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GM Gene Flow (B)

Horizontal gene transfer of viral inserts from GM plants to viruses

by

Jonathan Latham, PhD

Ricarda Steinbrecher, PhD

EcoNexus

P.O.Box 3279
Brighton BN1 1TL, UK
(+44) 01273 625 173
info@econexus.info
www.econexus.info

Amended review paper for the GM Science Review Meeting of the Royal Society of Edinburgh on
"GM Gene Flow: Scale and Consequences for Agriculture and the Environment"

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Jonathan R Latham, PhD and Ricarda A Steinbrecher, PhD
Econexus; PO Box 3279; Brighton BN1 1TL; UK; info@econexus.info

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Introduction

The use of GM plants containing viral inserts (as transgenes or promoters) raises the possibility that these inserts may lead to the creation of new viruses through recombination (HGT). Viruses commonly evolve by recombination and have been shown to recombine with transgenes which are derived from viruses in numerous laboratory-scale experiments (e.g. Greene and Allison 1994, 1996). These recombination events can generate viruses with new biological properties (e.g. Wintermantel and Schoelz 1996). The creation of new viruses by recombination with a transgene is therefore a plausible risk whose possibility has been a focus of concern in the scientific literature (de Zoeten 1991; Hull 1994; Gibbs 1994; UCS 1994; Allison et al 1997; Gibbs et al 1997). The creation of new viruses would have consequences not just for agricultural systems but also in the wider context of human health and environmental protection, as many viruses are only controlled by time-consuming precautions and extensive pesticide use (Rybicki and Pietersen 1999; Morales and Anderson 2001).

The risks of HGT posed by virus-derived gene constructs in transgenic crops have been reviewed before (de Zoeten 1991; Tepfer 1993; Falk and Bruening 1994; APHIS 1995; Allison *et al* 1996; Robinson 1996; Miller *et al* 1997; Aaziz and Tepfer 1999; DETR 1999; Hammond *et al* 1999; Ho *et al* 1999; Rubio *et al* 1999; Power 2002; Tepfer 2002).

We demonstrate here that the views of many scientists working in this area (as reflected in the scientific literature) are at odds with the policy of widespread commercialisation of virus-containing GM crops being pursued by the USDA. Neither do they support the conclusions of the recent DETR policy advisory report on the use of viral inserts in GM crops (UK DETR 1999).

The published scientific data best supports the conclusion that GM crops containing virus-derived transgenes should not at present receive commercial approval. This is because recombination (HGT) leading to the creation of new viruses is inevitable, while the consequences of such recombination cannot at present be predicted. Little or no effort has been made to minimise the risks posed by the use of viral transgenes in commercialized crops and their use is often entirely unnecessary (e.g. the use of the CaMV 35S promoter). Consequently, the use of viral sequences poses both substantial and unnecessary

risks. Commercialisation of crops containing virus-derived transgenes is therefore irresponsible at present.

Abbreviations: CaMV, Cauliflower mosaic virus; CMV cucumber mosaic virus; FMV, Figwort mosaic virus; HGT, horizontal gene transfer; PTGS, post-transcriptional gene silencing; PLRV, Potato leaf roll virus; PRSV, Papaya ringspot virus; WMV-2 Watermelon mosaic virus; ZYMV Zucchini yellow mosaic virus

Background

Commercial transgenic crops contain viral sequences for two main reasons, to regulate gene expression or to confer virus resistance. CaMV terminator sequences, the CaMV 35S promoter and promoters from other caulimoviruses are all used to engineer high constitutive levels of gene expression. Most commercialised GM varieties contain one or more virus promoters.

Viral sequences are also being used to engineer resistance to viruses. Virus-resistance in transgenic plants is called pathogen derived resistance (PDR). PDR is the expression of viral mRNAs or proteins which confer resistance to related (and sometimes unrelated) viruses. While much remains to be discovered, a consensus is emerging that most PDR observed in transgenic plants is obtained through a post-transcriptional, homology-based, gene silencing mechanism. in which both the transgene mRNA and the infecting virus are degraded (e.g. Ahlquist 2002). Thus, in principle, any virus sequence engineered into a transgenic plant may confer resistance to a virus carrying a homologous sequence. Transgenic plants containing virus coding sequences are much less common than transgenic plants containing viral promoter sequences but, as presently used, are considered to carry greater risks for viral recombination. To-date only varieties of squash, potato and papaya have been commercially approved to carry virus-resistance genes in the US (Table 1). Unlike natural virus infections, viral inserts are present in every cell of the transgenic plant containing them.

This review focuses on these two applications of viral inserts since their HGT risks illustrate or general concens. It concentrates primarily on virus-resistance transgenes.

The commercial background

In 1995 Upjohn released the first commercial transgenic virus-resistant plant: ZW-20 squash and later an upgrade: the CZW-3. This latter variety contains genes coding for coat proteins of the cucumovirus CMV and the potyviruses ZYMV and WMV-2. Also authorised for commercial use in the US are potatoes expressing potato leaf roll virus (PLRV, a polerovirus) replicase and potatoes expressing the potato virus Y (PVY, a potyvirus) coat protein. Papaya expressing PRSV (a potyvirus) and cucumber mosaic virus (CMV, a cucumovirus) fusion coat protein has also been approved and has been used in Hawaii.

Although most authorised field tests in the US have been of coat protein genes, some utilise viral replicase genes, viral movement protein genes, viral proteases, viral helper component (HC proteins) and others which are less well characterised. It is probable that most or all of these are being used to engineer virus resistance (Tepfer 2002). Field tests of similar crops have been conducted in the UK.

Natural virus recombination

The risks of transgene/virus recombination can only be understood in the context of the background level of recombination events between related and unrelated viruses in natural and agricultural settings. In nature, the majority of new viruses arising by recombination are non-viable, have low fitness or are indistinguishable from their progenitors. It is nevertheless well-established that new and successful variants of viruses do arise naturally by recombination with a frequency that varies depending on the virus family (e.g. Chenault and Melcher 1994; Revers *et al* 1996; Padidam *et al* 1999). Natural recombination is more common between closely related strains or species of RNA and DNA viruses. Like DNA recombination, RNA recombination is likely to be promoted by shared replication origins and DNA or RNA sequence homology (similarity) (Nagy and Simon 1997). Despite this bias towards homologous recombination most viruses contain in their phylogenetic history a record of multiple recombination events between more distantly related viruses (reviewed in: Koonin and Dolja 1993; Chenault and Melcher 1994; Simon and Bujarski 1994; Roosinck 1997). Some of them are also known to have recombined with non-homologous host genes (Mayo and Jolly 1991). This general pattern of recombination is also found in animal viruses (Lai 1992). Thus viral recombination occurs naturally and gives rise to viruses of great agricultural and economic importance. Any new technology that might enhance the rate of emergence of new viruses is a source of concern.

Evidence for recombination with transgenes

Many plant viruses have now been shown to be able to routinely recombine with transgenes. In laboratory studies recombination of a transgene with an infecting RNA virus has been demonstrated for a dianthovirus (Xiong and Lommel 1991), a bromovirus (Greene and Allison 1994; Greene and Allison 1996), a tombusvirus (Borja *et al*

1999), a tobamovirus (Adair and Kearney 2000) and a potyvirus (Varrelmann *et al* 2000). For the pararetrovirus CaMV, recombination of both its' RNAform (Schoelz and Wintermantel, 1993; Wintermantel and Schoelz 1996) and its' DNA form (Gal *et al* 1992) have been reported, as has recombination between a geminivirus (a DNA virus) and chromosomal DNA (Frischmuth and Stanley 1998). In some cases recombination occurred at very high rates-in up to 80% of all plants tested (Borja *et al* 1999).

Since repair of a defective virus was used in these experiments (to permit detection of recombinant progeny), in all cases (except one, Xiong and Lommel 1991) recombination was between a transgene and a virus with homologous (or nearly so) sequences. Whether recombination events with less homologous viral sequences will occur at comparable rates is a still-unresolved question. Other things being equal, recombination with more dissimilar viruses may occur but probably will be less common since homology (similarity) promotes recombination (Nagy and Simon 1997).

Although these experiments were restricted to greenhouses, they suggest no clear reason why field-grown virus-expressing transgenic plants should not also show recombination (but see below).

Predicting virus hazards from HGT

The principle danger (from an HGT perspective) inherent in the use of virus-derived transgenes in GM plants is that incoming viruses will acquire a transgene sequence to create a new virus strain or species. In a worst-case scenario this virus could cause either ecosystem or crop damage, necessitate intensive pesticide use (to control viral vectors) or require expensive and inconvenient containment procedures (Morales and Anderson 2001; Rybicki and Pietersen 1999). Both of these measures are currently used to control viruses.

Predicting which (if any) recombinant viruses will prove to be significantly pathogenic as a result of HGT is not presently feasible. This is because scientific understanding of virulence determinants and ecological fitness in viruses is almost non-existent. It is even not clear if recombination between closely related viruses should be of more or less concern than recombination between those that are distantly related. Among Caulimoviruses (the plant virus group most systematically tested) strains differ in symptomatology and titre in infected plants. Recombinants formed between two mild strains can nevertheless yield virulent progeny with high titre and severe symptoms (Anderson *et al* 1992). Thus viruses are complex systems, as are the plants they infect, and therefore one should not necessarily expect simple additive results from recombination events.

Are there limits to how related viruses must be in order to recombine with a transgene to create a viable new virus? The phylogenetic evidence for a recombinational origin of most virus families reflects the fact that many virus proteins are frequently not virus-specific. For example, the viral movement protein of one virus may be used by another virus in a shared host plant (e.g. Cooper *et al* 1995). We have found over 100 papers in the scientific literature demonstrating these complementation (or synergistic) effects of a virus (or part of one) on infection by a second virus from a different species[1]. Complementation covers almost the complete diversity of viral traits and viral proteins. This includes replication (e.g. Goldberg and Brakke 1987; Anjos *et al* 1992; Hormuzdi and Bisaro 1995; Cooper *et al* 1995), host specificity and movement (e.g. Carr and Kim 1983; Barker 1989; Malyshenko *et al* 1989; Giesman-Cookmeyer *et al* 1995; Solovyev *et al* 1996; Agranovsky *et al* 1998; Lauber *et al* 1998; Briddon and Markham 2001); vector specificity (e.g. Kassanis and Govier 1971; Briddon *et al*, 1990; Baulcombe *et al* 1993; Ryabov *et al* 2001) and disablement of host defences (e.g. Pruss *et al* 1997; Sunter *et al* 2001; Liu *et al* 2002). These reports cover complementation crossing all known viral phylogenetic boundaries, though not for all traits. Researchers have shown that even an animal virus can infect a transgenic plant that expresses plant virus movement proteins (Dasgupta *et al* 2001). The conclusion to be drawn from this is that whenever a virus can use proteins from a distinct or unrelated virus, it could also use them if it acquires them in a recombination event.

Thus the greatest phylogenetic distance across which potentially viable recombinant events may plausibly occur is unclear but may be very great. So far, there have been no reports of recombinants between DNA and RNA viruses or between geminiviruses (DNA) and pararetroviruses (DNA) such as CaMV. Thus, though there are considerable possibilities for virus recombination, there may also be limitations.

[1] This information derives from three types of experiment: a) synergisms between two wild-type viruses in which one or both viruses benefit from the presence of the other; b) from experiments in which virus proteins are exchanged leading to a complete or partial restoration of the wild-type phenotype, and; c) from complementation type experiments where a transgenic plant expresses a viral protein from one virus species which complements a lost function in a defective virus from a different species. In all, more than 100 peer-reviewed articles, many of which detail more than one instance, found synergism or complementation which crossed at least a species boundary. Articles showing complementation only between strains within a species were excluded from this list.

Commercialisation and biosafety assumptions

These experiments suggest that recombination between transgenes (as currently used) and viruses is inevitable. Consequently, justification for the commercial approval of GM crops incorporating virus sequences has taken the form of asserting that the recombinants which arise will have no significant ecological impact. i.e. that these recombination rates do not matter or should not be extrapolated to field situations. We discuss the evidence for these arguments in points -a) to -e).

a) It is argued that the likelihood of HGT from viral transgenes is no greater than that from mixed infections (Falk and Bruening 1994; USDA 1994; Hammond *et al* 1999). This argument is flawed because it ignores the fact that a transgenic plant is not equivalent to a plant infected with a virus and because most plants are not usually infected with any viruses. The relevant differences include:

1) For all commercialised virus-resistant plants the viral-derived transgene mRNA is expressed in every plant cell. Viruses, in contrast, often exhibit tissue tropisms which may not (or only partially) overlap with those of other viruses-e.g. they may be restricted to phloem cells (Barker and Harrison 1986; Latham *et al* 1997) while other virus infections may be restricted to surface tissues[2]. Thus transgenic crops will result in quantitatively and qualitatively enhanced opportunities for virus recombination (de Zoeten 1991; Gibbs 1994; Allison *et al* 1996).

2) In naturally occurring mixed infections, opportunities for recombination may be limited by intracellular compartmentalisation of viruses and antagonistic interactions between viruses. Animal viruses are known to often exclude each other from preinfected cells in a phenomenon known as superinfection exclusion (e.g. Simon *et al* 1990). Whether exclusion occurs widely between plant viruses is not known but the reported instances suggest it may be common. (e.g. Davis and Mizuki 1987; Sackey and Francki 1990; Allison *et al* 1997; Fraile *et al* 1997). This contrasts with viral transgenes which are present in every cell of each transgenic plant.

Thus, the possibilities for recombination between different viruses in a natural mixed infection are not clear. Thus it may be wrong to assume that natural mixed infections ill invariably give rise to opportunities for recombination.

b) Where observed levels of transgene mRNA are lower than those of genomic virus mRNA it is asserted that recombination frequencies will in turn be lower (e.g. Falk and Bruening 1994; USDA 1994; Rubio *et al* 1999). This argument is not well supported by data. In practice, recombination frequency is likely to be a function of the distribution of viral genomes within the cell as well as of their quantity (Gibbs 1994; UCS 1994). Not only are most viral genomes usually encapsidated, but most viruses also replicate in discrete foci, many of which are enclosed by membranes (e.g. Schwarz *et al* 2002). These factors may act to limit opportunities for recombination between viruses. Additionally, most instances of virus resistance rely on simultaneous repression of the transgene and virus replication by a post-transcriptional gene silencing mechanism (e.g. Gonsalves 1998). It has recently been shown that many viruses disable this system when infecting a host (Kasschau and Carrington 1998). Thus levels of transgene mRNA and protein may be much higher in virus-infected cells than in uninfected ones (Tepfer 2002). Consequently, we do not have a clear picture of whether recombination is more likely between viruses or between viruses and transgene mRNAs.

c) It is proposed that recombinant viruses 'are unlikely' to survive competition from pre-existing viruses or will not give rise to significant new strains (Falk and Bruening 1994; AIBS 1995; Aaziz and Tepfer 1999; Rubio *et al* 1999; Hammond *et al* 1999). These assertions are unsupported by either data, references or detailed arguments (Falk and Bruening 1994; Hammond *et al* 1999; Rubio *et al* 1999) nor are the situations they envisage precisely defined. Consequently, it is difficult to discern the basis of these claims and thus to evaluate them. They seem largely based on the adaptationist (and controversial) idea that newly arising organisms are necessarily less fit than preexisting ones (Pigliucci and Kaplan 2000). Space does not permit a detailed critique of this position except to say that 1) new and significant viruses do arise naturally by recombination (e.g. Briddon *et al* 1996; Zhou *et al* 1997; Moonan *et al* 2000) (as well as mutation) and 2) recombinant viruses created *in vitro* have been created which have superior fitness (in one or more respects) than their parents (Anderson *et al* 1992; Ding *et al* 1994; Fernandez Cuartero 1994). This argument also sidesteps the issue of whether a new and recombinant virus would find a new niche rather than compete with its progenitor. The damage caused by the many viruses which spread beyond their centre of origin, or to new crops, is testament to the ability of viruses to find new niches.

d) The presumed mechanism by which commercially approved virus-resistant plants are thought to work is that post transcriptional gene silencing targets the transgene mRNA and a virus carrying homologous sequences. It is suggested that PTGS will prevent or reduce HGT because a selective pressure will be directed against any virus which acquires the transgene (USDA 1994; AIBS 1995; Allison

[2] Unsuccessful infections by non host-adapted viruses are called subliminal infections. Their prevalence is not known.

et al 1996; Robinson 1996; DETR 1999; Rubio *et al* 1999; Hammond *et al* 1999; Varrelmann *et al* 2000; Power 2002).

This position was relied on by the USDA (USDA 1994) in deregulating the first virus-resistant plant variety (the ZW-20 squash). This argument has been undermined by the subsequent finding that infecting viruses can disable the post transcriptional gene silencing mechanism (PTGS) presumed to be the basis for all the commercially approved virus resistance transgenic traits (e.g. Voinnet *et al* 1999).

PTGS cannot therefore be relied upon to select against viruses which acquire the transgene in a recombination event.

e) A final argument used is that reduction of virus prevalence due to growing resistant plants will reduce the rate of recombination and HGT (Falk and Bruening 1994; Rubio *et al* 1999). It is difficult to assess the validity of this proposition and indeed the authors provide no supporting data. It is worth noting that virus-resistant transgenic crops are directed at excluding one or a few strains or species of virus (unless gene stacking is used). Much of the concern over HGT from transgenes to viruses focuses on viruses that do not routinely infect the transgenic host (Gibbs 1994). This is because this type of invading virus (which is *not* adapted to that host) would be acquiring a transgene that *is* adapted to that host and therefore useful to it. Even if acquisition of the new gene did not confer pathogenicity directly it could constitute a significant adaptive step in that direction, thus enabling a new virus to evolve. Virus adaptation to new hosts can be acquired by as little as a single base change (Ingham and Lazarowitz 1993; Weiland and Edwards 1996), thus the barriers to mounting an infection can be small for some viruses.

Questioning of these assumptions would be pedantic if they did not form the bedrock of successful biosafety arguments in commercial applications to the USDA or were not used by the USDA itself (USDA 1994). With the exception of point e) every application for new virus-resistant varieties explicitly makes the above dubious and unsubstantiated arguments and selectively uses the scientific literature for support.

Reducing the risk of HGT from transgene to virus

Various transgene modifications and safeguards might reduce the rate of HGT when making GM plants. These safeguards include avoidance of certain proteins known to interact with other viruses (AIBS 1995; Hammond *et al*; Power 2002); mutation or disablement of protein coding sequences (DETR 1999; Hammond *et al* 1999; Power 2002); removal of replication-associated sequences (Allison *et al* 1996; Miller *et al* 1997; DETR 1999; Hammond *et al* 1999); use of mild and endemic strains (Hammond *et al* 1999; Power 2002); and use of short viral sequences (Allison *et al* 1996). Almost no research has been devoted to investigating the feasibility or effectiveness of these possibilities and currently authorised commercial crops with viral inserts incorporate few of these precautions (see Table 1).

Variety name	Species	Constructs inserted	Notes
ZW-20	squash	3xCaMV 35s promoter 3x CaMV 35s terminator WMV-2 coat protein ZYMV coat protein	full length coat proteins, both detected in plants
CZW-30	squash	4x CaMV 35s promoter 4x CaMV 35s terminator CMV coat protein WMV-2 coat protein ZYMV coat protein	full length coat proteins, all 3 detected in plants
sunUp and 63-1	papaya	PRSV coat protein fused to short CMV coat protein sequence	full length protein product detected in plants
3 transgenic lines	potato	PVY coat protein FMV promoter	full length coat protein plus PVY 3' replication origin, mRNA only detected
7 transgenic lines	potato	PLRV replicase (ORFs 1 and 2) 2x FMV promoter	full length replicase (3.8Kb) mRNA only detected

Table 1. Varieties of GM virus-resistant crop approved for commercial use in the USA

Conclusions

It is our conclusion that too little is known at present about the evolution, ecology, biochemistry and pathogenicity of viruses to allay the concerns about horizontal gene transfer (HGT) from plants containing viral transgenes. Risk assessment of transgenic crops for HGT involves extrapolation to large scales and diverse real-world situations, which cannot at present be done without the use of crucial and unsubstantiated assumptions. For example, it is a fundamental tenet of risk assessment that extrapolation from limited knowledge requires a robust mechanistic understanding of a new technology. Failure to establish this basic information can lead to incorporation of false assumptions into risk-assessment procedures. In the case of virus-resistant transgenic crops this information should certainly include substantial knowledge of the mechanism of virus-resistance, its characteristics and limitations. For instance, it now seems likely that the resistance mechanism for virus-resistant lines is *via* post transcriptional gene silencing (PTGS) and it is now known that PTGS is turned off by many viruses (e.g. Voinnet *et al* 1999). Thus a key assumption relied on by the USDA to argue against the likelihood of HGT now appears to be invalid (USDA 1994). As we showed in this paper, this was not the only unvalidated assumption used to justify approval. Not only is it unscientific to rely on unvalidated assumptions it is also indefensible to at the same time argue (before the general public), that only well-understood transgenes and systems are used in the manufacture of transgenic crop varieties.

We are not alone in concluding that more information on plant virus biology is needed and many authors have argued the necessity for more information (de Zoeten 1991; Tepfer 1993; AIBS 1995; Miller *et al* 1997; Aaziz and Tepfer 1999; Power 2002; Tepfer 2002). None of these authors has however made explicit the link between inadequate baseline information and irresponsible regulation. Unlike the USDA and other national regulatory bodies, we believe that lack of such knowledge requires delaying applications and withdrawing approval for the release of GM virus sequence-containing plant varieties on the grounds that approval is incompatible with a prudent and precautionary approach.

Furthermore, most of the HGT risks carried by commercial transgenic varieties containing virus inserts are probably unnecessary ones. To reduce HGT, non-viral promoters could be used, transgene sizes reduced, replication origins removed, protein expression prevented and gene sequences disabled. These improvements would probably not impact on transgene effectiveness. By not requiring applicants to incorporate these safety features, or to justify why they have not done so, regulators are exposing third parties to unnecessary risks.

The question of monitoring the consequences of virus sequences in GM crops is crucial. The power of science derives from testing and refining its predictions. If we do not test predictions of a lack of impact of viral sequences in GM crops we will either learn nothing about the risks and hazards or we will learn it too late.

What is required above all is for the scientific community to make itself heard so that poor biosafety assessments are challenged in print and best practice is incorporated into commercial varieties. By not doing this the scientific community is perceived as failing to be independent of commercial pressures. It is difficult to see however, individual members of the scientific community, who wished to express such views, being able to do so explicitly and openly in the present climate.

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P.O.Box 3279, Brighton BN1 1TL, UK - Phone: +44(0)1273 625 173

Email: info@econexus.info Website: www.econexus.info