A, B, C and E, with very few mushrooms testing positive for the virus. Nonetheless, positive results for AbV16 were obtained for some mushroom samples for all strains, indicating that transmission had taken place (Fig 2). Thus, virus transmission between different *A. bisporus* strains, irrespective of their compatibility status would appear to be difficult to prevent. An *A. bisporus* 'virus breaker' strain may therefore be quite a challenge to develop.

Fig 1: Experimental set up for *in vitro* MVX transmission experiment between MVX-1153 'donor' culture ('D' on the left in dual cultures) and five different *A. bisporus* 'acceptor' strains ('A' on the right in dual cultures);



and RTPCR results for presence/absence of AbV16 (+/-) in subcultures taken on three occasions: the day of hyphal interaction and after a further 6 and 12 days.

Fig 2: RTPCR results for presence/absence of AbV16 (+/-) in mushrooms from crops of different strains of *A. bisporus* inoculated with MVX 1153 at spawning time (@ Sp) or at casing time (@ Ca).



Proteomics of inter-strain interactions: Unique proteomic responses were detected between co-cultures of the different *A. bisporus* strains. An array of cell-wall modifying enzymes, plus fungal growth and morphogenesis proteins were found in significantly ($P < 0.05$) altered abundances. Nitrogen metabolism dominated the intracellular proteome, with evidence of nitrogen starvation between competing, non-compatible cultures. Changes in key enzymes of *A. bisporus* morphogenesis were observed, particularly via

increased abundance of glucanoyltransferase in competing interactions and certain chitinases in vegetative compatible interactions only. Carbohydrate-active enzymes were expanded in antagonistic interactions. **Transcriptomic and proteomic responses to virus infection:** Virus infection also induces distinct transcriptomic and proteomic responses in the different strains. In the commercial Strain D, which was most susceptible to infection, this was reflected in cellular stress responses, manipulation of gene expression and myo-inositol biosynthesis, which is reported as a marker for mechanical damage. Strain C fruit bodies were less susceptible to MVX and demonstrated a variety of antiviral activity when AbV16 was detected, including high levels of ribonucleases, antiviral proteins, stress responses and autophagy. Strain E, though almost as unsusceptible to MVX as Strain C, mounted an entirely different transcriptomic and proteomic response. Antiviral strategies of this strain amounted to significant down regulation of gene expression. New insights into the differential response of interacting strains of *A. bisporus* will enhance our understanding of potential barriers to viral transmission through vegetative incompatibility. Our results suggest that a differential proteomic response occurs between *A. bisporus* at strain-level and findings from this work may guide future proteomic investigation of fungal anastomosis.

Visualisation of viruses by FISH: A Fluorescence *In-Situ* Hybridisation (FISH) method was successfully developed for *in situ* visualisation of the AbV16 and AbV6 viruses within *A. bisporus* mycelium. Fluorescently-labelled AbV16 showed high signal intensities within reticulating networks of hyphae in a highly compartmentalised manner (See main image on Page 1). Results indicate that FISH may be more sensitive at detecting viruses at low levels, compared to current molecular methods.

Wild *A. bisporus* genome. The whole genome of *A. bisporus* ARP23 was assembled and the final genome was found to be 33.49 Mb in length and had significant levels of synteny to other sequenced *A. bisporus* strains. Overall, 13,030 genes were annotated. Relative to other *A. bisporus* genome sequences, *A. bisporus* ARP23 is the largest in terms of gene number and genome size

5. Opportunity/Benefit:

The proteomic, transcriptomic and genome sequence data that have been generated in this project, and disseminated in peer-review publications, are the starting point for more detailed molecular analyses into understanding, and perhaps eventually eradicating, the undesirable symptoms manifesting in *A. bisporus* strains when infected with pathogenic viruses. This project has increased our understanding at a genetic level of how mushroom strains respond to each other and to virus infection. This information will enable mushroom breeders to identify interesting incompatibility and anti-viral traits that could be incorporated into new strains that offer greater protection against virus diseases.

6. Dissemination:

The information from this project will be incorporated into the advice currently given to the mushroom industry on a one to one basis and via workshops on how to control outbreaks of mushroom viruses in crops. Technical articles for popular publications are in progress. Three peer-review publications are already published/in press and a fourth is in progress.

Main publications:

- O'Connor E, McGowan J, McCarthy CGP, Amini A, Grogan H, Fitzpatrick DA. (2019) Whole Genome Sequence of the Commercially Relevant Mushroom Strain *Agaricus bisporus* var. *bisporus* ARP23. G3 (Bethesda, Md.). 2019 Oct;9(10):3057-3066. DOI: 10.1534/g3.119.400563. <https://www.g3journal.org/content/9/10/3057>
- O'Connor E, Owens RA, Doyle S, Amini A, Grogan H, Fitzpatrick DA. (2020) Proteomic investigation of interhyphal interactions between strains of *Agaricus bisporus*. **In press** Fungal Biology. <https://doi.org/10.1016/j.funbio.2020.02.011>
- O'Connor E, Coates CJ, Eastwood DC, Fitzpatrick DA, Grogan H. (2020). FISHing in fungi: Visualisation of mushroom virus X in the mycelium of *Agaricus bisporus* by fluorescence in situ hybridisation. **In press** Journal of Microbiological Methods.

Popular publications:

Technical articles that summarise the results presented here are in progress for publication in popular publications such as The Mushroom People, Mushroom Business and TRResearch.

7. **Compiled by:** Dr. Helen Grogan, Dr. David Fitzpatrick and Mr. Eoin O Connor.