Off-target Effects of Plant Transgenic RNAi: Three Mechanisms Lead to Distinct Toxicological and Environmental Hazards

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Abstract

Recent developments in the application of RNA interference (RNAi) to plants mean that the introduction of transgenes with defined sequences can now routinely result in the inhibition of target RNAs and therefore gene activity. Consequently, there are now greatly enhanced opportunities for the use of this technology in agriculture and speciality crops. Applications demonstrated so far include the manipulation of plant metabolism and behaviour, and resistance to pathogenic bacteria, viruses, insects, and other invertebrates. Realisation of this potential, however, will ultimately depend on the specificity with which transgene-derived RNAs act. Specificity is important within the crop plant itself but also towards exposed non-target organisms such as beneficial insects and mammals. There has been little discussion of off-target effects (OTEs) arising from plant transgenic RNAi. This review considers three classes of potential plant RNAi OTEs: (1) OTEs leading to non-specific downregulation of plant RNAs; (2) OTEs affecting non-target invertebrates feeding on plant material and; (3) potential effects on mammals. In mammals, long (>30bp) perfectly duplexed RNAs (such as are typically produced by plant RNAi transgenes) are Pathogen Associated Molecular Patterns (PAMPS) and are consequently highly potent triggers of innate anti-viral defences. The effects of long dsRNAs on mammalian cellular functions are typically profound and extend to complete inhibition of protein translation and cell death. Nevertheless, the implications of such molecules in the mammalian diet have hardly been tested.

Introduction

The discovery of multiple pathways of cellular regulation by small RNA molecules in eukaryotic organisms is almost certainly the most dramatic single development in cell biology in the last twenty years. We now know that superimposed on the traditional pathways of cellular regulation, such as gene expression and biochemical feedback loops, is a whole other layer of control based on the homologous base pairing of small (21-25nt) RNA molecules to either DNA or other RNAs (Bartel 2004). These diverse small RNA molecules, principally microRNAs (miRNAs) and short interfering RNAs (siRNAs), are thought to influence gene activity by three main mechanisms: by binding to DNA and altering its activity; by inhibiting protein translation through binding to mRNAs; and by targeting mRNAs for destruction (for a plant review see Ossowski et al. 2008; Table 1). These pathways, now called RNA interference (RNAi) pathways (but formerly gene silencing or co-suppression) provide, in principle at least, simple opportunities for precise intervention in cellular functioning. In theory, researchers can now artificially modulate any biological process for therapeutic or research purposes using molecules of RNA designed according to the simple rules of Watson-Crick base pairing.

Only a few years ago, this seemed likely to be an almost unconstrained therapeutic opportunity to cure human disorders or infectious diseases and an unmitigated boon to researchers studying gene function (Hannon and Rossi 2004). In the last few years, however, a series of flies have been discovered in this ointment. These flies are diverse reports of off-target effects (OTEs) attributable to short RNA molecules (Cho-Chung and Becker 2003; Saxena et al. 2003; Jackson and Linsley 2004; Scacheri et al. 2004; Lin et al. 2005; Jackson et al. 2006; Kulkarni et al. 2006; Ma et al. 2006). One of these reports asserts that as many as 70% of small RNAs can cause mis-expression of a single non-

target transcript (Scacheri et al. 2004), another that as few as 7 nucleotides of sequence homology can produce an off-target effect (OTE) (Lin et al. 2005). On the other hand, some authors have found OTEs to be rare (Chi et al. 2003).

Since the great interest in RNAi rests absolutely on the specificity with which small RNAs bind to their targets, the existence of OTEs constitutes a potentially major setback for the prospects of RNA interference. Therefore, despite some discrepancies in these findings, researchers in animal systems have generally accepted that the rules governing small RNA specificity may not be so simple after all and concerns over side effects have been an important reason no therapeutic product based on RNAi has yet reached the market (Juliano et al. 2008; Kleinman et al. 2008).

RNA interference in plant biotechnology

RNA interference is considered also to be a highly promising technology in the field of plant biotechnology. To achieve RNA interference, a transgene homologous to the target is constructed typically as inverted repeats separated by an intron or other non-functional sequence. When integrated and transcribed, the resulting RNA forms a hairpin loop structure, which in turn generates siRNAs that can alter plant phenotypes (Waterhouse et al. 1998). This hairpin transgene arrangement is so far the one most commonly used but other potential gene silencing systems also exist (e.g. Schwab et al. 2006). The remainder of this review, unless indicated, refers to hairpin (hpRNA) transgene constructs (Waterhouse et al. 1998).

The experimental achievements of transgenic RNAi in plants are already considerable. They include the downregulation of individual plant genes and the control of gene activity in a tissue-specific fashion, the second of which has yet to be achieved with conventional breeding (e.g. Sunilkumar et al. 2006). A further potential advantage of RNAi over conventional breeding is that inhibition of gene expression via silencing is a genetically *dominant* trait, while mutations in conventional breeding are typically *recessive* traits, meaning that only a single copy of the transgene is required. It is hoped that such manipulations will allow the nutritional alteration of crop products as well as perhaps the removal of plant allergens or toxins, although this can also be done with conventional breeding. An example, which may become the first deliberate commercial use of RNAi anywhere is a high oleic acid soybean (TREUS™) from Pioneer (DP-305423-1) which was developed using an hpRNA transgene (Delaney et al. 2008). TREUS™ is awaiting approval in the US, Australia and New Zealand.

Of perhaps still greater interest is that RNA interference can also enable plants to resist pests and pathogens. This has so far been demonstrated against viruses (e.g. Waterhouse et al. 1998), bacterial pathogens (Escobar et al. 2001), nematodes (Yadav et al. 2006; Huang et al. 2006; Steeves et al. 2006; Sindhu et al. 2009) and the chewing insects western corn rootworm and cotton bollworm (Baum et al. 2007; Mao et al. 2007). RNAi may also allow plants to resist parasitic weeds, which can be important pests (Tomilov et al. 2008). This general phenomenon, of small RNAs from one organism entering the environment and affecting other organisms, has been termed 'environmental RNAi' (Whangbo and Hunter 2007).

Consequently, transgenic RNAi has commercial appeal for many crop applications (Waterhouse et al. 1998; Eamens et al. 2008). This optimism over plant RNAi, however, is again conditional on specificity, a subject which has received only limited discussion in plants (Auer and Fredericks 2009). Recent progress, however, in the understanding of OTEs in animals, together with the discovery that artificial miRNAs and hairpin-loop transgenes both generate OTEs in plants, suggest that OTEs could also prove important in plant biotechnology (Table 2).

The discovery of OTEs in plants

The overwhelming majority of research measuring the specific OTEs produced by artificial or introduced RNAs has been performed by introducing small RNAs into animal cell cultures (Cho-Chung and Becker 2003; Saxena et al. 2003; Jackson and Linsley 2004; Scacheri et al. 2004; Lin et al. 2005; Jackson et al. 2006). However, demonstrations of OTEs in animals are not necessarily applicable to RNAi transgenic plants. This is partly due to the evolutionary distance separating plants from animals but also because of the various technical differences between transgenic plants expressing hairpin loop RNAs compared to experiments on animal cell cultures.

Nevertheless, despite this caveat, plant and animal OTEs so far show many similarities. In plants, as in animal systems, for naturally occurring RNAs, interference does not depend on perfect sequence complementarity between the miRNA and its target mRNA. This is true both for miRNAs that translationally repress their target (Aukerman and Sakai 2003) and for those that catalyse cleavage of their target mRNA (Tang et al. 2003). Though such promiscuous interactions are now firmly established in plants, they do not, however, constitute examples of OTEs since they are not inadvertent. Their significance instead was in providing evidence for non-specific interactions that we now know apply also to artificial RNAs. Indeed. *bona fide* OTEs by artificial plant miRNAs were discovered soon afterwards (Schwab et al. 2005; Schwab et al. 2006). This work established two important facts; that naturally occurring miRNAs could cause OTEs if expressed outside their natural tissues and, that artifical miRNAs could lead to unexpected phenotypes (Table 2).

Despite the difficulties of extrapolation, one animal cell culture OTE report does have particular relevance to transgenic plants on account of its substantial methodological similarities to plant RNAi using hpRNAs (Ma et al. 2006). In this report the authors individually inhibited (by RNAi) each of 20,000 genes of the *D. melanogaster* genome to look for novel members of the Wingless signal transduction pathway. To knock out individual genes the authors used long dsRNAs derived from cloned Drosophila cDNAs. To their surprise, the knockdown screen identified, not the novel pathway components hoped for, but instead only known Wingless pathway genes. In other words, although they tried (by removing known Wingless pathway genes from their dsRNA library) to prevent knocking out already-known Wingless genes, they failed to achieve this. Thus, in their experiments, dsRNAs from non-Wingless genes targeted Wingless genes, even though none had precise sequence homology. Their 'results', therefore, were all OTEs.

There is irony in these findings: the authors' use of longer RNA sequences stemmed in the first place from their hope

of avoiding the OTEs associated with shorter RNAs. Instead, the opposite occurred. Longer sequences affected more spurious targets than shorter ones.

One of the implications of this study was to heighten the probability that transgenic plants expressing hpRNAs (i.e. long dsRNAs rather than miRNAs) would also be found to generate OTEs. This expectation was confirmed almost simultaneously by a study on the plant *Arabidopsis thaliana* (Xu et al. 2006). In this report, a hairpin loop transgene construct (derived from the gene *BTI1)*, caused multiple OTEs compared to the *BTI1* null mutant, as measured by cellular mRNA levels (Xu et al. 2006). Moreover, the authors found that multiple silenced lines also had an unanticipated delay in flowering. Thus, the first deliberate effort, in a plant, to identify unanticipated consequences arising from a hairpin RNAi transgene, proved successful. Since then, a further set of diverse OTEs associated with hairpin constructs have been described, also in *A. thaliana* (Xing and Zachgo 2007). Taken together, these results imply that OTEs from transgenes may be common events.

Estimating the frequency of OTEs in plants

Two divergent approaches can be taken to estimate the frequency of OTEs. A theoretically oriented approach is to use the data available in completed genome sequences to calculate the frequency with which small RNAs will interfere with other RNAs. Applying this approach to plants Xu and colleagues calculated that, depending on the species, a typical full length plant gene would exhibit between 3 and 24 OTEs (Xu et al. 2006). This approach requires making some major assumptions, however. The most obvious of these was that complete sequence identity (21nt) is required to generate an OTE. When the authors tested their predictions experimentally (in *A. thaliana*) 3 of the 14 predicted targets were downregulated by the BTI1 hairpin transgene (Xu et al. 2006). This work demonstrated that sequence similarity searches in plants can identify OTEs. It also supports the hypothesis that OTEs in RNAi plants will be common. Nevertheless, there is one study that failed to find even one OTE in a transgenic plant carrying an hpRNA (Aelbrecht et al. 2006)

An alternative approach, based on direct measurement of general cellular mRNA downregulation, can be demonstrated using the *Drosophila* data of Ma et al. (2006). Using a threshold of a 2.5 fold reduction in mRNA expression levels as the minimum criterion for a downregulatory effect, Ma et al. showed that seven out of 20,000 (non-Wingless) dsRNAs affected the five known genes of the Wingless pathway. Assuming that the Wingless pathway is typical in its propensity to be targeted by OTEs, this calculates to an approximately 1 in 16,600 chance of a single dsRNA producing a 2.5 fold or more reduction to any single gene in the *D. melanogaster* genome. In a genome size of approximately 30,000 genes this means that any one dsRNA will downregulate, by 2.5 fold or more, on average approximately two non-target transcripts. If, instead, the threshold were set at any reduction at all in mRNA expression levels, then the same data set suggests that each dsRNA would on average downregulate seven genes in addition to its known target. Such small-fold effects are normally considered below the threshold of experimental interest but they may not be below biological interest since a 1.5 fold downregulation of an mRNA by a plant

transgene resulted in lethal effects in nematodes (Sindhu et al. 2009).

The frequency of OTEs implied by these detailed experiments is considerable, but it is not out of line with other similar studies (Kulkarni et al. 2006). Ma et al. (2006), however, believe that the true probability of an OTE may be higher than they observed, and for a reason that has implications for transgenic RNAi organisms in general. Ma et al. (2006) performed their experiments over a two day period; they noticed, however, that the probability of observing an OTE appeared to increase significantly with time, and they proposed that there can be a time delay such that cellular processes that cycle slowly show effects only after a time-lag. One implication is that a transgenic organism using RNA interference would have its whole lifespan to develop OTEs.

A related point is also worth noting. Only in plants have OTEs been studied under conditions that allow them to develop over the course of growth and differentiation. All other investigations into OTEs have been on specific cell types, which express only a subset of their total genome. The probability of OTEs will presumably be greater in whole organisms compared to single cell types, at least for constitutively expressed transgenes.

Our overall conclusion, therefore, is that although they are still relatively little studied, and one study failed to observe them at all, most evidence from studies of long dsRNAs in animals and hpRNAs and amiRNAs in plants predicts that multiple OTEs should be expected within any one RNAi transgenic plant line. Many important questions remain to be resolved, however. For example, are results from *A. thaliana* also true in crop species? And are particular sequences or particular genes especially prone or resistant to OTEs, either as target molecules or as effectors?

RNAs as novel pesticides

A novel and remarkable ability of plant hpRNA transgenes to lethally (and sub-lethally) harm pests and pathogens is now becoming apparent (Waterhouse et al. 1998; Escobar et al. 2001; Huang et al. 2006; Yadav et al. 2006; Baum et al. 2007; Mao et al. 2007). This ability invites a comparison between RNAi and pesticides and prompts the question of what extent transgenic RNAi effects will be specific to the organisms for which they are intended (Whangbo and Hunter 2008). This is a question that is especially important given the many non-pest species in agricultural systems that would be routinely exposed to RNAi through contact with crop plant tissues.

This organism specificity question has so far only been addressed among insects and nematodes (Huang et al. 2006; Baum et al. 2007; Sindhu et al. 2009). For each of these, however, it has been shown, using both dsRNAs produced *in vitro* and with hpRNAs from a plant transgene (consumed as part of plant tissues), that dsRNAs can cause mortality and reproductive failure in non-target organisms (Huang et al. 2006; Baum et al. 2007; Sindhu et al. 2009). Importantly, however, such non-target effects have so far been observed only in species related to the target organism (Table 2). These findings are discussed in detail below.

In the case of the *in vitro* produced dsRNAs, these were derived from the genome of the western corn rootworm

(WCR; *Diabrotica virgifera virgifera* LeConte), and were applied to the food of the non-target coleopteran species spotted cucumber beetle (*Diabrotica undecimpunctata howardi*) and also of the Colorado potato beetle (*Leptinotarsa decemlineata*) (Baum et al. 2007). Of the three WCR dsRNAs tested on spotted cucumber beetle and the two WCR dsRNAs tested on Colorado potato beetle, all possessed the ability to cause significant mortality, though a higher concentration of non-homologous RNAs was required to give the same percent mortality (Baum et al. 2007). To a third coleopteran species, the cotton boll weevil (*Anthonomous grandis* Boheman), however, neither WCR dsRNAs, nor dsRNAs derived from its own genome had any detected phenotypic consequence (Baum et al. 2007).

Effects on non-target species have also been obtained by feeding plant tissues expressing hpRNAi transgenes to root-knot nematodes. In reporting that an hpRNA transgene expressed in *A. thaliana* was active against the target species *Meloidogyne incognita,* Huang et al. (2006) also determined that the same transgenic plant tissues were also active against three other *Meloidogyne* species. A potentially highly important observation is that, unlike the results of Baum et al., the transgene was *more* effective against two of the three non-target species than it was against the target itself (Huang et al. 2006). Non-target RNAi activity has also been observed against cyst nematodes. Integrated hpRNA transgenes constructed using sequences from the genome of *Heterodera glycines* (a cyst nematode) protected *A. thaliana* against the related species *Heterodera schachtii* (Sindhu et al. 2009).

Many important questions remain, but the experiments above are significant in securely establishing that, even in the absence of perfect sequence complementarity, hpRNAs expressed from an integrated plant transgene or dsRNAs applied to food, can cause lethal and sub-lethal effects in non-target organisms (related at the family level or below). In some instances RNAi was less effective against non-targets and in other cases it was more effective, but the probability of RNAi affecting related non-targets (excepting of *A. grandis* which is apparently refractory to environmental RNAi) is thus far 100%. A key issue then becomes, from the point of view of non-target effects, at what evolutionary distance do OTEs cease? And how much does this depend on the gene chosen and the RNAi length? Answers to these questions will have to await further research.

OTEs in unrelated non-target organisms

Another important question in assessing the potential pesticidal effects of transgenic RNAi is whether OTEs can also affect organisms entirely unrelated to the target species. This question arises because the detailed studies of OTEs in animal cell cultures indicate that even siRNAs (which are considered more specific than hpRNAs) generally do not have just one mRNA target (Saxena et al. 2003; Jackson and Linsley 2004; Scacheri et al. 2004; Lin et al. 2005; Jackson et al. 2006). Instead, when interfering RNAs of any kind enter tissues in sufficient concentration to cause on-target effects, they also typically cause off-target ones, unless they are specifically selected to avoid them.

Many organisms are sensitive to the presence of small RNAs, either in their food or in the ambient environment (Whangbo and Hunter 2008). As well as viruses, bacteria, nematodes and insects, the list of species demonstrated to

be susceptible to environmental RNAi now includes protozoans, planarians, arthropods and cnidarians (Galvani and Sperling 2002; Newmark et al. 2003; Vayssié et al. 2004; Soares et al. 2005). The large number of RNAi-susceptible organisms, and the near-ubiquity of OTEs observed so far, together suggest the hypothesis that, if used in crop plants, transgenic RNAi might well have important ecological consequences, considerably beyond toxicity towards the close relatives of target pests.

In spite of this inference, the few direct experimental tests for phenotypic effects on unrelated organisms have so far proved negative. In one experiment the nematode *H. Schachtii* was allowed to feed on plants (*A. thaliana)* expressing hpRNAs capable of inhibiting reproduction and growth of the related nematode *M. incognita*, which is in the same superfamily. The authors reported (data not shown) no effect on the reproduction of *H. Schachtii* (Huang *et al*. 2006). A similar experiment (also data not shown), and also reporting no effect, was to feed *H. schachtii* on (*A. thaliana)* plants transgenic for an undescribed hpRNA construct derived from the *gfp* gene (Sindhu et al. 2009). These negative findings, though unsatisfactorily described, are consistent with the results of the many experimenters who have made transgenic RNAi organisms (plant and animal), mostly without observing any obvious unexpected phenotypic consequences (e.g. Huang et al. 2006; Sindhu et al. 2009, though see Xu et al. 2006 and Xing and Zachgo 2007). Although this is inevitably a self-selecting group (since we do not know to what extent lethal or disabling RNAi constructs are biologically selected against), it does at least suggest that unanticipated phenotypic consequences of RNAi may not be inevitable.

Thus, we arrive at something of a contradiction since the frequency of OTEs observed in most molecular analyses stands in some contrast to the general lack of observed phenotypic consequences of OTEs *in vivo.* However, there are obvious ways by which this might be resolved. Firstly, an OTE is not itself a phenotypic consequence. Just as gene deletion does not always lead to a readily observable phenotype, perhaps neither will OTEs. Secondly, the more detailed molecular or phenotypic assays have mostly detected OTEs while those limited to visual inspection or mortality under ideal conditions have not, making it probable that OTEs may have been missed.

There remain further reasons not to dismiss the importance of OTEs in unrelated non-targets. Firstly, there is wide variation in the susceptibility to environmental RNAi even among closely related organisms. For example, while *C. elegans* is the organism in which environmental RNAi was first discovered, its close relative *C. briggsae* is seemingly impervious to feeding or washing in RNAi (Winston et al. 2007). Among insects, many, including honey bees (*Apis mellifera*), western corn rootworm, colorado potato beetle and the apple moth (*Epiphyas postvittana*) specifically and efficiently take up dsRNAs from their gut. Others, however, do not (reviewed in Whangbo and Hunter 2008). These latter include *D. melanogaster* and the oriental leafworm moth (*Spodoptera litura*). The ease with which insects and other organisms have apparently lost and gained sensitivity to environmental RNAi is an interesting feature of these studies, and one that will likely complicate prediction of vulnerabilities to OTEs.

Secondly, some organisms, such as *C. elegans*, amplify (i.e. replicate) small RNAs acquired from their environment (Fire et al. 1998; Gordon and Waterhouse 2007). Amplification presumably increases susceptibility but, more interestingly, it is also a potential biological magnifier that might increase environmental exposure of specific

organisms to RNAi. This can happen in unexpected ways. One such example is the trans-specific gene silencing observed when an RNAi signal originating in one transgenic plant passed through a parasitic plant and downregulated gene expression in a third plant (Tomilov et al. 2008).

A third important source of uncertainty is the activity of small RNAs in dead and decaying plant tissues. Although it seems unlikely that small RNAs will remain active for long in dead tissues, they are potentially a highly important source of exposure since many ecologically important organisms are detritivores. The recent finding that RNA can persist for months at high temperature should serve as a timely reminder that the assumed fragility of RNA derives from experiments performed under a limited set of conditions (Michaud et al. 2007).

One final question relevant to this discussion is that there are significant grounds for questioning the specificity claimed for plant RNAi against pest animals. Plant hpRNA transgenes can clearly cause target pest mortality, and at the same time downregulate a specific target mRNA in the pests, but it has never been formally demonstrated that downregulation of the target mRNA (rather than an OTE) was the actual cause of mortality (e.g. Huang et al. 2006; Yadav et al. 2006; Baum et al. 2007; Sindhu et al. 2009). This data gap is potentially very significant, especially because there are reasons to question the standard account. Firstly, off-targets in animals frequently outnumber on-targets by many to one (Scacheri et al. 2004; Xu et al. 2006; Ma et al 2006). Secondly, off-target RNAs can be downregulated to a greater extent than on-target ones (Scacheri et al. 2004; Xu et al. 2006; Ma et al 2006). Furthermore, in some cases the effect on the target mRNA in the pest was marginal, suggesting that on-target effects were not the true mechanism of toxicity (Sindhu et al. 2009). If these doubts were to be borne out then the implications for OTEs would be profound because they imply that, in terms of targeting specific pests, hpRNAi might in practice not be specific at all.

Consequently, it is our view that several issues crucial to the specificity of RNAi towards pest organisms are unresolved. In contrast, the evidence that OTEs can mediate lethal effects against related non-target organisms is conclusive. Thus, in agriculture, as with human therapeutic RNAi, the issue of specificity is going to be central to whether plant RNAi will in the long term turn out to be a useful technology and a safer alternative to conventional pesticides.

Long dsRNAs are mammalian toxins

A well-known problem in RNAi therapeutics is the activation of the mammalian interferon response by dsRNAs (Kumar and Carmichael 1998; Sledz et al. 2003; Juliano et al. 2008). This interferon response occurs because, unlike invertebrates, mammalian cells are highly sensitive to the intracellular presence of perfect RNA duplexes of more than 30bps in length, regardless of their specific sequence (Cordell-Stewart and Taylor 1971; Hunter et al. 1975; Manche et al. 1992). As little as a single molecule of long dsRNA can trigger this innate immune response. Since dsRNAs alert the cell to the presence of viral replication intermediates. dsRNAs are thus considered classical pathogen associated

molecular patterns (PAMPs) (Kumar and Carmichael 1998).

The mammalian response to dsRNAs operates through multiple interdependent pathways. At the intracellular level, the best understood operate initially through the binding to dsRNAs of the dsRNA-dependent protein kinase (PKR), the oligo adenylate synthetases (OAS), retinoic acid inducible gene I (RIG-I) and the toll-like receptor 3 (TLR3), but other pathways also exist (Alexopoulou et al. 2001; de Veer et al. 2005; Kanneganti et al. 2006; Rehwinkel and Reis e Sousa 2010). Activation of each of these leads to separate but interconnected events ranging from global termination of protein translation, to the non-specific degradation of cellular RNAs via RNase L, and sometimes to cell death (Hunter et al. 1975; Karpala et al. 2005).

Largely because of their interferon-inducing capabilities, dsRNAs were originally expected to have therapeutic value against infectious diseases and cancers (Anonymous, 1969). Further investigation, however, revealed diverse toxicological properties of long duplexed RNAs and, as a result, effort in this direction was largely abandoned (Table 3). Writing in 1969 in the journal *Nature* of the embryotoxic effect of dsRNAs, Adamson and Fabro concluded "that PolyI-PolyC (a dsRNA analogue) may be of value in population control". The authors were among those who discovered that, depending on the site, dose, location and the mammalian species, injection of dsRNAs led to a variety of short term responses and longer term disorders. Immediate responses include temperature elevation, an inflammatory response and embryo abortion, behavioural changes and death; while longer term responses included ocular toxicity, angiogenesis suppression, teratogenicity and immune system effects (reviewed in e.g. Lindsay et al. 1969; Carter and De Clercq 1974; Freeman et al. 1977; Lin et al. 2006; Zhang et al. 2007; Kleinman et al. 2008). dsRNAs are further implicated in autoimmune diseases and even have been used to generate a mouse model for the autoimmune disease biliary cirrhosis (Okada et al. 2005). In humans, various Phase I and Phase II clinical trials demonstrated diverse side effects, including fever, hypotension, myalgia, bone pain, arthralgia, arthritis, abdominal pain, liver toxicity, thrombocytopenia and neurotoxicity (e.g. Lampkin et al. 1985).

These side effects are whole organism and whole tissue responses to dsRNAs and are probably primarily extracellular ones mediated by the TLR3 and TLR7 receptors (Alexopoulou et al. 2001; Hornung et al. 2005). Mammalian responses to dsRNAs are not uniformly negative, however, and PolyI-PolyC has been used experimentally as an adjuvant, an anti-tumour agent and an anti-viral agent with success usually attributed to its ability to induce interferons (e.g. Anonymous, 1969; Cui and Qiu 2006; Le et al. 2009). Without much doubt, dsRNAs are powerful biological agents in mammals (Table 3).

These effects of dsRNA would seem to be relevant to the majority of plant transgenic RNAi constructs that use hpRNAs. hpRNA transgene constructs tested in plants have so far invariably been designed to produce dsRNAs that exceed the (30bp) threshold required for activation of the interferon response and typically also the (85bp) threshold for maximal activation (Manche et al. 1992). Therefore, as presently conceived, the use of plant hairpin transgenes to produce dsRNAs constitutes a deliberate introduction of mammalian toxins into the food system.

An important mechanistic observation made as part of work to obtain pest resistance has been that while hpRNA

transgenes were designed to produce siRNAs, and these are still believed responsible for gene silencing within plants, it is the hairpin dsRNA precursor that is apparently the molecule responsible for toxicity towards invertebrate pests (Gordon and Waterhouse 2007; Mao et al. 2007). Therefore, transgene-derived dsRNAs enter through the gut and can cause high mortality in invertebrates. Furthermore, mortality occurs *via* a mechanism (RNA interference) that normally requires considerably higher doses than does the interferon response in mammals (Kumar and Carmichael 1998).

Besides preventive avoidance of the use of dsRNA sequences in transgenic plants, other potential courses of action can be identified. The first is to redesign hpRNA transgenes to evade the dsRNA/PAMP recognition apparatus (see discussion below). The second course, encouraged by the fact that there seems to be no direct data on the toxicological consequences of dsRNAs administrated orally to mammals, would be to attempt to establish that dsRNAs produced in plants are safe, by reason of their concentration, structure, physical route of entry, or some other property, and therefore incapable of triggering the interferon response and its consequences (Table 3). It should not be expected that this latter course will be a simple task, nor is it bound to succeed, since we cannot ahead of time know the answer. Nevertheless, the wisdom of this approach can be better judged by examining some possible lines of argument.

One possible avenue depends on presuming that human exposure to transgenic dsRNAs will be principally in the diet and that oral intake may constitute a safe route of exposure. This would presumably require establishing that dsRNAs (including hpRNAs) in the human diet are fully destroyed within the gastrointestinal tract (i.e. before absorption), or, by analogy with its tolerance of commensal bacteria, that the mammalian gut tolerates ingested dsRNAs even as it retains the ability to mount an effective response to viral pathogens (Cerovic et al. 2009).

Could dsRNAs in mammalian diets enter via the gut?

Mammalian digestion is a complex process in which food molecules are taken into the body by many routes (DeSesso and Jacobson 2001). It has been demonstrated in mammals that some of these pathways allow limited entry into the bloodstream of macromolecules such as DNA and intact proteins (Schubbert et al. 1994; Schubbert et al. 1997; Einspanier et al. 2001; Palka-Santini et al. 2003; Reuter and Aulrich 2003). Thus absorbed, macromolecules may enter internal organs, muscle tissue and even embryos (Hohlweg and Doerfler 2001). At least in some tissues, foreign DNA enters the nuclei of individual cells (Schubbert et al. 1997; Schubbert et al. 1998). The uptake of RNA macromolecules from the gut has nevertheless not specifically been investigated but ingested long dsRNAs would presumably follow similar pathways to these other macromolecules.

Are dsRNAs common in the diet?

If dsRNAs penetrate the gut, why then do mammals apparently not generate a gut-mediated interferon response? One possibility is that, although the typical mammalian diet contains diverse RNAs-from plants, animals, bacteria and

fungi, etc.-none of them meet the molecular requirements for activating an interferon response. We decided to test this hypothesis by surveying known and predicted RNA structures, in databases and the published literature, for the presence of perfectly duplexed dsRNAs.

As discussed above, activation of the interferon response (at low concentrations) requires perfect duplexes of RNA at least 30bp in length, with maximal activation occurring above about 85bp (Manche et al. 1992). Since, in terms of abundance, most RNAs are non-coding RNAs (ncRNAs), and ncRNAs are more likely to be highly structured (and therefore to contain duplexes), we searched the RNA STRAND non-coding RNA secondary structure database, which is the most comprehensive database of experimentally verified secondary RNA structures (Andronescu et al. 2008). RNA STRAND contains the validated structures of 4666 non-coding RNAs, and includes the secondary structures of bacterial and eukaryotic ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), signal recognition particle RNAs (SRP RNAs), group I and group II introns and transfer-messenger RNAs (tmRNAs) and many others. For many of these RNAs multiple versions (primarily homologues) are represented in the RNA STRAND database.

Searches of RNA STRAND yielded only one example of a perfect duplex exceeding 29bp, but only if G:U base pairs are counted as canonical. This was the *Plasmodium vivax* 16S rRNA, which has a 36bp duplex. G:U base pairings have not been tested for activation of the interferon response, and therefore it is not yet clear if this molecule satisfies the requirements of this search.

Certain types of ncRNAs are not well represented in the RNA STRAND database. This is primarily because their structures have not been experimentally determined and we examined these separately. One class is the eukaryotic small nuclear (spliceosomal) RNAs (U1, U2, U4, U4atac, U5, U6, U6atac, U11 and U12) and the U3 small nucleolar RNA, which are important because they are present in very high copy numbers - approximately 10^4 to 10^6 per cell (Montzka and Steitz, 1988). We also selected for examination the full complement of human H/ACA box snoRNAs (as listed in the Rfam structure database (Feb 1st 2010); http://rfam.sanger.ac.uk/family/snora1) (SNORA1, SNORA2, SNORA4, SNORA5, SNORA7, SNORA8, SNORA9, SNORA11, SNORA12, SNORA13, SNORA14, SNORA15, SNORA17, SNORA18, SNORA19, SNORA20, SNORA21, SNORA22, SNORA24, SNORA25, SNORA26, SNORA27, SNORA28, SNORA29, SNORA30, SNORA32, SNORA33, SNORA35, SNORA38, SNORA40, SNORA41, SNORA42, SNORA43, SNORA44, SNORA46, SNORA48, SNORA49, SNORA50, SNORA51, SNORA52, SNORA53, SNORA54, SNORA55, SNORA56, SNORA57, SNORA58, SNORA61, SNORA62, SNORA63, SNORA64/SNORA10 family, SNORA65, SNORA66, SNORA67, SNORA68, SNORA69, SNORA70, SNORA71, SNORA72, SNORA73, SNORA74, SNORA75, SNORA76, SNORA77, SNORA79) (Griffiths-Jones et al. 2003). We also selected the 187 miRNAs of *A. thaliana* (indexed in miRBase (accessed on 17th June 2009), since miRNAs are especially known to form hairpin structures (Griffiths-Jones et al. 2008). The predicted secondary structures of these 255 non-coding RNAs were visually inspected. Of these, one (MIR783 of *A. thaliana*) had a 34bp perfect duplex structure. Two other potentially noteworthy RNA structures were also found, a 33bp duplex in the same RNA and a 32bp duplex in MIR852, but both contained one G:U base pairing. No other perfect duplex exceeding 30bp was found. Therefore, in this second set of searches, a single sequence was found with the unambiguous potential to activate the interferon response in mammals.

Next, we searched the literature for examples of other naturally-occurring coding or non-coding duplexed RNA molecules. We found three non-coding RNAs with a predicted perfect duplex of 30bp or more from *C. elegans*. These were the product of the *rncs-1* gene, which contained two duplexes (one of 55bp, the other of 128bp) and the product of the *C. elegans* M05B5.3 gene (with a 50bp duplex) (Morse and Bass 1999; Hellwig and Bass 2008). A further example of (predicted) duplexing is the *4f-rnp* gene from *D. melanogaster*. *4f-rnp* appears to be regulated by convergent transcription, resulting in production of duplexed RNA (Peters et al. 2003). Convergent transcription has also been observed in mammals and plants, where the RNA products may be processed into siRNAs (Morris et al. 2008; Swiezewski et al. 2009).

Another potential source of perfectly duplexed RNAs are transcribed repetitive sequences. Primate genomes contain many Alu repeat sequences that sometimes are transcribed from the same promoter. Before splicing, these may transiently pair to form dsRNAs (Athanasiadis et al. 2004; Kim et al. 2004; Levanon et al. 2004). Similar repeats are also found in insects.

Interestingly, however, none of these RNAs (*rncs-1*, M05B5.3 and Alu-containing mRNAs) are fully convincing candidates for stimulating the interferon response. This is either because they are borderline in length or because they were originally identified as the substrates of nuclear-localised deaminase enzymes which act to modify the nucleotide bases of dsRNA molecules (Morse and Bass 1999). Thermodynamically, the predicted consequence of deamination (for perfectly duplexed dsRNAs) is destabilisation of the dsRNA structure (Morse and Bass 1999; Serra et al. 2004; Bass 2006). This suggests that deamination would prevent such molecules from stimulating the interferon response. This hypothesis, however, has not been directly tested.

Our inability to find more than a single convincing example (MIR783) of naturally-occurring perfectly duplexed dsRNA molecules might be interpreted as reflecting insufficient available data. Alternatively, it may indicate a more generalised absence, presumably for one or more biological reasons. Interestingly, several candidate biological explanations for the rarity of RNA duplexes are available. Among bacteria, DNA hairpin loop structures (which might produce dsRNAs) are typically genetically unstable, while in eukaryotes perfect dsRNAs would tend to become unwanted substrates of the RNA interference pathway or (in mammals) of the interferon response itself. They therefore may be highly selected against. Indeed, it is tempting to speculate that the rarity of dsRNAs in nature is precisely what allows them to act as such sensitive signals of viral intrusion.

There is one further known potential source of long duplexed dsRNAs,: phages and viruses. It is possible to purify interferon-stimulating viral nucleic acids from virus-infected microorganisms and all RNA viruses and RNA phages produce dsRNA as part of their normal lifecycle (Banks et al. 1968). Therefore, there is no doubt about the existence of these molecules and that dsRNAs will be an intermittent part of a mammalian diet but their significance is unclear. dsRNAs are typically a minor fraction of total viral nucleic acid but in some cases dsRNA is the encapsidated form of the virus. These encapsidated-dsRNA type viruses are relatively uncommon but some, such as rice dwarf virus, are individually important. There has been no research on their possible effects in the diet.

In conclusion, long duplexed dsRNAs have previously been discarded as medical therapies for the reason that they induce side-effects at low doses. Based on our analysis it seems unlikely that a convincing case can be made for their safe inclusion in food. Nevertheless, no conclusion should ever preclude further research. If, as a result of long-term safety studies, dsRNAs are in future deemed safe in the diet, other routes of vertebrate exposure (*via* pollen etc.) should also be considered in any risk assessment.

General considerations for plant biotechnology and its regulation

The application of RNAi to achieve longstanding medical and agricultural goals is a recent and radical new strategy in biology. In agriculture, possible applications include heritable and inducible control of plant biochemical and physiological functions as well as multitrophic pest and pathogen management. These startling technical possibilities of RNAi exist, however, for specific and noteworthy biological reasons that are relevant also to risk assessment.

Firstly, RNA is the biological macromolecule with the longest evolutionary history and this makes it the macromolecule most deeply integrated into biological processes. It is presumably not a coincidence that RNAs are the only class of molecule indispensable for all known life forms.

Equally important, more so than proteins, DNA or other biological molecules, RNA has a remarkable diversity of chemical forms, shapes and sizes and thus structural and functional possibilities. The size of functional RNA molecules in metazoans, for example, ranges over 6 orders of magnitude, while the distinctively flexible physico-chemical properties of RNA, which span structural arrangements, enzymatic properties, information content and replicative features have enabled RNA to be exploited by biological systems for a uniquely diverse set of roles. Thus RNAs are as diverse as ribosomes and tRNAs, enzymes, miRNAs, viruses and viroids. In this broad context of RNA flexibility and evolutionary history, the identification of three distinct hazard classes arising from the use of a single innovation (plant RNAi transgenes) is perhaps less surprising than it might be.

Off-target-effects in crop plants

The first of these hazards is the possibility of OTEs affecting the traits of the crop cultivar itself. Such a hazard requires the misdirected action of RNAi to affect gene expression and through this the predictability or wholesomeness of the resulting cultivar. The first of these, predictability, is certainly important for breeders and farmers but it is most important as a food security concern if it leads to unexpected crop loss. In transgenic breeding it is common to make thousands of primary transformed plants in order to obtain a single line or event suitable for commercialisation (e.g. Thomas et al. 1998; Hu et al. 2003). This number is necessary chiefly because the plant transformation process is subject to somaclonal variation, the insertion of transgenes at random and the complexity of the insertion event itself (reviewed in Wilson et al. 2006). Since initial data from plant RNAi suggests that OTEs within plants are common,

probably occurring multiple times per hpRNA transgene, OTEs will likely add further uncertainty to the event selection process for transgenic plants. In this context it is noteworthy, therefore, that the transgenic hpRNA cultivar first in line for commercialisation (DP-305423-1) has a clear unintended effect that results in the novel production of the fatty acids heptadecanoic acid (17:0) and heptadecenoic acid (17:1) (Delaney et al. 2008).

Effects on wholesomeness are the concern of risk assessment. A long-running subject of discussion in GMO biosafety has been over the question of whether the risk assessments of transgenic plants take unintended effects adequately into account (e.g. Millstone et al. 1999, Haslberger 2003; NRC/IOM 2004). The possibility of OTEs within plants will further intensify this issue since OTEs, by definition, are a source of unintended effects. Nevertheless, future research may at least decrease the unpredictability of OTEs. For example, recent work has shown that G:U base pairings are recognized by the RNAi machinery, a finding that has two implications (Du et al. 2005; Holen et al. 2005). Since guanosines can pair with either cytosine or uracil and uracil can pair with either adenine or guanosine, OTEs will be significantly more common than consideration of Watson-Crick pairings would suggest, but also more predictable since many OTEs will be explainable.

Environmental RNAi: off-target effects in other organisms

The second specific hazard discussed in this review arises from the discovery that hpRNAs produced by plant transgenes can be lethal to invertebrates consuming plant tissues. Just as pest organisms can be harmed, so, it seems, can their close relatives, provided they ingest plant parts, possess target gene homology and are susceptible in general to environmental RNAi. Close relatives of insect pest targets include *Lepidoptera*, mites and beetles whose survival is desirable and ecologically important and therefore effects on related non-targets are likely to be significant issues for risk assessment. The majority of exposed organisms, however, will be unrelated to the target pest. For these organisms there remain many unanswered questions.

The study of OTEs to date has shown that when silencing RNAs enter a living cell there is a high likelihood of fortuitous homology as well as a high probability of off-target interactions with partially-homologous mRNAs. Therefore, species unrelated to the target will likely be potentially affected by OTEs. To fit into this category, an organism needs only to be exposed to hpRNA in sufficient quantity and be susceptible to environmental RNAi. This is an extremely important prediction since many organisms will fall into this class. Clearly, direct experimental testing of effects on unrelated organisms will be necessary to test this hypothesis. In particular, it will be important to determine whether OTEs can result not just from RNAi transgenes intended to target pests but also those RNAi transgenes introduced for other purposes, such as nutritional alteration. If hpRNAs routinely induce phenotypically or ecologically important consequences in non-target organisms then this would clearly constitute a major obstacle to the safe and beneficial use of RNAi in plants.

One further consideration worth noting is the possibility that sequence-specific OTEs (i.e. not via the interferon

response) may affect mammals, including humans. Therapeutically, RNAi has so far been shown to be effective only when introduced through routes other than oral ingestion. The exceptions to this are when the RNA has been packaged in vesicles or engineered bacteria (Xiang et al. 2006; Wolfrum et al. 2007). Nevertheless, it would seem highly prudent to avoid exact homology between human genome sequences and RNAi sequences intended for commercial production.

The effects of dsRNAs on mammals

The final hazard analysed in this review is the potential toxicological consequence for mammals of exposure to dsRNAs originating from plant hpRNA transgenes. This hazard is unique in that the use of long dsRNAs in food crops breaks new ground in incorporating into edible crops and the human diet long dsRNAs that, by routes other than ingestion, are undisputed toxins (de Clerq et al. 1972). If establishing the safety of dsRNAs in the diet can be done, it will presumably have to be based in the first instance on establishing oral intake as a safe route. This will not necessarily be easy. (1) Animal testing is one possible avenue for establishing the safety, or otherwise, of dsRNA ingestion. However, it is noteworthy that there are some considerable differences in mammalian responses to dsRNAs, even within individual species, implying that the predictive value of such experiments may be low (Rice et al. 1971). (2) Consideration should also be provided for human inflammatory bowel diseases (Cerovic et al. 2009; see Table 3). These common diseases are thought to reflect dysfunction in the ability of the gut to balance its responses to harmless resident organisms versus genuine pathogens. Obviously, affected individuals may be particularly sensitive to dsRNAs in the diet since these are one of the molecular patterns (PAMPs) organisms associate with pathogenicity. (3) The likely delayed onset nature of some of the responses to dsRNAs (e.g. neurotoxicity and autommune diseases; e.g. Steinberg et al. 1969; Table 3). (4) Special attention should be paid to exposures experienced by newborn mammals, for example infant formula. In the first days or weeks of life, newborns have unusually permeable gastrointestinal tracts. This makes them particularly sensitive to certain toxins that would not otherwise be consequential. These and other difficulties make it hard to recommend approaches other than the preventive one of avoidance of transgenes capable of generating perfect RNA duplexes.

The importance of mechanistic understanding in risk assessment

This review has used a mechanistic approach to identify potential hazards of RNAi. Thus, it has emphasised the implications of what is known about RNAi (mostly in animals) to infer various specific consequences of the use of plant hpRNA transgenes. The mechanistic approach is not, however, the only possible one. Transgenic crops, dating back to the Flavr Savr tomato, and including all transgenic virus-resistant cultivars approved to date, have contained various, probably RNA-interfering, transgenes (Sanders and Hiatt 2005; Auer and Frederick 2009). However, these commercial cultivars were approved in the absence either of any mechanistic understanding of RNA interference or of

its relationship to the function of these transgenes. For example, the Flavr Savr tomato was approved even before RNAi was discovered and inhibition of ripening was at that time believed to be an antisense effect (Sheehy et al. 1988). Indeed, because limited information has been gathered, it is still not known for certain whether any of these commercial crops are truly examples of RNAi or are qualitatively or quantitatively comparable to hpRNA transgenes (e.g. Fitch et al. 1992; Sanders and Hiatt 2005). The effect of this regulatory indifference to transgene mechanism of action is that whereas these previously commercialised transgenes might have shed useful light on the safety (or otherwise) of RNAi, the lack of definitive mechanistic data and any animal feeding, off-target effect experiments or other monitoring, make it doubtful that they can retrospectively serve that purpose. The one potential exception is the recently approved TREUS™ (Pioneer) soybean for which there is one ninety day rat feeding study (Delaney et al. 2008). Whether this study is of value in this context is unclear, however, in part because the soybeans in the feed meal were ground and toasted prior to consumption.

Mechanistic approaches to determining safety bring three specific advantages. One, is that a basic framework of understanding can place any innovation into the context of known phenomena; and this aids risk assessment. Secondly, mechanistic understanding (in engineering and elsewhere) is indispensable to any rational risk assessment since it is a precondition for arriving at conclusions via a transparent process. In other words, unless the reasoning process is explicit it can neither be explained nor refuted (Phillips 2000). The third benefit of mechanistic understanding is to allow the rational design of preventive measures.

These benefits of mechanistic understanding for rational risk assessment are not in dispute. Nevertheless, as the above examples show, regulators in crop biotechnology routinely allow commercialisation in the absence of a clear understanding of a particular transgenes' mechanism of action (Sanders and Hiatt 2005; Wilson et al. 2006; Latham and Wilson 2008; Auer and Frederick 2009). The analysis in this paper therefore strongly supports the view that mechanistic understanding in risk assessment is as indispensable in crop biotechnology as it is to any other field of risk assessment. This lesson we believe is crucial to the safe use of biotechnology, especially if interventions in crop function become in the future more ambitious.

Risk reduction for plant RNAi technologies

In plant breeding and for other potential applications of transgenic RNAi, our analysis suggests there are opportunities for reducing the risks or avoiding the hazards described here (Table 4). These opportunities arise, broadly speaking, because RNAi permits (within limits) variation in sequence, structure, length and expression level and each of these variables provides possibilities for hazard avoidance or risk mitigation.

Some of the options, for instance shortening the RNA sequence (especially to below 29bp), should reduce the probability of all three of the hazards discussed here. Other options are specific to a single hazard; for example, the introduction of appropriately spaced bulges or mismatches perturbs RNA duplex structures and would specifically

prevent stimulation of the interferon response. Sequence selection might also have a role to play, especially for sequences with homology to human or host genes. Other options are likely to be more problematic to implement. For example, OTEs are concentration-dependent (Sledz et al. 2003; Hannon and Rossi 2004; Jackson et al. 2006). Therefore, keeping transgene expression levels low should reduce them. However, as with shortening the RNA sequence, this approach may compromise efficacy and, in any case, expression levels can be hard to control. Collectively, preventive strategies may require some ingenuity but they should be applied to RNAi transgenes in plants before commercial use is considered. If present understanding applies to crop species, these and other steps should greatly reduce the probability of OTEs of different kinds. Table 4 summarises the chief options for RNAi-specific risk reduction.

Conclusion

The preceding analysis represents the first comprehensive review of the potential for OTEs arising from plant RNAi transgenes (though see also Auer and Fredericks 2009). It has raised diverse and important questions concerning the biosafety of RNAi in plants but, for several reasons, it does not comprise a full risk assessment of RNAi, and it seems appropriate to conclude with some further questions. One of the more obvious is that characterisation of RNA as the biological ur-molecule implies there is a high potential for as-yet-undiscovered biological pathways that utilise RNAs and that may have additional risk implications (Table 5). Perhaps the leading candidate at the present time comes from evidence that small RNAs can upregulate as well as downregulate gene expression and indeed OTEs can also sometimes result in increases in abundance of mRNAs as well as decreases (e.g. Scacheri et al. 2004; Morris et al. 2008). A second source of uncertainty is the existence of 'known unknowns': for example, where OTEs have been observed, there is often no obvious mechanistic explanation for them, implying that current understanding in this field is significantly incomplete (Fedorov et al. 2006; Aleman et al. 2007). Lastly, a possible explanation of some observed OTEs is saturation of the RNAi machinery, a condition which in mice is lethal (Grimm et al. 2006). Whether saturation of the plant RNAi machinery could result from a plant transgene or might have any implications for plant transgene biosafety is currently not known.

RNAi is better understood in animals than in plants and among plants is better understood in *A. thaliana* than in any other species. Nevertheless, the first ever deliberate commercial application of RNAi is likely to be in agriculture - the TREUS™ high oleic acid soybean which is intended for human consumption. It is difficult not to conclude this situation is the reverse of the ideal. Medicine is the more compelling social need but in that discipline the availability of superior mechanistic understanding has instilled not faster commercialisation but a more careful and cautious approach (reviewed in Juliano et al. 2008). In agriculture, however, only the Weigel laboratory, in developing transgenic artificial miRNAs for plants, has consistently identified OTEs as a potential problem (Schwab et al. 2006; Ossowski et al. 2008).

As we have shown, however, RNAi in agriculture raises a spectrum of important biosafety issues. The extent to which

these can be satisfactorily resolved is important for their own sakes but will probably contribute substantially to the acceptance and widespread use of biotechnology more generally.

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