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in the US are needed to fully understand the early spread of HIV-1 in North America.

One of the most significant features of the work of Worobey *et al.* [3] lies in recovering the genome of the so-called 'Patient 0'. Worobey *et al.* present a detailed historical analysis of how the legendary status of 'Patient 0' was formed [3]. Let's make a long story short. According to the study of Worobey *et al.* [3], Patient 0 was initially numbered as Case 057, when the report reached the Centers for Disease Control and Prevention (CDC) in the US. An epidemiologic study that started with patients from California identified a cluster of homosexual men with AIDS-like symptoms, which was historically important in suggesting the sexual transmission of AIDS [6]. When Patient 0 was placed near the center of this cluster, he was renamed as 'Patient O', meaning a patient residing 'Out[side]-of-California'. Unfortunately, 'Patient O' was misinterpreted as 'Patient 0' when the CDC investigators rearranged the cluster cases by the disease onset dates. Although the identity of Patient 0 has never been disclosed by the CDC, the California-based journalist Randy Shilts revealed it (Gaétan Dugas, a highly sexually active Canadian airline steward) in his popular book *And the Band Played On* [7]. In this book, Dugas was claimed to deliberately spread the disease and was described as 'the Quebeçois version of Typhoid Mary' [7,8]. Subsequent media coverage of the book insinuated that Patient 0 was the source of the HIV/AIDS epidemic in North America. Although this idea has drawn criticism and clarification [8], it is still enjoying considerable attention by the public. The HIV-1 genome recovered from Patient 0 should directly test this idea. If it is true, the genome would be the earliest branching isolate among the US subtype B infections. However, Worobey *et al.* found that the viral sequence of Patient 0 was nested within the diversity of other US subtype B strains, which shows clear-cut evidence that Patient 0 was not the index case but is 'just one of many thousands infected prior to the recognition of HIV/AIDS' [3]. This finding should finally lay to rest the theory of Patient 0 as the source of the North American epidemic.

Although Worobey *et al.* succeeded in answering when and where the North American epidemic emerged, the question remains as to how the subtype B viruses entered North America. There are several plausible scenarios: (i) Caribbean immigrants brought the virus into North America; (ii) American individuals were infected by the virus when visiting the Caribbean, possibly via sex tourism; and (iii) contaminated commercial blood products imported from the Caribbean led to American infections [5,9]. To disentangle these possibilities, further virus archeological investigations of relevant samples should be performed.

The > 2500 serum samples which Worobey *et al.* serologically screened were collected for studying hepatitis B virus in the first place [3]. Worobey *et al.*'s work would not be possible without these archival samples. Archival samples play crucial roles in studying the emergence and evolutionary history of not only HIV-1 but also many other infectious agents, such as the 'Spanish flu' influenza virus [10]. Moreover, they are of potential importance in tracking the origin, diversification, and evolution of newly emerging diseases that circulate but have been neglected, such as Zika virus. Unfortunately, many archival samples are going to dwindle as their collectors fade away and thus will never be discovered. Therefore, the scientific community should conceive of ways to preserve archival samples.

Acknowledgments

G.Z.H. was supported by the Natural Science Foundation of Jiangsu Province (BK20161016) and the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

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http://dx.doi.org/10.1016/j.tim.2016.11.005

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Spotlight

Spray-Induced Gene Silencing: a Powerful Innovative Strategy for Crop Protection

Ming Wang[1] and Hailing Jin[1,*]

Plant pathogens cause serious crop losses worldwide. Recent new studies demonstrate that spraying double-stranded RNAs (dsRNAs) and small RNAs (sRNAs) that target essential pathogen genes on plant surfaces confer efficient crop protection. This so-called spray-induced gene silencing (SIGS) strategy of disease

control is potentially sustainable and environmentally friendly.

Plant diseases caused by eukaryotic pathogens, such as fungi and oomycetes, have a devastating worldwide economic and agronomic effect on crop production. For example, *Fusarium graminearum* causes Fusarium head blight and Fusarium seedling blight in important cereal crops such as rice, maize, and wheat, as well as other crops such as soybean. Mycotoxins produced during the progression of these diseases are harmful to animals and humans and compromise food safety, putting a strain on the world grain industry. Current disease control methods are still mainly dependent on chemical sprays, which potentially have harmful environmental and health side-effects, and induce fungicide-resistant pathogen strains [1]. With the increasing world population, reduced farmland, and need for heightened global food security come the need for new sustainable, effective, and environmentally-friendly solutions to control plant diseases. A new transgene-based host-induced gene silencing (HIGS) strategy, which involves host expression of hairpin RNAs or small RNAs targeting genes in interacting pathogens and pests, has been developed in multiple crop systems to effectively control diseases caused by insects, nematodes, fungi, and oomycetes [2]. However, HIGS is limited by several factors: the lack of available transformation protocols in many crop species, public concern for the production of genetically modified crops (GMOs), and the instability of engineered RNA silencing traits.

A recent study by Koch *et al.* published in the current issue of *PLoS Pathogens* demonstrated an effective RNA spraying method – the so called spray-induced gene silencing (SIGS) – for controlling *F. graminearum* infections on barley [3]. Koch *et al.* previously showed that *Arabidopsis* and barley ectopically expressing a double-stranded RNA (dsRNA) targeting three important fungal *CYP51* genes which encode cytochrome P450 lanosterol C14α-demethylase significantly enhanced plant resistance to *F. graminearum* species by disrupting fungal membrane integrity [4]. In the current study, Koch *et al.* elegantly demonstrated that even spraying detached barley leaves with a 791-nt long *CYP3*-dsRNAs that contains complementary sequences to *CYP51B, CYP51A*, and *CYP51C* prior to fungal infection could effectively inhibit disease and yield much smaller lesions, indicating reduced disease development [3]. Moreover, the levels of the three *CYP51* genes were also reduced as measured by reverse transcription quantitative PCR, indicating that the *CYP51*-targeting RNAs get into fungal cells to suppress the expression of fungal *CYP51* genes [3]. They also demonstrated that spraying the RNA fragments of jellyfish green fluorescent protein (GFP) on barley leaves effectively silenced the expression of GFP in a GFP-expressing *F. graminearum* strain, suggesting that such SIGS is not sequence selective, potentially allowing for targeting of any essential genes in various interacting pathogens [3]. Thus, SIGS is a new innovative strategy for protecting crops from pathogen infection. Indeed, similar disease control was also observed in another study by Wang *et al.* who showed that when externally applying dsRNAs and small RNAs (sRNAs) targeting Dicer-like protein genes *DCL1* and *DCL2* of *Botrytis cinerea* on vegetables, fruits, and flower petals, grey mold disease was effectively suppressed [5]. Both *Botrytis cinerea* DCL proteins are required for generating sRNA effectors [6]. These studies suggest that such RNA-based disease control strategy is effective on both monocots and dicots. SIGS is powerful, fast, and environmentally friendly, which also circumvents the problems in creating GMOs.

Strikingly, SIGS also conferred resistance against *F. graminearum* in unsprayed distal leaf parts. The relative amounts of fungal *CYP51* transcripts were also reduced in the unsprayed distal area, strongly suggesting that these dsRNAs were translocated into plant cells and tissues and the silencing signals were effectively spread to distal parts [3]. RNA uptake has been only observed in a few organisms, and most mechanistic studies were performed in nematodes [7]. Recently, Wang *et al.* also showed that the plant fungal pathogen *Botrytis cinerea* was capable of taking up external dsRNAs and sRNAs [5]. However, plant uptake of external RNA molecules was not reported. Here, Koch *et al.* have provided convincing evidence to show that green fluorescent dye ATTO 488-labeled dsRNAs were indeed taken up by barley cells when sprayed on the barley leaf surface and subsequently transported into other parts of the plants through the vasculature [3]. The labeled RNAs were observed in xylem, phloem parenchyma cells, companion cells, mesophyll cells, trichomes, and stomata. *CYP3*-dsRNAs were also detected by Northern blot analysis in both sprayed and non-sprayed leaf parts [3]. This is the first example of active RNA uptake by plant cells. Thus, the RNAs sprayed on the plant surfaces have at least two possible pathways to get into fungal cells (Figure 1): the RNAs are taken up by the plant cells first and then transferred into the fungal cells [3], and/or the RNAs are taken up by the fungal cells directly [5]. Fungal cells are likely to take up RNAs via both pathways spontaneously. Furthermore, Koch *et al.* showed that the *F. graminearum* DCL1 protein was required for *CYP3*-dsRNA processing and efficient SIGS in systemic leaf areas [3], indicating that translocated long dsRNAs are processed into sRNAs by fungal DCL1 proteins to induce silencing of fungal genes.

To serve as an efficient disease control agent, a reasonable duration of efficacy is desired. The Northern blot analysis showed that the expression of *CYP3*-dsRNAs was not reduced even at 168 hours post spray of the local sprayed site, suggesting either these external RNAs were stable for seven days on the surface of the leaves and/or they were efficiently taken up and remained stable in the plant cells [3]. Consistent with this, Wang *et al.* also demonstrated that dsRNAs and sRNAs could protect

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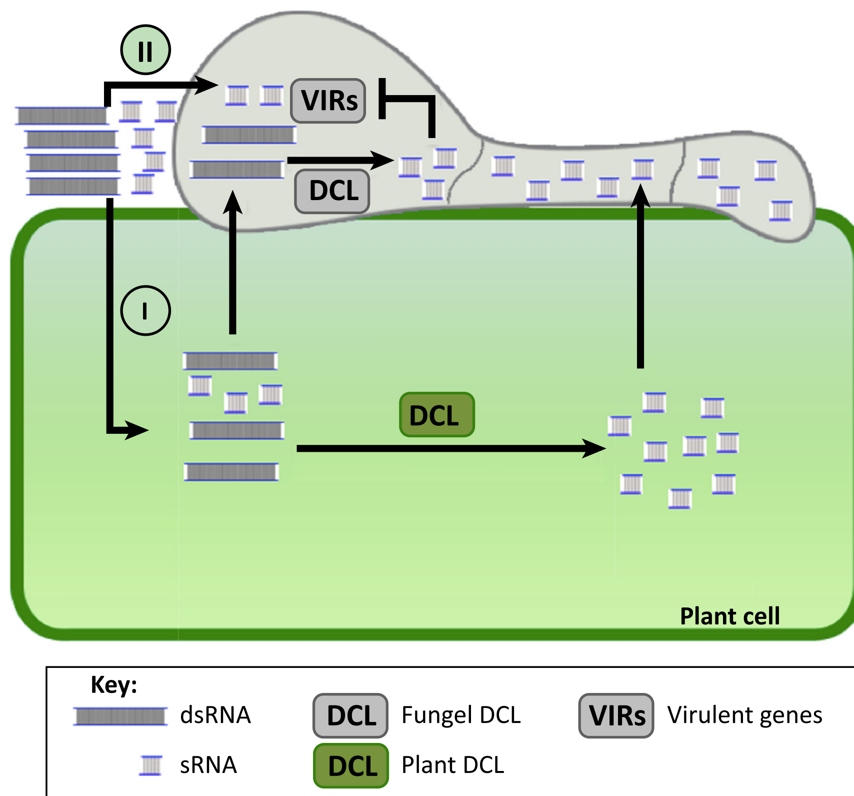

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Figure 1. Two Possible Pathways of Silencing Fungal Genes Induced by dsRNA- and sRNA-Spray. Pathway one: the external dsRNAs and sRNAs are taken up by the plant cells and then transferred into fungal cells (I). These dsRNAs are cleaved into sRNAs by either the plant DCL proteins or fungal DCL proteins. At the same time, the transferred dsRNAs and sRNAs in the plant cells also systemically spread and are transferred into fungal cells. The systemically spread dsRNAs are processed into sRNAs mainly by the fungal DCL1 protein. Pathway two: the external dsRNAs and sRNAs are directly taken up by the fungal cells (II), and the transferred dsRNAs are processed into sRNAs by the fungal DCL proteins.

vegetables and fruits against grey mold disease for up to 8 days [5].

Taken together, the study by Koch *et al.* highlighted the effectiveness of this novel SIGS disease control method by targeting genes vital to fungal integrity or pathogenicity. Indeed, spraying RNAs were also used as 'oral insecticides' to control plant pests [8–10]. SIGS can be tailored to be highly specific for fungal genes without disrupting host gene expression and can potentially be developed against an unlimited range of pathogens or pests. SIGS has opened an avenue of development of 'RNA fungicides' that are environmentally-friendly [3,5], because they are made of nucleotides that are present in all life and will not leave toxic residues in the soil and environment. Furthermore, because such RNA fungicides are sequence based rather than structure based, they are likely to be sustainable and should not induce resistant or tolerant mutated pathogen strains.

Further research is needed to determine how these external RNAs are taken up by plant and fungal cells, and how these RNAs are transported from plant cells into fungal cells, and vice versa. Application strategies can be improved by mixing the RNAs with chemical reagents to stabilize the RNAs and thus increase the strength and duration of plant protection. Overall, this new generation of RNAi-based fungicides looks promising to meet our world's increasing demands for increasing safety and quality of crop yields to feed the growing population.

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http://dx.doi.org/10.1016/j.tim.2016.11.011

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Spotlight

Decoding the Ecological Function of Accessory Genome

Michelle Qiu Carter[1,*]

Shiga toxin-producing *Escherichia coli* O157:H7 primarily resides in cattle asymptomatically, and can be transmitted to humans through