Of maize and men, or peas and people: case histories to justify plants and other model systems

David Baulcombe

One of the byproducts of molecular biology has been support for the 'model system' concept. All living organisms are based on the same genetic code, they have similar subcellular structures and they use homologous metabolic pathways. So, mechanisms can be investigated using organisms other than those in which the knowledge will be exploited for practical benefit. Model systems are particularly useful in the early discovery phase of a scientific endeavor, and recent progress in biomedical science has fully vindicated their use. Jacques Monod, for example, famously justified his work on a bacterial model system by stating that "what is true for *Escherichia coli* is also true for elephants." My fellow laureates, Victor Ambros and Gary Ruvkun, can defend the use of the worm *Caenorhabditis elegans* as a good model system and so I will focus on plants.

Probably the best example I could use is that of Barbara McClintock, who used corn in her Nobel Prize–winning work[1]. Her identification of mobile genetic elements has been enormously influential in microbiology and immunology, and continues to bear on the field of epigenetics that I refer to again below. She could have adapted Monod's aphorism to refer to maize and men. Gregor Mendel would, of course, have referred to peas and people.

There are many other historical examples, in addition to McClintock's and Mendel's work, that could justify the use of plants as models. Robert Hooke used his newly developed microscope in 1664 to show that cork comprises structures that he referred to as cells ("Our microscope informs us that the substance of cork is altogether filled with air, and that air is perfectly enclosed in little boxes or cells distinct from one another.")[2] (**Fig. 1**). Two hundred fifty years later, Beijerinck discovered a *contagium vivum fluidum* in extracts of diseased tobacco plants that he later referred to as a virus[3].

In contemporary science, a green alga—*Chlamydomonas reinhardtii*—is a useful model in the analysis of kidney disease[4]. However, in this article, I refer to the contribution of plant biology to a family of mechanisms that I refer to as RNA silencing. This topic has been reviewed comprehensively elsewhere[5,6], so here I focus on personal experience and my view of future potential from this work.

The early history of RNA silencing in plants

The most important development in plant biology in the 1980s was the introduction of methods for transfer of DNA into the nuclear genome[7]. Transgenic techniques were developed as a routine research tool, and the new technology prompted the introduction of genetic manipulation for crop improvement. The first generation of genetically modified plants used bacterial genes for herbicide tolerance or insect resistance[8,9]. There were also attempts from several laboratories, including my own, to develop virus resistance by transgenic expression of virus-derived genes in the host organism[10–12]. We were testing a concept known as parasite-derived resistance in which the replication or spread of a disease agent could be reduced by transgenic expression of its genes in a host. A third category of these early transgenic crops exploited an antisense approach to block expression of an endogenous plant gene[13]. Crops were to be improved in this approach by specific suppression of an endogenous gene that limited agronomic performance. The idea was simple: a duplex RNA structure would be formed between the mRNA of the target gene and a transgenic antisense RNA. This duplex structure would then either block translation of the mRNA or initiate its degradation by a nuclease specific for double-stranded RNA.

Figure 1 Robert Hooke's micrograph of cells in a sample of cork. Downloaded from http://www.nlm.nih.gov/exhibition/historicalanatomies/Images/1200_pixels/hooke_t11.jpg.

Many of the early transgenic plants had the desired traits, and they were the progenitors of transgenic crops that are now grown in large areas of different countries. However, there were anomalous phenotypes in some of the transgenic lines. For example, the bacterial and viral transgenes were not always expressed[8,9]. In the 'antisense plants', the suppression effect

David Baulcombe is Royal Society Research Professor and Professor of Botany, Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK.
e-mail: dcb40@cam.ac.uk

Richard Jorgensen

Figure 2 Variegation of silencing in flowers of petunia. The target of silencing by a sense transgene is chalcone synthase. The silencing effect manifested as the absence of pigment is present in only part of the flower. Reprinted with permission of the American Society of Plant Biologists.

was sometimes variegated—it was present on some branches of the plant and not in others, or it was absent in localized parts of a flower[14]. There was also a problem with some of the sense RNA controls in these antisense experiments. There was a silencing effect even when the transgene was designed to generate sense RNA that would not form a duplex with the target mRNA[15–17]. In some examples, there was variegation of this sense suppression as with the antisense plants (**Fig. 2**). Another strange process occurred when two transgenes carried the same promoter sequence: one of these transgenes could silence the other through a process operating at the transcriptional level[18].

In retrospect, I can see that these anomalies challenged the existing paradigm of genetic regulation, but initially, I made the mistake of thinking that they were due to multiple distinct mechanisms and that they were artifacts of transgenes. However, I became more interested when we characterized transgenic plants manifesting parasite-derived resistance. The plants with the strongest viral resistance were those in which the transgene RNA was present at a low abundance, whereas plants expressing the same gene at a high level were fully susceptible[19]. It seemed that the transgene silencing in this example of 'less is more' could not be dismissed as a side effect or artifact.

A new model to explain some of the counterintuitive properties of the virus-resistant plants was attractive—at least to us—because it could account for the anomalous results with many other transgenic plants, although it could not explain the variegation or transinactivation effects. The model proposed that certain transgenes, irrespective of whether they were designed to be expressed in sense or antisense orientation, have a silencing property that operates in a nucleotide sequence–specific manner. The silencing *in cis* would explain why the transgenes in certain lines were not abundantly expressed. Correspondingly, the silencing *in trans* would account for the ability of these transgenes to silence endogenous genes or viruses[20,21].

The hypothesis further proposed that the silencing mechanism operates at the RNA level because the targeted viruses had an RNA genome and did not have a DNA phase in their replication cycle. Originally, many of the silencing-related phenomena in transgenic plants were referred to as 'gene silencing'. However, at a seminar in Switzerland, I was interrupted by Ingo Potrykus—the inventor of 'golden rice'[22]—who pointed out that I was not talking about 'gene silencing'; it was 'RNA silencing'. I agreed and have since promoted 'RNA silencing' as a generic term to cover a family of mechanisms involving silencing and RNA.

Support for the model came from a series of tests carried out by Jim English with genetically modified viruses[20], and the remaining challenges were to find out about the details of the mechanism and its biological role. We were also interested in finding out whether the variegated silencing of transgenes and transcriptional silencing of promoter sequences could be accommodated in the model.

A central mystery in RNA silencing was its ability to act in a nucleotide sequence–specific manner. The simplest explanation required an antisense RNA that would guide a silencing machinery to its target. In the transgenic experiments described above, this antisense RNA would be present irrespective of whether the transgene was in a sense or an antisense orientation. Andrew Hamilton, who had been interested in antisense RNA ever since his PhD work on transgenic tomatoes[23], joined the lab to carry out the search for this elusive molecular species. It is testimony to his persistence and talent as a scientist that he eventually found them[24]. He tried several different methods, and his early successes used a sensitive but imprecise RNase protection method that generated rather ugly looking smears instead of the neat electropherograms that were eventually published in *Science* (**Fig. 3**). Our first estimate was that these antisense RNAs were 25 nucleotides long, but later, we refined this to 21–24 nucleotides[25]. They are now known as small interfering RNAs (siRNAs)[26].

The initial convergence of our work with that of Ambros and Ruvkun was because the methods we used to detect siRNAs were the same as those used to detect small temporal RNAs from *lin-4* in worms[27,28]. However, we did not immediately make the biological connection. In retrospect, I cannot say why—it seems obvious now. My failure to make the connection was probably because the mechanism in worms involved suppression of translation, whereas, in plants, we were dealing with a process that decreased stability of the targeted RNA.

Andrew Hamilton's experiments further demonstrated that the production of small antisense RNA required transcription of the corresponding sense strand. This involvement of both RNA strands provided an obvious connection between RNA silencing in plants and RNA interference in worms as revealed by the famous Andrew Fire and Craig Mello 1998 paper in *Nature*[29]. Once this connection was recognized, there was an opportunity for discoveries in plants to catalyze our understanding of animal systems and vice versa. It has been enormously satisfying to witness this exchange of information and the elucidation of variations on a molecular theme associated with different types of silencing mechanism in diverse organisms[5,6].

Much current RNA silencing research interest is focused on endogenous small RNAs. In plants, there are thousands of loci that produce these small RNAs[30–32]. A small proportion of the endogenous small RNAs are similar to the microRNAs of worms that were first discovered as short temporal RNAs by Ruvkun and Ambros[27,28]. Most microRNAs are negative regulators, although a positive regulatory microRNA has been recently described[33]. The other small RNAs in plants are siRNAs, the double-stranded RNA precursors, which are produced in various ways, including the annealing of complementary RNA, foldback of long inverted repeats and the action of RNA-dependent RNA polymerases on a single-stranded RNA[30,31].

It could be said that, now that we have the understanding of multiple variations on silencing mechanisms, RNA silencing research with a biomedical target should focus exclusively on people or at least on vertebrate experimental systems. However, for this view to be valid, the discovery phase of RNA silencing research would have to be played out. I do not consider this to be the case just yet because there are at least two areas—virus resistance and epigenetics—in which there is much to be discovered and in which RNA silencing in plants has the potential to inform biomedical research.

RNA silencing and virus resistance

Genetic engineering of virus resistance was my introduction to RNA silencing, and there is a nice irony from the subsequent discoveries that plants are naturally protected from viruses by RNA silencing. Frank Ratcliff, Tamas Dalmay and Olivier Voinnet joined my group to investigate these aspects of RNA

silencing together with Andrew Hamilton. Their work, alongside that of the Covey, Carrington and Vance groups, revealed a picture in which virus-specific siRNAs are produced using a viral RNA template in infected plant cells[34–40]. These siRNAs then target an RNA silencing effector protein to the viral RNA so that the replication cycle of the virus is blocked. Inevitably, in the arms race of defense and counter-defense between viruses and their hosts, plant viral genomes encode pathogenicity or virulence factors that are suppressors of silencing. Olivier's work, together with the work of those other groups, revealed that there are many different types of viral suppressor protein[36–40].

Associated with antiviral silencing, there is a mobile signal that mediates antiviral effects of silencing beyond the infected regions of the plant and that prevents or delays spread of the virus in the infected plant. The discovery of signaling[41,42] was particularly satisfying because, in addition to virus resistance, it explained the variegation anomaly in the early transgenic antisense and sense RNA experiments: signal produced in one part of the plant spread unevenly so that the silencing effect was manifested only in the parts in which it was received.

We now understand that RNA silencing influences many aspects of the interactions between viruses and their plant hosts. It explains, for example, how viruses are excluded from the meristem of infected plants, why some plants recover from viral disease and why virus-infected plants may be protected from secondary infection. Many aspects of symptom production may also be due to suppression of endogenous genes by viral siRNA. Additionally, in a biomedical context and consistent with the model system justification for plant work, RNA silencing can target viral RNA in animals.

Invertebrates may use RNA silencing as part of natural antiviral defense, as in plants[43]. In vertebrates, RNA silencing may not be part of the natural antiviral defense systems[44]. However, artificial RNA silencing can be effectively targeted against viruses[45]. Given the difficulty of developing small molecule therapeutic agents to target viruses, there is much interest in the possibility of delivering double-stranded RNA or siRNAs to protect against viral disease. A major challenge in such a task is to find ways to chemically modify the RNA so that it is stable until it is taken up by cells and targeted to the desired cell type. This challenge is therefore essentially pharmacological and probably outside the influence of plant biology. However, it may be possible, on the basis of an understanding of the plant viral systems, to optimize the intracellular efficacy of antiviral siRNAs. Nature has already done the experiments in plants to find out which regions of viral genomes are the most effective targets of siRNAs and whether the antiviral potential is influenced by base composition or other chemical characteristics of the siRNAs. Given the structural and genetic similarities of various groups of plant and animal viruses, it is likely that the understanding of these plant systems will facilitate development of novel antiviral therapy in medicine.

RNA silencing and epigenetics

The final anomaly in the original transgenic experiments—promoter silencing at the transcriptional level—was finally resolved by the Matzke group and others. They demonstrated that double-stranded RNA and siRNAs have the potential to guide epigenetic modification of DNA or the associated chromatin proteins in plants and other organisms[25,46,47]. The silencer transgene evidently produces siRNAs that can target the promoter of the silenced gene.

There is now a good understanding of this epigenetic RNA silencing mechanism[48], and we also know that many endogenous siRNAs are associated with epigenetic modification of the corresponding DNA[30,49]. The siRNA associated with epigenetic modification is 24 nucleotides long. However, consistent with the past history of RNA silencing research, a mystery remains because these 24-nucleotide siRNAs do not have an obvious role. Mutant plants that do not produce these 24-nucleotide siRNAs are able to grow and develop at a normal rate[50]. We can therefore rule out the possibility that these RNAs have a major role in normal growth and development. Many of these endogenous siRNAs correspond to transposons[30]. However, transposons are not mobilized in RNA silencing mutants, and it seems unlikely that these RNAs have the obvious role in protection against damage caused by mobile genetic elements.

Resolution of this mystery may not have immediate relevance to biomedicine because the answer may involve mechanisms in evolution. One possibility is that epigenetic RNA silencing by one parent may silence essential genes in the opposite parental genome and thereby influence hybrid formation. A second possible role is prompted by Louise Jones's finding that RNA-directed epigenetic changes may persist for several generations even in the absence of the initiator RNA[51]. This finding illustrates how there are separate initiation and maintenance phases to the RNA-directed epigenetic mechanism. It also illustrates how endogenous siRNAs that are induced by environmental stimuli could induce heritable phenotypic changes. Such changes would not be associated with genetic mutation but nevertheless could be subject to selection in certain environments.

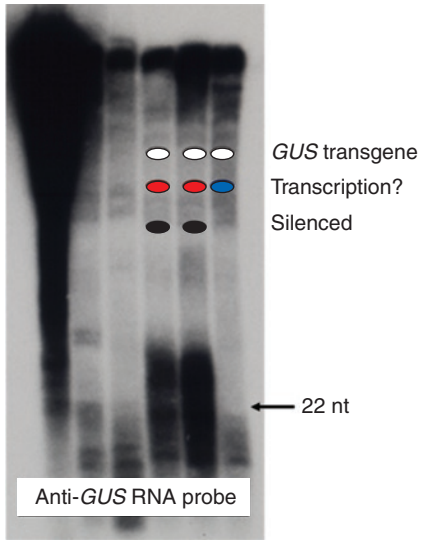

Figure 3 One of Andrew Hamilton's early RNase protection assays showing that small antisense RNA—the black smear corresponding to 22 nucleotides (nt)—was only present in plants that were silencing the target RNA. Production of the *GUS* reporter gene antisense RNA was suppressed if the sense transcription was blocked. One of Andrew Hamilton's early RNase protection assays showing that small antisense RNA was only present in plants that were silencing the target RNA. Production of the *GUS* reporter gene antisense RNA, detected by the anti-*GUS* RNA probe, was suppressed if the sense transcription was blocked. Far left, hybridization probe without RNase treatment. The two unmarked samples in the next two lanes were from plants without a *GUS* reporter gene; there is no evidence of any antisense RNA. The other three samples (white symbols) were from plants with a *GUS* transgene. The sample on the far right (blue symbol) had an untranscribed *GUS* transgene and there was also no antisense RNA. However, the two samples from a plant with a transcribed *GUS* transgene (red symbols) that was silenced at the RNA level generated a smeared signal centered on the 22-nucleotide length that was the first evidence for siRNAs.

If this RNA-directed epigenetic modification occurs in humans—and there are some indications that it may[52,53]—the existence of separate initiation and maintenance phases may allow long-term silencing of some targets. Delivery of promoter-targeted siRNA would initiate silencing of a selected disease-causing gene. The RNA-independent maintenance mechanism would then mediate long-term persistence of the silencing effect.

Postscript

The focus of this article should not be taken as special pleading for plants. It is more intended as an attempt to use a set of case histories to justify model systems in general, with plants given an equal footing alongside yeast, worms,

fungi and the rest. I realize that it is not easy for science policy makers and funding agencies to prioritize funding and also that basic scientists—myself included—do not always make their task easy because we often prefer to stay in the comfort zone of basic research. It is easier there than in the uncompromising world of technology, in which the only important consideration is whether something works in the field or patient. Nevertheless, I hope that some of the decision makers in biomedical science will bear in mind the small-RNA story when they decide how to allocate their funds. There will be other discoveries through work in model systems that will have application in diagnosis or treatment of disease.

I hope that this short account may encourage young scientists setting out on their career or deciding which systems to use as they set up their first research group. It may well be that plants will help you address whatever scientific questions you are attracted toward. There is also the possibility, of course, that your efforts with plant systems will help with the small problem of harvesting the sun and feeding the world.

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