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Not all GMOs are crop plants: non-plant GMO applications in agriculture

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Not all GMOs are crop plants: non-plant GMO applications in agriculture

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Abstract Since tools of modern biotechnology have become available, the most commonly applied and often discussed genetically modified organisms are genetically modified crop plants, although genetic engineering is also being used successfully in organisms other than plants, including bacteria, fungi, insects, and viruses. Many of these organisms, as with crop plants, are being engineered for applications in agriculture, to control plant insect pests or diseases. This paper reviews the genetically modified non-plant organisms that have been the subject of permit approvals for environmental release by the United States Department of Agriculture/Animal and Plant Health Inspection Service since the US began regulating genetically modified organisms. This is an indication of the breadth and progress of research in the area of non-plant genetically modified organisms. This review includes three examples of promising research on non-plant genetically modified organisms for application in agriculture: (1) insects for insect pest control using improved vector systems; (2) fungal pathogens of insects to control insect pests; and (3) virus for use as transient-expression vectors for disease control in plants.

Keywords Genetically modified organisms (GMOs) · GM insects · GM fungi · GM virus

The global adoption of genetically modified (GM) crops, engineered using the tools of modern biotechnology, has

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steadily increased over the last 30 years. GM crops are being grown in over twenty-five countries, and this includes eleven different crops (James 2012). Many other GM plants are the subject of ongoing research. The first environmental releases for GM crop plants were approved in the United States (US), for confined field trials in 1985 and for unconfined planting (deregulation) in 1992. GM plants have received significant attention in the media and have dominated the discussions regarding risk assessment and regulation among the scientific and regulatory communities (McHughen and Smyth 2008). However, the first permits for field trials with genetically modified organisms (GMOs) in 1985 (or earlier) were not for plants, but for genetically modified bacteria: *Pseudomonas syringae*—the 'ice-minus' bacteria (Lindow and Panoupoulus 1988; McHughen and Smyth 2008). There has been steady research on these and various other non-plant GMOs in the years since.

A good indication of historical and ongoing research and regulation with non-plant GMOs can be found in the record of permits issued for environmental release in the US (See Table 1). The US coordinated framework for the regulation of biotechnology was established in 1986 (OSTP 1986; McHughen and Smyth 2008). According to this framework, the US Department of Agriculture/Animal and Plant Health Inspection Service (USDA/APHIS) has responsibility for the regulation of all GM organisms that are a 'plant pest' or have been engineered using 'plant pests'. To date, most GMOs have fit within this 'plant pest' criteria for regulation by APHIS, although with recent advances of the technology including targeted gene modifications and cisgenics that do not use any 'plant pests', some GMOs are not regulated by APHIS (Kuzma and Kokotovich 2011). However, most GMOs in the US have required a permit from APHIS before any 'controlled' release into the environment. These permits are usually for the purposes of research in field trials, until the GM organism has been approved following a process of petition to APHIS for deregulation, after which a permit is no longer required (USDA 1997).

A comprehensive database (maintained by Information Systems for Biotechnology at Virginia Tech) including all of the permits, as well as deregulations, can be accessed through the APHIS website (http://www.aphis.usda.gov/biotechnology/status.shtml). This database is described as 'GE crop data' although it does include other organisms in addition to crops (e.g., bacteria, fungi, insects, nematodes, virus). Permits in this database come in two forms: 'release permits' and 'notifications'. 'Release permits' were required for all organisms originally and now only for certain GMOs not eligible for the more common 'notification'. 'Notifications' are an abbreviated version of a permit initiated by APHIS starting in 1993 for GMOs that meet certain criterion to be eligible for this simplified process (USDA 1997). The earliest permits in the database were issued in 1985. At the time of this review (June 18, 2013), the database included 18,789 permit requests, of which 16,438 were notifications and 2,351 were release permits. Although some ($\sim$1,500) of these permit requests were withdrawn or denied, most have been issued ('acknowledged', in the case of notifications). The earliest release permits, in 1985 and 1986, were 'accepted' rather than 'issued'.

Almost all of these permits have been for plants; only 155 permit requests are for GMOs that are not plants. Of these, 20 were withdrawn and two were denied. Since 1985, 133 'release permits' have been issued for non-plant GMOs. Of the total permits/notifications that have been approved, less than one percent are for non-plant organisms, while approximately ten percent of permits that were issued in the first 5 years (1985–1989) were for organisms other than plants. It should be noted that all non-plant organism permits issued have been 'release permits'; none have been by the simplified 'notification'. It is not entirely clear, but it appears from the APHIS regulations that one eligibility requirement for a permit under the 'notification' option is that the organism (regulated article) is a plant species (USDA 1997).

A summary from the database of the non-plant organisms that have been issued environmental release permits by APHIS is provided in Table 1. There are 230 different total organisms (plant and non-plant) listed to search in the database; 38 of these are non-plant organisms. These numbers somewhat exaggerate the actual number of organisms because a few organisms are listed by different descriptors e.g., *Xanthomonas campestris* pv. *vesicatoria* is also listed as Bacterial Spot of Tomato, *Cryptonectria parasitica* is also listed as Chestnut Blight, tobacco mosaic virus is also listed as TMV. In some cases, the database shows more than one species together with a forward slash between them, which is as it appears in the permit application. The descriptors are entered in the

Table 1 Non-plant GM organisms issued permits for environmental release by USDA/APHIS

	Total # Permits Issued	Years Issued			APHIS Permit No. linked to EA in ISB database
		1985-1994	1995-2004	2005-present	
BACTERIA					
Bt	1				
Bacterium	1				
Clavibacter[a]	7				7[a]
Rhizobium[b]	6				3[b]
Erwinia[c]	3				2[c]
Xanthomonas[d]	15				2[d]
Pseudomonas[e]	26				6[e]
FUNGI					
Cephalosporium stripe	2				
Aspergillus flavus	5				
Fusarium[f]	14				3[f]
Cryphonectria parasitica[g]	5				94-010-01r
Neotyphodium[h]	2				05-152-01r
VIRUS					
TEV (Tobacco etch virus)	1				98-120-01r
Citrus viroid iii	2				99-032-03r
Citrus tristeza virus	5				
Tobacco mosaic virus (TMV)[i]	21				4[i]
INSECTS					
Western orchard predatory mite	1				
Pink bollworm	15				2[j]
NEMATODES					
Heterorhabditis bacteriophora	1				

A note is included if there is more than one description in the database list of organisms, or if there is more than one permit linked to an Environmental Assessment

[a] *Clavibacter*, *Clavibacter xyli* 87-355-01r, 88-355-01r, 89-053-01r, 90-016-01r, 90-333-01r, 91-343-01r, 92-329-01r

[b] *Rhizobium*, *Rhizobium etli/Rhizobium leguminosarum*, *Rhizobium etli/Rhizobium leguminosarum/Rhizobium meliloti*, *Rhizobium fredii/Rhizobium leguminosarum* 90-164-03r, 94-207-02r, 97-071-01r

[c] *Erwinia amylovora*, *Erwinia carotovora*, *Pectobacterium carotovorum* 03-279-01r, 05-097-01r

[d] *Xanthomonas*, *Xanthomonas campestris*, *Xanthomonas campestris* pv. *vesicatoria*, Bacterial Spot of Tomato 89-290-01r, 96-071-06r

[e] *Pseudomonas*, *Pseudomonas Syringae*, *Pseudomonas Syringae* pv. *syringae*, *Pseudomonas putida* 90-135-01r, 91-023-06r, 93-026-04r, 95-130-01r, 97-023-02r, 97-023-01r

[f] *Fusarium graminearum*, *Fusarium graminearum/Fusarium sporotrichioides*, *Fusarium moniliforme*, *Fusarium verticillloides* 94-006-01r, 95-003-01r, 98-355-01r

[g] *Cryphonectria parasitica*, Chestnut Blight

[h] *Neotyphodium* sp., *Neotyphodium* sp. *Lpl Endophyte*

[i] Tobacco Mosaic Virus (TMV), TMV 91-007-08r, 94-081-01r, 95-041-01r, 96-051-04r

[j] 01-029-01r, 05-098-01r

database according to how they are described in the permit application that was submitted to APHIS. Where more than one descriptor in the database has been combined into one row in Table 1, the different descriptors are given in a footnote. Some organisms are grouped by genus for the table. Note that in the database the list of all organisms will show some non-plant organisms there that are not included in Table 1, such as *Agrobacterium tumefaciens* and *Eschericia coli*. These organisms are not included in the table because the permit requests were withdrawn before they were issued. Table 1 only includes organisms for which permits have been issued.

The total number of permits that have been issued for each organism, and the years in which the permits were issued are also shown in Table 1. Some organisms were issued permits early on, but there have been no additional requests for a permit in the last 10 years, including all permits for *Rhizobium* species which only occurred before 2001, and only one permit for a nematode in 1996. Organisms with the most permits issued include 22 for *Pseudomonas syringae* (spanning the entire period from 1985 to 2013), 21 for Tobacco Mosaic Virus (from 1991 to 2013), and 15 for pink bollworm (from 2001 to 2013). There have been permits for several different bacteria and fungi over the years, but only two insects have been the subject of a release permit, as well as four viruses/viroids.

Table 1 also provides the permit number for any permit for these organisms that is linked to an environmental assessment (EA) in the database. APHIS does not prepare an EA for every release permit issued, usually only those that raise new risk issues. There is at least one EA on record for every organism or similar organisms, with a few exceptions. For some organisms, multiple EAs have been prepared. The full record in the database (opened by selecting the permit number) includes the name of the submitting institution and a brief (although very vague) description of the phenotype and genes, as well as location, and acres if available. More information about these particular cases of nonplant GMOs, including the purpose of the transformation, can be found by accessing the EA, if there is one, from the full record in the database, or in the literature. It should also be noted that these EA are all for 'controlled' environmental releases and consider the adequacy of the confinement measures to minimize the risks that could be associated with an unconfined release. APHIS has not 'deregulated' a non-plant organism to date. It is likely that some of these non-plant organisms are intended to only be used under a permit.

The following review provides three examples of research by the authors, on non-plant GMOs being developed for agricultural purposes: (1) insects for insect pest control using improved vector systems; (2) fungal pathogens of insects to control insect pests; and (3) virus for use as transient-expression vectors for disease control in plants. Each of these examples is a summary of a presentation that was given by the authors in a plenary session on the topic of non-crop applications of GMOs at the 2012 ISBGMO12 conference in St. Louis, MO USA. These examples demonstrate the potential of using genetically engineered "non-plant" organisms and suggest that the regulatory community should be prepared to consider the risks and benefits of these organisms.

Development of transgenic strains for biologically-based control and ecological safety in tephritid pest insects

The development of transgenic insect strains has advanced rapidly, with nearly 30 species within five orders of insects being genetically transformed (O'Brochta and Handler 2008). Gene transfer provides the opportunity to create transgenic strains that may be used directly to control the population size or behavior of agriculturally and medically important insects. Transgenic strains may be created to improve existing biologically-based control strategies, such as the sterile insect technique (SIT; Knipling 1955), or to provide the means for new strategies for biologically-based control based on conditional lethality systems (see Alphey 2002; Handler 2002). For beneficial insects, their fitness, reproductive capacity, and behavior, or their ability to produce and process proteins, may be improved. For vectors of disease, an alternative to suppressing population size is their transformation into inhospitable hosts for the parasites or pathogens they normally transmit (James 2005).

The potential applications of genetically transformed insects must, however, be viewed in light of limitations that are inherent to the gene-transfer vector systems used to integrate transgenes into the host

genome (O'Brochta and Handler 2008). All of the heritable germ-line transformations in insects have been achieved with vectors derived from transposable elements, which currently include *Hermes, mariner, Minos*, and *piggyBac*. While these elements provide advantages over other types of vectors and transformation strategies, a major consideration is their potential for re-mobilization by an unintended source of transposase, and the random nature of transposon integration into host genomes, both of which can adversely affect program effectiveness and ecological safety (Handler 2004; FAO/IAEA Report 2002).

First, while the transposase enzyme required for transposon excision/insertion is typically eliminated after integration, the unintended or undetected presence of genes for the transposase, or related enzymes, within the host can result in vector re-mobilization (Sundararajan et al. 1999). While generally not considered to be problematic for small-scale laboratory studies, the rearing and release of many millions (if not billions) of insects for bio-control programs increases the probability that such rare events may occur. Secondly, the random nature of genomic transposon integrations, including sites within coding and enhancer/promoter regulatory regions, result in insertional mutations (Horn et al. 2003; Wilson et al. 1989) that often have deleterious effects on the transformed host's fitness and viability (Catteruccia et al. 2003; Irvin et al. 2004). Localized genomic position effects on gene expression also result in variable transgene expression depending upon the integration site (Schotta et al. 2003). Thus random integrations make true comparative gene expression studies impossible, and host fitness and viability is often negatively affected, as is the efficiency of creating optimal strains for applied use. Importantly, variable expression of lethal effector genes could result in the unintended survival of transgenic insects in the field, or suppression of marker genes used for identification of transgenic individuals.

To address these limitations on transgenic insects, we developed new transgene vectors that can be immobilized post integration for enhanced stability, and which can provide acceptor sites for subsequent targeted transgene integrations. After their initial integration, optimal genomic target sites would include those that do not negatively affect transgene expression by position effects, or result in insertional mutations. These vectors were first tested individually and then in combination in an integrated targeting/stabilization system in *Drosophila melanogaster* (Handler et al. 2004; Horn and Handler 2005). We have since modified these vectors for more efficient and flexible use in tephritid fruit fly species to test their function (Schetelig et al. 2009), and have initiated the creation of a series of stabilized target-site strains that can be used to create transgenic strains for biological control and functional genomics analysis (Meza et al. 2011). This new generation of transformation vectors is expected to increase the efficiency of transgenic strain development and strain effectiveness, while improving their ecological safety. It is likely that use of these strategies, if not the genetic components themselves, may be extended to other transgenic organisms as well.

Vector stabilization by post-integration terminal sequence deletion in *Drosophila*

To stabilize transposon vectors subsequent to genomic integration we took an approach that was initially tested in *Drosophila* (Handler et al. 2004), and is simply applicable to all species subject to transposon-mediated transformation. This was achieved by introducing an internal tandem duplication of one of the transposon inverted terminal sequences (ITR), either from the 5′ or 3′ terminus, which are both required for transposon mobility. If the 5′ ITR and 3′ ITR are designated as L1 and R1 (for left- and right-arm), respectively, and the internal ITR sequence is L2, then the stabilization vector configuration would be L1-L2-R1. Within this vector, distinguishable marker genes (M1 and M2) are placed between ITR sequences, and genes of interest (GOI) to be stabilized are placed in between the duplicated ITRs. Thus the final construct may be represented as L1-GOI-M1-L2-M2-R1. Once this stabilization vector has been transformed into a host genome, it may re-mobilized in two ways after providing exogenous transposase. Mobilization of the L1 and R1 ITRs together would result in complete loss of the vector, as determined by loss of both markers. However, mobilization of R1 with the internal L2 ITR would result in the loss of only the L2-M2-R1 sequence, with the L1-GOI-M1 construct remaining as a genomic transgene insertion. In this instance, the absence of a 3′ R1 terminal sequence would prohibit any future mobilization of L1-GOI-M1 by an unintended source of transposase.

This system was first tested for stability in *Drosophila* using the *piggyBac* transformation vector, by mating a stabilized L1-PUbDsRed1 line to a jumpstarter strain (having a chromosomal source of transposase) which showed that no remobilization of the remaining transgene occurred (by loss of phenotype) in more than 7,000 progeny assayed. This was compared to a ~5 % remobilization rate in the original L1-PUbDsRed1-L2-3xP3-ECFP-R1 vector, indicating that the L1-PUbDsRed1 transgene was stabilized owing to the loss of the 3′ *piggyBac* terminus.

Modified stabilization vectors were subsequently created for tephritid species and tested in the medfly, *Ceratitis capitata* (Schetelig et al. 2009), mexfly, *Anastrepha ludens* (Meza et al. 2011), and caribfly, *Anastreph suspensa* (A. M. Handler, unpublished data). These vectors were stabilized by either mating to a jumpstarter strain in medfly, or injecting a transposase helper plasmid in the *Anastrepha* species. Stability in the presence of transposase was confirmed quantitatively in *C. capitata* by testing more than 70,000 progeny (Schetelig et al. 2009).

RMCE targeting and stabilization vectors

An efficient gene targeting method for insects was based on a recombinase-mediated cassette exchange (RMCE) strategy first developed for genomic targeting in mammalian stem cells (Baer and Bode 2001). We first tested RMCE in *Drosophila* (Horn and Handler 2005) using the *FRT*/FLP recombination system (Andrews et al. 1985; Siegal and Hartl 1996) by designing a transposon acceptor vector that once integrated by *piggyBac* transformation (Handler et al. 1998), could act as a target-site for subsequent donor vector insertions by double-recombination of variant *FRT* sites (see Horn and Handler 2005, Fig. 2). In this system, the RMCE transformation vector has an acceptor target site that consists of hetero-specific *FRT* and *FRT3* recombination sites (RSs), that can only recombine with their identical RS, but not with each other (i.e. *FRT* × *FRT* and *FRT3* × *FRT3*, but not *FRT* × *FRT3*). These were placed in a tandem orientation, flanking a marker gene to create an exchange cassette. Once integrated by transposon-mediated transformation, the *FRT/FRT3* cassette was targeted for exchange by a donor vector having the same hetero-specific RSs, but with a different internal marker. After injection into target strain embryos with plasmid-encoded FLP recombinase, RMCE was observed in the progeny of the injected embryos by loss of the acceptor vector marker, and appearance of the donor vector marker, which was verified by target-site sequencing and DNA hybridization. The validation of *FRT/FRT3* RMCE in an insect species suggests that, depending on the number of hetero-specific RSs and their position in a target site, a variety of cassette exchange, insertion and deletion events may be achieved, and continued repetitively by the insertion of new RS sequences.

Stabilization of the target-site vector by RMCE was then tested by incorporating a *piggyBac* 3′-ITR (pBacR2) sequence linked to a PUb-DsRed marker within an *FRT/FRT3* donor cassette. After RMCE, the donor pBacR2 sequence was remobilized with the acceptor vector's *piggyBac* 5′-ITR (pBacL1), resulting in genomic stabilization of the original acceptor vector *piggyBac* 3′ (pBacR1) sequence and associated genes of interest. In this way, stabilization of a desired genomic target site could be achieved in a two-step process.

A *loxP-based* RMCE system was also tested in *Drosophila* by Oberstein et al. (2005) using the *P* transformation system to genomically integrate an acceptor target-site vector, that recombined with a donor cassette in the presence of Cre recombinase using eye color mutant-rescue to mark recombinants. Donor/target cassette exchange occurred reliably, though the system was specific for use in *Drosophila* (Oberstein et al. 2005) in terms of the *P* vector system used, and the visible mutation markers used to follow the process. Recently we tested a new Cre/*lox* RMCE system that showed function for the first time in a nondrosophilid species, *A. suspensa*, as well as in *Drosophila* (Schetelig and Handler 2013). The unidirectional phiC31 integrase recombination system (Groth et al. 2004) has also been tested for site-specific attB/attP genomic targeting in *C. capitata* (Schetelig et al. 2009), as well as in several mosquito species.

The premise of this work is that vectors that allow RMCE-based genomic targeting with a post-integration stabilization component will greatly enhance the effectiveness and safety of transgenic strains used in field release for biocontrol. They will also greatly reduce the time and effort needed to create these strains by avoiding genomic integration sites that negatively effect strain fitness and transgene expression, and will allow direct "allelic" comparisons of

different recombinant DNA strategies by comparative gene expression studies that identify and optimize ideal promoter and effector sequences. The ability to stabilize transgenes will minimize ecological risks by ensuring predictable transgene marker expression that could be compromised by intra-genomic movement, and by limiting the potential for inter-genomic movement that could result in transfer of transgenes to unintended host species. The RMCE strategy will be equally important to functional genomics analysis, both for model systems and insects important to agriculture and human health. Transposon-based vectors are inherently limited by the size insert they can carry, but recombinase systems are not (and indeed, they facilitate chromosomal rearrangements). Thus, repetitive anchoring (or replacement) of different transgene cassettes at identical loci will be possible, without restriction on gene size or number, allowing continued modification of established strains for improved efficacy and ecological safety (e.g. improved marker and lethality systems) of genetically modified insects.

Strain improvement of the insect pathogen *Metarhizium*

Fungi are the most common pathogens of insects in nature, and perhaps the most well suited microbes for development as biopesticides because unlike bacteria and viruses they infect insects by direct penetration of the cuticle and so function as contact insecticides. While many of the approximately 1,000 known species of entomopathogenic fungi have narrow host ranges, collectively they target most if not all insect species including sucking insects, and the many coleopteran and orthopteran pests, among others, which have few known viral or bacterial diseases. Industrial production of *Metarhizium* spp. is highly automated and the price of commercialized *Metarhizium acridum* for locust control in Africa, Australia, and China works out at US$20/ha for 50 g/ha, which is similar to the price of conventional chemical insecticides (Langewald and Kooyman 2007). However, fungal pathogens have a small market share because of inconsistencies in performance and low virulence (slow kill and high inoculum load) compared to the chemicals with which they compete. Low efficacy could be inbuilt because an evolutionary balance may have developed between microorganisms and their hosts so that quick kill, even at high doses, is not adaptive for the pathogen, in which case cost-effective biocontrol will require genetic modification of the fungus (Gressel 2007). Better understanding of fungal pathogenesis in insects and the availability of efficient tools for genetic manipulation is alleviating efficacy limitations by allowing construction of transgenic strains with improved ability to kill insects, tolerate adverse conditions and tackle vector-borne diseases. With increasing public concern over the continued use of synthetic chemical insecticides, these new types of biological insecticides offer a range of environmental friendly options for cost-effective control of insect pests (Federici et al. 2008).

Genetic engineering to improve virulence has focused on reducing both lethal spore dosage and time to kill. Reducing spore dosage improves infection rates allowing control to be achieved with less product. It also increases effective persistence of the biopesticide because as spores decay there is a greater probability that an insect will come into contact with enough propagules of the genetically modified fungus to exceed the inoculums threshold (Thomas and Read 2007). Most studies to date have exploited the insect pathogenic fungi themselves as a resource of genes for strain improvement. In the first example of a recombinant fungal pathogen with enhanced virulence, additional copies of the gene encoding the regulated cuticle degrading protease Pr1 were inserted into the genome of *Metarhizium anisopliae* and constitutively overexpressed. The resultant strain showed a 25 % mean reduction in survival time (LT50) toward *Manduca sexta* as compared to the parent wild-type strain (St. Leger et al. 1996). Importantly, a Pr1 overexpressing strain of *M. anisopliae* was used in the first EPA approved field trial of a transgenic fungal pathogen, thus setting a precedent and paving the way for future trials (Hu and St. Leger 2002). The complete sequencing of *M. acridum* and *Metarhizium robertsii* has been completed and is helping determine the identity, origin, and evolution of traits needed for diverse lifestyles and host switching (Gao et al. 2011). Success in developing transgenic organisms will benefit from knowledge of the signal transduction pathways that regulate pathogenesis, particularly host range, and the availability of a wide range of suitable genes that can be used to increase virulence. An esterase involved in mobilizing internal

nutrients in the broad host range *M. robertsii* was used to transform the locust-specific *M. acridum* into a caterpillar pathogen (Wang et al. 2011).

In spite of their potential for strain improvement, microbial genes have not yet produced the leap to hypervirulence necessary for a breakthrough product. Arthropod neuropeptides are a particularly attractive alternative to microbial toxins as they offer a high degree of biological activity, and rapidly degrade in the environment providing environmental safety (Edwards and Gatehouse 2007). Over one million peptide toxins have been isolated from arachnids and scorpions, but their use for pest control has been limited since they are not toxic by oral administration, and require a means of delivery into the circulatory system. We combined some of these toxins with the natural ability of insect pathogenic fungi to penetrate into insects. We initially tested AaIT (an insect specific sodium channel blocker) because it is well studied and very potent and so would provide a benchmark for efficacy (Zlotkin et al. 2000). The modified *M. anisopliae* expressing AaIT under the control of a hemolymph-specific promoter (to prevent expression outside an insect) achieved the same mortality rates in tobacco hornworm (*M. sexta*) at 22-fold lower spore doses than the wild type (Wang and St. Leger 2007). Similar results were obtained with mosquitoes (LC50 reduced ninefold) and Broca (coffee berry borer beetle; LC50 reduced 16-fold) (Pava-Ripoll et al. 2008). Toxins from funnel web spiders have proven to be even more potent than AaIT against some insects (Fang, St. Leger, unpublished data).

There are many international crop pest and disease problems that are amenable to biotechnology solutions. Many of these problems could require transgenic technology for which there is only a beginning precedent being set. There is willingness in the regulatory community to take on these issues, but what is most needed are clear and compelling needs, such as malaria control. As described above, the virulence of *M. anisopliae* can be increased to a remarkable extent by expressing a scorpion toxin (AaIT) (Pava-Ripoll et al. 2008). However, mosquitoes are notoriously adept at out-evolving control strategies, and a slow speed of kill that enables mosquitoes to achieve part of their lifetime reproductive output could reduce selection pressure for mosquitoes to develop resistance to the biopesticide (Thomas and Read 2007, Read et al. 2009). Fungal strains that greatly reduce mosquito infectiousness could improve disease control without increasing the spread of resistance (Thomas and Read 2007). To achieve this effect, we produced recombinant strains expressing molecules that target sporozoites as they travel through the hemolymph to the salivary glands. Our best strain reduced the sporozoite intensity approximate 98 %. Additional benefits included decreased host feeding (and therefore transmission potential) and increased mosquito mortality (Fang et al. 2011).

Metarhizium anisopliae's ability to express a functional single-chain antibody fragment is notable (Read et al. 2009) because recombinant antibodies provide a vast array of potential antiparasite and anti-arthropod effectors that could target, for example, insect hormone receptors. These would facilitate construction of very effective, highly specific, biopesticides with minimal increased potential for negative environmental impact relative to their parental wild-type strains. The rich arsenal of antiparasite and anti-insect proteins makes it possible that new transgenic strains can be developed that stay one step ahead of the insect or parasite evolving resistance. Given their ease of genetic manipulation, *Metarhizium* and *Beauveria* provide a tractable model system for screening novel effectors or fusion products produced by gene shuffling. The most potent anti-insect or antimicrobial effectors could then be delivered by the fungus, another microbe, and/or in a transgenic insect or plant. Likewise, insect pathogenic fungi could be used to test various metabolic pathways for their ability to enhance tolerance to abiotic stresses. Given the increasing public acceptance of GMOs, particularly crops expressing *B. thuringiensis* toxins (Federici et al. 2008), field application of GM insecticidal microbes should have a bright future if care is taken to ensure social acceptance through rigorous risk benefit analysis.

Changing an enemy into an ally to manage citrus diseases

The ability to express foreign genes or to silence endogenous genes in plants has revolutionized both basic and applied plant biology. Foreign genes can be expressed in plants either by permanent insertion into the genome or by transient expression using virus-based vectors. Each approach has distinct advantages. While the insertion of genes into the plant genome is generally permanent, expression by viral vectors

occurs only for a limited period of time. In genetically improving plants, these systems are complementary. The potential value and applications of each of these approaches varies broadly, especially comparing annual to woody plants (Dawson and Folimonova 2013).

Citrus tristeza virus (CTV) is an endemic virus in most citrus industries (Bar-Joseph et al. 1989; Bar-Joseph and Dawson 2008). Although some isolates of the virus cause serious economic losses, many isolates cause little damage (Dawson et al. 2013). For example, most trees in Florida are infected with isolates of CTV that cause little damage as long as trees are not grown on the sour orange rootstock. By recombinant DNA technologies, we built a transient-expression vector based on CTV (Folimonov et al. 2007) that provides systemic expression of foreign genes in citrus. This vector contains an additional subgenomic RNA promoter from a related closterovirus to control expression of a foreign gene of choice in citrus trees. Using a green fluorescent protein (GFP) as a reporter, we examined several engineered vector prototypes for their ability to infect, move, and express GFP throughout citrus trees. The CTV vector is limited to phloem-associated cells. The vector provides expression of a foreign protein at very high levels and has been unusually stable, having continually produced foreign proteins in trees for more than 10 years so far. This vector can be graft transmitted and expressed in a range of citrus varieties of different ages.

The original objective was to build a vector as a tool for citrus improvement. With the emergence of the citrus greening (huanglongbing: HLB) disease into Florida, we began screening genes for activity against the bacterial pathogen or the psyllid vector, with the intention of building transgenic citrus with the genes found to be effective. However, the spread of greening in Florida has been more rapid than expected and the disease is debilitating trees. The majority of citrus produced in Florida is for juice. Since juice processing plants require a minimum amount of fruit for processing to remain open and the fear is that production will start declining, it is possible that processing plants will have to close unless developments are made to stabilize fruit production. Although it is likely that transgenic citrus trees will be a long-term answer to the citrus greening problem, there is concern that they will not be available in adequate numbers in time to save the industry. There is desperate need to find solutions more quickly than transgenic trees. For those reasons, the CTV vector is being considered as a temporary measure to protect trees until transgenic trees become available. The advantage of the CTV vector with an anti-HLB gene over transgenic plants is that the vector can be deployed sooner. A major reason is that since the virus does not affect the production and quality of trees, there is no need for evaluation as is needed with transgenic trees and the virus can utilize trees that are beyond juvenility. Another advantage of the CTV vector is that if effective genes or RNAi molecules can be found, the vector could be used to treat trees in the field that are already infected with HLB (Dawson and Folimonova 2013). From an environmental standpoint, the CTV vector adds nothing permanently to the environment. With time, the vector deletes all of the inserted sequences and becomes identical to the endemic virus already prevalent in most citrus trees.

The approach is to find genes that can control the bacterial pathogen or the psyllid vector to be expressed by the vector. Since psyllids suck large amounts of fluid from citrus phloem, the CTV vector can be used to produce compounds in the citrus phloem to reduce the number of psyllids. Two different methods are being considered: antimicrobials to prevent endophytic bacteria in psyllids and the use of RNAi molecules to target specific enzymes of psyllids.

Trees can be productive for many years—over 100 years in some cases. The question arises: Why just use a virus-based vector against pathogens and pests? New technologies are always being produced and value-added products are being developed. With transformation, to respond to these developments, the existing trees have to be removed and the new transgenic trees be planted. A transient-expression vector could be used to improve the existing trees in the field. Another question is 'Why just citrus?' Similar vectors could be developed for a range of tree crops. Virus-based vectors have been used for several years to produce specialty products in herbaceous plants. Similar vectors could have completely different uses in perennial crops.

Conclusion

The USDA/APHIS database is an easily searchable/sortable database that includes authorizations of both crop and non-crop GMOs for agricultural applications.

It seems probable that the research and progress with non-crop GMOs in the US is representative of this type of research worldwide, if not more advanced. Regulatory agencies in some other countries maintain databases of permits and approvals similar to the USDA/APHIS, although these may not be easy to search, may only be available in the language of the country, and may only be for GM crops. There is not a global database available that captures this information. There are several very useful global databases available for GM crop approvals (e.g., cera-gmc.org and www.isaaa.org) but these do not include nonplant organisms.

The biosafety clearing house (BCH), maintained by the secretariat of the Convention on Biological Diversity as part of the Cartagena Protocol on Biosafety (bch.cbd.int), should include information about approvals for intentional releases into the environment of any GM organism in countries that are party to the protocol. However, the BCH database is currently likely not representative of global approvals because several countries that are leaders in GM approvals are not parties to the protocol (including the US, Canada, Argentina, Australia), and many countries that are parties are not up to date in their entries to the BCH. The BCH is difficult to search, and the level of detail seems to vary by entry. A search of all recipient organisms in the BCH revealed very few nonplant organisms; Only three organisms (*Pseudomonas fluorescens*, pink bollworm, and olive fly) from this search appeared to be for agricultural applications.

Our review does not include organisms that are being engineered for applications outside of agriculture specifically. We do not include GM organisms being engineered for human or animal vaccines (which are typically regulated as a drug), or GM mosquitos being engineered for controlling the spread of human diseases such as malaria or dengue. We also do not include GM mammals or fish. It should be noted that there is also significant progress in these areas of research in the US and in other countries, and such GMOs are also the subject of biotechnology regulation.

The noncrop GM organisms listed in Table 1 and the three detailed examples provided in this review demonstrate the significant potential for applications in agriculture of genetic engineering in organisms other than plants. Some of this research could lead to methods of control for agricultural diseases and pests that are more economical or more environmentally benign than current options of control, or may provide control where no other solutions are available. This review highlights research that involves a release into the environment (i.e., field test), which could be considered as an indication of advanced progress of a project toward a product. However, this review must only represent a portion of the total effort toward this type of research, because more of this kind of research is being conducted under contained use conditions in laboratories or artificial environments where a permit (in the US) is not required.

As the technology continues to advance and new tools of biotechnology become available, this kind of work can be expected to increase in the future. Before this research will be translated into meaningful applications, there will be regulatory hurdles, risk assessment issues, and public acceptance issues, as there are for GM crops. Product developers and regulators should consider how these issues may be similar or different from those encountered with GM crops, to prepare for successful applications of these non-plant GMOs.

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References

Alphey L (2002) Re-engineering the sterile insect technique. Insect Biochem Mol Biol 32:1243–1247

Andrews BJ, Proteau GA, Beatty LG, Sadowski PD (1985) The FLP recombinase of the 2 micron circle DNA of yeast: interaction with its target sequences. Cell 40:795–803

Baer A, Bode J (2001) Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. Curr Opin Biotechnol 12:473–480

Bar-Joseph M, Dawson WO (2008) Citrus tristeza virus. In: Mahy, BWJ, van Regenmortel, MHV (eds) Encyclopedia of virology, 3rd edn. Evolutionary biology of viruses. Amsterdam: Elsevier Ltd, Vol. 1, pp 161–184

Bar-Joseph M, Marcus R, Lee RF (1989) The continuous challenge of citrus tristeza virus control. Ann Rev Phytopathol 27:291–316

Catteruccia F, Godfray HC, Crisanti A (2003) Impact of genetic manipulation on the fitness of *Anopheles stephensi* mosquitoes. Science 299:1225–1227

Dawson WO, Folimonova SY (2013) Virus-based transient expression vectors for woody crops: a new frontier for vector design and use. Ann Rev Phytopathol 51:321–337

Dawson WO, Garnsey SM, Tatineni S, Folimonova SY, Harper SJ, Gowda S (2013) Citrus tristeza virus-host interactions. Front Microbiol 4:88. doi:10.3389/fmicb.2013.00088

Edwards MG, Gatehouse AMR (2007) Biotechnology in crop protection: towards sustainable insect control. In: Gressel J, Vurro M (eds) Novel biotechnologies for biocontrol agent enhancement and management. Springer, Berlin, pp 1–24

Fang W, Vega-Rodrı′guez J, Ghosh AK, Jacobs-Lorena M, Kang A, St. Leger RJ (2011) Development of transgenic fungi that kill human malaria parasites in mosquitoes. Science 331:1074–1077

FAO, IAEA Report (2002) Status and risk assessment of the use of transgenic arthropods in plant protection. IAEA, Vienna 48

Federici BA, Bonning BC, St. Leger RJ (2008) Improvement of insect pathogens as insecticides through genetic engineering. In: Hill C, Sleator R (eds) PathoBiotechnol Landes Biosci pp 15–40

Folimonov AS, Folimonova SY, Bar-Joseph M, Dawson WO (2007) A stable RNA virus-based vector for citrus trees. Virology 368:205–216

Gao Q, Jin K, Ying SH, Zhang Y, Xiao G et al (2011) Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. PLoS Genet 7(1):e1001264. doi:10.1371/journal.pgen.1001264

Gressel J (2007) Failsafe mechanisms for preventing gene flow and organism dispersal of enhanced microbial biocontrol agents. In: Vurro M, Gressel J (eds) Novel biotechnologies for biocontrol agent enhancement and management. Springer, Berlin, pp 353–362

Groth AC, Fish M, Nusse R, Calos MP (2004) Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. Genetics 166:1775–1782

Handler AM (2002) Prospects for using genetic transformation for improved SIT and new biocontrol methods. Genetica 116:137–149

Handler AM (2004) Understanding and improving transgene stability and expression in insects for SIT and conditional lethal release programs. Insect Biochem Mol Biol 34:121–130

Handler AM, McCombs SD, Fraser MJ, Saul SH (1998) The lepidopteran transposon vector, *piggyBac*, mediates germline transformation in the Mediterranean fruit fly. Proc Natl Acad Sci USA 95:7520–7525

Handler AM, Zimowska GJ, Horn C (2004) Post-integration stabilization of a transposon vector by terminal sequence deletion in *Drosophila melanogaster*. Nat Biotechnol 22:1150–1154

Horn C, Handler AM (2005) Site-specific genomic targeting in *Drosophila*. Proc Natl Acad Sci USA 102:12483–12488

Horn C, Offen N, Nystedt S, Häcker U, Wimmer EA (2003) PiggyBac-based insertional mutagenesis and enhancer detection as a tool for functional insect genomics. Genetics 163:647–661

Hu G, St Leger RJ (2002) Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. Appl Environ Microbiol 68: 6383–6387

Irvin N, Hoddle MS, O'Brochta DA, Carey B, Atkinson PW (2004) Assessing fitness costs for transgenic *Aedes aegypti* expressing the GFP marker and transposase genes. Proc Natl Acad Sci USA 101:891–896

James AA (2005) Gene drive systems in mosquitoes: rules of the road. Trends Parasitol 21:64–67

James C (2012) Global status of commercialized biotech/GM crops: 2012. ISAAA brief No. 44. ISAAA: Ithaca, NY

Knipling EF (1955) Possibilities of insect control or eradication through the use of sexually sterile males. J Econ Entomol 48:459–462

Kuzma J, Kokotovich A (2011) Renegotiating GM crop regulation. EMBO Rep 12(9):883–888

Langewald J, Kooyman C (2007) Green muscle, a fungal biopesticide for control of grasshoppers and locusts in Africa. In: Vincent C, Goettel MS, Lazarovitis G (eds) Biological control, a global perspective. CABI, pp 311–327

Lindow SE, Panoupoulus NJ (1988) Field tests of recombinant ice-pseudomonas syrinage for biological frost control in potato. In: Sussman M, Collins CH, Skinner FA, Stewart-Tull DE (eds) Release of genetically-engineered micro-organisms. Academic Press Publishing, London, pp 121–138

McHughen A, Smyth S (2008) US regulatory system for genetically modified [genetically modified organism (GMO), rDNA or transgenic] crop cultivars. Plant Biotechnol 6:2–12

Meza JS, Nirmala X, Zimowska GJ, Zepeda-Cisneros CS, Handler AM (2011) Development of transgenic strains for the biological control of the Mexican fruit fly, *Anastrepha ludens*. Genetica 139:53–62

O'Brochta DA, Handler AM (2008) Perspectives on the state of insect transgenics. Adv Exp Med Biol 627:1–18

Oberstein A, Pare A, Kaplan L, Small S (2005) Site-specific transgenesis by Cre mediated recombination in *Drosophila*. Nat Methods 2:583–585

Office of Science and Technology Policy (1986) Coordinated framework for the regulation of biotechnology. Federal Register 51:23302

Pava-Ripoll M, Posada FJ, Momen B, Wang C, St Leger RJ (2008) Increased pathogenicity against coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae) by *Metarhizium anisopliae* expressing the scorpion toxin (AaIT) gene. J Invertebr Pathol 99:220–226

Read AF, Lynch PA, Thomas MB (2009) How to make evolution-proof insecticides for malaria control. PLoS Biol 7:e1000058. doi:10.1371/journal.pbio.1000058

Schetelig MF, Handler AM (2013) A functional comparison of the 3xP3 promoter by recombinase-mediated cassette exchange in *Drosophila* and a tephritid fly, *Anastrepha suspensa*. G3 Genes Genomes Genet. doi:10.1534/g3.112.005488

Schetelig MF, Scolari F, Handler AM, Kittelmann S, Gasperi G, Wimmer EA (2009) Site-specific recombination for the modification of transgenic strains of the Mediterranean fruit fly *Ceratitis capitata*. Proc Natl Acad Sci USA 106: 18171–18176

Schotta G, Ebert A, Dorn R, Reuter GP (2003) Position-effect variegation and the genetic dissection of chromatin regulation in *Drosophila*. Semin Cell Dev Biol 14:67–75

Siegal ML, Hartl DL (1996) Transgene coplacement and high efficiency site-specific recombination with the Cre/loxP system in *Drosophila*. Genetics 144:715–726

St. Leger RJ, Joshi L, Bidochka MJ, Roberts DW (1996) Construction of an improved mycoinsecticide over-expressing a toxic protease. Proc Natl Acad Sci USA 93:6349–6354

Sundararajan P, Atkinson PW, O'Brochta DA (1999) Transposable element interactions in insects: crossmobilization of *hobo* and *Hermes*. Insect Mol Biol 8:359–368

Thomas MB, Read AF (2007) Can fungal biopesticides control malaria? Nat Rev Microbiol 5:377–383

USDA (1997) Introduction of organisms and products altered or produced through genetic engineering which are plant pests or which there is reason to believe are plant pests. 7CFR340. http://ecfr.gpoaccess.gov

Wang C, St. Leger RJ (2007) A scorpion neurotoxin increases the potency of a fungal insecticide. Nat Biotechnol 25: 1455–1456

Wang S, Fang W, Wang C, St. Leger RJ (2011) Insertion of an esterase gene into a specific locust pathogen (*Metarhizium acridum)* enables it to infect caterpillars. PLoS Pathog 7:e1002097. doi:10.1371/journal.ppat.1002097

Wilson C, Pearson RK, Bellen HJ, O'Kane CJ, Grossniklaus U, Gehring WJ (1989) P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*. Genes Dev 3:1301–1313

Zlotkin E, Fishman Y, Elazer M (2000) AaIT: from neurotoxin to insecticide. Biochemie 82:869–881