Something new to explore

Plant viruses infecting and inducing gene silencing in filamentous fungi

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Functional genomics in plants has been facilitated greatly by the use of plant viruses to carry segments of host genes that can then promote the silencing of the RNAs expressed from the corresponding host genes; a process called virus-induced gene silencing (VIGS). The silencing of genes in filamentous fungi is either technically more problematic or labor-intensive, especially if transgenic plants need to be generated first. However, a recent paper from our team demonstrated that a plant virus could infect three related fungal species, as well as express a reporter gene ectopically, and also silence the correspondingly expressed reporter transgene. The gene expression and RNA silencing of the reporter gene was maintained for six passages in culture and also persisted in plants infected by the virus-infected fungus. Here, we consider how the virus can enter and migrate within the fungus, whether the virus can move back and forth between the fungus and the plant and the ramifications of this, the prospects for VIGS being used to silence fungal endogenes and possible biotechnological or therapeutic applications of using plant viruses for expressing foreign proteins in fungi or silencing fungal endogenes.

Plant viruses have proven to be quite versatile as tools for biotechnology. They have been used as vectors for protein expression in plants,1 as well as for expression of fragments of plant genes to silence endogenes.2 The latter process, termed virus-induced gene silencing (VIGS),2 depends on the plant RNA silencing system to generate small RNAs from both the viral RNAs and the expressed plant gene sequence, such that both the viral genome RNA and the host gene RNA sequences are targeted for degradation, leading to gene silencing.3 As this process is not complete, infection by the virus continues allowing the same process to occur in newly formed leaves.4,5 VIGS has been used for high throughput screening for the functional genomics associated with specific processes in plants.2 The ability to use fungal viruses for VIGS in fungi is more limited, due to a number of factors, including the less developed tools available, the less amenable systems for transfection of many fungi, and stability of the fungal virus vectors.6 Thus, in the cases of plant pathogenic fungi, plants have been either infected by plant viruses expressing fungal genes,7,8 or made transgenic to express fungal genes.9 In both cases, the small RNAs generated in the plants can then enter the fungus during infection of the plant to silence the fungal endogene and the effects can be observed on the course of infection. This process is termed host-induced gene silencing, or HIGS,9-15 and also has a number of drawbacks prohibiting high throughput screening.6,16 This prompted a mycologist colleague of one of us (DG) to lament that it is too bad that plant viruses cannot infect fungi. Well, who says that they cannot?

It has been known since 1958 that fungal spores could vector some plant viruses.17 In some cases the virus was
inside the spore, but it was not known how the virus got into the spore. There was no direct evidence for the replication of plant viruses in fungi, although it was also known that both tobacco mosaic virus (TMV)\textsuperscript{18,19} and tobacco necrosis virus\textsuperscript{19} could infect Pythium sp.; oomycetes formerly classified as fungi. In fact, attempts to infect two fungal species (Gaeumannomyces graminis and Aureobasidium bolleyi) and Pythium ultimum with barley stripe mosaic virus were unsuccessful.\textsuperscript{20} Recently, however, it was shown that TMV could indeed infect and replicate in fungi (Fig. 1), specifically three species of Colletotrichum (C. acutatum, C. clavatum, and C. theobromicola).\textsuperscript{21} Infection by TMV did not alter the growth rate, morphology or pathogenicity of C. acutatum, while virus re-isolated from C. acutatum was able to infect plants. Infection of C. acutatum by TMV engineered to express the jellyfish gene encoding green fluorescent protein (GFP), TMV-GFP, resulted in the expression of GFP in both fungal hyphae and conidia (Fig. 2).\textsuperscript{21} The fluorescence was maintained for six passages, but was lost in most mononiodial cultures on the seventh passage. Furthermore, infection of C. acutatum expressing a transgenic GFP gene by TMV-GFP resulted in the silencing of both the GFP transgene and the virally-expressed GFP gene, which again persisted for six passages, with the transgene fluorescence regained in a few of the mononiodial cultures at the seventh passage.\textsuperscript{21} TMV-GFP infectious to plants was not recoverable from the fungus at the seventh passage. Thus, both overexpression of foreign genes and VIGS of endogenes are possible in fungi using the TMV vector system, although these effects may not be permanent.

How does the virus get in and move within the fungus? TMV gets into plant cells when cells are damaged.\textsuperscript{22} TMV infection can be established, during root growth in TMV-infected soil, from an infected plant source among plants grown hydroponically, and by infected leaves rubbing against non-infected leaves.\textsuperscript{23} In the case of tomato seeds obtained from infected fruit, TMV is not inside the seed coat, but the seedlings become infected when they germinate out of the seed coat.\textsuperscript{23} Thus, TMV and several other plant viruses, such as potato virus X and tomato bushy stunt virus, are referred to as being highly contact transmissible plant viruses, vs. those plant viruses that can be transmitted by rubbing high concentrations of inoculum onto leaves, but not by leaves contacting against each.\textsuperscript{23} Infection of fungi by TMV was shown to be an efficient process, approaching nearly 100%.\textsuperscript{21} Hence, it is likely that when hyphae grow in/through a medium, some cell membrane damage occurs and the virus enters the cell (Fig. 3), especially at the growing tip, where the cell wall is in dynamic equilibrium between being broken down to accommodate the growing end and cell wall materials being added to the lengthening cell. Two other entry options are (a) the shaking of virus and either conidia or regenerating hyphae in the incubator may have led to some cell membrane damage allowing TMV to

Figure 1. (A) Electron micrograph of the tip of a hypha of Colletotrichum acutatum not exposed to TMV inoculum. (B) Accumulation of TMV particles at the tip of a hypha of C. acutatum 20 dpi with TMV. White arrows point to aggregates of TMV-like particles while intense vesiculation is visible on the background. (C) In situ localization of TMV particles by immunogold labeling (IGL). The cellular ultrastructure is poorly resolved due to the IGL treatment. Scale bars: 200 nm. Images: courtesy of Dr Angelo De Stradis.
enter the fungal cells, or (b) virus might be taken up by pinocytosis (Fig. 3). Once inside a hyphal strand, the virus should be able to spread throughout the entire mycelium, since the septa separating hyphal cells have openings large enough for organelles to pass through, and thus should not present a barrier to the virus (Fig. 3). However, no TMV accumulation was detected in older hyphae, suggesting either that virus replication was limited to the growing tip of the hyphae, or that the virus was turned over in older hyphae that also undergo autophagy. This also explains the high concentration of TMV in young hyphae, but an overall concentration much lower than an equivalent weight of plant tissue.

We would expect other so-called highly contact transmissible plant viruses to be able to infect filamentous fungi, while those plant viruses that require other biological vectors for their transmission probably would not be able to do so. The extent to which plant viruses that can be transmitted to plants by rubbing virus mechanically onto leaves also can be transmitted to filamentous fungi is unknown, but is the subject of ongoing investigation.

Can the plant virus move back and forth between the fungus and the plant? This has yet to be established conclusively. When *C. acutatum* infected with TMV was inoculated to *Nicotiana benthamiana* flowers or apple fruit tissues, the virus did not move and multiply into other plant tissues outside fungal-infected areas and probably was limited to the mycelia. However, while TMV has been found in apple trees, apparently the virus cannot infect the fruit. In addition, since *C. acutatum* cannot infect *N. benthamiana* vegetative tissues and the flowers represent a strong sink for photosynthate (sugars), TMV, which moves through the phloem to infect distal tissues, would likely be unable to enter the phloem in flowers and move to vegetative tissues against the phloem sap stream. Other fungi, viruses, or virus-fungus-plant combinations will need to be examined to establish whether such virus movement occurs. If the virus cannot exit the fungus and infect the host, this represents another advantage of our approach as the effects of silencing fungal genes by VIGS on plant pathogenicity can be examined without the additional effects that could be caused by infection of the plant by the virus. On the other hand, if the virus can enter the fungus from infected plant cells, then for those fungi which are obligate parasites and thus cannot be grown in liquid medium, like oidia and rust fungi, the ability to enter the hyphae while they grow in planta could be a means of establishing VIGS. The subsequent transfer of the fungus to healthy plants should eliminate any interference from virus infection of the plant host.

Can the VIGS system be used to silence fungal endogenes and examine the effect of this silencing on growth and development in culture and the pathogenicity of fungi in their plant hosts? These have all yet to be established for VIGS, although since they have been established for HIGS, we would expect the same to be true. Of course, there may be some fungi that are missing components of the RNA silencing machinery, in which case this would be more challenging, also requiring the expression of the missing genes needed for RNA silencing. In addition, the extent of fungal gene silencing, which has been quite variable in HIGS, could also affect the outcome, if the same problems occur with VIGS. However, this seems less likely with VIGS, given the high levels of viral RNA expression. Another requirement is that the genes shown to be silenced by VIGS in fungi grown in culture would have to remain silenced after inoculation to the plant. This has been demonstrated indirectly, in that the GFP transgene in *C. acutatum* silenced by infection with GFP was still silenced for GFP expression, after the virus-infected fungus was inoculated to olive seedlings, propagated in planta, recovered from infected leaves and propagated in culture. This could not be shown directly in the fungal-infected plants because *C. acutatum* caused necrosis, which autofluoresces. The modification of several plant viruses (TMV, potato virus X and tobacco rattle virus) by inserting the Gateway cloning system into their genomes can facilitate the high throughput screening of fungal genes for various roles, as has been done for VIGS in plants.

Does overexpression of foreign genes have any effects on the fungus? In the case of overexpressing GFP in *C. acutatum*, there was a reduction in the growth rate of the fungus, but this occurred whether the GFP was expressed from the transgene or from the virus. In fact, overexpression of GFP from either source led to the appearance of electron dense inclusions, which did not occur when the transgenically-expressed GFP was silenced.
by infection with TMV-GFP. Thus, overexpression may lead to aggregation of the protein product, as also often occurs in bacteria. How overexpression affects fungal growth is not clear, since the transgenic fungus still showed a slower growth rate when expression of the GFP transgene was silenced by infection with TMV-GFP, while electron dense inclusions were still visible in fungal hyphae.21 Transgenic expression of GFP, and to a lesser extent from the virus vector, led to changes in the morphology of the fungus. Nevertheless, overexpression of GFP from TMV did not affect the ability of the viral-infected fungus to propagate in apple and olive tissues.21

Is it possible to use plant virus infection of fungi for biotechnological or therapeutic purposes? Some such uses might be predicted. Biotechnological applications...
could include the expression of enzymes in fungi that improve their functionality in food processing, use for control of other pathogens, enhanced mycorrhizal activity (bringing nitrogen and phosphorus to plants), or bioremediation. The therapeutic uses could include the silencing of genes involved in either the production of toxins by fungi or fungal pathogenicity. If virus movement within the fungus does not require the virus movement protein, then virus vector lacking part of its movement protein gene could be released into specific environments to control fungal pathogens, with no risk of the virus being able to infect its natural host.

Does infection of fungi by plant viruses occur in nature? Except for the experimental evidence involving TMV and Typhon in *Colletotrichum* species,21 this has not been recorded. This may be due to a lack of examining fungi for the presence of plant viruses, or perhaps opportunities for infection by plant viruses do not occur as readily in nature. The latter may be because the virus cannot enter the fungus in planta, although has yet to be established. Certainly, plant viruses cannot cross membranes without damage to those membranes, and hence, this may be a limiting factor in planta.23 Nevertheless, the finding that in some cases plant viruses have the ability to infect organisms in a different kingdom offers new opportunities for both a better understanding of what host factors are required for plant virus multiplication, as well as the development of new technologies for better understanding the biology of fungi and their potential uses in serving the dominant species.

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Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest.

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