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Review

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Letting the gene out of the bottle: the population genetics of genetically modified crops

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Contents

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Summary

Genetically modified (GM) plants are rapidly becoming a common feature of modern agriculture. This transition to engineered crops has been driven by a variety of potential benefits, both economic and ecological. The increase in the use of GM crops has, however, been accompanied by growing concerns regarding their potential impact on the environment. Here, we focus on the escape of transgenes from cultivation via crop × wild hybridization. We begin by reviewing the literature on natural hybridization, with particular reference to gene flow between crop plants and their wild relatives. We further show that natural selection, and not the overall rate of gene flow, is the most important factor governing the spread of favorable alleles. Hence, much of this review focuses on the likely effects of transgenes once they escape. Finally, we consider strategies for transgene containment.

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I. Introduction

Transgenic plants are rapidly becoming a common feature of modern agriculture in many parts of the world. In 1996, 1.7 million hectares (M ha) of genetically modified (GM) crops were grown world-wide, and by 2004 this figure had increased to 81.0 M ha (Fig. 1). In 2003, the USA alone grew 42.8 M ha of GM crops, comprising 81% of the soybean (*Glycine*

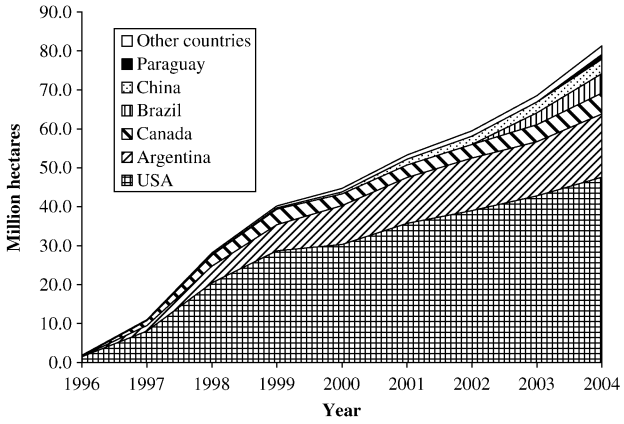

Fig. 1 Global increase in the total area planted with genetically modified (GM) crops from 1996 to 2004. Data were combined for countries with < 1 M ha of GM crops in 2004.

max), 73% of the cotton (*Gossypium* spp.) and 40% of the corn (*Zea mays*) produced in the country (Global Knowledge Center on Crop Biotechnology, www.isaaa.org/kc/). The dramatic overall increase has been driven by a variety of potential benefits, including increased yields, easier and more effective weed/pest control, lower prices to consumers, a wider variety of produce available throughout the year, and the production of nutrient-enriched staple crops (James, 2003). GM crops can have other beneficial effects. For example, increasing the yield per hectare of crops by genetic modification (also abbreviated as 'GM') might prevent further conversion of natural habitat into agricultural fields, and the introduction of herbicide- or pest-resistant crops could result in a transition to a more environment-friendly mode of weed or pest control (McGaughey *et al.*, 1998; Trewavas & Leaver, 2001; Dale *et al.*, 2002).

Because traditional breeding approaches are limited by the reproductive compatibility of crops and their wild relatives, GM is often the only alternative for introducing traits from one taxon into another. This approach is much faster than conventional breeding, and also removes the potentially undesirable effects of linked alleles, which could be inadvertently introduced into a crop gene pool in a traditional breeding program. The types of traits that are typically targeted for GM often relate to the tolerance of a variety of stresses, including both biotic (e.g. pests and pathogens) and abiotic (herbicides and environmental extremes) factors, or cause the crop to synthesize a novel compound. In some cases, dubbed 'biopharming', crop plants are used as platforms for the efficient and cost-effective production of vaccines, antibiotics and industrial proteins (Giddings *et al.*, 2000), although it currently seems unlikely that any such crops will be deregulated for large-scale use by commercial farmers.

As noted above, many of the first engineered crops were created to produce pesticides or tolerate herbicides. This means that traditional pesticide applications may no longer be necessary for the control of herbivorous insects, or that effective weed control might be achieved via chemical applications without a detrimental effect on the crop. Insect herbivory is a major factor affecting agricultural productivity, reducing world-wide crop yields by as much as 30–40% year^{-1} (Oerke *et al.*, 1994), and crop yields are known to be reduced by the presence of weeds (e.g. O'Donovan *et al.*, 1988, 1989; Manitoba Agriculture, 2002). In fact, the transition to GM soybean and cotton has resulted in a decrease in the application of herbicides/pesticides (Fernandez-Cornejo & McBride, 2000; Bennett *et al.*, 2004) and, for GM soybean, the major herbicide that is now applied (glyphosate) is much less toxic and persistent than several of the common pre-GM herbicides (Fernandez-Cornejo & McBride, 2000). Another advantage of herbicide-resistant crops is that effective weed control can often be achieved without plowing (zero-till), thereby reducing damage to the soil ecosystem and preventing topsoil loss (Trewavas & Leaver, 2001).

The potential benefits of GM notwithstanding, the rapid increase in GM farming has been accompanied by growing concerns regarding the large-scale release of engineered crops. These concerns include possible nontarget effects of GM crops on the local biota, as well as the possibility that transgenes might escape from GM crops into their wild relatives. In the latter case, the specific concern is that the transgene might increase the invasiveness of the recipient population (Raybould & Gray, 1994; Burke, 2004).

The foregoing concerns have been amplified in recent years, sometimes unnecessarily, by a few notable mistakes and confusions. For example, the report by Quist & Chapela (2001) that transgenic constructs had been found in a native maize landrace in Oaxaca, Mexico, where transgenic maize had not been previously grown, was used by *Greenpeace* and *Friends of the Earth* as evidence that GM crops are not safe (Hodgson, 2002). Following criticisms of the techniques used for the detection of the transgene (e.g. Metz & Futterer, 2002), this paper was ultimately retracted. By this time, however, public concern over the possibility of transgene escape had already been heightened. A recent analysis of over 150 000 maize kernels from the same region failed to find evidence of the presence of the transgene (Ortiz-Garcia *et al.*, 2005). A second example concerns the presence of the transgene from Starlink corn (only approved for release as animal feed) in taco shells and a number of other related products destined for human consumption (Dorey, 2000; Fox, 2001). While this sort of contamination is clearly a cause for concern, it remains unclear whether it resulted from hybridization between GM and non-GM crops in the field, or whether batches of non-GM seed were contaminated by GM seed before planting or after harvest.

1. Direct effects of GM crops on natural habitats

While numerous herbicide-resistant GM crops are now available to farmers, most have been modified to be resistant to one of only a handful of the many herbicides available (e.g.

glyphosate or glufosinate). Thus, the growing of several crops engineered to be resistant to the same herbicide, and the concomitant consistent use of that herbicide, will increase the selection pressure on nearby wild species. This, in turn, increases the likelihood of herbicide resistance evolving in a local weed population, as has been reported in both annual ryegrass (*Lolium rigidum*) (Powles *et al*., 1998) and horseweeds (*Conyza canadensis*) (VanGessel, 2001; Koger *et al*., 2004).

There is a similar concern with regard to the evolution of pesticide resistance in herbivores that are consistently exposed to toxin-producing crops. Ultimately, this would reduce the efficacy not only of the GM crop, but of any pesticide based on the same toxin. Of particular concern in this context are members of a group of endotoxins isolated from the soil bacterium *Bacillus thuringiensis* (*Bt*). These toxins affect lepidopteran larvae (e.g. European corn borer (*Ostrinia nubilalis*)), and are the most common toxins engineered into crops. In fact, *Bt* pesticides have been applied for over 40 years, and *Bt* GM crops were grown on over 15 M ha in 2004 (http://www.isaaa.org/kc/); thus, any detrimental effects of *Bt* toxin on the environment are likely to be of major consequence. Thus far, the evolution of *Bt* resistance has been documented in only two cases (diamondback moths (*Plutella xylostella*) in Hawaii (Tabashnik *et al*., 1990) and cabbage loopers (*Trichoplusia ni*) in glasshouses in British Columbia (Janmaat & Myers, 2003)), and the selective pressure in both instances was the use of *Bt*-containing pesticides, not a *Bt*-producing GM crop. Interestingly, *Bt*-resistant moths and cabbage loopers show reduced fitness in the absence of the toxin (see section III for a discussion of the 'cost of resistance'), and resistance in wild populations has been shown to decline rapidly under such conditions (Tabashnik *et al*., 1994; Janmaat & Myers, 2003).

Given the potential costs associated with resistance, it has been suggested that crop rotation might be an effective means of reducing the likelihood that resistance will evolve in response to herbicide/pesticide use. Indeed, Baucom & Mauricio (2004) found that glyphosate tolerance in the agricultural weed *Ipomea purpurea* (morning glory) carries a strong fitness cost in the absence of the herbicide, and concluded that crop rotation (along with parallel rotation of the herbicides applied to the fields) could have delayed or even prevented the evolution of tolerance. Similarly, the presence of refugia may allow the maintenance of susceptible source populations (Rausher, 2001). These sorts of considerations are of paramount importance in light of the scale at which GM crops are now being grown. For example, *Bt* cotton is currently being planted on such a large scale in India (Jayaraman, 2005) that resistance of the target pest, cotton bollworm (*Helicovera armigera*), is predicted to evolve within a few years (Kranthi & Kranthi, 2004).

Apart from the direct effect of a pesticide-producing crop on a target herbivore, it is possible for a transgene to negatively affect nontarget organisms. For example, Losey *et al*. (1999) reported that monarch butterflies (*Danaus plexippus*) fed on milkweed (*Asclepias curassavica*) leaves dusted with pollen from *Bt* corn showed increased mortality; however, a subsequent investigation showed that such high concentrations of *Bt* pollen are unlikely to be encountered in the wild (Sears *et al*., 2001). Moreover, the effects of standard pesticide applications on monarch butterfly populations may be more detrimental than the endogenous production of *Bt* toxin in a GM crop (Pimentel & Raven, 2000). A related example of the possible nontarget impacts of GM crops involves the increased mortality and delayed development of lacewings (*Chrysoperla carnea*) when reared on *Bt* corn-fed insects (Hilbeck *et al*., 1998). Again, however, the amount of *Bt* toxin fed to the insects was greater than that expected to be encountered in the field, in this case by over 30 times. Another potential concern is that *Bt* (or other toxins) may be exuded from plant roots and hence get into the rhizosphere, thereby causing detrimental effects on the soil biota. To date, there is evidence that *Bt* toxin is exuded from the roots of some, but not all, *Bt* crops (Saxena *et al*., 1999, 2004). However, the presence of *Bt* in the rhizosphere appears to have little effect on earthworms, nematodes and soil microbes (Saxena & Stotzky, 2001a). Clearly, more risk assessments need to be carried out before conclusions regarding the possible nontarget effects of *Bt* (or other toxins) can be drawn.

2. Indirect effects – GM crops and invasiveness

Concerns regarding the long-term consequences of GM crops were first voiced in the mid-1980s (Colwell *et al*., 1985; Goodman & Newell, 1985), with attention focusing on: (1) whether or not genetic modification will make the crop itself more likely to become a pest species, and/or (2) the potential for transgene escape via hybridization to result in the evolution of an increasingly weedy or invasive wild plant species. Unfortunately, it is difficult to either measure or predict invasiveness. Part of the problem stems from the fact that the term 'invasive' refers to a complex, and largely unclear, set of characteristics. Invasive plants have been defined as '[Naturalized plants that] produce reproductive offspring in areas distant from sites of introduction' (Richardson *et al*., 2000, p. 93). Following Sax *et al*. (2005), we further restrict the term to include only those species that have caused economic or ecological damage. Beyond the difficulties associated with adequately defining invasiveness, it turns out that seemingly related phenomena, such as increases in fecundity, are not necessarily good predictors of population expansions and/or biological invasions (e.g. Bergelson, 1994; Cummings & Alexander, 2002).

Exotic invasive species are often responsible for displacing native species and, in the USA alone, invasive plants have invaded *c.* 40 M ha at an estimated cost of $35 billion per year to control (Pimentel *et al*., 2000). As a consequence, the US Department of Agriculture (USDA) will not approve a GM crop for commercial release if it appears to have the potential to become invasive. However, as reported by Purrington & Bergelson (1995), companies wishing to have GM crops deregulated are governed by vague guidelines when it comes

to 'proving' that their plants do not present a threat of invasiveness, and these guidelines do not appear to have been updated in the past 10 years (http://www.gpoaccess.gov/ecfr/). An additional concern highlighted by Purrington & Bergelson (1995) is that a number of the experiments that are performed to gain quantitative data on the relative performance of GM and non-GM lines for submission to the USDA may be flawed. For example, the parental lines are often not included in these studies as a control.

The question of whether or not a GM crop is more invasive than its non-GM counterpart has been investigated in a comparison of the population dynamics of several (GM and non-GM) lines of oilseed rape (*Brassica napus*), maize (*Zea mays*), sugar beet (*Beta vulgaris* spp. *vulgaris*) and potato (*Solanum tuberosum*) (Crawley *et al.*, 1993, 2001). In short, the authors did not find any instances in which the transgenic crop persisted longer than its non-GM counterpart and, in all but one case (a non-GM potato variety), both the GM and non-GM lines went extinct within 3 years (Crawley *et al.*, 2001). The risks associated with a wild species becoming increasingly invasive as a result of transgene introgression from a crop are, however, more difficult to predict. Clearly, introgression of a transgene could have detrimental effects in both an environmental and an economic context as a result of: (1) competition between the recipient plants (assuming that they become more invasive) and other neighboring species, and (2) the expense associated with controlling the newly formed pest species (Raybould & Gray, 1994; Li *et al.*, 2004). It must, however, be kept in mind that introgressive hybridization between non-GM crops and their wild relatives has resulted in the transformation of populations of certain wild species into agricultural weeds, including relatives of sugar beet, millet, rice, radish and rye (Ellstrand, 2003). Similarly, an increase in invasiveness or a range expansion of a wild species as a result of introgression (again, not associated with a GM trait) has been shown for *Sorghum halapense* (de Wet & Harlan, 1975), *Rhododendron ponticum* (Milne & Abbott, 2000), and *Manihot reptans* (Nassar, 1984). Thus, this problem is clearly not restricted to the realm of genetic modification.

While gene flow between crop plants and their wild relatives has been taking place since the dawn of agriculture, the advent of genetic modification has introduced an entirely new variable that must be considered. Recent reviews on crop × wild gene flow have concentrated on: (1) the extent of hybridization between crops and wild species (Ellstrand *et al.*, 1999; Dale *et al.*, 2002; Stewart *et al.*, 2003; Pilson & Prendeville, 2004), and/or (2) whether or not a transgene can be prevented from escaping (Gressel, 1999; Daniell, 2002; Stewart *et al.*, 2003). In this review we seek explicitly to unite these two areas of inquiry. We begin with a brief overview of the literature on natural hybridization, and we further relate this to gene flow between crop plants and their wild relatives. We then explore the existing population genetic framework for the prediction of gene flow, ultimately concluding that natural selection is the most important factor governing the flow of favorable alleles. Hence, much of the remainder of this article focuses on the likely effects of transgenes once they escape. We highlight these issues with two detailed case studies in which the rate of crop × wild gene flow has been investigated, as have the fitness effects of transgenes following their transfer into a wild genetic background. Finally, we consider strategies for the containment of transgenes.

II. How do transgenes escape? – Hybridization, gene flow, and introgression

1. Natural hybridization

Interspecific hybridization is a common phenomenon amongst plants and, depending on a variety of factors such as the rate of hybridization and the fitness of hybrids, a number of outcomes are possible (Harrison, 1993; Arnold, 1997). At one extreme, if the hybrids are inviable or sterile, no further gene flow (introgression) can occur, and the species will remain genetically distinct. Alternatively, if the hybrids are viable and at least partially fertile, and if gene flow is persistent, then (1) one population may be driven to extinction (particularly if hybridization is asymmetric – e.g. from a large to a small population; Wolf *et al.*, 2001; reviewed in Rhymer & Simberloff, 1996), (2) bilateral hybridization may result in the demise of both species and the establishment of a hybrid swarm in their place, or (3) introgression (i.e. the transfer of alleles from one taxon to another via backcrossing; Anderson & Hubricht, 1938) may occur. The following is a brief overview of the factors that must be satisfied before hybridization and introgression can occur.

1 Geographic proximity. Hybridization can only occur if the taxa in question are situated near enough one another for pollen exchange to occur. While the majority of pollen often travels short distances, it must be kept in mind that a small minority of the pollen grains can disperse over sometimes vast distances (Kirkpatrick & Wilson, 1988; Klinger *et al.*, 1991; Arias & Rieseberg, 1994). In fact, pollen dispersal has been detected over distances as great as 21 km in field trials involving GM plants (*Agrostis*; Watrud *et al.*, 2004), so geographic overlap (in a strict sense) is not necessarily required for hybridization to occur.

2 Phenological overlap. The populations in question must overlap at least partially in flowering time for pollen from one population to find a mate in another.

3 Pollinator overlap. For two taxa to hybridize, they must share pollinators. This condition is perhaps most easily satisfied for wind-pollinated species.

4 Reproductive compatibility. The taxa in question must exhibit some degree of reproductive compatibility; if the pollen grains fail to effect fertilization, hybridization will be prevented even if the foregoing requirements are satisfied.

5 Hybrid viability/fertility. For the alleles from one population to introgress into another, the initial hybrid generations must be viable and at least partially fertile. However, Piálek &

Barton (1997) showed that even strong genetic barriers, such as extremely low F1 fertility, can be overcome by the persistent flow of favorable alleles.

Assuming that these criteria are met, the likelihood and outcome of hybridization can still be influenced by factors such as the number of individuals in each of the parental populations. For example, a small population of one type surrounded by a much larger population of another type will likely be the recipient of a substantial amount of pollen flow. In fact, this is just the sort of situation that might be encountered in the context of crop × wild hybridization. As an example of the impact that this might have on the outcome of hybridization, a simulation of reproductive contact between 100 cultivated and 100 wild sunflowers resulted in hybrids being generally eliminated within a few generations (Wolf *et al.*, 2001). In contrast, when the number of cultivated individuals was increased to 10 000 while the number of wild individuals was held steady, there was a 75% chance that the wild population would be swamped by hybrids within the same time-frame (Wolf *et al.*, 2001).

As already alluded to, the fitness of hybrids can have a significant impact on the outcome of hybridization. While crosses between populations of the same species typically result in progeny with relatively high fitness, the progeny of interspecific crosses often exhibit reduced fitness (but see Arnold & Hodges, 1995). That being said, the fitness of crop × wild hybrids has been found to vary greatly across taxa (Ellstrand, 2003), and it is also clear that hybrid fitness might vary widely across different environments and in different seasons (Stebbins & Daly, 1961; Cruzan & Arnold, 1993, 1994; Grant & Grant, 1993; Arnold, 1997).

2. The frequency of crop × wild hybridization

In many cases, crop plants and their wild relatives overlap, at least partially, in terms of both geography and phenology (Ellstrand, 2003). Moreover, many crop–wild pairs exhibit similar floral structures, meaning that they may well share pollinators and, while it is possible that domesticated lineages have been selected to possess some degree of reproductive isolation from their wild progenitor (Ladizinsky, 1985), isolation between crops and their close relatives is usually not complete. In fact, the majority of crop plants are thought to exist as part of a crop–weed–wild complex, within which hybridization occurs at a low level, allowing for the regular exchange of alleles (DeWet & Harlan, 1975; Jarvis & Hodgkin, 1999; Ellstrand, 2003). Indeed, introgressive hybridization has been documented in numerous crop–wild pairs, with 22 of the world's 25 most important crops (*c.* 90%) showing evidence of hybridization with at least one wild relative (Ellstrand *et al.*, 1999; Ellstrand, 2003). While five of these cases are based solely on morphological data, which may or may not be a reliable indicator of hybridity, the remaining 17 cases are supported by molecular data.

While the potential for crop × wild hybridization seems to be high for a number of crops across the globe, this does not necessarily mean that a large percentage of all crops will hybridize wherever they occur. For example, 18 of the 20 most important crops (in terms of area planted) in the USA can hybridize with wild relatives somewhere in the world; however, only 11 of these have a compatible wild relative present within the USA. It is therefore important to consider what proportion of crops grown in a given locale can hybridize with wild relatives in that area. Based on studies that have explicitly addressed this issue, it appears that *c.* 25–50% of the most commonly cultivated species in the UK, the Netherlands, Norway, and Switzerland have the potential to hybridize with at least one wild species (reviewed in Ellstrand, 2003).

3. Selection, gene flow, and introgression

Over the years, gene flow both within and among species has been a topic of great interest to evolutionary biologists. Hence, a sound theoretical framework for the investigation and prediction of gene escape from crop plants into their wild relatives already exists. In short, gene flow between two populations can either act conservatively, preventing diversification of the populations in question, or it can serve as a creative force, promoting the spread of favorable alleles (Slatkin, 1987). With regard to the former, Wright's (1931) island model of gene flow demonstrates that differentiation as a result of genetic drift will be prevented if the number of migrants (Nm, where N is the effective population size and m is the migration rate) between two populations per generation is ≥ 1. Conversely, if $Nm < 1$, then interpopulation differences will accrue. Gene flow at a given locus is, of course, also influenced by the fitness effects of the alleles in question. More specifically, gene flow will prevent selective differentiation (i.e. local adaptation) unless the strength of selection (s, the fitness difference between alternative alleles) exceeds the migration rate (m). Note that, unlike genetic drift, which affects all loci equally, the effects of selection will vary across the genome.

Unfortunately, there are no simple rules governing the spread of favorable alleles. The primary difficulty here is that most models fail to incorporate the discontinuous population structure and occasional long-distance dispersal that is typical of most species. While discontinuous population structure reduces the rate of spread of an allele, long-distance dispersal can greatly increase the rate of spread. In fact, ecological models of biological invasions have shown that even rare long-distance dispersal can greatly influence the speed of an invasion (Neubert & Caswell, 2000). One model that accounts for both discontinuous population structure and occasional long-distance dispersal is that of Slatkin (1976). This model, which is based on the 'stepping-stone' model of gene flow, allows one to predict the time required for a favorable allele to spread across the range of a species based on estimates of Nm and s. Inspection of Fig. 2, which provides a response surface for this

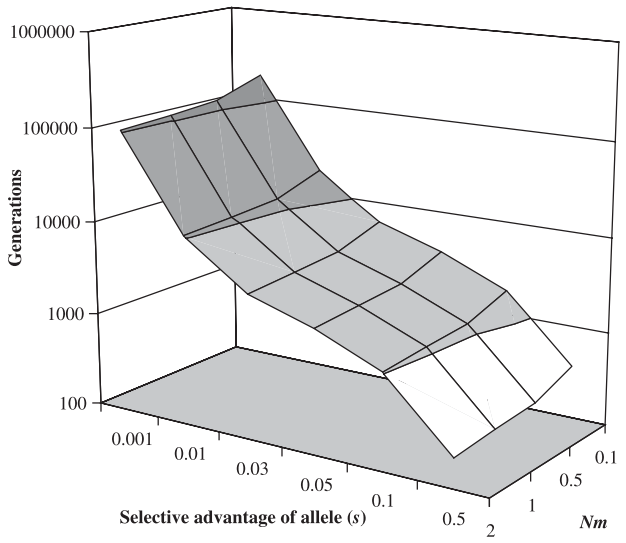

Fig. 2 The effect of the selective advantage of an allele (*s*) and the migration rate (*Nm*) on the rate of spread of an advantageous allele across a species range (reprinted with permission from Morjan & Rieseberg, 2004; Blackwell Publishing).

model, reveals that: (1) the magnitude of the migration rate has relatively little effect on the rate of spread, and (2) the selective advantage of the allele is the primary factor governing its spread. The inclusion of long-distance dispersal results in a similar overall response surface, although the rate of spread is greatly enhanced (Rieseberg & Burke, 2001).

In view of the above, it is clear that the parameter with which we should be most concerned in the context of transgene escape is the selective advantage of the transgene, as opposed to the overall rate of hybridization (see also Hails & Morley, 2005). Indeed, even if the initial hybridization event is rare, moderately advantageous alleles will readily spread from the crop into compatible wild populations. Similarly, even if early generation hybrids suffer from decreased fitness, recombination can easily separate the transgene from the parental alleles that are responsible for the fitness reduction and, once an allele has been transferred from one taxon into another, the problem becomes one of intraspecific as opposed to interspecific gene flow. In further support of the view that it is the effects of the allele, and not the rate of hybridization, that we should be most concerned about, a recent theoretical assessment revealed that even very low rates of transmission (on the order of 0.1%) are sufficient for the escape and establishment of a moderately advantageous (s = 0.10) transgene (Haygood *et al.*, 2004).

III. Assessing selection on transgenes – costs and benefits

Given that selection, and not the overall rate of hybridization, will be the primary factor governing the spread (or not) of any particular transgene, we now turn our attention to factors influencing the effects of transgenes in the wild.

1. Fitness costs and benefits

While it is not hard to imagine that a transgene that affords some level of protection against certain biotic or abiotic stress might provide a selective advantage in the wild, it is important to keep in mind that the strength and direction of selection in such cases may well be context dependent. Consider, for example, a transgene that affords protection against a certain pest species. In the presence of the target pest, the transgene is likely to provide a benefit, increasing the fitness of the individuals that carry it. In the absence of the pest, however, any such advantage would disappear. When this is combined with the fact that toxin synthesis often comes at a cost (e.g. Coley *et al.*, 1985; Bazzaz *et al.*, 1987), those individuals that carry the transgene might actually find themselves at a relative disadvantage when reared in a pest-free environment. This phenomenon – known as a 'cost of resistance' – highlights the importance of carefully considering the various effects that a transgene might reasonably have when investigating its likely impact on a wild plant population.

Most studies of the cost of resistance have been carried out by making comparisons between herbicide-resistant and -susceptible plants. In one of the earliest such studies, Bergelson (1994) showed that, in the absence of herbicides, a chemically-induced herbicide-resistant mutant of *Arabidopsis thaliana* produced fewer seeds, especially at high density, than a susceptible line. Cloning of the gene responsible for the herbicide resistance in *A. thaliana* allowed a comparison to be made between the EMS-derived line and four transgenic lines created by the insertion of the mutant gene (Bergelson *et al.*, 1996). Comparisons were also made with untransformed isolines and lines transformed with an empty vector. This latter comparison ensured that any possible phenotypic effects were the result of the resistance gene itself, and not simply a byproduct of the transformation *per se*. Seed output under controlled conditions was 34% lower in the transformed lines and 40% lower in the original mutant line as compared to their wild-type counterparts, and lines transformed with only the vector showed no reduction in seed production (Bergelson *et al.*, 1996). Further investigation revealed that the cost of resistance in this case was likely a result of an overall increase in amino acid production (Purrington & Bergelson, 1999). While resistance resulted in reduced fecundity in the absence of an herbicide challenge, this decrease did not result in reduced 'invasiveness' (= population size), suggesting that fecundity may not be a good predictor of invasiveness (Bergelson, 1994).

In general terms, direct resistance costs are known to vary substantially, with costs ranging from 6 to 45% having been reported (Strauss *et al.*, 2002). Costs may even vary between different insertion events of the same transgene into a common genetic background (e.g. Jackson *et al.*, 2004). Assuming that they were conferred by a transgene in a wild population, these sorts of costs would presumably help to counterbalance the benefits that might be afforded by the transgene, perhaps

decreasing the likelihood that it will spread following escape from cultivation. This seems especially likely in weedy populations, where competition amongst individuals is high (Gressel, 1999) and a small reduction in viability or competitive ability may well limit transgene movement because the initial introgressed weed could not compete and produce seed.

2. Unintended effects

Another important consideration when assessing the risks associated with transgene escape are the unintended advantages that might be associated a particular gene. For example, although Bergelson and colleagues provided clear evidence of a cost of herbicide resistance (see previous section), the outcrossing rate of the transgenic line was found to be much greater than that of the control plants (*c.* 6% vs 0.30%, respectively; Bergelson *et al.*, 1998). Another example of unintended consequences comes from GM maize, wherein the *Bt* transgene has been found to produce a pleiotropic increase in lignin content of 33–97% (Saxena & Stotzky, 2001b). High lignin content not only retards litter degradation and decomposition, but also has the potential to confer mold resistance. Thus, while the effect that this might have on a natural ecosystem remains unclear, one might envision the *Bt* transgene providing an additional benefit in the wild in the form of incidental disease resistance.

IV. The effects of transgenes – case studies

Two study systems in particular have provided us with an opportunity to investigate the likelihood of transgene escape and persistence. The first is *Helianthus annuus* (sunflower), and the second is *Brassica napus* (oilseed rape or canola). Early work in these taxa focused primarily on whether and how frequently the crop × wild hybridization occurs. For sunflower, the fitness of traditional crop × wild hybrids and, more recently, transgenic crop × wild hybrids carrying disease or pest resistance transgenes has also been evaluated, and inferences can now be made regarding the likely impact of transgene escape on the natural environment. In the case of oilseed rape, herbicide-resistant GM lines were commercially released in 1996, and a number of pest-resistant (i.e. *Bt*-expressing) lines have also been produced.

1. Sunflower

Sunflower is cultivated primarily as a seed oil crop, although it is also a major source of confectionery seeds. In 2004, 21.4 M ha of sunflower were planted world-wide, with Argentina, India, the Russian Federation and Ukraine each growing over 1 M ha (http://faostat.fao.org). In the USA, sunflower is grown on nearly 700 000 ha, and the vast majority of this area is contained within the range of the wild, common sunflower (Burke *et al.*, 2002). Despite being morphologically distinct (see next paragraph), cultivated and common sunflower are considered to be members of the same species, and are completely interfertile. In regions where they overlap, they typically exhibit extensive phenological overlap (Burke *et al.*, 2002), and have been shown to hybridize readily under natural conditions (Arias & Rieseberg, 1994). In fact, detailed genetic analyses of gene flow between wild and cultivated sunflower have revealed that hybridization can occur over distances of > 1000 m (Arias & Rieseberg, 1994), and Whitton *et al.* (1997) found that presumably neutral crop-specific alleles can be maintained in wild populations well after the cessation of reproductive contact.

Cultivated and common sunflower differ in a number of phenotypic traits associated with domestication (e.g. decreased branching and increased seed size). In a study focusing on the fitness of F1 crop × wild sunflower hybrids, Snow *et al.* (1998) found that hybrids germinate earlier and produce fewer branches, flower heads and seeds than do wild individuals, suggesting that F1 hybrids will have somewhat reduced fitness in the field. In one locale, however, Snow *et al.* (1998) found that crop × wild hybrids were resistant to a rust that infected over half of the wild plants, showing a potential benefit of 'traditional' crop alleles in a wild genetic background. Seeds from crop × wild hybrid sunflowers were significantly larger than were pure wild seeds (Alexander *et al.*, 2001), and this increase in size appears to translate into increased pre- and postdispersal seed predation. Indeed, predispersal seed predation was *c.* 20-fold higher in F1 hybrids than in wild plants (Cummings *et al.*, 1999), whereas postdispersal seed predation was *c.* 1.5-fold higher in the hybrids (Alexander *et al.*, 2001). However, Cummings & Alexander (2002) found that seed predation had no detectable effect on seedling recruitment, suggesting that the effect of predation is not sufficient to alter long-term population dynamics.

To date, the fitness effects of two cultivated sunflower transgenes have been investigated in crop × wild hybrids. In the first study, Snow *et al.* (2003) backcrossed a *Bt* crop × wild hybrid to wild sunflower and the resulting BC1 generation was analyzed in both the field and the glasshouse. As expected, lepidopteran damage was greatly reduced on hybrids carrying the transgene, resulting in an average increase in seed production of 14% in Colorado and 55% in Nebraska. Moreover, the *Bt* transgene had no effect on fecundity in the glasshouse, suggesting that it does not confer a cost of resistance (Snow *et al.*, 2003). While these data suggest that the *Bt* transgene would spread rapidly through wild sunflower populations if it ever got out, this does not necessarily mean that the escape of this gene would result in an increasingly weedy or invasive common sunflower, as sunflower populations do not appear to be seed limited (see the previous paragraph; Cummings & Alexander, 2002).

The second study examined the effects of a disease-resistance transgene following three generations of backcrossing into a wild sunflower genetic background (Burke & Rieseberg, 2003). The gene in question, oxalate oxidase (*OxOx*), confers resistance to the fungal pathogen *Sclerotinia sclerotiorum* (white

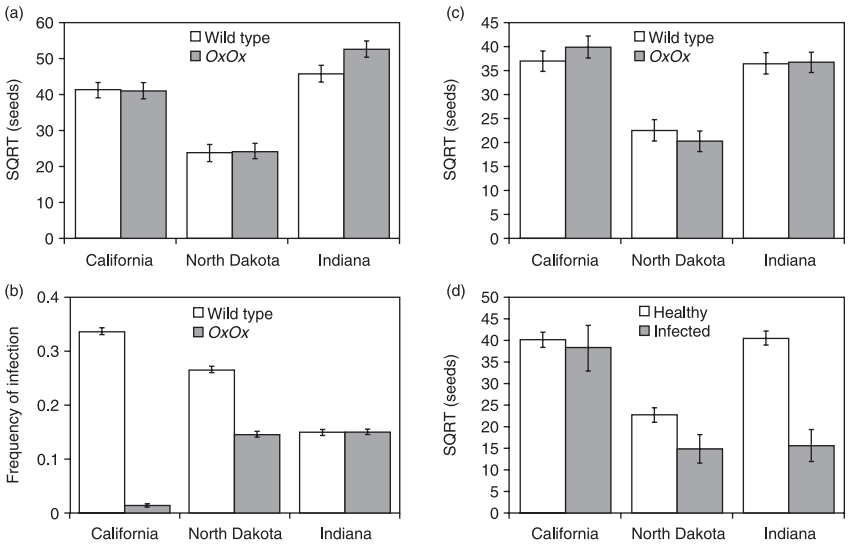

Fig. 3 Effects of the oxalate oxidase (*OxOx*) transgene and white mold (*Sclerotinia sclerotiorum*) infection on crop × wild sunflower hybrids. (a) Seed output of control individuals (i.e. not inoculated with white mold). (b) Frequency of infection following inoculation with white mold. (c) Seed output following inoculation with white mold. (d) Effect of white mold infection on seed output. All values in (a), (c) and (d) are square root (SQRT) transformed and expressed as least-squares means ± 1 standard error (SE), whereas (b) is based on means ± 1 SE (reprinted with permission from Burke & Rieseberg, 2003; copyright 2003 American Association for the Advancement of Science).

mold). This study, which was replicated in three different locales, revealed that the *OxOx* transgene had no detectable effect on fitness, even in the face of a severe pathogen challenge (Fig. 3c). This story is, however, somewhat more complex because of site-to-site variation in the effect of the transgene. In California, for example, the *OxOx* transgene greatly reduced the likelihood of white mold infection, but the disease itself had no detectable effect on seed production (Fig. 3b,d). Conversely, white mold infection had a major detrimental impact on seed production in Indiana, but the *OxOx* transgene had no effect on the likelihood of infection at that site. Control plants with and without the transgene at all three locations did not differ in seed production, implying that there was no cost of resistance (Fig. 3a). Thus, it was ultimately concluded that, should the transgene escape, it would do little more than diffuse neutrally throughout the recipient population (Burke & Rieseberg, 2003). This work also illustrates the importance of quantifying fitness directly, rather than relying on a presumptive correlate, such as disease incidence. Indeed, any conclusions drawn based solely on infection rates would have been quite different from those based on actual reproductive output.

2. Oilseed rape/canola

Oilseed rape (*Brassica napus*) was grown across 26.4 M ha in 2004 (Australia, Canada, China, France, Germany and India each grew more than 1 M ha; http://faostat.fao.org). This species is an allotetraploid (genome AACC and $2n = 38$ chromosomes) and can hybridize with both of its parental species, *Brassica rapa* (AA, $2n = 20$) and *Brassica oleracea* (CC, $2n = 18$), as well as with wild radish (*Raphanus raphanistrum*) and other weedy relatives (Warwick *et al.*, 2003; Chèvre *et al.*, 2004). Feral oilseed rape is also a common weed of disturbed land (e.g. Crawley & Brown, 1995), although it is a poor competitor in undisturbed habitat (Crawley & Brown, 1995; Stewart *et al.*, 1997). In terms of the fitness of transgenic plants, Crawley *et al.* (1993) found that herbicide-resistant oilseed rape lines varied in fitness across sites/years under natural conditions, but in no cases were the transgenic lines more invasive than their nontransgenic counterparts. Conversely, transgenic *Bt* oilseed rape produced significantly more seed under herbivore pressure than did nontransgenic oilseed rape (Stewart *et al.*, 1997).

Hybrids between *B. napus* and *B. rapa* have been observed under natural conditions in both Denmark (Jørgensen & Andersen, 1994) and the UK (Scott & Wilkinson, 1998). In the latter case, hybridization rates were found to be low (only 0.4–1.5% of all seeds produced on *B. rapa* were hybrids), and transgene escape was considered unlikely from *B. napus* (see also Scott & Wilkinson, 1999). Similarly, a survey of 48 million seedlings derived from herbicide-susceptible oilseed rape which had grown in sympatry with (nontransgenic) herbicide-resistant rape for one season in Australia revealed very low levels of gene flow (= 0.07%). Rare instances of pollen flow were, however, detected over distances of up to 3 km (Rieger *et al.*, 2002). Finally, Wilkinson *et al.* (2003) estimated that 49 000 *B. napus* × *B. rapa* hybrids are formed annually throughout the UK; however, as the authors point out, this represents only the first step in quantifying the risk that is posed nationally.

In a study of nontransgenic *B. napus* × *B. rapa* hybrids, Hauser *et al.* (1998a) found that the fitness of F1 individuals was intermediate to that of their parents, and fitness declined (on average) in F2 and backcross hybrids (Hauser *et al.*, 1998b). Some of these later generation hybrids were, however, as fit as their parents, and could therefore act as a bridge for the flow of alleles into wild populations. Moreover, Mikkelsen *et al.* (1996) found that transgenic *B. napus* × *B. rapa* hybrids can be similar to *B. rapa* in terms of both morphology and chromosome number and may have relatively high fertility, suggesting that transgenes from oilseed rape could pass into wild *B. rapa* with relative ease.

In terms of the fitness of transgenic *B. napus* × *B. rapa* hybrids, Vacher *et al.* (2004) found that *Bt*-producing F1 hybrids produced 1.4 times more seed than non-*Bt* hybrids in the presence of herbivores, although they produced 6.2 times less seed in the absence of herbivores. This clear cost of resistance runs contrary to the findings of Snow *et al.* (1999), who found that the presence of the *Bt* transgene had no effect on pollen fertility, seed production, or survival in third-generation *B. napus* × *B. rapa* backcross hybrids reared in the absence of herbivory. As far as competitive ability goes, Halfhill *et al.* (2005) found that *B. napus* × *B. rapa* F1 hybrids and backcrosses carrying the *Bt* transgene showed similar growth and nitrogen use efficiency when compared with transgenic *B. napus*, but that these levels were lower than that of *B. rapa*. This result suggests that these hybrids might have reduced competitive ability when grown with wild *B. rapa* (Halfhill *et al.*, 2005). This reduction in competitive ability was also evident when transgenic *B. napus*, wild *B. rapa* and their hybrids were grown alongside wheat and the hybrids were found to be less successful than their parents.

In contrast to the case of hybridization between *B. napus* and *B. rapa*, where early generation hybrids are sometimes quite fertile, F1 hybrids between *B. napus* and wild radish exhibit very low fertility (Chèvre *et al.*, 1997, 1998), although higher levels of fertility are regained in later generation backcrosses. Moreover, Gueritaine *et al.* (2002) found that the direction of the initial cross is likely to play a major role in the outcome of hybridization between herbicide-resistant oilseed rape and wild radish. Indeed, when transgenic oilseed rape was backcrossed to wild radish for six generations, those lines with a wild radish cytoplasm were 100 times more fit than were those with an oilseed rape cytoplasm. In terms of a cost of resistance, the resulting hybrids showed similar growth patterns regardless of whether the transgene was present or absent, although fecundity (pollen fertility, seed output and seedling emergence) was reduced by *c.* 50% in the presence of the transgene (Gueritaine *et al.*, 2002).

3. Summary of the case studies

In the two foregoing case studies, crop × wild hybridization appears to be a fairly common occurrence. In general, hybrids between the cultivated and wild forms exhibit a fairly high level of fitness, although F1 progeny from the wider cross of oilseed rape × wild radish suffer from very low fertility. In both sunflower and oilseed rape, the *Bt* transgene appears to provide an advantage in the presence of the herbivores, although the extent of this advantage will likely vary depending on the severity of herbivore pressure. By contrast, the *OxOx* transgene had no detectable effect on fitness in wild sunflower populations, even in the face of a severe pathogen challenge. Regarding the costs associated with the various transgenes, neither the *OxOx* nor the *Bt* transgene conferred a cost of resistance in sunflower, whereas there was fairly clear evidence of a cost of resistance associated with *Bt* in one of two *B. napus* × *B. rapa* studies. Similarly, herbicide resistance appears to reduce reproductive output in oilseed rape × wild radish hybrids when grown in the absence of herbicide. Clearly, the conclusions drawn from this sort of work are likely to vary across different sorts of transgenes, as well as different taxa. Thus, it seems most prudent to consider the likely effects of various transgenes on a case-by-case basis.

V. Can we prevent transgene escape?

In view of the prevalence of crop × wild hybridization, it seems likely that transgenes will be transmitted, at least occasionally, to wild populations (e.g. Colwell *et al.*, 1985; Goodman & Newell, 1985; Raybould & Gray, 1994; Ellstrand *et al.*, 1999; Stewart *et al.*, 2003; Pilson & Prendeville, 2004). Given the potential for many such transgenes to increase the fitness of wild plants, attention has turned to the development of gene containment strategies. In this section, we provide a brief overview of the most prominent theoretical and empirical advances that have been made in this regard, and we further consider the likelihood that such approaches will provide a suitable barrier to transgene escape into wild species. Additional details can be found in a number of recent reviews (e.g. Gressel, 1999; Daniell, 2002; Stewart *et al.*, 2003).

1. Keeping the transgene in the crop

Several approaches have been proposed to prevent transgenes from 'escaping' into wild populations and/or non-GM crops. Some of these strategies, such as the production of apomictic or cleistogamous crops (Daniell, 2002), are still in their infancy. Others, such as those detailed below, are somewhat more well developed, but all have their shortcomings.

In the case of a polyploid crop (e.g. cotton, oilseed rape, or wheat (*Triticum* spp.)), it has been suggested that targeting the transgene to a specific subgenome will prevent, or at least substantially reduce, gene flow into a wild relative that does not share this genome. While this strategy has the potential to reduce the flow of transgenes into wild relatives, it is only suitable for crops that differ in their genomic composition from local wild populations. For example, the targeting of transgenes to the C genome of *B. napus* (AACC) was suggested as a means for preventing transgene introgression into the diploid *B. rapa* (AA; Metz *et al.*, 1997). However, chromosomes from the A and C genomes have been shown to undergo homoeologous recombination in the progeny of such crosses (Chen *et al.*, 1990), and genetic markers derived from the C subgenome of *B. napus* have been found to introgress into *B. rapa* (Halfhill *et al.*, 2001). Thus, in this instance, transgene introgression would be only slightly reduced. This conclusion has gained further support from mathematical models that have shown that the resulting barrier to gene flow between *B. napus* and *B. rapa* will be weak (Tomiuk *et al.*, 2000). It therefore

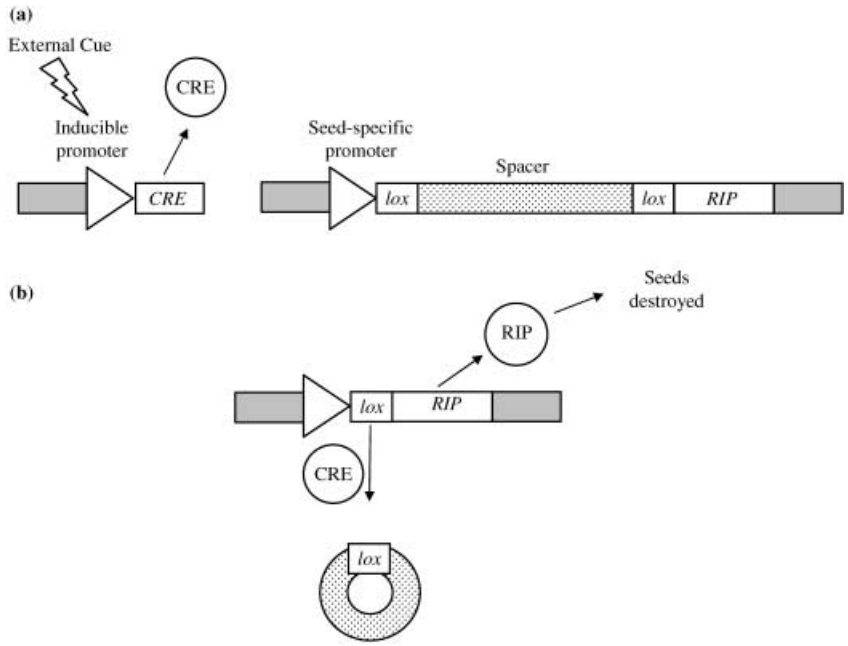

Fig. 4 Schematic diagram of the seed-suicide strategy for preventing transgene escape. Promoters are shown as triangles, genes as white boxes, host plant DNA as gray boxes and proteins as circles. (a) An external stimulus induces the *CRE* promoter. (b) Expression of *CRE* results in the excision of the 'spacer' flanked by *lox* sequences, thereby bringing together a seed-specific promoter and the ribosome-inhibitor protein (*RIP*) gene, resulting in seed inviability. See text for further details.

remains unclear whether or not this strategy will be generally effective.

Another logical strategy would be to target the transgene to the chloroplast or mitochondrial genomes. Indeed, in species with strict maternal inheritance, this sort of strategy would prevent transgene escape via pollen flow. In fact, this strategy has been successfully implemented in both tobacco (*Nicotiana tabacum*) (Daniell *et al.*, 1998) and tomato (*Lycopersicon esculentum*) (Ruf *et al.*, 2001). Unfortunately, although maternal inheritance is widely assumed to be the rule in most angiosperms, rare paternal leakage has been detected in a number of cases (reviewed in Smith, 1989) including, ironically, tobacco (Avni & Edelman, 1991). In fact, one would have to survey > 3000 progeny in order to be 95% certain that the rate of paternal leakage is no higher than 0.10% (Milligan, 1992) and, as noted in section II, even very low levels of leakage may be sufficient for the escape and spread of a moderately advantageous transgene (Haygood *et al.*, 2004). Another drawback of this approach is that it would do nothing to stop transgene escape via seed. Thus, if any seeds were to escape or be left behind following the harvest, the transgene could easily be incorporated into a wild population via chloroplast (or mitochondrial) capture.

An alternative method of preventing transgene escape via pollen flow would be to insert the gene into a male-sterile line (Mariani *et al.*, 1990). In the case of seed crops, this approach would require the planting of nontransgenic pollen donors to ensure seed set. As was the case for organellar transgene containment, however, this strategy would do nothing to prevent gene escape via seed – even in the case of nonseed crops where no pollen donors are grown, seed can be produced on male-sterile crops when they are pollinated by compatible wild species.

There are also a variety of molecular 'tricks' that can be used to prevent transgene escape by inducing seed sterility. For example, the seed-specific gene activation system described by Odell *et al.* (1994) could be used to induce seed suicide. More specifically, an external cue (in this case, treatment with tetracycline) can be used to induce a site-specific recombinase (Cre) which excises 'spacer' sequence flanked by *lox* sites (Fig. 4). Removal of the spacer brings together a seed-specific promoter with a target gene that is turned on during seed development. Assuming that a lethal gene such as a ribosome-inhibitor protein (RIP) was incorporated into this system, induction would result in the production of inviable seeds. A similar system has been suggested to allow transgene removal in seeds or pollen (Fig. 5; Keenan & Stemmer, 2002). One major disadvantage of these approaches is that they rely on an external cue to induce the system. Thus, unless all relevant cells are induced, some fraction of pollen grains and/or seeds might still be able to serve as vehicles for transgene escape.

To combat this possibility, Kuvshinov *et al.* (2001, 2004) suggested the use of a 'recoverable block of function' (RBF) system to induce seed sterility. Specifically, the transgene is flanked by a blocking sequence and a recovering sequence. The blocking construct prevents some vital biological process in the seed, rendering it inviable. The blocking construct can, however, be repressed by the activation of the recovering construct by a chemical or heat treatment which would not be encountered under natural conditions (Kuvshinov *et al.*, 2001). In this case, the multigene construct must remain intact. To solve this problem, Kuvshinov *et al.* (2004) showed that the blocking sequence can be inserted into an artificial intron within the transgene, thereby preventing the two from being separated by recombination. The advantage of RBF over the

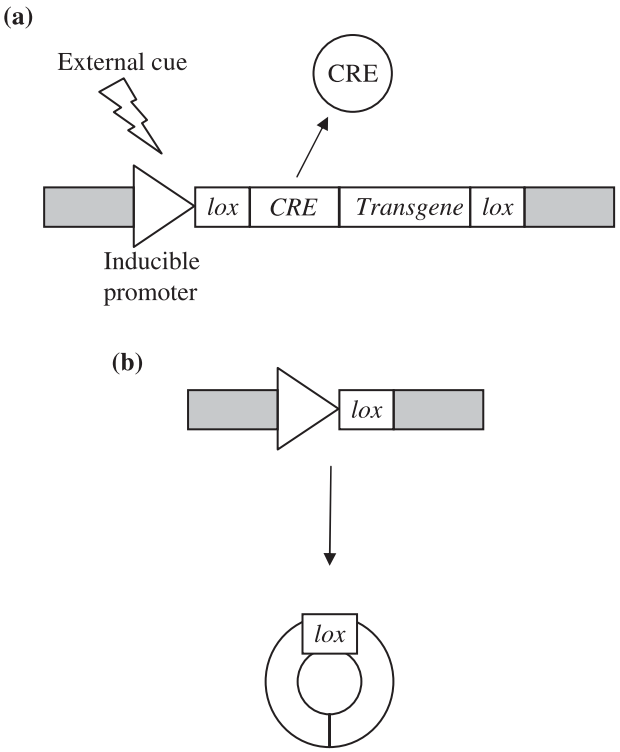

Fig. 5 Schematic diagram of transgene excision using the *Cre/lox* system. Promoters are shown as triangles, genes as white boxes, host plant DNA as gray boxes and proteins as circles. (a) Induction of the promoter by an external stimulus or in a specific tissue causes expression of *CRE*. (b) CRE excises both the transgene and the *CRE* gene, which are flanked by the *lox* sites.

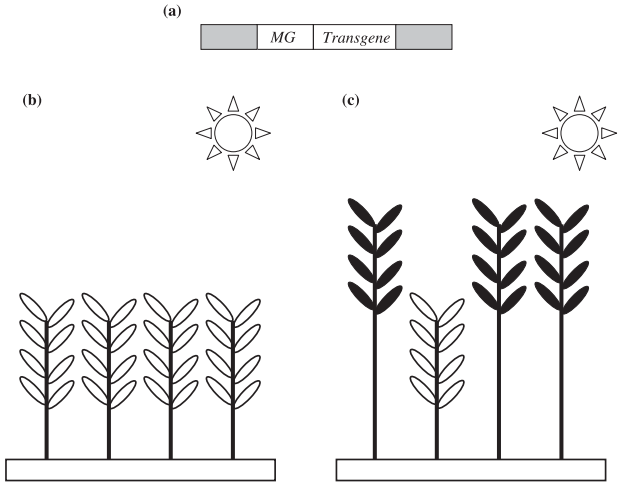

Fig. 6 Schematic diagram of 'transgenic mitigation.' (a) The transgene is linked to a mitigation gene (*MG*) which has a neutral or beneficial effect in the crop, but is disadvantageous in the wild. In this case, the mitigation gene causes a dwarfed phenotype. (b) All crops individuals (white leaves) are transgenic and thus dwarfed. (c) An introgressed wild individual (white leaves) is dwarfed as a result of the presence of the mitigation gene, and is therefore outcompeted by its nontransgenic counterparts (black leaves).

inducible seed-suicide mechanism is that the former system is 'on' until the trigger turns it 'off'. Hence, incomplete induction is not a concern in the context of transgene escape.

2. Transgenic mitigation

Each of the above strategies for transgene containment has certain disadvantages and, to a varying degree, may not completely eliminate the possibility of gene flow. Because even a low level of gene flow can be sufficient to allow the spread of a moderately advantageous allele (e.g. Burke & Rieseberg, 2003; Haygood *et al.*, 2004), a strategy that reduces the rate of gene escape to a low but nonzero level may not be enough to prevent the establishment and spread of transgenes. A promising alternative to the above approaches would be to couple a potentially advantageous transgene with a gene that is neutral or beneficial in an agricultural setting, but selectively disadvantageous in the wild. This basic approach has been dubbed 'transgenic mitigation' (TM; Gressel, 1999), and a simple example is shown in Fig. 6. In this case, the transgene is directly linked to a gene conferring dwarfing (Fig. 6a), which is not detrimental in an agricultural setting (Fig. 6b). However, if this construct were to be passed to a weedy population, the recipient individual(s) would be less able to compete with 'normal' plants (Fig. 6c), thereby limiting the spread of the transgene.

The success of TM relies on: (1) the mitigation gene being tightly linked to the transgene, such that the chance of recombination between the two is extremely low, and (2) the fitness disadvantage of the mitigation gene being at least as great as the advantage provided by the transgene. An additional concern is that the mitigation gene might be silenced, via either mutation or methylation. However, the insertion of the transgene between two copies of a mitigation gene in a so-called 'tandem construct' greatly reduces the likelihood of the transgene recombining away from the TM construct, and the presence of two mitigation genes makes the inactivation of both copies exceedingly unlikely (Gressel, 1999). Proposed mitigation genes include those conferring agricultural traits such as dwarfing, a loss of shattering, and a lack of seed dormancy, as these sorts of traits are likely to be deleterious in the wild (Gressel, 1999).

Recent work in *A. thaliana* has resulted in the identification of a gene (*GAI*) that responds to gibberellic acid; mutation of this gene (*gai*) renders the plant dwarfed (Peng *et al.*, 1997). The *GAI* gene is homologous to the mutant genes conferring dwarfing in 'green revolution' wheat (Peng *et al.*, 1999) and the mutant version has become a candidate for testing the efficacy of TM (Al-Ahmad *et al.*, 2004). In this case, a herbicide-resistance gene coupled with *gai* was transformed into tobacco, and the competitive abilities of the backcross progeny (semidwarf, herbicide-resistant) were evaluated in competition with wild-type tobacco under glasshouse conditions. At high density, no dwarf individuals survived to flower, whereas

at lower density only those dwarf plants on the periphery managed to flower, indicating a very poor ability to compete with wild-type plants (Al-Ahmad *et al.*, 2004). Because this work was performed in a glasshouse, however, it remains unclear whether or not these results will transfer to the field. Thus, while TM appears to hold great promise as a strategy for reducing the risks associated with transgene escape, the general applicability of this approach awaits further verification.

VI. Conclusions and future directions

In recent years, it has become increasingly clear that hybridization between crop plants and their wild relatives is the rule, as opposed to being an exception. Moreover, population genetic theory has shown us that the likelihood of establishment and rate of spread of an allele are governed primarily by the strength of selection, as opposed to the migration rate. Thus, even if crop × wild hybridization is a rare occurrence, a moderately advantageous transgene would be expected to spread quickly following its escape. Although increased individual fitness does not necessarily translate into increased invasiveness, fitness remains the best predictor of allelic spread. Thus, the fitness effects of a gene in the wild are a far more important consideration than the overall rate of gene flow (see also Hails & Morley, 2005).

With this in mind, it seems that efforts to assess the risks associated with transgene escape should be primarily directed at quantifying the costs and benefits associated with a given transgene, as well as investigating the possibility that it might provide recipient individuals with unintended (i.e. pleiotropic) benefits. Such work should, of course, be based on direct estimates of fitness, as indirect estimates (such as disease incidence in the case of white mold resistance in sunflower; Burke & Rieseberg, 2003) may not be reliable. Adding to the difficulty of this sort of work is the fact that fitness costs and benefits are likely to vary across environments, taxa, genes, and even insertion events (e.g. Jackson *et al.*, 2004). Indeed, research to date show that the effects of transgenes can be highly variable, indicating a clear need to replicate studies across space and time, and to consider the risks and benefits of GM on a case-by-case basis.

Despite the great progress that has been made in the development of approaches to reduce or prevent transgene escape, most gene containment strategies have their weaknesses, and in no case have these methodologies been field-tested and/or been shown to be 100% effective. Given that it is virtually impossible to contain genes under field conditions, the idea of countering the advantage provided by a transgene via linkage to one or more selectively deleterious mitigation genes holds great promise. While this strategy has already been tested and shown to be effective in a glasshouse trial (Al-Ahmad *et al.*, 2004), however, it still has not been proved effective in the field. It may well be that the best strategy going forward will be to employ a combination of these strategies – for example the use of a TM construct in conjunction with organellar transformation.

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