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RISK ASSESSMENT RESEARCH

CONTENTS

Does Recombination in Virus-Resistant Transgenic Plants Lead to Emergence of Novel Viral Diseases

Mark Tepfer and Fernando García-Arenal

Background

Over past decades, decisions on the release of genetically engineered organisms, also referred to as GMOs, have been largely science-based. But recently, the primacy of science-based risk assessment has begun to erode, as exemplified by the EU's recent decision, in Directive 2015/412, to allow individual EU member states to forbid environmental release of GMOs on non-scientific grounds[1]. If the scientific community hopes to maintain and enhance the use of scientific risk assessment in decision-making, it will be vitally important to present large, complex bodies of scientific research in a manner that is satisfactory to the research community, to participants in decision making, but also to various stakeholder groups, including the general public. From a global perspective, this challenge has become even more important, as greater numbers of developing and emerging countries have put in place regulatory procedures regarding GMOs that are adapted to their national and regional circumstances.

Through numerous workshops organized in response to the needs of its member states, the Biosafety Unit of the International Center for Genetic Engineering and Biotechnology (ICGEB) has developed a particularly flexible tool for presenting a scientific approach to risk assessment[2]. The ICGEB tool kit is based on the same principles that have been used in other formulations of risk assessment strategies[3, 4], but it was particularly designed to be easy to use by non-specialists, while maintaining scientific rigor. Although conceived as an aid in risk assessment in a decision-making context, it can also be used as a framework to pinpoint what research needs to be carried out to evaluate a particular risk, for instance whether gene flow from GM camelina to two of its closest wild relatives, shepherd's purse and arabidopsis, should be of concern[5]. More recently, as will be developed here, Tepfer *et al.*[6] have used this framework in a review article presenting in synthetic fashion the conclusions that can be drawn from nearly 20 years of research published in specialized scientific journals that is pertinent to an exceptionally complex biosafety question, "Will recombination in transgenic plants expressing viral sequences lead to appearances of new viral diseases?"

Evaluation of the potential risk associated with recombination in virus-resistant transgenic plants (VRTPs)

The risk assessment tool developed at ICGEB is based on several successive steps. The first is to state the risk hypothesis as a simple sentence that clearly identifies the initial cause and the potential harm being considered. In the case presented[6], the risk hypothesis was formulated as "Recombination in transgenic plants expressing viral sequences will lead to emergence of novel viral disease." They have considered a specific situation in which the initiating step is

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unrestricted release into the environment of plants expressing the coat protein (CP) of Cucumber mosaic virus (CMV), since CMV is one of the most important plant viruses[7], and it is also the virus for which pertinent results are most abundant.

The next step is to develop a "pathway to harm" in a chain of cause and effect that describes how the initiating action can lead to the harm of concern. This makes it possible to evaluate whether the link between the initiating step and the harm is in fact possible, since it forces development of an explicit, plausible scheme for exactly how the cause could lead to harm. Then consideration of the scientific evidence that defines the likelihood of each step in the pathway facilitates a rigorous evaluation of the overall likelihood of the harm to occur. It should be noted that in the light of attempting to refute the risk hypothesis, it would be sufficient to break irrevocably even a single link in the pathway. If none of the steps in the pathway has a zero likelihood to occur—which would constitute a formal refutation of the risk hypothesis—then evaluation of the likelihood of all steps in the pathway leads to an overall evaluation of the risk hypothesis.

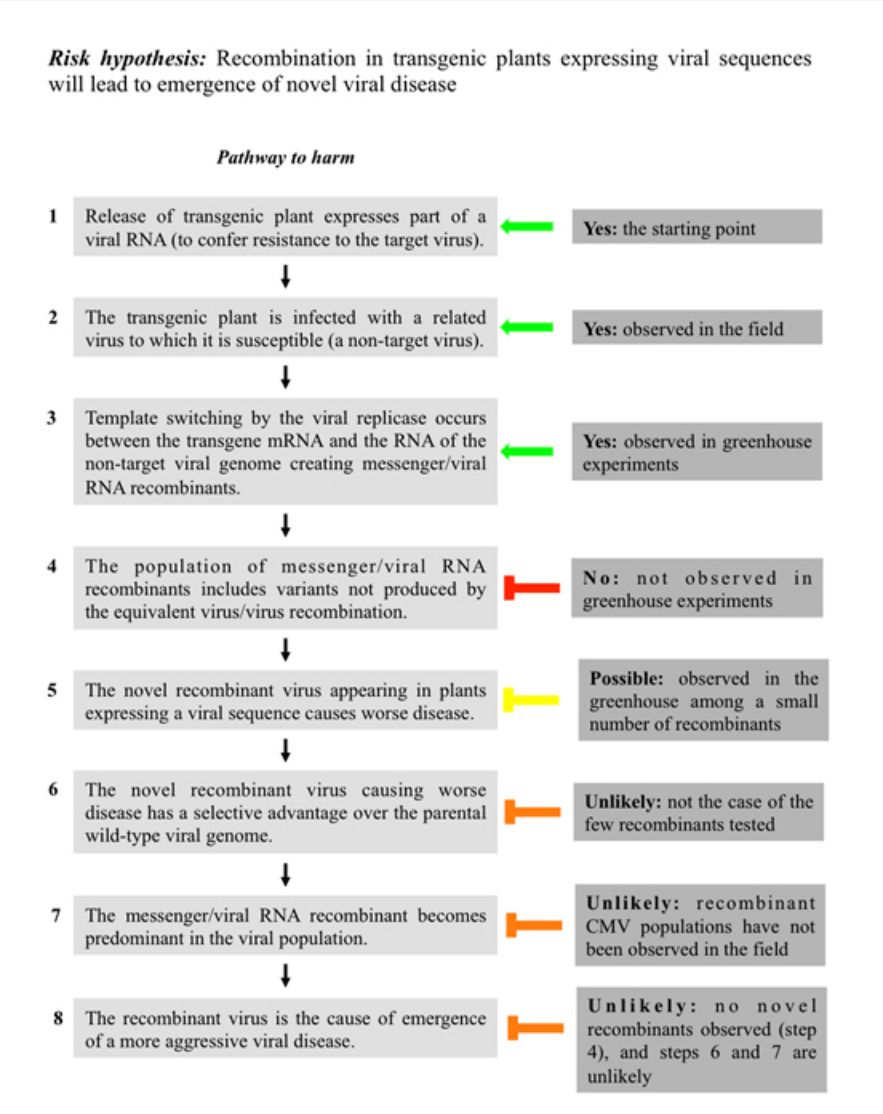
The **Figure** shows how the risk hypothesis "Recombination in transgenic plants expressing viral sequences will lead to emergence of novel viral disease" was broken down into a pathway to harm, and in summary form, whether each step was likely to occur or not. What emerges from the figure is that, as indicated by green arrows, steps 1–3 are known to occur, the occurrence of step 5 is supported by a small amount of laboratory research (yellow), steps 6–8 are unlikely (orange), and step 4 is very unlikely (red). This analysis shows that the most critical point of evidence is the testing of step 4. For this step, the essential result was a comparative high-throughput sequencing study of CMV recombinants that occur in transgenic plants and non-transgenic controls, which showed that all the recombinant CMV genomes observed in transgenic plants were also observed in the controls. For further details, the state of the evidence concerning each step is considered in depth in the review article[6]. From consideration of all the available data, the authors conclude that the risk hypothesis, within the framework considered (plants expressing a CP of CMV), is highly unlikely.

The next point that needs to be addressed is the quality of the risk assessment. Since no step in the pathway to harm was found to be impossible, this means that the risk hypothesis was not formally refuted, yet the authors concluded that the risk of the harm occurring was extremely slight. Although when formal refutation is not possible, it could always be argued that more research is necessary to reach a firmer conclusion, in this case it was suggested that the key to cutting short this potentially unending chain of requiring more data was the observation that, not only were no recombinants observed in transgenic plants that were not also present in non-transgenic ones, but in addition there was no evidence of novel mechanisms of recombination occurring in the transgenic plants that could possibly create novel viral genomes.

Conclusions

Although at the present time VRTPs are minor elements in world agriculture, this case study serves well to illustrate how a highly complex question regarding potential risks can be addressed in a clear and scientifically rigorous fashion. And while the presentation in the review article[6] was designed to condense a large body of results to a few pages to be easily understood by readers with some level of scientific literacy, its message

Figure



Presentation of the risk hypothesis and evaluation of the pathway to harm. The pathway to harm is shown in the left column of boxes as a chain of successive steps linking cause and effect. The present state of knowledge concerning CMV presented in the corresponding paragraphs in the text is summarized in the right column of boxes (for references see the body of the text). The colored symbols between the columns indicate the likelihood of the step, shaded from most likely (green) to least likely (red).

can be further condensed, as was done here. Depending on the people involved and the time available, the conclusions could be reduced even further to a presentation of the figure, and the authors of the review article suggested that the story could even be reduced if necessary to a single sentence: "The likelihood of emergence of novel viral diseases in VRTPs is low, since an in-depth analysis showed that all recombinant viruses found in VRTPs were also present in non-transgenic plants." The aim is to be able to present a clear view of the likelihood of a particular risk in a manner that is appropriate to the circumstances, but that can easily be referred to more complete, more complex presentations of the situation, to create a valid chain between the primary research results and—in the extreme case—a single-sentence sound bite.

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RNAi Silencing of a Chitin Synthase Gene Protects Wheat Plants against *Fusarium graminearum* and Mycotoxins

Yu-Cai Liao and He-Ping Li

Background and scope

Fusarium graminearum is a fungal species responsible for *Fusarium* head blight (FHB) in wheat and barley and *Gibberella* ear rot (GER) in maize, which are economically devastating diseases worldwide. *F. graminearum* species can produce various types of trichothecene mycotoxins that are directly accumulated in cereal grains, including deoxynivalenol, nivalenol, and their derivatives. The grain-contaminating mycotoxins enter the food/feed chains and are often associated with chronic or acute mycotoxicoses in domestic animals and humans. FHB epidemics frequently occur in the middle and lower regions of the Yangtze River and in Heilongjiang Province in northeastern China. FHB in wheat and barley has re-emerged as a serious threat to agriculture in Europe and North America since the mid-1990s, resulting in huge losses. The best control strategy is the prevention of infection in the field and during storage through expression of resistance genes in plants. However, innate FHB-resistance germplasm is inadequate in nature, and it is a challenge to develop resistant wheat varieties with suitable agronomic traits. To date, fungicides have been the primary means of control used for toxigenic *Fusarium* pathogens, generating undesirable environmental consequences and fungicide-resistant *Fusarium* strains. Therefore, it is necessary to identify new FHB-resistance genes and to use them to protect plants against *Fusarium* pathogens and to reduce mycotoxin production.

RNA interference (RNAi) silencing of genes important for various pathogens such as viruses, insects, and fungi has been shown to enhance plant resistance. To use RNAi-mediated gene silencing for control of FHB pathogens, we identified highly efficient RNAi constructs from a chitin synthase gene of *F. graminearum*. Chitin synthase catalyzes the biosynthesis of chitin. Chitin is a vitally important structural component of the cell walls of filamentous fungi. Neither chitin nor the chitin synthase are present in plants or mammals, thereby theoretically obviating most of the off-target effects that are associated with the implementation of new pest control measures. Therefore, chitin synthases have long been considered to be tantalizingly ideal targets in the development of antifungal agents. In spite of many suggestions in the literature for the use of fungal chitin physiology as a

target for control measures, no previous report has shown that a fungal pathogen can be controlled by targeting its chitin synthase. Plant pathogenic fungi have multiple chitin synthase genes, and the various chitin synthase gene family members play discreet roles in development and virulence. We reasoned that the chitin synthase genes, which are essential for fungal survival and infection, would be among the best possible RNAi targets for reducing fungal disease development in plants.

Experimental approaches and results

Chitin synthases (Chs) in fungi are classified into seven categories; the *F. graminearum* genome contains eight *Chs* genes that belong to the seven classes. Deletion of individual *Chs* gene members generated viable knockout mutant strains for seven *Chs* genes but no *F. graminearum* strain lacking *Chs3b* gene was generated. Further expression analyses of *F. graminearum Chs* genes during the fungal colonization of different wheat cultivars revealed that among the eight *Chs* genes, *Chs3b* was expressed at the highest levels during the entire colonization period. These results demonstrate that *Chs3b* is essential for fungal survival and infection. Thus, *Chs3b* was selected as the target for RNAi, and its cDNA was divided into five segments (Chs3b-1, -2, -3, -4 and -5) to generate five hairpin-RNAi constructs (Chs3bRNAi-1, -2, -3, -4, and -5) that were then introduced into *F. graminearum*.

Transgenic *F. graminearum* strains that expressed RNAi constructs Chs3bRNAi-1, -3, and -5 had clearly retarded, sparse mycelium growth when cultured on potato dextrose agar media and substantially altered growth under various stress conditions. The strain with Chs3bRNAi-2 showed a lesser degree of alteration than the strains containing the Chs3bRNAi-1, -3, or -5. The growth pattern of a strain with Chs3bRNAi-4 did not differ from that of the non-transgenic *F. graminearum* strain. As for the chitin content, a reduction of 3% to 33% was observed in the strains containing Chs3bRNAi-1, -2, -3 or -5, while a significant, 9.3% increase was observed in the strain expressing Chs3bRNAi-4.

Microscopic analyses revealed that strains containing Chs3bRNAi-1 or -5 had clearly restricted, severely distorted and crooked mycelia with increased widths and conglobated structures along the mycelia. These two strains produced smaller conidial spores. Moreover, lesion lengths in wheat seedlings were reduced by 21% to 47% for all RNAi-expressing strains except the strain containing Chs3bRNAi-4 that had a significant, 15% increase in lesion length; a similar pattern of infected spikelets at wheat flowering stages was seen for these five strains compared with their non-transgenic control. Further qRT-PCR showed that the expression levels of the targeted *Chs3b* gene in transgenic strains were significantly reduced in the RNAi strains: by 33% to 73%. Taken together, these experiments demonstrated that Chs3bRNAi-1, -3, and -5 constructs had the strongest overall interfering effects on mycelium growth, stress sensitivity, chitin biosynthesis, infectivity, and *Chs3b* expression in transgenic *F. graminearum*.

Since plant-expressed short interfering RNA (siRNA) molecules have to migrate from plant cells into the invading fungal cells, RNAi-mediated silencing of fungal genes would take place *in planta* to restrict fungal growth. To prove that siRNAs from outside can enter into *F. graminearum* cells, commercial non-*Fusarium* fluorescein siRNAs (19-21 nt) were incubated in potato dextrose broth with conidial spores of *F. graminearum* and then assayed with a fluorescence microscope. Clear fluorescence in the spores treated with the siRNA was observed, demonstrating that siRNAs present in a culture medium can actively migrate into *Fusarium* cells. These results prompted us to select the most effective Chs3bRNAi molecules for further expression in wheat plants to test whether expressing the Chs3bRNAi constructs *in planta* could confer resistance to *F. gramienarum.*

To increase efficacy of RNAi silencing, the three most effective RNAi constructs, Chs3bRNAi-1, -3, and -5, were mixed and co-transformed into the elite Chinese wheat cv. Yangmai15 by bombardment. This commercial wheat cultivar has a good quality and yield potential with moderate FHB resistance. Transgenic wheat plants were selected and identified by RNAi construct-specific PCR, and the stable integration of the RNAi constructs into the wheat genome was verified by Southern blottings using three probes (Chs3b-1, -3, and -5); these blots were used to confirm that each transgenic line contained the three RNAi constructs, with different integration patterns.

Only the transgenic wheat plants that contained all three RNAi constructs, displayed normal morphology, and set viable seeds were selected for selfing and propagation.

Two independent transgenic lines, L1 and L3, were generated. To verify that the transgenic wheat plants expressed and processed siRNA molecules, northern blot analyses were performed with small RNA isolated from 5-d-old seedlings and lemma/palea of spikes at the flowering stage of the two T_5 transgenic wheat lines and non-transgenic Yangmai15 and evaluated with the three probes that were used in the Southern blots. All three probes detected a single band with an expected size of ~21 nt in each of the transgenic wheat line samples; no signals were detected in the non-transgenic control plants. These results confirmed the presence of Chs3b-1, -3, and -5 siRNAs in the transgenic wheat organs that are directly exposed to *F. graminearum* spores at the onset of colonization and demonstrated the proper expression and processing of fungus-derived RNAi constructs in a host plant.

To assay the resistance of transgenic wheat plants to Fusarium seedling blight (FSB) and FHB, T_3 to T_5 generations of transgenic wheat lines L1 and L3 were inoculated with *F. graminearum*; non-transgenic FHB-susceptible Yangmai15 were also inoculated and served as controls. The results of the FSB resistance assays showed that the seedlings of the T_3 transgenic wheat plants were highly effective in restricting the spread of the fungus; the two transgenic lines displayed significant disease reductions of 75% and 45%, respectively, compared with non-transgenic control. In the T_4 and T_5 generations, transgenic line L1 had significantly reduction of lesion lengths, by 84% and 89%, respectively, compared with non-transgenic control. For transgenic line L3, the lesion lengths showed a significant reduction of 50% (T_4) and 75% (T_5) relative to those of control. These results indicated that the RNAi molecules expressed in stably transformed transgenic wheat plants were able to confer genetically stable resistance to the spread of *F. graminearum* in seedlings, i.e., type II resistance.

Single-floret inoculation of the spikes of T_3 to T_5 transgenic plants of the same lines showed that in the T_3 transgenic plants, the percentages of infected spikelets in lines L1 and L3 ranged from 15% to 20% at 21 dpi, which is a significant reduction of 61% and 49% compared with non-transgenic Yangmai15 (39%). In the T_4 and T_5 generations, the two lines had significant disease reductions of up to 61% and 70%, respectively, compared with the non-transgenic control. These results indicated that the type II resistance conferred by the expression of Chs3bRNAi molecules in stably transformed wheat plants is consistently effective in both seedlings and spikes.

Natural inoculation under field conditions showed that the two transgenic wheat lines had 8% to 10% (T_4) and 7% to 11% (T_5) infected spikelets at 30 days post-anthesis, respectively, with reductions of up to 74% and 76% compared with the non-transgenic Yangmai15 (31% and 29%). Thus, the RNAi molecules enhanced genetically stable resistance of wheat plants to initial infection by *Fusarium* pathogens, i.e., type I resistance. Furthermore, gas chromatography-mass spectrometry analyses of trichothecene mycotoxins from the harvested grains of T_5 transgenic wheat lines and the non-transgenic control revealed that the grains of the single-floret injection experiment for the L1 and L3 lines had significant reduction in deoxynivalenol (DON) content of 62% and 58%, respectively, compared to the non-transgenic control. More significantly, in the natural inoculation, the L1 and L3 transgenic lines had reductions in DON content of 85% and 78%, respectively, relative to the control. Thus, the expression of the RNAi molecules in stably transgenic wheat reduced mycotoxin accumulation in grains, thereby displaying a type III resistance.

qRT-PCR analyses of the fungal *Chs3b* transcripts in *F. graminearum*-infected non-transgenic control and T_5 transgenic wheat plants expressing the RNAi constructs showed that the Chs3b transcripts were reduced by 57% and 42% in the *F. graminearum*-infected wheat seedlings of two transgenic lines, respectively, relative to *F. graminearum*-infected control seedlings. Similarly, in *F. graminearum*-infected wheat spikes, the relative transcript levels of *Chs3b* were reduced by 52% and 49%, respectively, in the two lines, compared to the control spikes. These results demonstrated that transgenic wheat plants expressing RNAi constructs could produce siRNA molecules that efficiently down-regulate the *Chs3b* gene in colonizing *F. graminearum*.

Implications

Our results demonstrate that co-expression of the most efficient RNAi sequences targeting the same fungal gene in transgenic wheat plants can confer genetically-stable resistance in wheat seedlings and spikes under

field conditions and reduce mycotoxins in harvested grains. The targeted *Chs3b* gene was the only member of the *Chs* gene family in *F. graminearum* genome that was essential for fungal survival and also the only member to be expressed at the highest levels during the colonization of Fusarium pathogens on wheat. The *Chs3b* gene and/or its homologs in pathogenic fungi could be ideal targets for controlling fungi via RNAi-mediated approaches in plants and mammals, while avoiding off-target side effects, as *Chs3b* is absent in those organisms.

Comparative analyses of the silencing efficacy of five RNAi constructs derived from the *Chs3b* gene identified three RNAi constructs, Chs3bRNAi-1, -3, and -5, that have the most effective *Chs3b* silencing in transgenic *Fusarium* strains, and one strain expressing Chs3bRNAi-4 that had significant increases of chitin content and virulence compared with the control strain. Therefore, functional screening for silencing constructs from a candidate fungal target is essential for the identification of efficient RNAi sequences for subsequent use in transgenic plants.

Our results illustrate an efficient approach for the identification of novel resistance resources for the improvement of fungal resistance. This may be particularly vital in situations where there is a lack of naturally resistant germplasm to use in conventional breeding efforts. RNAi-mediated silencing of fungal genes may become a powerful approach to control FHB, FSB, and the associated mycotoxins in cereal crops.

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