Mobile Genetic Elements 4, e29782; January–December 2014; © 2014 Landes Bioscience

Something new to explore

Plant viruses infecting and inducing gene silencing in filamentous fungi

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Keywords: Fungal transfection, host species jump, plant pathogen adaptation, protein over expression, virus-induced gene silencing

Abbreviations: GFP, green fluorescent protein; HIGS, host-induced gene silencing; RNAi, RNA interference; TMV, tobacco mosaic virus; VIGS, virus-induced gene silencing

Submitted: 06/20/2014

Accepted: 06/30/2014

Published Online: 07/07/2014

Citation: Gallitelli D, Palukaitis P, Mascia T. RE: PNAS paper Gene silencing and gene expression in phytopathogenic fungi using a plant virus vector. Mobile Genetic Elements 2014; 4:e29782; http://dx.doi.org/10.4161/mge.29782

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Commentary to: Mascia T, Nigro F, Abdallah A, Ferrara M, De Stradis A, Faedda R, Palukaitis P, Gallitelli D. Gene silencing and gene expression in phytopathogenic fungi using a plant virus vector. Proc Natl Acad Sci U S A 2014; 111:4291–6; PMID:24594602; http://dx.doi.org/10.1073/pnas.1315668111

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

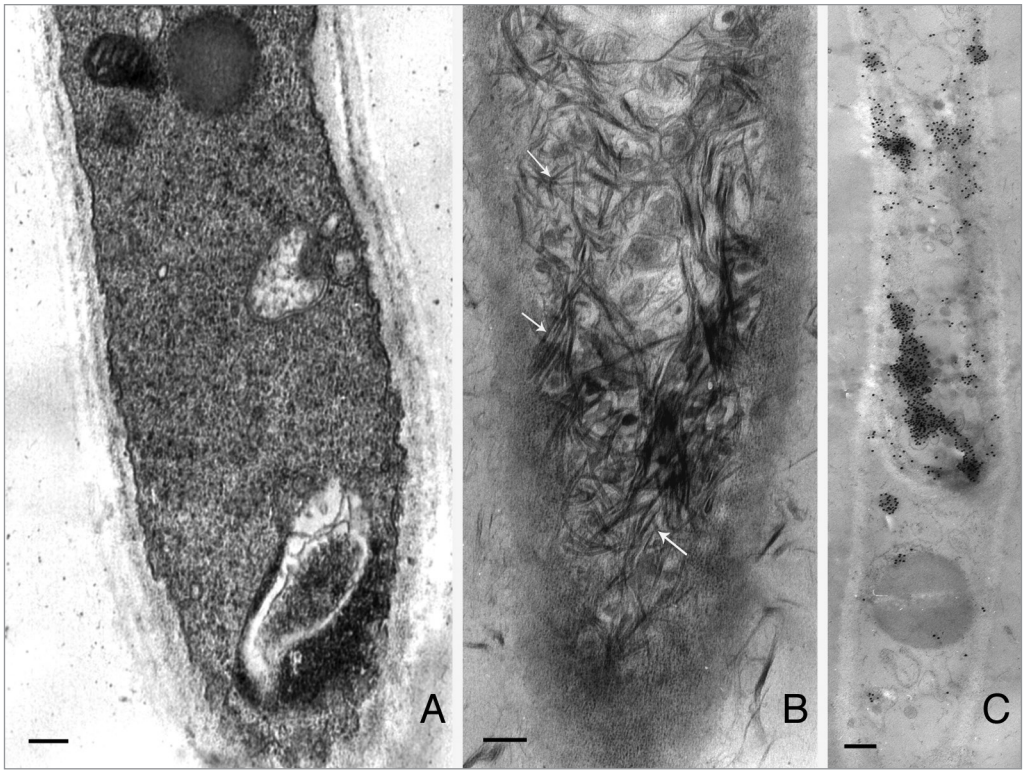

Figure 1. (**A**) Electron micrograph of the tip of a hypha of *Colletotrichum acutatum* not exposed to TMV inoculum. (**B**) Accumulation of TMV particles at the tip of a hypha of *C. acutatum* 20 dpi with TMV. White arrows point to aggregates of TMV-like particles while intense vesiculation is visible 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.

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)[18,19] and tobacco necrosis virus[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.[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*).[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**).[21] The fluorescence was maintained for six passages, but was lost in most monoconidial 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 monoconidial cultures at the seventh passage.[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.[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.[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.[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.[23] Infection of fungi by TMV was shown to be an efficient process, approaching nearly 100%.[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

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[24] (recycling of cellular materials) (**Fig. 3**). This also explains the high concentration of TMV in young hyphae, but an overall concentration much lower than an equivalent weight of plant tissue.[21] 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.[25,26] 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,[22] 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 grows 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.

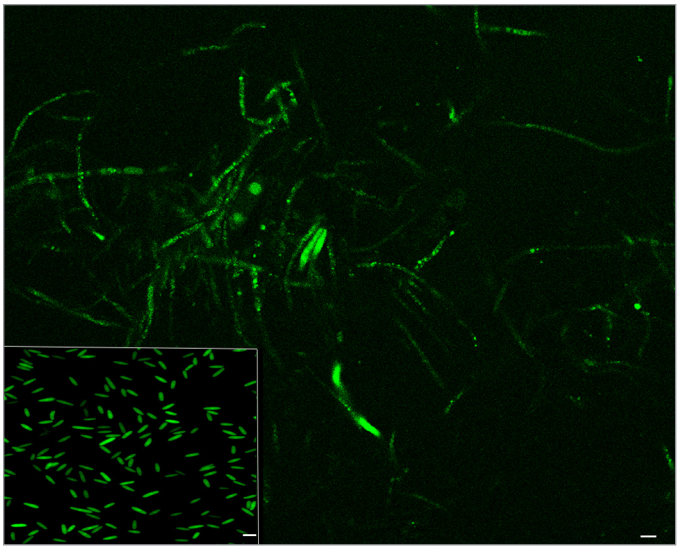

Figure 2. Hyphae and conidia (inset) of *Colletotrichum acutatum*, strain C71, emitting florescence from the ectopic expression of GFP vectored by the plant virus TMV. Scale bar: 10 μ. Image: courtesy of Prof. Franco Nigro.

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,[9-15] we would expect the same to be true. Of course, there may be some fungi that are missing components of the RNA silencing machinery,[9] 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.[27-29]

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.[21] 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

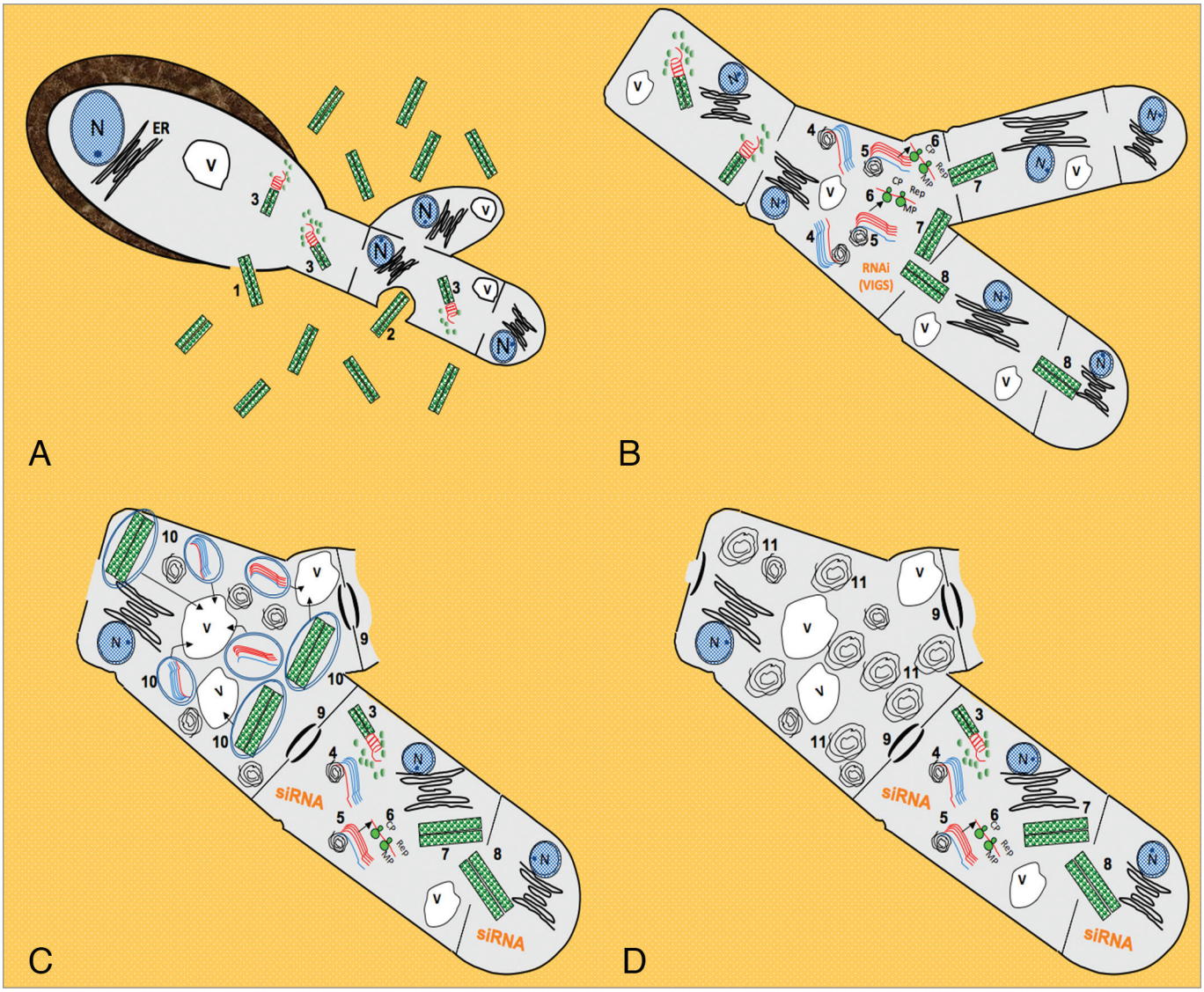

Figure 3. Hypothetical pattern of TMV entrance, replication and movement in fungal hyphae. (**A**) TMV particles added to liquid growing medium penetrate germinating conidia either through damage in the plasma membrane (1) or by pinocytosis after damage of the cell wall (2). After entering, TMV particles disassemble by uncoating and viral nucleic acid is released (3). (**B**) Uncoated viral nucleic acid (red line) associates with cellular membranes in which it induces strong vesiculation and is replicated by a viral-encoded RNA polymerase, probably associated with host components as in plants (4). Replication first produces minus strands (blue lines), which associate with other cellular membranes/vesicles (5) to synthesize plus-strand RNA (red lines). At this stage, double-stranded viral RNA is produced, which very likely activates an RNAi mechanism. If a recombinant viral vector is used to introduce a gene with sequence homology to a fungal endogene, the latter is silenced by a sequence homology-dependent mechanism (VIGS). Small interfering RNAs (siRNA) then move systemically through all fungal cells, spreading the silencing signal. The run-off plus strand RNA transcripts (red lines in 5) are translated to viral proteins (6) by fungal ribosomes and mature TMV particles are assembled (7). The newly produced particles are passively transported into new cells (8) as they are produced at the tips of the hyphae. (**C**) In the newly infected cells, virus replication begins again (3, 4, 5, 6, 7). The old cells, full of vesicles, virus particles and viral proteins and enzymes are insulated from the new ones by the synthesis of parenthesomes (9). Then, autophagosomes (10) are produced around remnants of viral replication and virus particles to carry them to the vacuoles for degradation. (**D**) The old cells show extensive vesiculation (11) and ultrastructure alterations but no virus particles. Picture components are not drawn to scale. N, nucleus; V, vacuole; ER, endoplasmic reticulum.

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 hypae.[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 TNV in *Pythium* species[18,19] and TMV 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 plants 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.

Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

The authors thank Dr Angelo De Stradis, Istituto per la Protezione Sostenibile delle Piante del CNR, Bari, Italy and Prof Franco Nigro, Dipartimento di Science del Suolo della Pianta e degli Alimenti, Università degli Studi di Bari Aldo Moro, Bari, Italy for supplying the images shown in Figures 1 and 2. PP was supported by grant number RDA-PJ009568 from the Next Generation BioGreen21 Program of the Rural Development Administration, Republic of Korea, and grant number NRF-2013R1A2A2A01016282 from the Korean National Research Foundation.

References

1. Hefferon KL. Plant virus expression vectors set the stage as production platforms for biopharmaceutical proteins. Virology 2012; 433:1-6; PMID:22979981; http://dx.doi.org/10.1016/j.virol.2012.06.012
2. Senthil-Kumar M, Mysore KS. New dimensions for VIGS in plant functional genomics. Trends Plant Sci 2011; 16:656-65; PMID:21937256; http://dx.doi.org/10.1016/j.tplants.2011.08.006
3. Ding S-W. RNA-based antiviral immunity. Nat Rev Immunol 2010; 10:632-44; PMID:20706278; http://dx.doi.org/10.1038/nri2824
4. Melnyk CW, Molnar A, Baulcombe DC. Intercellular and systemic movement of RNA silencing signals. EMBO J 2011; 30:3553-63; PMID:21878996; http://dx.doi.org/10.1038/emboj.2011.274
5. Wang M-B, Masuta C, Smith NA, Shimura H. RNA silencing and plant viral diseases. Mol Plant Microbe Interact 2012; 25:1275-85; PMID:22670757; http://dx.doi.org/10.1094/MPMI-04-12-0093-CR
6. Nuss DL. Mycoviruses, RNA silencing, and viral RNA recombination. Adv Virus Res 2011; 80:25-48; PMID:21762820; http://dx.doi.org/10.1016/B978-0-12-385987-7.00002-6
7. Yin C, Jurgenson JE, Hulbert SH. Development of a host-induced RNAi system in the wheat stripe rust fungus *Puccinia striiformis* f. sp. *tritici*. Mol Plant Microbe Interact 2011; 24:554-61; PMID:21190437; http://dx.doi.org/10.1094/MPMI-10-10-0229
8. Lee W-S, Hammond-Kosack KE, Kanyuka K. *Barley stripe mosaic virus*-mediated tools for investigating gene function in cereal plants and their pathogens: virus-induced gene silencing, host-mediated gene silencing, and virus-mediated overexpression of heterologous protein. Plant Physiol 2012; 160:582-90; PMID:22885938; http://dx.doi.org/10.1104/pp.112.203489
9. Meyer V. Genetic engineering of filamentous fungi--progress, obstacles and future trends. Biotechnol Adv 2008; 26:177-85; PMID:18201856; http://dx.doi.org/10.1016/j.biotechadv.2007.12.001
10. Nowara D, Gay A, Lacomme C, Shaw J, Ridout C, Douchkov D, Hensel G, Kumlehn J, Schweizer P. HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. Plant Cell 2010; 22:3130-41; PMID:20884801; http://dx.doi.org/10.1105/tpc.110.077040
11. Tinoco MLP, Dias BBA, Dall'Astta RC, Pamphile JA, Aragão FJL. In vivo trans-specific gene silencing in fungal cells by in planta expression of a double-stranded RNA. BMC Biol 2010; 8:27; PMID:20356372; http://dx.doi.org/10.1186/1741-7007-8-27
12. Panwar V, McCallum B, Bakkeren G. Endogenous silencing of *Puccinia triticina* pathogenicity genes through in planta-expressed sequences leads to the suppression of rust diseases on wheat. Plant J 2013a; 73:521-32; PMID:23110316; http://dx.doi.org/10.1111/tpj.12047
13. Panwar V, McCallum B, Bakkeren G. Host-induced gene silencing of wheat leaf rust fungus Puccinia triticina pathogenicity genes mediated by the Barley stripe mosaic virus. Plant Mol Biol 2013b; 81:595-608; PMID:23417582; http://dx.doi.org/10.1007/s11103-013-0022-7
14. Nunes CC, Dean RA. Host-induced gene silencing: a tool for understanding fungal host interaction and for developing novel disease control strategies. Mol Plant Pathol 2012; 13:519-29; PMID:22111693; http://dx.doi.org/10.1111/j.1364-3703.2011.00766.x
15. Ghag SB, Shekhawat UKS, Ganapathi TR. Host-induced post-transcriptional hairpin RNA-mediated gene silencing of vital fungal genes confers efficient resistance against Fusarium wilt in banana. Plant Biotechnol J 2014; 12:541-53; PMID:24476152; http://dx.doi.org/10.1111/pbi.12158
16. Suzuki N, Geletka LM, Nuss DL. Essential and dispensable virus-encoded replication elements revealed by efforts To develop hypoviruses as gene expression vectors. J Virol 2000; 74:7568-77; PMID:10906211; http://dx.doi.org/10.1128/JVI.74.16.7568-7577.2000
17. Grogan RG, Campbell RN. Fungi as vectors and hosts of plant viruses. Annu Rev Phytopathol 1966; 4:29-52; http://dx.doi.org/10.1146/annurev.py.04.090166.000333
18. Brants H. Tobacco mosaic virus in Pythium spec. Neth J Plant Pathol 1969; 75:296-9; http://dx.doi.org/10.1007/BF02015491
19. Nienhaus F, Mack C. Infection of *Pythium arrehnomanes* in vitro with tobacco mosaic virus and tobacco necrosis virus. Z Pflanzenkrankh 1974; 81:728-31
20. Lange L. Experiments on establishing a BSMV infection in three phytopathogenic fungi. Phytopathol Z 1977; 90:184-8; http://dx.doi.org/10.1111/j.1439-0434.1977.tb03235.x
21. Mascia T, Nigro F, Abdallah A, Ferrara M, De Stradis A, Faedda R, Palukaitis P, Gallitelli D. Gene silencing and gene expression in phytopathogenic fungi using a plant virus vector. Proc Natl Acad Sci U S A 2014; 111:4291-6; PMID:24594602; http://dx.doi.org/10.1073/pnas.1315668111
22. Liu C, Nelson RS. The cell biology of Tobacco mosaic virus replication and movement. Front Plant Sci 2013; 4:12; PMID:23403525; http://dx.doi.org/10.3389/fpls.2013.00012
23. Hull R. Matthews' Plant Virology. San Diego, California: Academic Press 2002; 1001 pp
24. Voigt O, Pöggeler S. Self-eating to grow and kill: autophagy in filamentous ascomycetes. Appl Microbiol Biotechnol 2013; 97:9277-90; PMID:24077722; http://dx.doi.org/10.1007/s00253-013-5221-2
25. Kirkpatrick HC, Lindner RC. Recovery of tobacco mosaic virus from apple. Pl Dis Reptr 1964; 48:855-7
26. Ragozzino A, van der Meer FA, Maat DZ. Does tobacco mosaic virus occur in the Netherlands? Neth J Plant Pathol 1970; 76:229-33; http://dx.doi.org/10.1007/BF01976580
27. Liu Y, Schiff M, Dinesh-Kumar SP. Virus-induced gene silencing in tomato. Plant J 2002; 31:777-86; PMID:12220268; http://dx.doi.org/10.1046/j.1365-313X.2002.01394.x
28. Avesani L, Marconi G, Morandini F, Albertini E, Bruschetta M, Bortesi L, Pezzotti M, Porceddu A. Stability of Potato virus X expression vectors is related to insert size: implications for replication models and risk assessment. Transgenic Res 2007; 16:587-97; PMID:17216546; http://dx.doi.org/10.1007/s11248-006-9051-1
29. Lacorte C, Ribeiro SG, Lohuis D, Goldbach R, Prins M. Potatovirus X and Tobacco mosaic virus-based vectors compatible with the Gateway cloning system. J Virol Methods 2010; 164:7-13; PMID:19903495; http://dx.doi.org/10.1016/j.jviromet.2009.11.005