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PERSPECTIVE

Problems in monitoring horizontal gene transfer in field trials of transgenic plants

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Transgenic crops are approved for release in some countries, while many more countries are wrestling with the issue of how to conduct risk assessments. Controls on field trials often include monitoring of horizontal gene transfer (HGT) from crops to surrounding soil microorganisms. Our analysis of antibiotic-resistant bacteria and of the sensitivity of current techniques for monitoring HGT from transgenic plants to soil microorganisms has two major implications for field trial assessments of transgenic crops: first, HGT from transgenic plants to microbes could still have an environmental impact at a frequency approximately a trillion times lower than the current risk assessment literature estimates the frequency to be; and second, current methods of environmental sampling to capture genes or traits in a recombinant are too insensitive for monitoring evolution by HGT. A model for HGT involving iterative short-patch events explains how HGT can occur at high frequencies but be detected at extremely low frequencies.

Today's commercial applications of transgenic organisms pose some of the same types of risk to environment and health as previous applications, such as the massive release of antibiotics into the environment[1,2]. When assessing the impact of transgenic organisms, most risk assessments will consider HGT (gene reproduction and segregation to organisms or cells separately from the reproduction and segregation of the genome as a whole), ecological lag times and toxicity of the product (if it is to be consumed; e.g., see refs. 3,4). These same issues were pertinent to the wide-scale deployment of antibiotics 50 years ago, even if they were not fully apparent to those who were assessing the risks at the time.

The impact of the medical and agricultural use of antibiotics is well understood and described, giving nearly complete retrospective explanation for the global spread of antibiotic resistance genes by HGT[5–9]. The question of gene transfer is not 'will it happen?' but 'when and where will it happen?' A more sophisticated understanding of the way genes transfer and ultimately settle into new genomes is required to reconcile divergent claims about the risks of HGT from transgenic crops.

Descriptions of genomes make clear that HGT has deeply influenced their structures[10–16]. Yet attempts to confirm HGT from transgenic plants to soil microorganisms in the broader environment have failed[3,17–19]. HGT from a transgenic organism into the genome of a recipient organism has been detected in the environment, but not without the use of recipient bacteria carrying special constructs (e.g., an allele of the neomycin phosphotransferase II gene (*nptII*) with an internal deletion) with significant sequence similarity to plant transgenes (e.g., an intact *nptII*), thus influencing the event through the use of homologous recombination to boost the detection of transfer[17–21]. These studies have been important demonstrations that gene transfer occurs, even if HGT was influenced by the methods used to observe it.

In this article, we use the best measurements of frequencies of gene transfer and the inferred histories of antibiotic-resistant bacteria to critique contemporary HGT risk assessments of transgenic crops. We show, using the evolution of penicillin-binding protein genes as an example, that experimental limitations preclude measuring HGT with the sensitivity necessary to dismiss eventual environmental harm. Therefore, existing data do not justify confidence in the statements that HGT happens, but at "exceptionally low frequencies[3]" and that it is "so rare as to be essentially irrelevant to any realistic assessment of the risk involved in release experiments involving transgenic plants"[22]. We offer a different view of the mobile gene ecosystem and a model of HGT that we believe is more relevant to assessing environmental risks (**Fig. 1**).

Lessons from Streptococcus pneumoniae

The penicillin-binding proteins (PBPs)—targets of the drug—of *Streptococcus pneumoniae* with reduced susceptibility to penicillin differ from those of wild-type *S. pneumoniae*[23–25]. Loosely speaking, five PBPs contribute to killing and resistance at some concentrations of penicillin (discussed in refs. 26–28). All five PBPs have been changed in some viridans streptococci isolated from the clinic[29], suggesting that, *in situ*, more than just the two most important PBPs (2b and 2x) might contribute to resistance. Four *S. pneumoniae pbp* genes, through five independent mutations[24,30], are reported to change to raise *S. pneumoniae*'s tolerance of penicillin to 2 μg/ml, the levels observed in some clinical isolates[25].

Mosaic genes. Clinical isolates resistant to high levels of penicillin have *pbp* genes that are mosaics (for an explanation of mosaic genes, see **Fig. 1**) of DNA sequences of *pbp* genes from at least two (the recipient and a donor), and possibly more, species[24,25]. Donor species have one or more *pbp* genes that produce proteins with naturally low affinities for penicillin. Regions of those genes are found interspersed in the sequences of resistant *S. pneumoniae*'s *pbp* genes.

The history of mosaic *pbp* genes in *S. pneumoniae* illustrates why HGT is profoundly difficult to measure. First, penicillin resistance was assimilated into the *S. pneumoniae* genome through successive

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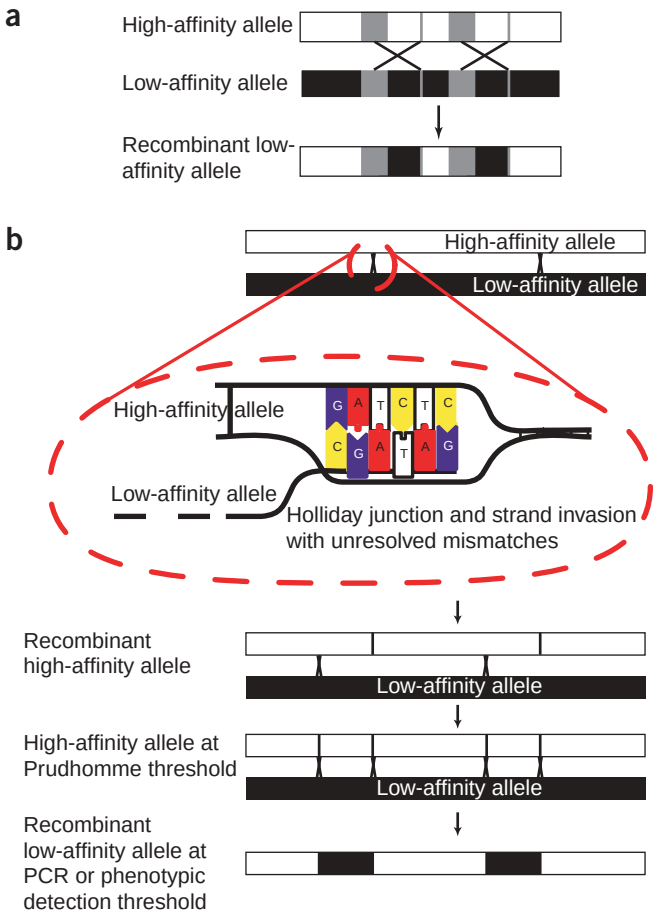

Figure 1 Evolution of mosaic alleles by molecular massage. HGT domain swapping involves moving genes or sub-genes between genomes. In nature, domain swapping extends to significantly different nucleotide sequences. (**a**) The homology-directed illegitimate recombination model of Prudhomme *et al.*[63] illustrates how homologous recombination leads to the insertion of nonhomologous DNA. In this model, insertion of donor DNA (solid boxes) follows the legitimate crossover events of homologous recombination, with concomitant deletion of intervening sequences of recipient DNA (open boxes). Single-stranded DNA corresponding to perhaps a highly divergent allele of a *pbp* gene, that encodes a PBP with low affinity for penicillin, is taken up by a competent and penicillin-susceptible strain of *S. pneumoniae*. Short stretches of DNA of near or absolute identity (≥153 nucleotides, gray boxes) in the otherwise highly divergent donor DNA initiate invasion of the donor strand. Extremely short stretches of sequence identity ('microhomology,' 3–10 nucleotides, gray lines) suffice to define the end of the length of heterologous DNA inserted. (**b**) The short stretches of highly similar DNA that bring a region to the threshold of 'recombination' are either present by chance or by conditioning. We propose that sequence conditioning begins when biochemical barriers, primarily mismatch repair (MMR)[64–67], fail to remove mispaired DNA during recombination (inset). Depending on the proficiency of mismatch repair (MMR), stretches of DNA from homologous (5%–30% divergent) sources may initially be paired, with the invading strand subsequently degraded. MMR can saturate under stress[49,68–70] or falter through mutation, allowing some mispairs to escape repair. Stretches of the recipient strand could be massaged into a closer match with the donor DNA over short intervals (black lines in recipient gene). High-frequency HGT could thus leave iterative small changes that would be mistaken for variation from polymerase errors, if detected at all. This model illustrates the importance of measuring gene transfer frequencies, not just inheritance (transmission) frequencies, for estimating the impact of HGT in the environment.

introductions and replacements of nucleotides sourced from highly diverged donors (**Fig. 1b**). Gene transfers between species most frequently result in short stretches of recombination, the mosaicism observed in *pbp* genes, which are invisible to most analyses (see chapter by J.A.H in ref. 31). Second, the lag[32] between environmental impact and genesis of the recombinant phenotype is an unpredictable variable. Although it took 50 years for high-level penicillin-resistant *S. pneumoniae* to become 21.5% of the isolates in the United States[33], that outcome could not have been predicted in 1950 anymore than in 2004.

Ecological lag time. The time taken for a trait to emerge is partly a function of the adaptive value of a new gene, but the strength of selection or absence of selection cannot always be known in advance[34,35], and any adaptive value must overcome the inhibiting effect of the dominant flora[36]. In some cases, the emergent phenotype may be seen only when the environment changes, or the microbe changes environments, as in the evolution of antibiotic resistance before selection (e.g., see reviews from J.A.H. group[8,37]). Only recently have formal experiments attempted to begin measuring the influence of selection on the frequency of HGT[19,35].

HGT introduces another complexity in attempts to measure the lag time. When genes evolve by transfer rather than through organismal reproduction, neither the generation time nor the geographical range of the organism necessarily limits the lag time. This last point is particularly relevant to attempts to measure HGT in field trials: the combinatorial development of mosaic genes in decade time scales follows from the flow of genes across the globe, not through the generation of variation within plots. In the case of *S. pneumoniae*, once one low-affinity *pbp* allele was made, it could be transferred between strains with much higher efficiency (by homologous recombination), as could combinations of recombinant genes assembled in one or more different strains[38]. The speed at which penicillin resistance spread by subsequent gene transfer events could have accelerated exponentially from the point in time at which the alleles were first assembled, far exceeding the speed at which emergent clonal lineages reproduced or colonized new environments.

Implications for monitoring

The purpose of a transgenic crop field trial designed to assess HGT is to produce meaningful measures of potential harms arising from gene transfer and estimate the safety margins needed to avoid them. A verified trial would, either through scale or other design features, produce outcomes that are both qualitatively comparable to the range, and proportional to the magnitude, of impacts that could be expected from full releases.

Detection limits. Could past trials have detected HGT at a frequency below which any environmental harm would arise? Techniques being used to monitor HGT in soil have sampling limits of about one recombinant bacterium in 10^8–10^{11}, and these experiments uniformly yield no detectable recombinants unless special conditions are applied[4,17–21,39–41]. Some authors have imposed additional assumptions about barriers to HGT and extrapolated an estimated frequency of many orders of magnitude less than their sampling limits, that is, to less than one event in 10^{16}–10^{17} (refs. 22,39). Trials verified as relevant to a risk assessment would therefore have features that permitted detection of recombinants at HGT frequencies $<10^{-17}$. Clearly, no published trials have been that powerful.

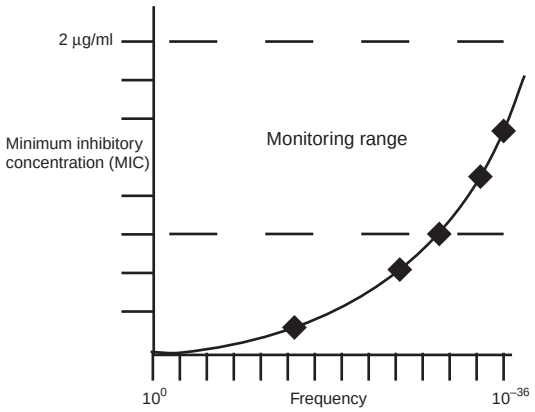

Figure 2 Implicit assumptions in HGT monitoring. The search for recombinant microorganisms that could arise in the soil surrounding transgenic plants invariably incorporates a step where the bacteria are isolated and cultured and those displaying a phenotype that can be selected (e.g., antibiotic resistance) or screened (e.g., PCR or intensity of fluorescence[54]) are taken as recombinants. Every selection/screen has a threshold monitoring range (dotted horizontal lines perpendicular to the *y*-axis). Recombinants that do not display in this range will not be detected. For example, an investigator-imposed threshold penicillin concentration would miss recombinant strains of *S. pneumoniae* that are resistant to other levels of penicillin but may arise at much higher frequencies and would lead to false confidence that the use of penicillin at such concentrations would be of low risk to the evolution of clinically important penicillin-resistant strains. The challenge for future monitoring proposals is to justify that the monitoring range is within the reach of the population at the temporal and population scales being tested.

Even if they had been, would this detection limit verify the trial? Existing knowledge of *S. pneumoniae pbp* genes can be used to answer this question. Majewski *et al.*[42] recovered single-gene *S. pneumoniae* recombinants at a frequency of approximately 10^{-6} using DNA from donor sources that have diverged by 17%–18% in DNA sequence. The degree of sequence divergence between donor and recipient *pbp* alleles used in this study was in the middle of the range seen in alleles actually donated to penicillin-susceptible clinical isolates of *S. pneumoniae* (14%–25%[25,30]). Using this transmission frequency as a guide, the predicted frequency of *S. pneumoniae* with one recombination event per *pbp* gene is 1×10^{-24} [$(1 \times 10^{-6})^4$]. (This estimate would be valid even if only PBP2b and 2x had to change because a minimum of two changes in each of PBP2b and 2x are thought to be required[24].) For strains with six events (e.g., possibly the South African isolate described in refs. 25,27), the frequency would be 1×10^{-36} [$(1 \times 10^{-6})^6$]. In theory, the evolution of penicillin resistance and its consequences has resulted from events predicted to be 10^7–10^{19} times rarer than frequencies of HGT estimated to be occurring in soil.

Whereas the low-affinity alleles of all the different mosaic *pbp* genes in a given strain of *S. pneumoniae* were probably not built into their final complexity at each locus from one lucky scoop out of the pool of DNA surrounding them, contemporary experiments on transgenic organisms impose that requirement on the organisms being monitored in a relatively small area for comparatively short times, and rely on a high frequency event to overcome unpredictable lag times so that at most only a trillion culturable organisms would be needed to reveal HGT. Gene transfers that result in intermediate phenotypes or phenotypes different from those expected by the investigator are lost (**Fig. 2**).

A verified trial for studying HGT, therefore, would require a protocol to screen approximately 10^{25}–10^{37} bacteria for penicillin resistance. Even if all wild bacteria could be cultured and plated at densities of 10^{10}/Petri dish, a minimum of 10^{15} and 10^{27} Petri dishes, respectively (and many times more if other culturable bacteria from the environment also grow on the plates) would be required to detect one recombinant arising *de novo* in the field trial.

Ecological issues. A new respect for the scale of the microbial world is required to appreciate the detection problem. In some soils, like rich top soils, there are approximately two billion microorganisms per gram[43]. The total global count is approximately 5×10^{30}, with an average turnover of three years[43]. Gene transfer magnitudes are conservatively 100 times this already impressive scale because each organism may host, over its lifetime, 10–100 horizontally mobile elements (e.g., viruses or conjugative plasmids). These approximations are also consistent with estimates of gene transfer derived from observations of the viral load in the world's oceans (see chapter by J.A.H[31] in book).

The number of transgene transfers to soil microbes thus can be estimated based on derived HGT frequencies and the size of the microbial population (**Table 1**). For example, a transmission frequency of 10^{-12} could result in 4,000 recombinants per square meter of top soil (based on 5×10^{28} bacteria per 1.4×10^{13} m^2 of top soil[43]). Ten recombinants could be expected in 250 m^2 if the gene transmission frequency were 10^{-17}, with upwards of a trillion recombinants among the nearly 70 million hectares of transgenic crops[44]. Were HGT truly rare, on the order of the inverse of Avogadro's number (10^{-24}), 5,000 recombinants would be expected in the estimated 11.4 million hectares currently planted in *Bacillus thuringiensis* (*Bt*) corn[45]. Although it might seem that these numbers should be big enough for trials to detect recombinants, distributed among the normal flora a minimum of 4×10^8 m^3 (500 million metric tons) of soil would have to be sampled to find one (this estimate is based on 5×10^{27} bacteria per 1.14×10^7 hectares of top soil; see **Table 1**).

Implications for risk assessment

When HGT is considered in relation to risk assessments, we must consider not only whether HGT is occurring, but also the critical issue of its consequences for health and environmental safety. The latter is

Table 1 Estimated number of HGT events from transgenic plants to soil microbes on the basis of derived HGT frequencies and microbial population size

	Transmission frequency[a]					
	10^{-3}	**10^{-8}**	10^{-10}	10^{-15}	10^{-20}	10^{-25}
Recombinants/m^2 field[b]	10^{12}	10^7	10^5	1	<1	<1
Recombinants in *Bt* corn fields (global)[b]	10^{24}	10^{19}	10^{17}	10^{12}	10^7	10^2
Size of soil sample for one recombinant[a,b,c]	3×10^{-6} g	0.3 g	**30 g**	**3 T**	**3×10^5 T**	**3×10^{10} T[d]**
Size of soil sample for one	3×10^{-4} g	**30 g**	**3 kg**	**300T**	**3×10^7 T**	**3×10^{12} T**
Culturable recombinant[a,e]						

[a]Boldface text for frequencies indicates frequencies higher than any reports of environmental HGT that we are aware of (unless special recipients were used); boldface text for sample sizes indicates sample sizes larger than those in any studies that we know of that have examined the full genomic content of the sample. [b]Based on the following calculation: 5×10^{27} bacteria/5,000 recombinant bacteria) × (g soil/2×10^9 bacteria) × (m^3/1.3×10^6 g soil). [c]g, grams; kg, kilograms; T, metric tons. [d]This amount of soil would fill a train of 500 million (standard 70 US tons) boxcars, long enough to encircle the equator 192 times. [e]Assuming 1% of soil microorganisms are culturable[71,72].

dependent on the likely impact of the newly acquired trait in its ecological and geographical context. In the case of transgenic plants expressing *Bt* toxins, for example, the ubiquity of *B. thuringiensis* in soil was considered by the US Environmental Protection Agency (EPA; Washington, DC, USA) to reduce the environmental impact of recombinant *Bt* toxin transgenes, even if they did transfer to soil microorganisms[41]. However, it is useful to invoke what is known about *S. pneumoniae* for evaluating the EPA assessment.

Bt is a shorthand for the *cry* toxin genes, modified from those first isolated from the soil bacterium *B. thuringiensis*, that confer resistance to various insect pests of plants. The *cry* genes appear to be of mosaic construction, like the *pbp* genes[46]. The combinations of domains distributed among the various *cry* genes alter the range of species that find the protein toxic. In this regard, it is noteworthy that *B. thuringiensis* has "a significant history of mammalian pathogenicity"[46] and is thus not irrelevant to food safety or other environmental issues. Before human application of penicillin, the EPA might have similarly dismissed concern about the trafficking of the extremely small PBP protein domains because *pbp* genes are ubiquitous in human flora (low-affinity alleles originate in normal human commensals, such as the viridans streptococci). Moreover, large fragments of the modified forms of *cry* transgenes, which may not be identical to the gene found in the soil bacteria[47], persist through digestion in pigs and exit with feces[48]. DNA from *cry* genes was detected only when their source was *Bt* corn, suggesting that normal soil organisms are less likely to contribute to any recycling through animals, making the transgene DNA relatively more available to gut and soil bacteria.

Mosaic genes are becoming the norm in antibiotic-resistant microbial flora[49]. For example, three genes (*parC, parE* and *gyr*A) must change in *S. pneumoniae* for it to become resistant to clinical levels of the drug ciprofloxacin. Resistant strains carry mosaic alleles of each of these genes, with the viridans streptococci again being the most likely donors[50]. The mosaic regions vary from 0.6%–12% in DNA sequence from susceptible strains, and some resistant strains have eight putative interspecies domains distributed over the three genes[50]. Domain swapping can be a powerful route to protein diversity that is revealed only by the introduction of new selective pressures and niches, not always predictable from known biochemical function and apparent role in current niches.

Concluding remarks

The consequences, if not the precise frequencies, of HGT in microorganisms are becoming well known to those who suffer from bacterial infections that resist treatment by the least toxic and least expensive antibiotics and to governments that must pay for treatments with more expensive antibiotics and intensive medical interventions. The lessons of antibiotic resistance should not be lost in the haste to introduce transgenic crops with new traits, nor in considering the impact of existing approved transgenic crops that contain antibiotic resistance genes (which nearly all do[51]).

To paraphrase Curtis *et al.*[52]: "Microbial ecology, which drives the ecology of the planet, urgently requires…descriptions of the whole to complement the trend to ever more perfect experimental descriptions of the parts" because the parts created by HGT will otherwise always be outside the resolution of our experiments.

Are there any alternatives to existing low-resolution techniques for monitoring? Molecular detection techniques, such as PCR, do not increase detection sensitivity more than a few orders of magnitude and are also blind to small changes in nucleotide sequence (see our review[53]). The most obvious alternative is not a technique but a decision to consider the scientific uncertainty surrounding environmental applications that introduce HGT risks and adjust the pace of their release to match developments in our ability to monitor at relevant sensitivities, recognizing that the technology of safety monitoring lags behind the technology of genetic engineering. The slowdown may not be for long.

From a technological standpoint, some groups are already developing vectors carrying genes that are expressed in a larger number of species, including those that cannot be cultured, thus extending our understanding of gene movement in the context of the larger soil biodiversity[54]. Even more intriguing are developments that map the mobile gene landscape and thus develop indirect measures of HGT activity and gene diversity in particular places and times[55–57]. As one of us (J.A.H) has previously noted[58], these approaches look promising because they capture the novel gene diversity predicted to emerge from HGT. The detection limits of these new developments are still not known, and will probably fall short of detecting the early bouts of short-patch interspecies recombination (**Fig. 1b**), but their innovation removes the need to find a particular DNA sequence within a sea of DNA, the very factor that limits conventional approaches.

HGT binds the microbial community into a complex network[59,60] even at intuitively 'low' frequencies of transfer ($\leq 10^{-17}$), allowing alleles of genes to evolve on a global scale[7,30,61]. HGT has been dismissed by commentators in response to concerns about the possible impact of transgene migration from released transgenic crops, even though the scale of commercial production already makes such risks plausible. Gene transfer is facilitated by many different kinds of vectors and environmental conditions and is not restricted to microbes. Among a number of different and plausible transgene vectors are viruses capable of crossing even the plant-animal divide[62]. The variety of transfer paths and vectors, and the number of genomes that could serve as temporary or permanent homes for transgenes (or parts thereof), make the speculative calculations presented here highly conservative. Contrary to the conclusion of others[3], we believe that new approaches to monitoring environmental scale applications of transgenic organisms are urgently needed.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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