MUSHROOM VIRUS X – THE IDENTIFICATION OF BROWN CAP MUSHROOM VIRUS AND A NEW HIGHLY SENSITIVE DIAGNOSTIC TEST FOR PHASE III COMPOST

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ABSTRACT

Mushroom Virus X (MVX) first observed in 1990 causes a range of yield and quality symptoms and is associated with double-stranded RNA (ds-RNA). Evidence is accumulating that MVX is caused by a collection of different viruses. Previous research has centred largely on ds-RNAs; this paper presents research based on transcript changes during MVX infection.

Suppression subtractive hybridization and microarrays identified 25 up-regulated and 32 down-regulated genes during MVX infection. Sixteen highly up-regulated transcripts were absent from the *A. bisporus* genome and hybridize to a subset of MVX ds-RNA elements and so are inferred to be viral. It is hypothesized that the mushroom cap browning symptom is caused by a distinct *Partitivirus*-like virus of two ds-RNAs, tentatively named Brown Cap Mushroom Virus (BCMV).

A second project was aimed at developing a highly sensitive detection method for MVX at low levels in compost, casing, and potential sources of infection. The current test based on separation of ds-RNAs on gels is insensitive requiring whole mushroom fruitbodies. All known MVX and mushroom genes were screened using microarrays and Quantitative PCR to identify the best indicator genes. This resulted in a combined PCR test based on two sequences which detects both forms of MVX (browning and pinning disruption symptom) at low levels in compost.

These quantitative results revealed that small amounts of early infection leads to 100-fold increases of transcript levels in the compost during cropping. Viral transcript levels were lower in the casing indicating low viral activity compared with compost.

Keywords: MVX; ds-RNA; Virus; Diagnostic test; Transcription

INTRODUCTION

The Mushroom Virus X (MVX) syndrome has been present in the industry for at least 20 years and reached epidemic levels some 5-6 years later. It was documented in 2000 by Gaze [1] who described the spread of the disease from 1996. However Gaze has since found earlier detailed notes of the description of disease symptoms which accurately match those of MVX from August 1990 (Richard Gaze, personal communication). Gaze observed that the symptoms were found on shelves, were specific to bulk compost and that similar symptoms had been observed previously. MVX disease causes a diverse a range of symptoms, including pinning disruption, crop delay, premature veil opening, various fruitbody abnormalities, and discoloured mushroom

caps, ranging from off-white to brown (which for the sake of clarity will be referred to here as the brown symptom). These effects on yield and quality have resulted in economic difficulties and even farm closures. The disease symptoms differ by geographical location – the 'patch' problem (discrete areas without mushrooms) more prevalent in the UK while browning symptoms are seen more in Ireland, Belgium, The Netherlands and Poland. This suggests that the term "MVX" is a catch-all term to describe more than one disease with uncharacterised causal agents of unknown origin.

The association between MVX disease and double-stranded RNA (ds-RNA) led to the hypothesis that a novel ds-RNA virus may be the cause of MVX disease however viral particles could not be observed by electron microscopy [1], [2]. Grogan identified 26 ds-RNA bands separated by gel electrophoresis with different banding patterns occurring in different samples [2]. The maximum number of ds-RNA bands from a single sample was 17. Two research groups found an association between the four low molecular weight ds-RNA bands (0.6- 2 kbp) and the browning symptom but no obvious correlation between the banding patterns and other symptoms [2], [3]. The authors conclude that it was not possible to prove Koch's postulates and so it was not possible to prove that the disease was caused by the ds-RNA molecules [2]. As transmission electron microscopy failed to reveal viral particles consistently associated with the disease, it was concluded that the infective agents are ds-RNAs, non-encapsidated, naked genetic elements [2].

Rao *et al* proposed a bacterial explanation for the cause of the brown symptom of MVX disease [4]. These authors suggest that *Pseudomonas tolaasii* may be the cause for the symptom as this bacterial species was detected on the skin of diseased mushrooms. *P. tolaasii* is the causative organism of 'brown blotch' disease on mushrooms manifesting as discrete sunken lesions with brown discolouration. This bacterium produces an 18 amino acid peptide called tolaasin which forms pores in plasma membranes which allow mixing of the contents of intra-cellular compartments [5], [6]. Tissue browning caused by disease such as brown blotch disease, mechanical damage or senescence is the result of oxidation of phenolics by the enzymes tyrosinase which is subject to activation by proteinases. The phenolics, tyrosinase and proteinase are believed to be located in different cellular compartments and the tolaasin or mechanical damage and ageing allow these components to mix and so the browning reaction takes place [7]. However, the Rao hypothesis was questioned on the grounds that the symptoms of bacterial blotch disease caused by *P. tolaasii* and MVX disease are very different in several aspects and that the association between MVX disease symptoms and the presence of *P. tolaasii* is from a limited sample size [8].

It is now clear that the collection of MVX ds-RNAs represents a complex of different viruses which accounts for the diversity of symptoms. Sonnenberg and Lavrijssen found that the sequence of a portion of the 17 kbp band shared significant similarity with *Cryphonectria parasitica* hypovirus [3]. The 12.75 kbp band of MVX has also been sequenced and described as an Endornavirus, AbEV1 [9]. Strong associations were made between the 4 smaller ds-RNAs and the browning symptom, and it was speculated that these bands may represent a single virus [2], [3].

Transmission of the MVX viruses is thought to be via infected spores and/or infected mycelium and symptom expression from mycelial infection appears to depend on the time and degree of infection and type of the infecting mycelium [2], [10]. Hygiene measures involving these principles have resulted in some limited success in controlling the disease. However MVX still represents a largely uncharacterised disease with little known about the causative agents. The current detection method for MVX by visualizing gel separated double-stranded RNA bands has shed some light on the disease and its epidemiology. However the method has low sensitivity (requiring whole mushroom fruitbodies) and so it is too late in the cropping cycle to be commercially useful and is unable to identify low level sources of infection.

This paper describes two research projects aimed at further understanding of the biology of MVX by examination of the transcript changes (single-stranded RNA) produced during infection, and then using the findings to develop a highly sensitive Quantitative PCR test to detect MVX at low levels in compost.

MATERIALS AND METHODS

To examine the transcripts changes in the mushroom after MVX infection and to develop a high-sensitivity detection method, five molecular methodologies were employed.

Suppression Subtractive Hybridization (SSH). RNA was isolated from non-infected, and brown coloured MVX-infected mushroom fruitbodies (strain A15) using TRI reagent, and enriched for poly A m-RNA. Subtractive hybridisation was performed followed by suppressive PCR to enrich for differentially expressed transcripts. Comparisons were made between RNA from non-infected and infected fruitbodies in both directions to identify transcripts which increase and decrease upon infection. Each gene fragment putatively identified as differentially expressed by SSH was Sanger sequenced.

Custom Microarrays. Two custom microarray designs were used in these studies. The first of these (version 3) contained probes to 1,300 genes/transcripts identified from the SSH work (above), other research projects from the Burton research group (from initiation, diooxygenase and post-harvest studies) and 550 *Agaricus bisporus* genes from the EMBL data-base. Version 4 microarrays were based on the 60,000 probe format and consisted of probes to the above 1,300 genes (replicated 3 times) and the 10,438 Open Reading Frames ORFs, (5 oligo probes per ORF) identified from annotation of the *A. bisporus* genome sequencing project (public release in May 2010). The microarrays were manufactured by Agilent Technologies and the probes were 60-mer *in situ* synthesized on the array. RNA was isolated from mushroom samples, converted to cRNA, fluorescently-labeled and then hybridized to a microarray. To determine the amount of hybridization to each probe the arrays were laser scanned and the resulting images analysed using commercially available image analysis software. Comparisons between treatments were made and statistically analysed using GeneSpring software (Agilent Technologies).

For crop experiment one ('Identification of transcripts changing during MVX infection'): 3 batches of non-infected mushrooms were compared with infected mushrooms from 5 different commercial farms. Four sample replicates were used for each batch/treatment.

For experiment two ('Development of a PCR-based test to detect MVX infection in compost'): microarray screening examined 4 different developmental states of the mushroom (i.e. as fruitbodies and as mycelium growing in axenic culture, compost and casing) and 2 infection states (MVX infected and non-infected). Four replicates per treatment.

Quantitative reverse transcriptase-PCR (Q-PCR). RNA samples were DNase treated (RQ1 RNase free DNase, Promega) according to manufacturer's instructions. DNase treated samples then underwent reverse transcription using Superscript II (Invitrogen) reverse transcriptase according to manufacturer's instructions producing cDNA samples ready for PCR analysis.

Primers were designed, using Primer Express (Applied Biosystems), to specifically amplify sections of each of the identified bands/transcripts based on their previously established sequence. These primers were then optimised using Sybr Green for detection and the ABI 7900HT thermocycler (Applied Biosystems). Standard curves were run, which were assessed for linearity and efficiency to ensure accurate quantification.

Bioinformatics. Transcript sequences identified during the course of these experiments were compared with known sequences available on open access and proprietary data-bases. Comparisons were made with *A. bisporus* genome sequence after its public release in May 2010.
Autogradiograph Northern methodology is described in the text.

RESULTS AND DISCUSSION

Identification of transcripts changing during MVX infection

Suppression Subtractive Hybridization (SSH). SSH analysis successfully generated putative differentially expressed transcripts associated with MVX disease. These transcript fragments were sequenced and a total of 197 unique sequences were identified. Probes were designed to the sequences and incorporated onto the microarray.
Custom Microarrays. Changes in gene expression from infected mushroom from 5 different farms were compared with non-infected mushrooms. Statistical analysis of all the microarray data identified 25 transcripts up-regulated during MVX infection and 32 down-regulated during infection. Of the 25 up-regulated transcripts, 16 were found by comparison with the *A. bisporus* genome sequence to be not derived from the *A. bisporus* host and are therefore to be presumed viral. All 32 down-regulated transcripts were host genes.

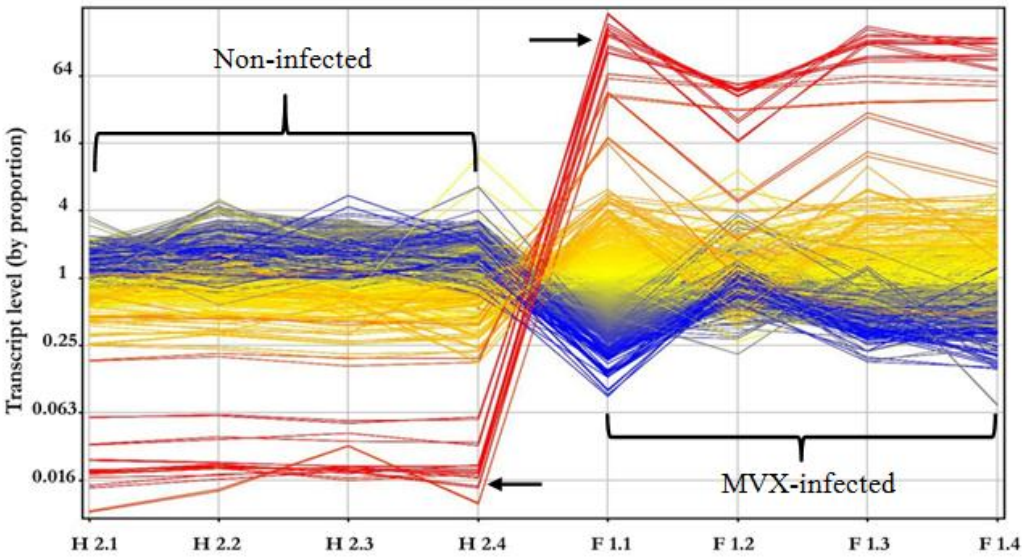

Figure 1: The expression of transcripts in non-infected and MVX-infected mushrooms determined by version 3 microarrays. Four separate non-infected samples were compared with four MVX-infected samples. Expression values are shown as normalised values relative to average expression. Y-axis is shown on $\log_2$ scale.

A typical microarray result is shown in Figure 1 which presents gene expression of four control (non-infected) mushrooms with four infected mushrooms from farm number one. The figure also demonstrates that most transcripts show little or no change in expression levels between infected and non-infected states. However there is a group of presumed viral transcripts (indicated with arrows in figure 1) which are at very low levels in non-infected mushrooms and very high levels in infected mushrooms. These up-regulated transcripts show average increases in infected fruitbodies from 150 to 4,000 fold and were found to accumulate to very high levels

in MVX-infected brown mushrooms and at lesser levels in white infected mushrooms. There appeared to be a correlation between the degree of tissue discoloration and the level of transcript.

Quantitative reverse transcriptase-PCR. The microarray analysis of the 10 transcripts showing the greatest change was confirmed independently by Q-PCR, however the fold change between non-infected and infected samples was much higher for the Q-PCR data compared with microarrays reflecting the greater dynamic range of PCR (Table 1).

Table 1: Comparison of the average fold-change values (MVX-infected relative to non-infected) of transcript levels between microarray and Quantitative PCR (Q-PCR) analyses

Viral transcript	Fold-change values	
	Microarray	Q-PCR
1	4,020	240,100
2	3,700	88,000
3	3,470	24,200
4	2,790	240,400
5	2,770	1,060,000
6	2,650	106,000
7	2,550	179,000
8	1,730	3,010,000
9	1,130	83,000
10	150	105,100

Autoradiograph Northern blots. Experiments were then performed to ascertain whether the 16 up-regulated and presumed viral transcripts originated from ds-RNA bands. Double stranded-RNA was extracted from a number of mushrooms showing a diversity of symptoms (to maximise the number of ds-RNA bands). The ds-RNAs were then gel separated in a number of identical tracks and blotted and fixed onto a membrane. Each track was then dissected out and separately hybridised with a radiolabelled probe specific to each of the 16 transcripts. The position of the probe was detected by autoradiography and compared against reference gel and markers.

All 16 probes hybridised onto ds-RNA bands as discrete bands. This strongly suggests that these transcripts are encoded by ds-RNA and so are presumably of viral origin. All of the hybridisations were to bands of size of 2 kbp (band 18) or less. These include the ds-RNA bands previously associated with tissue browning and 5 additional bands revealed by the greater sensitivity of radioactive detection used in autoradiography. These transcripts were originally identified from the SSH experiment comparing fruitbodies non-infected and infected with the browning form of MVX. Twelve of the 16 probes hybridised to two or more bands, indicating close sequence similarity between many of the bands and suggesting that the smaller ds-RNAs probably originate from the larger ones.

The hybridisation results suggest that the functional infective unit may be just the 2.0 and 1.8 kbp ds-RNAs (bands 18 and 19 respectively). These are of sufficient size to contain the coding potential required for independent existence. The lower molecular weight bands are likely to be satellite ds-RNAs which represent fragments of the 2.0 and 1.8 kpb molecules which are replicated with the 2.0 and 1.8 ds-RNAs but do not have any coding function [11]. Satellites can

be capable of modulating disease expression [12]. It is hypothesized that the 2.0 and 1.8 kbp molecules represent a single virus in the *Partitivirus* class and it is proposed that this should be named Brown Cap Mushroom Virus, BCMV, [13], Green *et al*, paper in preparation). Typically a *Partitivirus* has two ds-RNA components coding for an RNA-dependent RNA polymerase (RDRP) enzyme and the coat protein. Recently a virus which causes brown discoloration in *Flammulina velutipes* has been characterised by sequencing and named *F. velutipes* Browning Virus [14]. This virus consists of two ds-RNAs, 1.9 and 1.7 kbp encoding putative RDRP and coat proteins. Data-base comparisons of the limited sequences of the *A. bisporus* BCMV investigated in this study failed to find clear similarity to RDRP or coat protein. Full sequencing of all of the MVX bands is currently underway using Next Generation Sequencing technology.

Development of a PCR-based test to detect MVX infection in compost

All of the available sequences for *A .bisporus* and MVX were screened against non-infected and MVX infected samples to identify gene sequences which could be used in a PCR test to detect the presence of MVX in mycelia from a range of growth environments. Whole genome microarrays (version 4) were used for this experiment based on the 60,000 probe format. RNA was extracted from non-infected and MVX infected fruitbodies, compost and casing taken from farms and experimental crops and also from axenic (laboratory) culture. These were hybridised to microarrays and the results compared between non-infected and infected samples.

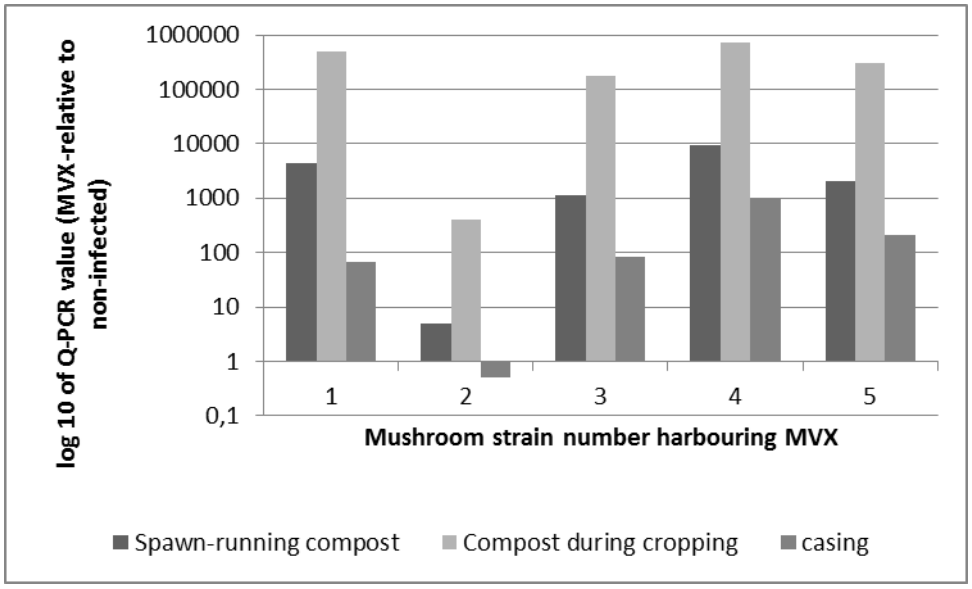

Figure 2: Bar chart showing transcript levels (Q-PCR data) of MVX-infected mycelia sampled from different growth environments: spawn-running compost, compost during cropping and casing. The data shown are the increase in Q-PCR signal relative to non-infected controls on a $\log_{10}$ scale. The five strains were originally collected from commercial farms; strains 1-3 cause the 'patch' symptom and strains 4 & 5 produce the browning symptom.

A number of probes showing at least 100-fold difference between control (non-infected) and MVX infected samples were identified: 97 probes in fruitbody samples; 102 probes in mycelium of colonised casing; 88 probes in mycelium of colonised compost; and 31 probes in mycelium grown in axenic culture. All but three of the identified probes related to non-*Agaricus* genome transcripts i.e. probably of MVX origin. The 3 genes of *Agaricus* origin were identified

from the compost samples. A selection was then made of 8 genes that showed the most consistent and significant up-regulation and the best 'spread' of detection across the range of MVX types examined.

PCR primers were designed to the 8 selected genes based on the full sequence known for each band or gene. Using these primer pairs Quantitative PCR reactions were carried out with the RNA extracted from samples of compost (during spawn-run and cropping) and casing of the 5 strains harbouring MVX and the control non-infected mushroom strain. The five strains used harbour different types of MVX, three strains causing the MVX 'patch' symptom (a discrete area with no mushroom production) and two strains producing the brown symptom.

A combined test of two quantitative PCR reactions (based on different primer pairs) was found to be successful in detecting all of the MVX forms that were investigated. The quantitative results of this project also show that small amounts of early infection at the time of spawning leads to massive increases (100 fold) in infection in the compost during cropping (Figure 2). This strongly infers that any amount of infection, no matter how small, is likely to lead to serious disease development. The transcript levels of the virus in the casing were consistently low suggesting that MVX does not thrive in the mycelium of casing and that compost is the better substrate for testing for MVX (Figure 2).

CONCLUSIONS

The hypothesis is made that the browning symptom of MVX is caused by a distinct virus consisting as a minimum of a 2.0 and 1.8 kbp ds-RNAs in the *Partitivirus* class. These ds-RNAs are actively transcribed to very high levels and satellites of these molecules are also present in infected tissues but it is unknown what effect these have on disease progression or symptom development.

A new diagnostic technique based on PCR, has been developed that can detect both forms of MVX (browning symptom and pinning disruption symptom) at low levels in spawn-run or Phase III compost. This test can be *predicative* to detect the presence of MVX in compost providing advanced warning to growers (i.e. before cropping), it can be used to identify the sources of infection and it could be used to certify compost as MVX-free.

This research is now focussed on fully sequencing all of the bands which will allow the viruses to be characterised and classified, and diagnostic tests will be developed for all ds-RNAs and viruses.

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