Mycoviruses mediate mycotoxin regulation in *Aspergillus ochraceus*

Running Title

Mycoviruses mediate mycotoxin regulation

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Competing Interests

The authors declare that they have no conflict of interest. This article does not contain any studies performed by any of the authors with human participants or animals.

Abstract

To date, no demonstration of a direct correlation between the presence of mycoviruses and the quantitative or qualitative modulation of mycotoxins has been shown. In our study, we transfected a virus-free ochratoxin A (OTA)-producing isolate of *Aspergillus ochraceus* with purified mycoviruses from a different *A. ochraceus* isolate and from *Penicillium aurantiogriseum*. Among the mycoviruses tested, only Aspergillus ochraceus virus (AoV), a partitivirus widespread in *A. ochraceus*, caused a specific interaction that led to an overproduction of OTA, which is regulated by the European Commission and is the second most important contaminant of food and feed commodities. Gene expression analysis failed to reveal a specific viral upregulation of the mRNA of genes considered to play a role in the OTA biosynthetic pathway. Furthermore, *AoOTApks1*, a polyketide synthase gene considered essential for OTA production, is surprisingly absent in the genome of our OTA-producing isolate. The possible biological and evolutionary implications of the mycoviral regulation of mycotoxin production are discussed.

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Originality-Significance Statement

We report the first evidence of differential mycotoxin accumulation associated with specific mycovirus infection. Ochratoxin A (OTA) is a mycotoxin produced by a number of *Penicillium spp.* and *Aspergillus spp.* and is considered the second most important contaminant in food and feed commodities after aflatoxin. The costs of maintaining OTA contamination below the legal threshold are considerable. OTA is regulated by the European Union, and the results presented by our work may be taken into account to update provisional models to predict OTA contamination. Furthermore, from an ecological point of view, we herein describe an interaction between a virus and its fungal host that suggests evolutionary advantages for virus-infected isolates. Our results support the recent interest in mutualistic virus-host interactions, as opposed to the classical binary host-pathogen relationship.

Introduction

Mycoviruses, widespread in all major fungal phyla, were first reported as a pathogen of cultivated mushrooms (i.e., *Agaricus bisporus*) (Hollings, 1962). Most reports have studied the ability of mycoviruses to reduce the virulence (i.e., hypovirulence) of pathogenic fungi (Nuss, 2010) and the broad spectrum of their biotechnological applications (Nuss, 2005; van de Sande et al., 2010; Ghabrial et al., 2015). Contrary to most bacterial viruses, mycoviruses do not cause lysis of the fungal host cell and accumulate to high levels without specific cytotoxic effects as persistent and often cryptic infections (Ghabrial et al., 2015). The idea that multi-level interactions between mycoviruses and fungi, involving molecular and biochemical targets able to modulate host behaviour to successfully spread and survive in the environment (Son et al., 2015) and provide adaptive advantages, is well-established (Mehle et al., 2012; Selman et al., 2012).

Many studies have tried to decipher the molecular mechanisms concerning mycovirus-induced hypovirulence using different approaches (Allen et al., 2003; Li et al., 2008; Cho et al., 2012). The model system for these studies is the ascomycetous fungus *Cryphonectria parasitica*, the causal agent of chestnut blight (Hepting, 1974). Many reports have demonstrated that infection by Cryphonectria hypovirus 1 (CHV1), a naturally occurring virus, leads to reduced virulence of the fungal pathogen (Elliston, 1985; Allen et al., 2003). In addition, this virus-induced phenotype has been exploited in Europe as a natural biocontrol agent against *C. parasitica* (Nuss, 1992). In the same fungus, we recently showed an increased osmotic stress tolerance when it was infected with Penicillium aurantiogriseum partiti-like virus 1 (PaPLV1) (Nerva et al., 2017b), highlighting a further level of complexity in the fungus-virus-environment interactions that needs elucidation. Despite the vast array of interesting features discovered in specific mycovirus-host combinations, the vast majority of mycoviruses appear to be cryptic, at least under laboratory conditions, based on phenotype and/or virulence evaluations (Pearson et al., 2009).

The fungal genus *Aspergillus* contains species of economic importance for industry, agriculture and medicine. The presence of mycoviruses infecting *Aspergillus* spp. was first demonstrated in 1970 for *A. foetidus* (Banks et al., 1970; Ratti and Buck, 1972), followed by a number of mycovirus reports in the *Aspergillus* sections *Nigri* (Varga et al., 1994), *Flavi* (Schmidt et al., 1986), *Circumdati* and *Fumigati* (Varga et al., 1998). Most of these studies were focused on viral epidemiology, whereas others addressed the possibility of using mycoviruses as biocontrol agents for the human and insect pathogenic fungal species *A. fumigatus* (Bhatti et al., 2011; Özkan and Coutts, 2015). Some *Aspergillus* species have been used also for food fermentation, taking advantage of their ability to improve food quality, as in the case of *A. oryzae* (Hong et al., 2004) and *A. sojae* (Matsushima et al., 2001). A major industrial species is *A. niger*, which is used to manufacture citric acid (Currie, 1917)

and numerous commercial enzymes (Bentley and Bennett, 2008). *A. terreus* is known for the production of the statin lovastatin, a cholesterol-lowering agent used for the treatment of hypercholesterolemia (Alberts, 1988). Conversely, there are also species with negative impacts on human and animal health. For instance, many *Aspergillus* spp. are common contaminants of agricultural crops; these species are involved in food and feed decay and often produce metabolites highly toxic and carcinogenic to mammals, known as mycotoxins. Among these species, *A. flavus* and some other *Aspergillus* spp. produce aflatoxin B1 (Davis et al., 1966), one of the main natural carcinogenic substances threatening human and livestock health (Eaton and Gallagher, 1994). Another important mycotoxin-producing species is *Aspergillus ochraceus*, which is able to produce ochratoxin A (Harris and Mantle, 2001) (OTA, a dihydrocoumarin moiety amide linked to a molecule of L-β-phenylalanine derived from the shikimic acid pathway), a contaminant regulated by the European Commission for food and feed commodities (EC1881, 2006). OTA has teratogenic, embryotoxic, genotoxic, neurotoxic, immunosuppressive and carcinogenic properties (IARC group 2B), in addition to nephrotoxic effects (FAO/WHO, 2001). OTA contamination can also have a huge economic impact: a Canadian study estimated that if OTA residues would be considered a food contaminant, the potential cost for Canadian food producers could reach 240 million dollars per year (Wu et al., 2014). *A. ochraceus* was described for the first time by the German botanist and mycologist Karl Adolf Wilhelm in 1877. *A. ochraceus* is an ubiquitous fungus usually described as a soil inhabitant (Domsch et al., 1980) but has also been reported to live in the marine environment (Cui et al., 2010). *A. ochraceus* was exploited for the industrial production of xylanase, β-xylosidase, and some antibacterial molecules able to inhibit human pathogens (Meenupriya and Thangaraj, 2011; Michelin et al., 2012). Moreover, *A. ochraceus* is one of the main contaminants of grapes and coffee and its derivatives in warm climates (Patiño et al., 2005). *A. ochraceus* is able to grow and synthesise OTA at a low water activity level (Pardo et al., 2004).

Mycoviruses infecting *Aspergilli* have been studied extensively because of the medical, economic and ecological importance of this fungal genus. Several studies have focused on hypovirulence traits for a possible biotechnological exploitation against the human, animal and insect pathogen *A. fumigatus* (Kotta-Loizou and Coutts, 2017) without success. Additionally, several studies attempted to identify mycovirus-mediated mechanisms involved in the qualitative or quantitative production of aflatoxins in *A. flavus*. Initially, a possible correlation between the presence of a Chrysovirus and the production of aflatoxins in *A. flavus* NRRL5565 (Schmidt et al., 1986) was observed but was not confirmed in the same or different virus-host systems (Elias and Cotty, 1996; Silva et al., 2001; Kotta-Loizou and Coutts, 2017).

Here, we decided to investigate the possibility of a metabolic shift in the presence of different mycoviruses in *A. ochraceus*. In the present work, two different isolates of *A. ochraceus* were selected from the *Mycotheca Universitatis Taurinensis*: MUT2036 and MUT2096. These fungi were isolated from the same ecological niche and are endophytes of *Holoturia polii* (also known as sea cucumber), a marine invertebrate widely distributed in the Mediterranean Sea. The two isolates differ from each other in their ability to produce OTA; MUT2036 is the only isolate able to synthesize OTA. MUT2096 is infected with a partitivirus, Aspergillus ochraceus virus (AoV), but cannot synthesize OTA. We previously characterized the virome of a marine fungus, *Penicillium aurantiogriseum* var. *viridicatum* (MUT4330), harbouring six different viruses belonging to six different viral families in the positive single-stranded RNA (+ssRNA) and double-stranded RNA (dsRNA) lineages (Nerva et al., 2016). Here, we used partially purified virus particles from both MUT4330 and MUT2096 isolates to transfect the virus-free and OTA-producing isolate MUT2036. These investigations of mycoviruses in a new host following viral transfection proved to be a useful tool to decipher aspects of the virus-host interaction (Chiba et al., 2016; Nerva et al., 2017b). We demonstrate here for the first time an effect of a mycovirus, Aspergillus ochraceus virus (AoV), on OTA production. This finding illustrates new perspectives in mycovirus-fungus-environment interaction studies, highlighting complex and possibly mutualistic interactions beyond the common concept of mere host exploitation for replication.

Results and Discussion

Virus detection and OTA production assay

Virus detection was achieved following dsRNA purification from two different *A. ochraceus* isolates from the same geographical origin and original substrate, stored at *Mycotheca Universitatis Taurinensis*. Both isolates were isolated from *Holoturia polii* (sea cucumber) tissue. Isolate MUT2096 contained two dsRNA elements, whereas isolate MUT2036 contained no dsRNA elements. The dsRNAs present in isolate MUT2096 were identified as the genomic segments of AoV (Kim and Bozarth, 1985; Liu et al., 2008).

To assess OTA production by MUT2096 and MUT2036, we first grew the two isolates under the same conditions with and without an inducer of OTA, carbon tetrachloride (CCl_4). Under these conditions, only MUT2036 had the ability to produce OTA as confirmed by HPLC-DAD analysis (Supplementary Fig. 1).

Transfection of A. ochraceus MUT2036 and OTA quantification

Partially purified virus particles of AoV obtained from isolate MUT2096 and from *Penicillium aurantiogriseum* var. *viridicatum* (MUT4330) containing a mixed virus infection were used to transfect protoplasts of the OTA-producing isolate MUT2036. Forty-eight colonies from each individual transfection (using virions from MUT2096 and MUT4330 and using water as a negative control) were screened for viral replication using quantitative reverse transcription PCR (qRT-PCR). All MUT2036 colonies transfected with AoV virions were successfully infected, as confirmed by northern blot analysis (Fig. 1a), and three isolates, namely, T1, T3 and T24, were randomly selected for further study. Conversely, only one isolate of those transfected with viruses from MUT4330, isolate T20, was stably infected with Penicillium aurantiogriseum totivirus 1 (PaTV1), as assessed by qRT-PCR and northern blot analysis (Fig. 1b). One colony transfected with water and confirmed negative for all the viruses used for transfection was named TH_2O and was used as a negative control in further experiments.

To evaluate OTA production in the presence of viral infections, we used the original MUT2036 isolate as a control, the derived isolates (T1, T3, T24, T20) as virus-infected isolates, and the TH_2O isolate as a virus-free control isolate. Extracts from each of three biological replicates for each isolate were analysed for OTA quantification in an HPLC-DAD apparatus using a reference dilution curve.

The results showed that isolates T1, T3 and T24 produced at least 3 times more OTA than the original MUT2036 or TH_2O isolates. Conversely, isolate T20 (harbouring viruses from MUT4330), isolate MUT2036 (the original virus-free isolate) and isolate TH_2O (transfected with water only) showed a similar pattern of OTA production without any significant difference (Fig. 2 and Supplementary Tab. 1).

Relationships between mycovirus infection and metabolic changes were previously reported for *C. parasitica* infected by CHV1 (Allen and Nuss, 2004), where an extensive modulation of metabolites belonging to classes of amino acids, carbohydrates, lipids, nucleotides and polyamines was observed (Dawe et al., 2009). Moreover, we recently observed that *C. parasitica* isolates infected by Penicillium aurantiogriseum partiti-like virus 1 display higher resilience to osmotic stress than do isogenic virus-free isolates (Nerva et al., 2017b). As previously suggested, it is possible that some biotic/abiotic-mediated stress-response pathways lead to the expression of partially overlapping suites of genes and metabolite production, inducing crosstalk signalling (Bostock, 2005). Moreover, Okada et al. (Okada et al., 2018) recently reported mycovirus-mediated effects on *Alternaria alternata* disease development in Japanese pear. The authors demonstrated a Chrysovirus-mediated enhancement of *A. alternata* pathogenicity through the overproduction of an effector (AK-toxin), which is a host-specific toxin able to induce necrotic spots in leaves.

Nevertheless, to our knowledge, no correlation between mycotoxin production and mycoviral infection has been demonstrated to date. Here, we report for the first time a mycovirus effect on the production of a mycotoxin. AoV is a known mycoviral species reported ca. 40 years ago (Kong, 1979), but its ability to modify host metabolism has not been investigated.

Following the transfection of a virus-free isolate of the fungus, we demonstrated a host-specific ability of AoV to modulate OTA production when virus-infected and virus-free isolates sharing a genetic background were compared. The specificity of the interaction between *A. ochraceus* and AoV was further demonstrated since the isogenic isolate T20, infected with a different mycovirus, did not show significant differences in OTA production. Several *A. ochraceus* isolates have been reported to be infected by AoV (Kim and Bozarth, 1985; Liu et al., 2008), supporting the hypothesis of a long and widespread co-evolutionary relationship (Pearson et al., 2009) between the two biological entities, potentially providing an evolutionary and ecological advantage for AoV-infected isolates. In the last decade, the intriguing concept of "good viruses" (Roossinck, 2011; Virgin, 2014) has been proposed to highlight that the relationships between viruses and their host are often mutualistic. Indeed, several studies in fungal biology showed that many species of filamentous fungi can rapidly produce complex substances that are not directly involved in the survival of the organism but whose production can provide a competitive edge in a range of different environmental conditions (Magan and Aldred, 2007). It has been postulated that secondary metabolites produced by long, complex and energetically expensive biosynthetic pathways represent an intrinsic competitive benefit for the fungus (Williams et al., 1989; Vining, 1990). This ecological concept results from the observation that several of the genes involved in the production of secondary metabolites were positively selected over time, highlighting strong evidence for evolutionary selection (Stone and Williams, 1992). Moreover, the production of mycotoxins was also linked to a further competitive advantage, favouring substrate colonization by limiting the surrounding microbial competitors (Magan and Aldred, 2007). Consequently, it has been recently suggested that OTA biosynthesis represents a strategy to colonize substrates by providing a territorial advantage (Magan and Aldred, 2007). The ability to exploit the same substrate by other microorganisms is conditioned by the ability to detoxify OTA, which requires an active, expansive set of enzymes (Calvo et al., 2002). Taking all these aspects into consideration, we speculate that AoV infection could provide a competitive advantage to its fungal host.

OTA-related gene expression and AoV *quantification*

To assess whether the amounts of OTA measured are correlated with increased expression of genes putatively involved in the biosynthetic pathway, we performed RT-qPCR analysis on MUT2036

and T1 isolates at 48, 96 and 144 h post inoculation. We chose to perform gene expression analysis on these two isolates because they displayed the greatest differences in OTA production. The OTA biosynthetic pathway is not as well-defined as the aflatoxin pathway. Several genes have been reported in the literature as being putatively involved in OTA biosynthesis. Two of these genes belong to the polyketide synthase (pks) family (Wang et al., 2015), one belongs to the cytochrome P450 family, and one is an oxygenase (Sartori et al., 2014). Polyketide synthase 1 (*AoOTApks1*) was not amplified in any of the samples analysed, while polyketide synthase 2 (*AoOTApks2*) was equally expressed in the two isolates (Fig. 5a). The levels of cytochrome P450 (*AoOTAP450*) and oxygenase 1 (*AoOTAOxi1*) were significantly lower in isolate T1 than in MUT2036 (Fig. 5b and 5c).

To confirm the absence of *AoOTApks1* transcripts, we also performed a BLASTn search with an e-value of e-20 as the threshold for selected genes (*AoOTApks1, AoOTApks2, AoOTAP450, AoOTAOxi1, AoβTub*) in the fungal transcriptome assembled from our RNA-seq analysis. All the searched genes were confirmed to be present in the transcriptome assembly except for *AoOTApks1*. For these reasons, the molecular mechanism(s) by which AoV can induce OTA overproduction remain(s) unclear, because none of the genes putatively involved in the biosynthetic pathway displayed virus-associated upregulation at any of the time points we checked.

The intriguing absence of *AoOTApks1* transcripts in an OTA-producing strain and the presence of the other three genes confirmed in the transcriptome assembled from the total RNA sequencing characterization suggest the possible involvement of a molecular pathway to synthetize OTA different from that previously suggested (Sartori et al., 2014; Wang et al., 2015). Furthermore, the lack of virus-induced differential transcript accumulation suggests a possible AoV-mediated direct/indirect post-transcriptional or post-translational modification of the genes analysed, including a potential interference with mycotoxin secretion, as is the case for the hydrophobin cryparin in the CHV1-*C. parasitica* system (Kazmierczak et al., 2012). Alternatively, we can envision a possible viral impact on still-uncharacterized genes (downstream and/or upstream in the biosynthetic pathway) leading to a perturbation of OTA production. To better characterize the enzymes differentially involved in OTA biosynthesis in AoV-free and AoV-infected *A. ochraceus* isolates, a more comprehensive transcriptomic and proteomic approach is ongoing.

Given the statistically significant differences among the three AoV infected isolates regarding OTA production, we wanted to determine whether this observation could be correlated with the partitivirus titre. Relative quantification by RT-qPCR for each of the viral RNAs was performed, but no correlation was observed between OTA production and the differential accumulation of viral RNA (Fig. 3). The absence of a correlation between the viral titre and the strain-specific OTA accumulation suggests further regulatory elements not directly linked to the AoV titre.

Phenotypic evaluation

To evaluate the macroscopic phenotypes of MUT2036 (TH_2O) and the four transfected isolates (T1, T3, T24 and T20), all the isolates were grown on 18 different growth media in combinations of six different media (ACM, YES, YPD, MEA, CYA and CREA) and three different salt (NaCl) concentrations (0%, 3% and 10%) for each substrate. The colony diameters of each isolate were measured after five and ten days and were statistically analysed.

Minimal phenotypic differences were observed on all media, and no significant differences in colony diameter were observed at either 5 or 10 days post inoculation (dpi) (Supplementary Tab. 2 and 3). The phenotype of the T20 isolate, which was infected with PaTV1, was lightly pigmented on the reverse side of solid cultures of YES containing no salt, with an absence of exudate and significantly weaker sporulation than all the other isolates (Fig. 4, Supplementary Tab. 4).

Fungal phenotypic features mediated by mycovirus infection are largely undetectable in laboratory axenic cultures (Ghabrial et al., 2015). In our study, the absence of phenotypic differences between virus-infected and virus-free isolates confirms the cryptic behaviour of mycoviruses at least in axenic conditions (Pearson et al., 2009). Noticeable differences were observed in pigmentation and conidial production only for the PaTV1-infected strain, a virus unrelated to AoV and only experimentally introduced in *A. ochraceus* through protoplast transfection. Since PaTV1 is not a naturally occurring virus infecting *Aspergillus* species, the observed relatively strong virus-induced phenotypic effects could be explained by recent host adaptation, whereas longer co-evolution times would allow the minimization of symptoms, as is the case with AoV-*A. ochraceus* interactions.

Virome analysis

To confirm viral infection in MUT2036 and to confirm the absence of further viruses not detected following dsRNA isolation, we analysed the virome of isolates T1 and T20 using Illumina next generation sequencing. Assembled contigs were used for BLASTx searches against a curated viral database. Only the two anticipated viral genomes were identified in these studies, confirming that isolate MUT2036 was not infected with other viruses.

Moreover, the data from RNA-seq were used to assess any possible nucleotide and/or amino acid alterations to the PaTV1 genome upon the infection of a new host species. More than 100,000 reads were mapped to the viral sequence (NC_028948.1), with an average nucleotide coverage of 2,000×.

The results reported in Supplementary Tab. 5 reveal that seven nucleotide substitutions were detected, one in the 5' UTR sequence and six in the coding region, two of which resulted in amino acid changes. There was one change at position 745 in the reference genome (NC_028948.1) that resulted in an altered hydrophobicity pattern.

In contrast, following RNAseq, the nucleotide sequence of AoV after transfection into the new isolate of the same fungal species was confirmed to be identical to that observed in the original *A. ochraceus* isolate.

Since mycoviruses are transmitted through hyphal anastomosis, their natural host range is thought to be limited to vegetative compatible isolates of the same species, but the host range can be artificially expanded through transfection, as demonstrated by several authors (Kanematsu et al., 2010; Nerva et al., 2017b; Nerva et al., 2017a). In addition, viral features such as a short generation time and high mutation rate support the quasispecies theory (Domingo and Holland, 1997), in which a viral infection in a single host is represented by a modal distribution of different sequences. Often, when new host adaptation occurs, a new modal distribution is selected to improve viral fitness, as previously observed for Penicillium aurantiogriseum partiti-like virus infecting *C. parasitica* (Nerva et al., 2017b). Here, we observed stable nucleotide changes in the PaTV1 genome sequence following infection of the new host species, *A. ochraceus* isolate MUT2036, confirming the selection of the best-fitting viral genome sequence from quasispecies variants. In our work, the original amino acid leucine, which is non-polar (hydrophobic), is substituted by serine, which is polar and acidic. Hydrophobic patterns play an important role in protein structure (secondary and tertiary structures) and in protein-protein interactions (Huang et al., 1996).

Conclusions

In this work, we demonstrated for the first time that mycovirus infection induces enhanced toxin production in the presence of AoV, a virus that likely has coevolved with its host. Our results provide valuable information regarding possible evolutionary and ecological advantages for AoV-infected isolates. Mycovirus-mediated metabolite overproduction provides an additional parameter to exploit the production of secondary metabolites from fungi. In addition, these results reveal a new factor that must be considered when predicting OTA contamination in food and feed commodities. In fact, mycotoxin modulation should be considered on a case-by-case basis when considering mycoviruses as biological control agents, especially where myco-toxigenic fungi are considered targets. The beneficial effect of virus-associated hypovirulence could be countered by an increase in mycotoxin production.

Material and methods

Fungal maintenance

The two *A. ochraceus* isolates (MUT2036 and MUT2096) were maintained on solidified Aspergillus complete medium (ACM) (Bhatti et al., 2011). For increased RNA yield and for AoV purification, fungi were cultured in liquid ACM at 26°C in a flask with shaking at 130 rpm for 48 to 72 h.

P. aurantiogriseum (MUT4330) was maintained on solidified CY medium (Nerva et al., 2016). The large amounts of mycelia required for virus purification were grown in liquid CY media at 2[illegible]°C in a flask with shaking at 120 rpm for 72 h.

Virus detection

To detect the viral infection of *A. ochraceus,* dsRNA was isolated and analysed as described previously (Nerva et al., 2016).

Isolated dsRNA was used as a template for cDNA synthesis, and the cDNA was then amplified by PCR using an anchored primer to generate random amplified fragments that were cloned and sub-sequentially sequenced as previously described (Nerva et al., 2016). The sequences obtained were used in BLASTp searches against the non-redundant protein database.

Protoplast transfection

Partially purified virus particles from MUT4330 and MUT2096 were obtained using a differential centrifugation protocol (Nerva et al., 2016) and visualized by electron microscopy using carbon-formvar coated copper grids to confirm virion abundance.

Protoplasts from MUT2036 were obtained following a previously described method (Tilburn et al., 1983) with minor modifications. An inoculum of 10^6 to 10^7 conidia was germinated in Aspergillus minimal medium (AMM) for 16 h. Mycelia were washed twice with 0.6 M $MgSO_4$ and then re-suspended in 1.2 M $MgSO_4$ (1 g in 5 ml), and the cell walls were digested with 1 mL Glucanex (Sigma-Aldrich, Saint Louis, MO, USA), 0.15 ml β-glucuronidase (Sigma-Aldrich) and 5 mg bovine serum albumin (Sigma-Aldrich, Saint Louis, MO, USA). The solution was shaken at 30°C and 100 rpm for 120 min. Protoplasts were then filtered through a disposable sterile 30 µm mesh cell filter, washed twice in 1 M sorbitol (pH 7.5) and re-suspended in 500 µL 1 M sorbitol, 10 mM $CaCl_2$ and 10 mM Tris-HCl (pH 7.5). Using 100 µl aliquots of protoplasts, transfection was performed by adding 20 µl virions to 1 mL of PEG solution (25% PEG 4000, 10 mM $CaCl_2$ and 10 mM Tris-HCl pH 7.5). Protoplasts were incubated for 20 min; washed with 1 M sorbitol, 10 mM $CaCl_2$ and 10 mM Tris-HCl (pH 7.5); and plated in solidifying AMM (36°C) supplemented with 1 M sucrose.

Fifteen days after transfection, the regenerated colonies were transferred from 24-well plates and cultured on ACM. The regenerated colonies were then grown in 3.5 ml liquid ACM medium in 12-well plates. Forty-eight hours post-inoculation, mycelia were harvested and lyophilized in 2 ml tubes for 24 h. A crude extract of the transfected cells was obtained by adding 0.5 mL glass beads (0.5 mm diameter) and 1 mL TE buffer, followed by vigorous shaking with a FastPrep bead beater. The extract was then diluted 1:5 in sterile water and directly used as a template for RT-PCR amplification using TaqMan™ Fast Virus 1-Step Master Mix (Thermo Scientific, Waltham, MA, USA).

Further confirmation of successful viral infection followed at least 5 subcultures of selected isolates. For this, total RNA was extracted from each transfected isolate, and northern blot analyses with radioactively labelled probes were performed as previously described (Nerva et al., 2017b; Nerva et al., 2017a) for the detection of both AoV and PaTV1.

OTA quantification

To test the OTA production capability, MUT2036 and MUT2096 were grown in 150 mm Petri dishes with 50 ml ACM medium with or without 20 mM CCl_4 in the dark under static conditions at 26°C for 6 days. Mycelia were then harvested, lyophilized and extracted with a three-step solid-liquid extraction by using 1.5 mL methanol:chloroform (1:2 v/v), ethyl acetate and 2-propanol, each for 30 min in an ultrasonic bath. After each passage, the organic phase was collected in a flask. The final extract was evaporated to dryness in a rotary evaporator at 35°C. The residue was dissolved in 500 µL H_2O:CH_3CN (1:1 v/v) for HPLC-DAD analysis. The original standard for OTA (Merck KGaA; Darmstadt, Germany; purity ≥99%) was used for the identification by comparing retention time and the UV spectrum. The external calibration method was used.

Nine biological replicates of each *A. ochraceus* isolate (MUT2036, T1, T3, T24, T20 and TH_2O) were grown in 150 mm Petri dishes with 50 ml ACM supplemented with 20 mM carbon tetrachloride (CCl_4) in the dark under static conditions at 26°C for 6 days. At the end of the growth period, the nine biological replicates of each isolate were pooled together three by three to minimize individual differences but to retain three distinct biological replicates for further analyses. Mycelia were lyophilized, and then 1 g of each replicate was extracted with the method described above and quantified using an HPLC-DAD apparatus.

The HPLC apparatus was an Agilent 1220 Infinity LC system (Agilent®, Waldbronn, Germany) model G4290B equipped with a gradient pump, auto sampler, and column oven set at 30°C. A 170 Diode Array Detector (Gilson, Middleton, The USA) set at 230, 276, 300 and 330 nm was used as the detector. An XTerra Shield RP18 analytical column (150×4.6 mm i.d., 3.5 µm, Waters Corporation, MA, USA) was used. The mobile phases consisted of water acidified with formic acid

0.1% (A) and acetonitrile (B) at a flow rate of 0.800 mL/min in gradient mode, 0-5 min: 5% B, 5-45 min: from 5% to 50% B, 45-60 min: from 50% to 80% B, and 60-70 min: from 80% to 100% B. Twenty microliters of each sample was injected and analysed.

Phenotype evaluation

The phenotypes of the virus-infected isolates T1, T3, T24 and T20 were compared with that of the isogenic virus-free TH_2O isolate. Six different media (ACM, YES, YPD, MEA, CYA and CREA) were selected from those commonly used for *Aspergillus* spp. (Samson et al., 2004), and three different NaCl percentages (0%, 3% and 10%) were used, obtaining up to 18 different conditions. Each isolate was inoculated in three biological replicates, and each biological replicate was observed for pigmentation, conidiation, exudate production and colony diameter at 5 and 10 days post inoculation (dpi). A total of 270 colonies were measured. Colony diameters were measured on the reverse side of the plate in duplicate for each biological replicate. The average diameters and standard deviation were calculated for each isolate, and ANOVA and Tukey post hoc tests were used to assess statistical differences among isolates.

Conidial counting was performed by harvesting conidia from each plate at the end of the growing experiment, using 2 ml water supplemented with 0.01% Tween 20. Each biological replicate was counted independently, and then statistical analyses were performed as described above.

Viral genome re-sequencing

The total RNA of isolates T1 and T20 was purified using the Total Spectrum RNA reagent (Sigma-Aldrich, Saint Louis, MO, USA), and RNA concentration was quantified with a NanoDrop 1000 spectrophotometer. Ribosomal RNA (rRNAs) was depleted with the Ribo-Zero™ Gold kit (Epicentre, Madison, USA), and after library assembly, samples were sequenced in a single lane of Illumina NovaSeq (100 M, 100 bp, paired-end reads). The raw reads were then mapped against reference genomes using BWA (Li and Durbin, 2009) and SAMtools (Li et al., 2009) as previously described (Matsumura et al., 2017). Integrative Genomics Viewer was used for data visualization and exploration (Thorvaldsdottir et al., 2013). Nucleotide changes were annotated, and the ORF translation was analysed to identify amino acid changes.

AoV *quantification*

Total RNA from the previously obtained mycelia was extracted with Total Spectrum RNA reagent (Sigma-Aldrich). For each replicate of the six isolates, cDNA was synthetized following the manufacturer's instructions provided for the High-Capacity cDNA Reverse Transcription kit (Applied

Biosystems). iTaq universal SYBR Green supermix (Bio-Rad) and specific primers (Supplementary Tab. 6) were then used to amplify β-tubulin (*AoβTub*), as a housekeeping gene, and the three viral RNAs. For each biological replicate, three technical replicates were analysed, and the relative quantification to MUT2036 was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

OTA gene expression analysis

Isolates MUT2036 and T1 were grown in OTA-inducing media as described above. Nine replicates for each isolate and time point (48 h, 96 h and 144 h) were pooled (three by three) to obtain three biological replicates for each isolate. Total RNA was extracted, cDNA was synthetized and iTaq universal SYBR Green supermix (Bio-Rad) with specific primers (Supplementary Tab. 6) was used to amplify four genes of the OTA metabolic pathway: *AoOTApks1* (Wang et al., 2015), *AoOTApks2* (Wang et al., 2015), *AoOTAP450* (Sartori et al., 2014), and *AoOTAOxi1* (Sartori et al., 2014). β-Tubulin (*AoβTub*) was used as a housekeeping gene. The relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001); three technical replicates were run for each biological replicate, and the expression of transcripts was quantified after normalization to the expression of the housekeeping gene *AoβTub*. The results were calculated as an expression ratio (relative quantity, RQ) to MUT2036 at 48 h, as previously described (Chitarra et al., 2017).

In addition, the presence of the selected genes in the fungal transcriptome was assessed using TBLASTn with a custom database containing the nucleotide coding sequences. The threshold e-value was fixed at e^{-20} to retain only significant alignments.

Statistical analysis

Significant differences among isolates were statistically analysed by one-way ANOVA, and mean separation was done using Tukey's post hoc test only when the ANOVA results were significant ($P < 0.05$). Significant differences in pairwise comparisons were assessed by Student's *t* test. The SPSS statistical software package (version 22; SPSS) was used for statistical analyses.

Author contributions

L.N. designed the experiments, carried out the experiments and drafted the first manuscript. W.C. provided most of the material, helped refine the experimental setup and helped draft the manuscript. I.S. helped by designing and interpreting the HPLC experiments. M.C. and M.F. helped with experiments and data analysis. F.G. helped with data analysis and carefully reviewed the manuscript. G.C.V. carefully reviewed the manuscript and provided the original fungal isolates. M.T. supervised the experiments and edited the manuscript.

Competing Interests

The authors declare that they have no conflicts of interest. This article does not contain any studies with human or animal participants.

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REFERENCES

Alberts, A.W. (1988) Discovery, biochemistry and biology of lovastatin. *Am J Cardiol* **62**: J10-J15.
Allen, T.D., and Nuss, D.L. (2004) Linkage between mitochondrial hypovirulence and viral hypovirulence in the chestnut blight fungus revealed by cDNA microarray analysis. *Eukaryot Cell* **3**: 1227-1232.
Allen, T.D., Dawe, A.L., and Nuss, D.L. (2003) Use of cDNA microarrays to monitor transcriptional responses of the chestnut blight fungus Cryphonectria parasitica to infection by virulence-attenuating hypoviruses. *Eukaryoti Cell* **2**: 1253-1265.
Banks, G., Buck, K., Chain, E., Darbyshire, J.E., Himmelweit, F., Ratti, G. et al. (1970) Antiviral activity of double stranded RNA from a virus isolated from Aspergillus foetidus. *Nature* **227**: 505-507.
Bentley, R., and Bennett, J.W. (2008) A ferment of fermentations: reflections on the production of commodity chemicals using microorganisms. *Adv Appl Microbiol* **63**: 1-32.
Bhatti, M.F., Jamal, A., Petrou, M.A., Cairns, T.C., Bignell, E.M., and Coutts, R.H. (2011) The effects of dsRNA mycoviruses on growth and murine virulence of Aspergillus fumigatus. *Fungal Genet Biol* **48**: 1071-1075.
Bostock, R.M. (2005) Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annu Rev Phytopathol* **43**: 545-580.
Calvo, A.M., Wilson, R.A., Bok, J.W., and Keller, N.P. (2002) Relationship between secondary metabolism and fungal development. *Microbiol Mol Biol Rev* **66**: 447-459.
Chiba, S., Lin, Y.H., Kondo, H., Kanematsu, S., and Suzuki, N. (2016) A novel betapartitivirus RnPV6 from Rosellinia necatrix tolerates host RNA silencing but is interfered by its defective RNAs. *Virus Res* **219**: 62-72.
Chitarra, W., Perrone, I., Avanzato, C.G., Minio, A., Boccacci, P., Santini, D. et al. (2017) Grapevine Grafting: Scion Transcript Profiling and Defense-Related Metabolites Induced by Rootstocks. *Front Plant Sci* **8**: 654.
Cho, W.K., Yu, J., Lee, K.-M., Son, M., Min, K., Lee, Y.-W., and Kim, K.-H. (2012) Genome-wide expression profiling shows transcriptional reprogramming in Fusarium graminearum by Fusarium graminearum virus 1-DK21 infection. *Bmc Genomics* **13**: 173.
Cui, C.-M., Li, X.-M., Meng, L., Li, C.-S., Huang, C.-G., and Wang, B.-G. (2010) 7-Nor-ergosterolide, a pentalactone-containing norsteroid and related steroids from the marine-derived endophytic Aspergillus ochraceus EN-31. *J Nat Products* **73**: 1780-1784.
Currie, J.N. (1917) The citric acid fermentation of Aspergillus niger. *J Biol Chem* **31**: 15-37.
Davis, N.D., Diener, U., and Eldridge, D. (1966) Production of aflatoxins B1 and G1 by Aspergillus flavus in a semisynthetic medium. *Applied Microbiol* **14**: 378-380.
Dawe, A.L., Van Voorhies, W.A., Lau, T.A., Ulanov, A.V., and Li, Z. (2009) Major impacts on the primary metabolism of the plant pathogen Cryphonectria parasitica by the virulence-attenuating virus CHV1-EP713. *Microbiol* **155**: 3913-3921.

Domingo, E., and Holland, J. (1997) RNA virus mutations and fitness for survival. *Ann Rev Microbiol* **51**: 151-178.
Domsch, K.H., Gams, W., and Anderson, T.-H. (1980) *Compendium of soil fungi. Volume 1*: Academic Press (London) Ltd.
Eaton, D.L., and Gallagher, E.P. (1994) Mechanisms of aflatoxin carcinogenesis. *Annu Rev Pharmacol Toxicol* **34**: 135-172.
Elias, K.S., and Cotty, P.J. (1996) Incidence and stability of infection by double-stranded RNA genetic elements in Aspergillus section flavi and effects on aflatoxigenicity. *Can J Botany* **74**: 716-725.
Elliston, J.E. (1985) Characterisation of dsRNA-free and dsRNA-containing strains of Endothia parasitica in relation to hypovirulence. *Phytopathol* **75**: 151-158.
European Commision, Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuff. (2006R1881-EN-01.09. 2014-014.001-1, 2006).
Ghabrial, S.A., Castón, J.R., Jiang, D., Nibert, M.L., and Suzuki, N. (2015) 50-plus years of fungal viruses. *Virol* **479**: 356-368.
Harris, J.P., and Mantle, P.G. (2001) Biosynthesis of ochratoxins by Aspergillus ochraceus. *Phytochemistry* **58**: 709-716.
Hepting, G.H. (1974) Death of the American chestnut. *J Forest Hist* **18**: 60-67.
Hollings, M. (1962) Viruses associated with a die-back disease of cultivated mushroom. *Nature* **196**: 962-965.
Hong, K.-J., Lee, C.-H., and Kim, S.W. (2004) Aspergillus oryzae GB-107 fermentation improves nutritional quality of food soybeans and feed soybean meals. J Med Food **7**: 430-435.
Huang, Enoch S., S. Subbiah, and Michael Levitt. (1995) Recognizing native folds by the arrangement of hydrophobic and polar residues.*J Molec Biol* 252.5: 709-720.
Joint FAO/WHO Expert Committee on Food Additives. Safety Evaluation of Certain Mycotoxins in Food. 2001; WHO Food Additivies Series 47; FAO Food and Nutrition Paper 74.
Kanematsu, S., Sasaki, A., Onoue, M., Oikawa, Y., and Ito, T. (2010) Extending the Fungal Host Range of a Partitivirus and a Mycoreovirus from Rosellinia necatrix by Inoculation of Protoplasts with Virus Particles. *Phytopathol* **100**: 922-930.
Kazmierczak, P., McCabe, P., Turina, M., Jacob-Wilk, D., and Van Alfen, N.K. (2012) The Mycovirus CHV1 Disrupts Secretion of a Developmentally Regulated Protein in Cryphonectria parasitica. *J Virol* **86**: 6067-6074.
Kim, J.W., and Bozarth, R.F. (1985) Intergeneric occurrence of related fungal viruses: the Aspergillus ochraceous virus complex and its relationship to the Penicillium stoloniferum virus S. *J Gen Virol* **66**: 1991-2002.
Kong, T. (1979) Biophysical and biochemical characteristics of viruslike particles in Aspergillus ochraceous. In. Indiana State University.
Kotta-Loizou, I., and Coutts, R.H. (2017) Mycoviruses in Aspergilli: A Comprehensive Review. *Front Microbiol* **8**: 1699.
Li, H., and Durbin, R. (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**: 1754-1760.
Li, H., Fu, Y., Jiang, D., Li, G., Ghabrial, S.A., and Yi, X. (2008) Down-regulation of Sclerotinia sclerotiorum gene expression in response to infection with Sclerotinia sclerotiorum debilitation-associated RNA virus. *Virus Res* **135**: 95-106.
Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N. et al. (2009) The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**: 2078-2079.
Liu, W., Duns, G., and Chen, J. (2008) Genomic characterization of a novel partitivirus infecting Aspergillus ochraceus. *Virus Genes* **37**: 322-327.
Livak, Kenneth J., and Thomas D. Schmittgen. (2001)Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods 25.4: 402-408.

Magan, N., and Aldred, D. (2007) Why do fungi produce mycotoxins?
Maplestone, R.A., Stone, M.J., and Williams, D.H. (1992) The evolutionary role of secondary metabolites—a review. *Gene* **115**: 151-157.
Matsumura, E.E., Coletta-Filho, H.D., Nouri, S., Falk, B.W., Nerva, L., Oliveira, T.S. et al. (2017) Deep sequencing analysis of RNAs from citrus plants grown in a citrus sudden death-affected area reveals diverse known and putative novel viruses. *Viruses* **9**: 92.
Matsushima, K., Yashiro, K., Hanya, Y., Abe, K., Yabe, K., and Hamasaki, T. (2001) Absence of aflatoxin biosynthesis in koji mold (Aspergillus sojae). *Applied Microbiol Biotechnol* **55**: 771-776.
Meenupriya, J., and Thangaraj, M. (2011) Analytical characterization and structure elucidation of metabolites from Aspergillus ochraceus MP2 fungi. *Asian Pac J Trop Biomed* **1**: 376-380.
Mehle, A., Dugan, V. G., Taubenberger, J. K., & Doudna, J. A. Reassortment and mutation of the avian influenza virus polymerase PA subunit overcome species barriers. J Virol. 2012; 86.3: 1750-1757.
Michelin, M., Maria de Lourdes, T., Ruzene, D.S., Silva, D.P., Ruiz, H.A., Vicente, A.A. et al. (2012) Production of xylanase and β-xylosidase from autohydrolysis liquor of corncob using two fungal strains. *Bioprocess Biosyst Eng* **35**: 1185-1192.
Nerva, L., Varese, G.C., Falk, B.W., and Turina, M. (2017a) Mycoviruses of an endophytic fungus can replicate in plant cells: evolutionary implications. *Sci Rep* **7**: 1908-1908.
Nerva, L., Silvestri, A., Ciuffo, M., Palmano, S., Varese, G.C., and Turina, M. (2017b) Transmission of Penicillium aurantiogriseum partiti-like virus 1 to a new fungal host (Cryphonectria parasitica) confers higher resistance to salinity and reveals adaptive genomic changes. *Env Microbioly* **19**: 4480-4492.
Nerva, L., Ciuffo, M., Vallino, M., Margaria, P., Varese, G.C., Gnavi, G., and Turina, M. (2016) Multiple approaches for the detection and characterization of viral and plasmid symbionts from a collection of marine fungi. *Virus Res* **219**: 22-38.
Nuss, D.L. (1992) Biological-control of chestnut blight - an example of virus-mediated attenuation of fungal pathogenesis. Microbiological Rev **56**: 561-576.
Nuss, D.L. (2005) Hypovirulence: Mycoviruses at the fungal-plant interface. *Nat Rev Microbiol* **3**: 632-642.
Nuss, D.L. (2010) Mycoviruses. In *Cellular and Molecular Biology of Filamentous Fungi*: American Society of Microbiology, pp. 145-152.
Okada, R., Ichinose, S., Takeshita, K., Urayama, S.-i., Fukuhara, T., Komatsu, K. et al. (2018) Molecular characterization of a novel mycovirus in Alternaria alternata manifesting two-sided effects: Down-regulation of host growth and up-regulation of host plant pathogenicity. *Virol* **519**: 23-32.
Ozkan, S., and Coutts, R.H. (2015) Aspergillus fumigatus mycovirus causes mild hypervirulent effect on pathogenicity when tested on Galleria mellonella. Fungal Genet Biol 76: 20-26.
Pardo, E., Marın, S., Sanchis, V., and Ramos, A. (2004) Prediction of fungal growth and ochratoxin A production by Aspergillus ochraceus on irradiated barley grain as influenced by temperature and water activity. *Int J Food Microbiol* **95**: 79-88.
Patiño, B., González-Salgado, A., González-Jaén, M.T., and Vázquez, C. (2005) PCR detection assays for the ochratoxin-producing Aspergillus carbonarius and Aspergillus ochraceus species. *International J Food Microbiol* **104**: 207-214.
Pearson, M.N., Beever, R.E., Boine, B., and Arthur, K. (2009) Mycoviruses of filamentous fungi and their relevance to plant pathology. *Mol Plant Pathol***10**: 115-128.
Ratti, G., and Buck, K. (1972) Virus particles in Aspergillus foetidus: a multicomponent system. *J Gen Virol* **14**: 165-175.
Roossinck, M.J. (2011) The good viruses: viral mutualistic symbioses. *Nat Rev Microbiol* **9**: 99-108.
Samson, R.A., Hoekstra, E.S., and Frisvad, J.C. (2004) *Introduction to food-and airborne fungi*: Centraalbureau voor Schimmelcultures (CBS).

Sartori, D., Massi, F.P., Ferranti, L.S., and Fungaro, M.H.P. (2014) Identification of genes differentially expressed between ochratoxin-producing and non-producing strains of Aspergillus westerdijkiae. *Indian journal of microbiology* **54**: 41-45.
Schmidt, F.R., Lemke, P.A., and Esser, K. (1986) Viral influences on aflatoxin formation by Aspergillus flavus. *Applied Microbiol Biotechnol* **24**: 248-252.
Selman, M., Dankar, S. K., Forbes, N. E., Jia, J. J., & Brown, E. G. Adaptive mutation in influenza A virus non-structural gene is linked to host switching and induces a novel protein by alternative splicing. Emerging microbes & infections, 2012; 1.11: e42.
Silva, V.N., Durigon, E.L., Pires, M.d.F.C., Lourenço, A., Faria, M.J.d., and Corrêa, B. (2001) Time course of virus-like particles (VLPs) double-stranded rna accumulation in toxigenic and non-toxigenic strains of Aspergillus flavus. *Braz J Microbiol* **32**: 56-60.
Son, M., Yu, J., and Kim, K.-H. (2015) Five questions about mycoviruses. *PLoS Path* **11**: e1005172.
Stone, M., and Williams, D. (1992) On the evolution of functional secondary metabolites (natural products). *Mol Microbiol* **6**: 29-34.
Thorvaldsdottir, H., Robinson, J.T., and Mesirov, J.P. (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform* **14**: 178-192.
Tilburn, J., Scazzocchio, C., Taylor, G.G., Zabicky-Zissman, J.H., Lockington, R.A., and Davies, R.W. (1983) Transformation by integration in Aspergillus nidulans. *Gene* **26**: 205-221.
van de Sande, W.W.J., Lo-Ten-Foe, J.R., van Belkum, A., Netea, M.G., Kullberg, B.J., and Vonk, A.G. (2010) Mycoviruses: future therapeutic agents of invasive fungal infections in humans? *Eur J Clin Microbiol Infect Dis* **29**: 755-763.
Varga, J., Kevei, F., Vágvölgyi, C., Vriesema, A., and Croft, J.H. (1994) Double-stranded RNA mycoviruses in section Nigri of the Aspergillus genus. *Can J Microbiol* **40**: 325-329.
Varga, J., Rinyu, E., Kevei, E., Toth, B., and Kozakiewicz, Z. (1998) Double-stranded RNA mycoviruses in species of Aspergillus sections Circumdati and Fumigati. *Can J Microbiol* **44**: 569-574.
Vining, L.C. (1990) Functions of secondary metabolites. *Ann Rev Microbiol* **44**: 395-427.
Virgin, H.W. (2014) The virome in mammalian physiology and disease. *Cell* **157**: 142-150.
Wang, L., Wang, Y., Wang, Q., Liu, F., Selvaraj, J.N., Liu, L. et al. (2015) Functional characterization of new polyketide synthase genes involved in ochratoxin A biosynthesis in Aspergillus ochraceus fc-1. *Toxins* **7**: 2723-2738.
Williams, D.H., Stone, M.J., Hauck, P.R., and Rahman, S.K. (1989) Why are secondary metabolites (natural products) biosynthesized? *J Nat Prod* **52**: 1189-1208.
Wu, F., Bui-Klimke, T., & Naumoff Shields, K. (2014). Potential economic and health impacts of ochratoxin A regulatory standards. World Mycotoxin Journal, 7(3), 387-398.

FIGURE LEGENDS

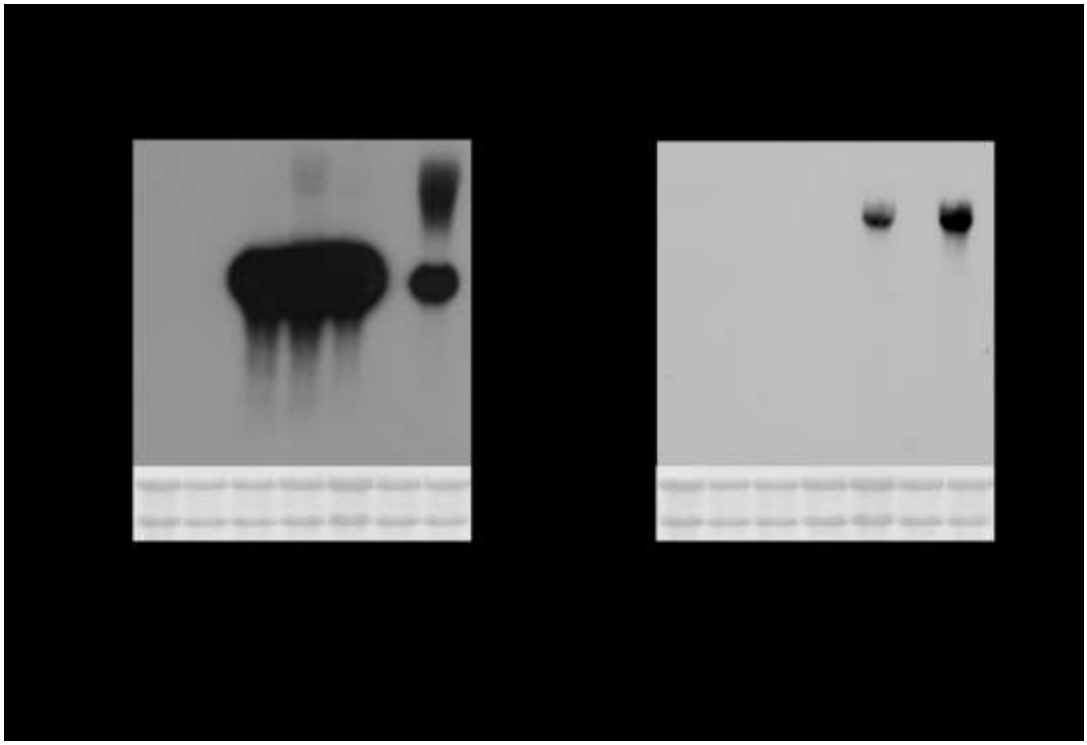
Figure 1. Northern blot analysis of total RNA extracted from MUT2036 and isogenic transfected isolates. In panel **a**, the probe matching the RdRP sequence of Aspergillus ochraceus virus (AoV) was used. Isolates T1, T3 and T24 had the same band as the positive control MUT2096 (used as the viral inoculum source). In panel **b**, the probe matching the RdRP of Penicillium aurantiogriseum totivirus 1 (PaTV1) was used. Only isolate T20 showed evidence of infection (MUT4330 is the virion source used as a positive control).

Figure 2. Ochratoxin A (OTA) quantification in MUT2036 and derived isolates. Isolates T1, T3 and T24 infected with Aspergillus ochraceus virus had an overproduction of OTA compared with that of MUT2036. Isolates TH_2O (virus free) and T20 (infected with Penicillium aurantiogriseum totivirus 1) were not significantly different from the isogenic virus-free MUT2036. The lower-case letters above the bars denote significant differences according to Tukey's HSD test ($P < 0.05$). The data are expressed as the means ± SEs (n = 3). DW = dry weight.

Figure 3. Quantification of Aspergillus ochraceus virus RNA1 (Panel **a**), RNA2 (Panel **b**) and RNA3 (Panel **c**) in the different infected isolates. The lower-case letters above the bars denote significant differences according to Tukey's HSD test ($P < 0.05$). The data are expressed as the means ± SDs (n = 3).

Figure 4. Phenotype evaluation of virus-free (TH_2O) and virus-infected isolates on YES media 10 days post-inoculation. In panel **a**, isolate T20, harbouring Penicillium aurantiogriseum totivirus 1, had a lighter colony colour, an absence of exudate production and reduced conidiation. The reverse of the colony in panel **b** showed a lighter colour for isolate T20.

Figure 5. Relative quantification of genes putatively involved in ochratoxin A production in the original MUT2036 isolate and in isolate T1 infected with Aspergillus ochraceus virus. Panel **a**: *AoOTApks2*, a polyketide synthase, shows no significant difference in expression between MUT2036 and isolate T1. In panel **b**, *AoOTAP450*, an enzyme in the cytochrome P450 family, showed statistically different expression between the two isolates. Starting at 96 hours post inoculation (hpi), MUT2036 showed higher expression levels of *AoOTAP450* than T1. In panel **c**, the oxygenase *AoOTAOxi1* had an increase in transcript accumulation at 96 hpi, followed by a decrease at 144 hpi. The expression trend is similar for both isolates, but in MUT2036, expression was upregulated at 96 hpi. The asterisks (*) denote significant differences supported by a two-tailed Student's *t* test for each time point ($P < 0.05$). The data are expressed as the means ± SDs (n=3).



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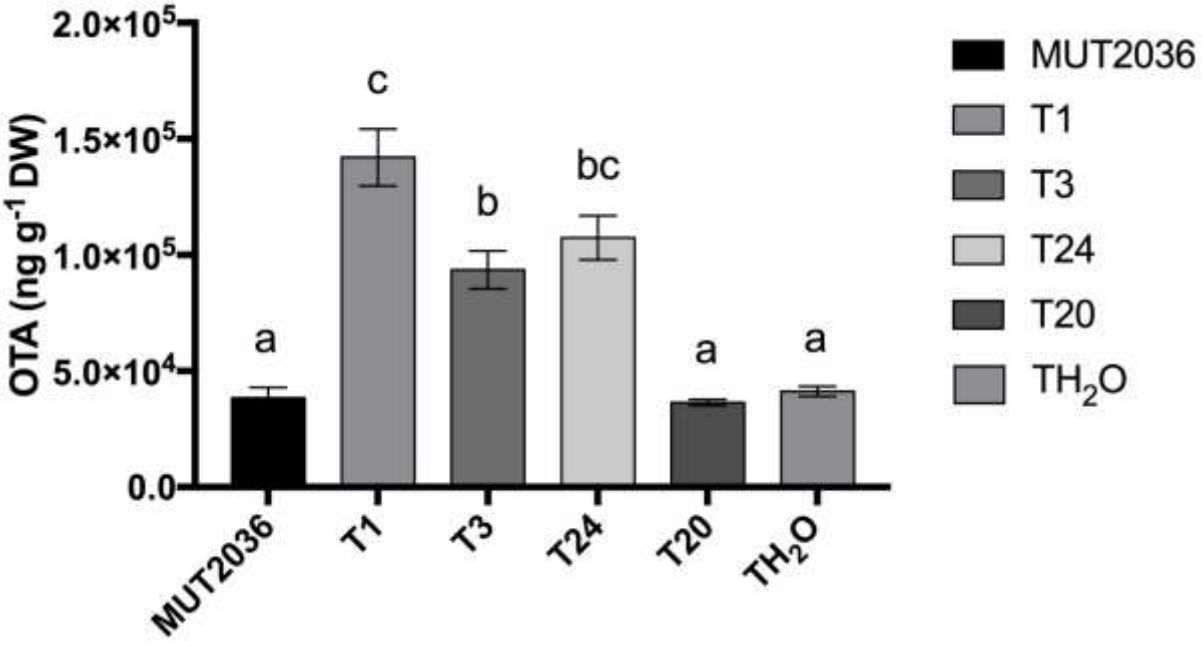

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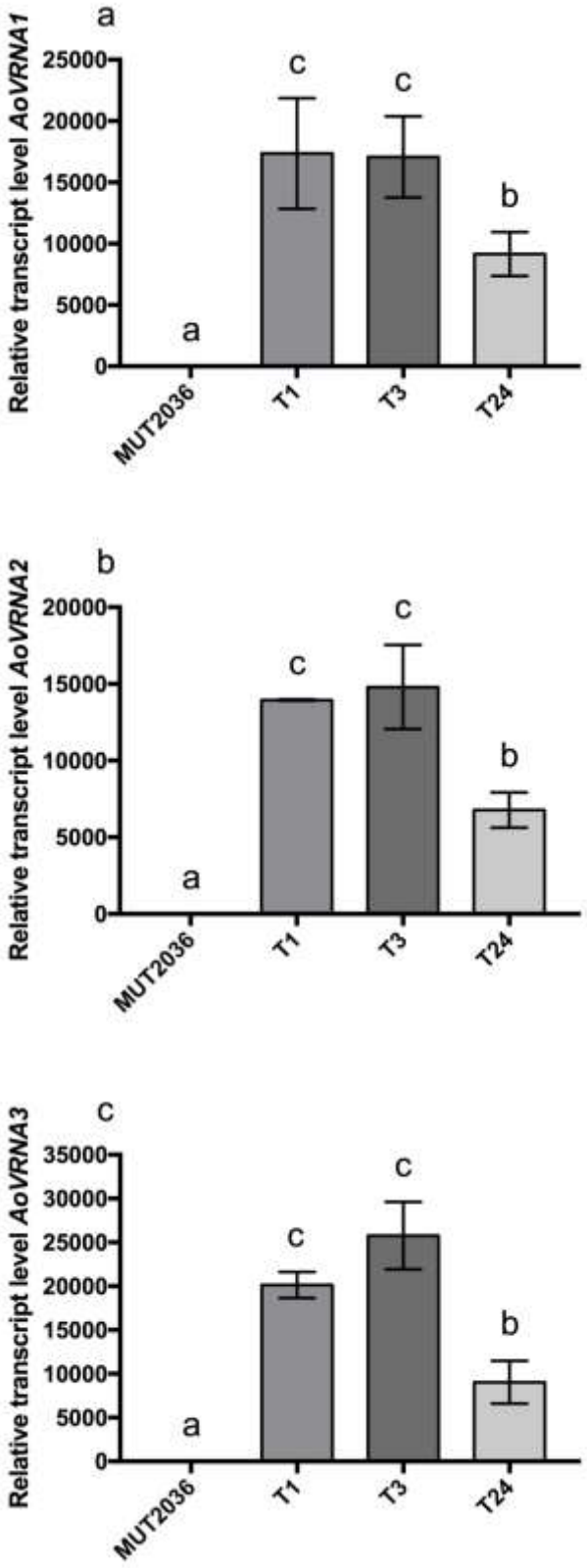

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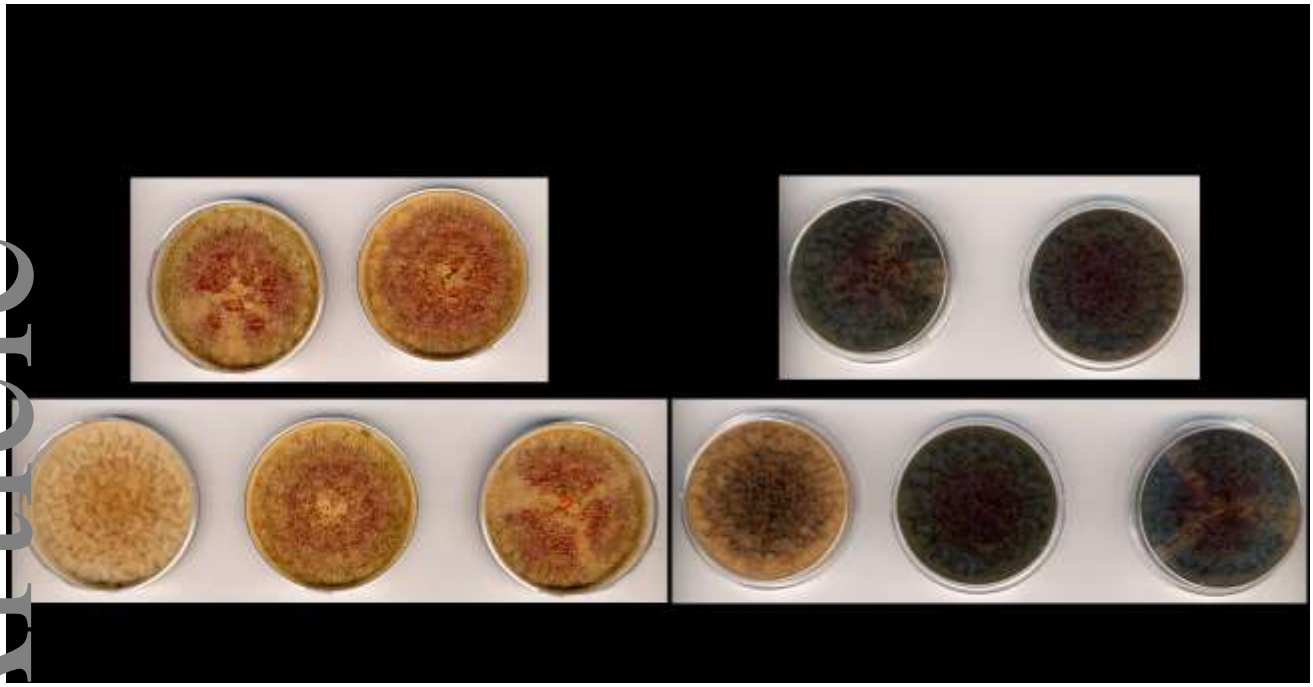

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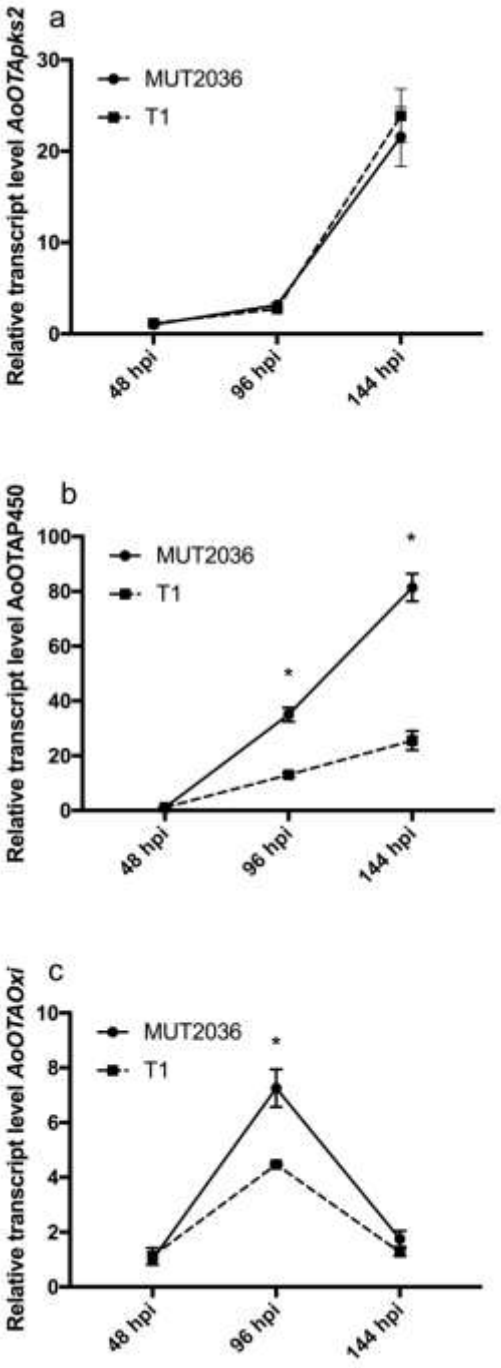

Figure 5. Relative quantification of genes putatively involved in ochratoxin A production in the original MUT2036 isolate and in isolate T1 infected by *Aspergillus ochraceus virus*. Panel **a**: *AoOTApks2*, a polyketide synthase, shows no significant differential expression between MUT2036 and isolate T1 over the time course. In panel **b**: *AoOTAP450*, an enzyme in the cytochrome P450 family, showed statistically supported differential expression between the two isolates: starting from 96 hours post inoculum (hpi) MUT2036 showed higher expression levels when compared to T1. In panel **c**: the oxygenase *AoOTAOxi1* showed an increase in transcript accumulation at 96 hpi followed by a decrease at 144 hpi. The expression trend is similar for both isolates, but in MUT2036, expression was up-regulated at 96 hpi. Asterisks (*) denote significant differences supported by two-tailed Student's *t* test for