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Opinion piece

A silenced spring?

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There seemed to be a well-thumbed copy of *Silent Spring* (Carsen, 1962) in every biology classroom in which I was taught. This was the late 1970s/early 1980s, the environmental movement was in full swing and lapel badges with pressure group logos were the designer labels showing an individual's allegiance. Chemical industries were amongst the pariahs, not helped by infamous cases, such as dichlorodiphenyltrichloroethane (DDT) and the Bhopal pesticide plant disaster. Therefore, at the time, I had no idea that I might end up in a career which includes aspects of plant pathology and crop protection. Molecular genetics was the new big thing and, importantly, it gave coherent simple answers without resorting to statistics. Therefore, that was the subject area I focused on at university. Genes were straightforward to explain. They were segments of DNA. There was a promoter region at the start, controlling on/off and volume. Then came the open reading frame (ORF), encoding the protein, and, finally, a terminator region to ensure correct processing of the transcript. Gene ORFs were transcribed to mRNA, these were translated into protein and proteins were enzymes doing things in the cell, all summed up in the 'central dogma of molecular biology'.

DNA was obviously the important component. Yes, admittedly, there were ideas about RNA having been the primitive self-replicator from which all life derived, but things had moved on in the intervening 4 billion years or so. RNA was now of minor importance; rRNA helped ribosomes to function, tRNA carried amino acids for precise translation and mRNA was a messenger; roles all readily explainable. There were RNA viruses, but viruses were weird, and could not be thought of as normal, or even alive.

Through my career, it has become increasingly clear that this simplistic view of molecular biology is woefully inadequate, with RNA-based processes gaining more and more prominence, yet triggering little debate.

Several recent papers highlight how this could impact on plant pathology.

Koch *et al*. (2013) showed that the expression of a silencing construct, targeting all three *Fusarium cyp51* genes in either transgenic *Arabidopsis* or barley, could give excellent control of *Fusarium graminearum*. This fungus is an important pathogen, not just because of yield losses, but also because of mycotoxin production in infected grains, and thus its efficient control has long been desirable.

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Why highlight this paper in particular? After all, virus control by gene silencing has been going on for years. I guess this is partly my bias against viruses, but mostly that viral replication is within the host cytoplasm, and so readily accessible to host-induced gene silencing. The control of free-living external pests seems to be far more challenging; plus, it has been the traditional remit of the agrichemical industry, with all sorts of compounds developed for their control.

Previously, there have been similar reports of efficient host-induced gene silencing (HIGS) control of various invertebrate crop pests, such as nematodes (e.g. Huang *et al*., 2006) and insects (Baum *et al*., 2007), but these were built on well-founded mechanisms of gene silencing in such organisms, where it was well established that ingestion of the silencer construct (usually a hairpin RNA) was sufficient to trigger silencing, at least in related model systems, such as *Drosophila melanogaster* or *Caenorhabditis elegans*; thus, the mechanism for crop protection was readily apparent.

The control of a fungus is another matter. Conversations at conferences had suggested that several groups had tried to feed silencing constructs to fungi, but without any apparent success. Thus, an *in planta* silencing mechanism against fungi was far from obvious. That was until Nowara *et al*. (2010) showed that HIGS-based silencing was effective against the powdery mildew *Blumeria graminis*, followed by others reporting similar successes on rusts (e.g. Panwar *et al*., 2013a, b). These authors used viral or transient *Agrobacterium* systems to drive the expression of the silencing RNA, and so it was only a matter of time before a true transgenic plant was made to target an important pathogen. That is what was delivered by Koch *et al*. (2013). And the target involved? Well, what better choice than *cyp51*, the target of the azole fungicides, a well-characterized system known to be essential for efficient fungal growth and development. Great news, plant-derived silencing can be effective against pathogens. What an innovative system. Congratulations to all involved!

In the following month, Weiberg *et al*. (2013) published their study on *Botrytis*. Here was a fungal pathogen blocking the plant's defence reactions to assist with disease progression. The molecules responsible for this were fungal-derived RNA. In this case, *Botrytis* was using silencing-dependent mechanisms to target host processes. Using sequencing-based methods, Weiberg *et al*. (2013) had identified in excess of 800 fungal sRNAs from *Botrytis cinerea*. Certainly, some of these were important for the infection

of *Arabidopsis* or tomato, but, with in excess of 800 sRNA molecules made, how many of these might be important for the infection of the hundreds of other plant species for which *Botrytis* is a pathogen? How many such sRNAs might there be in other fungi? It is noteworthy that our current genome annotation tools still sometimes struggle to identify traditional protein-encoding genes. Thus, the likelihood of predicting the sRNA complement of a fungus from the genome sequence alone is still some way off, let alone the means to then predict the functionality of such molecules.

Virus-induced gene silencing (VIGS) has also had considerable success in helping to understand gene function in plant physiology. Thus, it is no great surprise that it has been similarly successful in the study of plant pathogens. However, an article by Mascia *et al*. (2014) has gone a step further. Recombinant *Tobacco mosaic virus* (TMV) [a green fluorescent protein (GFP)-expressing strain] has been shown to be taken up by several species of *Colletotrichum* and to replicate asymptomatically in these fungi. Yes, that is correct, a plant virus entering into and replicating in a plant-pathogenic fungus. In a wild-type fungus, GFP fluorescence was observed, demonstrating that virally encoded proteins could be expressed, whereas viral transduction into a GFP-expressing transgenic fungus caused silencing of a fungal-encoded GFP. This clearly opens up new ways to manipulate the expression of fungal genes, possibly including those involved in infection processes, but raises all sorts of questions about the potential use of modified viruses in the biological control of plant pathogens.

FUTURE PROSPECTS

So where will things go from here? Clearly, there is lots of scope for the use of these techniques in laboratory studies to more rapidly ascertain gene function and to obtain better insights into infection or resistance. However, this is moving into new territory and there is much still to be discovered about these RNA-based mechanisms. To me, at least, it is perhaps less clear how quickly such approaches should be used in commercial agriculture. Do we need to fully understand these processes before they can be exploited for commercial purposes or is it acceptable to simply demonstrate no appreciable persistence or toxicology, as is the case for some current agrichemicals for which modes of action may still be unclear?

In terms of the mechanisms, there have been numerous studies in model organisms looking at silencing. Yet, as far as I am aware, it is difficult to predict the likely strength of silencing in a transformant. Indeed, we know that different transformants show different degrees of silencing when transformed with the same construct. Why should this be the case in a system that involves both a component of self-perpetuation and of amplification? Is it simply a result of the variation in the level of expression of the silencer, or is it a more complex interaction involving the physiological state of the recipient at the time at which silencing is triggered. Thus, will each infection event be prone to the same degree of silencing-based control, or might this vary?

For these HIGS-based mechanisms, how much silencer needs to be present to be effective, and how well does this work in a real situation in which plants are not maintained under constant environmental conditions? If we think about chemical control systems, there has been considerable effort placed into ensuring that sufficient active ingredient reaches the correct location to obtain effective control of the pathogen, much of which has been achieved by the careful design of spray application methods, coupled with deliberate manipulation of the compounds to ensure appropriate partitioning in lipids versus the aqueous phase, or even some volatility. How do we ensure that silencing-based control systems can present the sRNA to the pathogen wherever it might be on the plant, and at the required concentration? As with other transgenic approaches, should these constructs be expressed constitutively in all tissues, thus increasing the likelihood of non-target effects, or should they be deployed in a more careful manner, but with the risk of incomplete control?

Which pathogens should be targeted for control? Chemical fungicides often display broad-spectrum control, with activity against minor pathogens as well as the major targeted species. Silencing could be far more specific, giving control only of those species which have targets with sequence identity to the siRNA. This has the benefit of probably being able to control root-infecting pathogens, which is still a challenge for conventional chemical control methods. However, to achieve a good breadth of control, it will either be necessary to stack together numerous siRNA constructs (expressed individually or in chimaera) or to attack highly conserved targets. Either of these strategies could potentially have the same issues as broad-spectrum chemicals in impacting on non-target organisms, or indeed beneficial fungi, if not carefully and thoroughly investigated in advance.

Which targets should be selected for effective durable disease control? Will it be the core processes common to many of the current fungicides, such as complex II or III of the electron transport chain? Will it be against fungal-specific processes, such as ergosterol or chitin synthesis? Or, will we see new targets developed? It is notable that Koch *et al*. (2013) targeted the *cyp51* genes of *Fusarium*. These are well-characterized fungicide targets, and the degree to which they need to be impacted for effective control is likely to be known. In comparison, the targets used against coleopteran pests (Baum *et al*., 2007) are core functions, many of which are not currently accessible to chemical intervention and so are less well understood.

Similarly, what does this mean for resistance management? With such selection pressures, presumably resistance is inevitable, but by what mechanism? Will we see mutant versions of the target genes arise for which sequence-specific silencing is no longer effective, and, if so, will these mutations be predictable? Resist-

ance to a single specific siRNA is likely to be manageable, simply by changing the specific control measure. However, might we see the loss of the uptake of the siRNA by the pathogen, or perhaps impairments in gene silencing within the fungus, reducing the efficacy of any silencing-based control? After all, some fungi, such as *Ustilago maydis*, prosper without having the necessary machinery for efficient gene silencing, and some oomycete plant pathogens, such as *Phytophthora parasitica*, seem not to be amenable to such silencing techniques. If we do not understand how silencing functions and how it might be compromised, the design of a plan to best use such crops, whilst minimizing the risk of resistance, is going to be a challenge. The article by Weiberg *et al*. (2013) shows that, in some cases, such as *B. cinerea*, complete loss of the gene silencing pathway in the fungus results in reduced virulence, and so this might yet turn out to be a durable form of control.

RNA clearly plays far more roles than those implied within the 'central dogma of molecular biology'. However, our understanding of RNA-mediated silencing mechanisms and their control is still in its comparative infancy. Any predictions on how silencing could be evaded are likely to be mostly guesswork, unless considerable effort is placed into this area of research. Should the release of such crops be delayed until we have a better understanding? If so, how much is sufficient information? Likewise, should the use of these mechanisms be managed by something similar to a Fungicide Resistance Action Group to maximize their longevity, or are these simply dominant resistance genes just like all the others deployed by plant breeders?

I expect that the rise of gene silencing is likely to continue in both laboratory studies and commercial planting. Indeed, these sorts of traits might become the acceptable face of genetically modified (GM) crops for European farming, provided that they give reliable disease control with reduced pesticide inputs. There is certainly scope for far more research into these mechanisms and how they should best be deployed. Thus, no doubt, there will be a flood of papers emerging over the coming years about this sort of technology. It certainly provides a new twist to crop protection, perhaps delivering fields of silenced spring wheat rather than Carsen's warning of a *Silent Spring*. However, I doubt that the critics of such GM technologies will ever be silenced.

Perhaps, we should have discovered all of this RNA weaponry much earlier. Twenty years ago, I read a paper by Celerin *et al*. (1994). They were looking at the structure of fimbriae, the fungal equivalent to bacterial pili, in the anther smut *Microbotryum violaceum*. These fimbriae apparently had a 30-nucleotide single-stranded RNA molecule associated with them. Now, what would a fungus be doing with a short RNA molecule, on a spike, aiming at a plant cell?

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