Transgene Escape Monitoring, Population Genetics, and the Law

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There has been little discussion about how to apply population genetics methods to monitor the spread of transgenes that are detected outside the agricultural populations where they are deployed. Population geneticists have developed tools for analyzing the genetic makeup of individuals in hybrid zones, estimating migration and selection of genes, studying the influence of migration and selection on the shape of clines, and assaying the fitness of hybrids and backcrossed individuals. These tools may prove useful for monitoring the dynamics of escaped transgenes, but their effective application is likely to require access to information on the genetic makeup of transgenic organisms—information that is often proprietary. At present, depending on the jurisdiction involved, developers and regulators of transgenic organisms may be under no obligation to provide such information, thereby impeding independent public research of transgene escape and the refinement of methods used to study it.

Keywords: transgenic plants, gene flow, selection, molecular markers, access to information

Transgenic crop species have been adopted by a growing number of countries in the past decade, and the acreage devoted to transgenic species has expanded considerably (James 2005). In the United States alone, the National Agricultural Statistics Service of the US Department of Agriculture (USDA) reported that corn acreage planted with transgenic hybrids expanded from 26 percent of the crop in 2001 to 52 percent in 2005. Furthermore, transgenics now account for the majority of the US canola, cotton, papaya, and soybean crops. Given the continuing pace of advances in plant biotechnology, transgenic agriculture is likely to continue to grow in popularity in years to come.

Much has been written about both the advantages associated with the culture and consumption of transgenic plants and the ecological and agricultural problems that might accompany unintended hybridization of transgenic plants with wild relatives (e.g., Royal Society et al. 2000, Pilson and Prendeville 2004, Snow et al. 2005). As for potential problems, it has been postulated that the migration of transgenes coding for herbicide or pest resistance into native or naturalized weedy populations of plants may result in the evolution of weeds that are more difficult to control (Kareiva et al. 1994, Ellstrand 2003a, Snow et al. 2005); that migration of maladaptive transgenes into populations of wild species may reduce their fitness and lead to local population decline (Muir and Howard 1999, Hedrick 2001, Wolf et al. 2001, Ellstrand 2003a, Haygood et al. 2003); and that transgenic plants and their hybrids may be harmful to nontarget consumer organisms, such as native plant-feeding insects and their predators (Losey et al. 1999, Sears et al. 2001, Zangerl et al. 2001, Groot and Dicke 2002).

In the past few years, reports of transgene migration from agricultural to wild populations have begun to emerge. For example, transgenic canola (*Brassica napus*) in Quebec has been reported to hybridize with weedy *Brassica rapa* (Warwick et al. 2003); and more recently, in Oregon, transgene escape has been reported from field trials of creeping bentgrass (*Agrostis stolonifera*) into natural populations of three compatible *Agrostis* species (Watrud et al. 2004, Reichman et al. 2006). Such reports highlight the potential necessity of developing monitoring methods to determine whether and why a particular transgene may change in frequency once it

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has escaped from farm or test plots. Moreover, monitoring for transgene escape should, in principle, be a component of testing technological approaches designed to mitigate the unintended spread of transgenes into wild populations (NRC 2004, Al-Ahmad et al. 2006).

Population geneticists, in the course of conducting a variety of evolutionary and ecological studies, established many of the underlying basic theoretical perspectives required to develop tools that could be used to monitor the spread of escaped transgenes. These include (a) marker-based characterization of hybridization and introgression between transgenic crops and wild populations; (b) marker-based estimation of the rate of migration of the transgene into wild populations, and the strength of selection once it is there; and (c) the analysis of clinal variation in transgene frequency to infer informative characteristics of migration and selection.

Although transgene escape has been discussed from the perspective of gene flow and selection (Chapman and Burke 2006), and ecological issues associated with monitoring transgene escape have been reviewed (Marvier et al. 1999), the use of population genetics approaches in monitoring has not received much attention. Our focus is on the biological and legal constraints that may exist when applying and refining such genetically based monitoring tools. The study of transgene escape bears some similarity to other environmental problems (e.g., the spread of invasive species, population decline, and the spread and impact on natural systems of particular environmental pollutants), but a number of unique logistical and legal challenges may complicate the task of monitoring transgene escape—notably, developers of transgenic organisms may restrict access to the very information that could be useful for monitoring population genetics. Moreover, regulators also can restrict access to this information to protect the intellectual property rights of developers. As part of this review, we discuss some of these logistical and legal constraints and indicate how they may impede the study and monitoring of escaped transgenes.

Marker-based characterization of hybridization and introgression

If the first-generation hybrids of a particular crop-wild pair of taxa have extremely low survivorship or are highly sterile, then, for that pair, the impacts of transgene flow, if any, will be limited spatially to the F_1 hybrids. However, if F_1 individuals typically survive and have some male or female fertility, then the production of advanced-generation hybrids, or introgressants, is likely. In plants, hybrid survivorship and fertility vary considerably with the taxa involved; in some cases, the fitness and fertility of the F_1 hybrid may be markedly reduced, whereas in others, the F_1 hybrid may be robust and capable of backcrossing with the local natural population (Ellstrand 2003a). Thus, once transgene escape has been detected, the question is, has it progressed beyond the F_1 stage? In some cases, fitness assays of artificial crop-wild plant hybrids may be informative predictors. For example, hybrids between *B. napus* and *B. rapa* suffer reduced pollen fertility associated with their triploid state (Warwick et al. 2003). But low fitness of F_1 hybrids does not necessarily translate into a permanent barrier to further spread of the transgene, as hybrid descendants beyond the F_1 may show substantial fitness recovery (Mikkelsen et al. 1996). Backcrossing of F_1 hybrids with parents from the wild population is of special concern, because once a barrier of low F_1 fitness has been overcome, introgression of the transgene into the wild population may follow. Introgression may indicate a more severe environmental problem if the transgene in question reduces the fitness of the wild population, or if the population receiving the transgene is a weed and is thereby rendered more invasive and difficult to control (Ellstrand 2003a).

In cases in which the hybridizing species differ markedly in ploidy level, flow cytometry can be applied to transgene-bearing individuals to infer whether transgene flow has progressed beyond the F_1 hybrid stage (Warwick et al. 2003); however, this approach reveals only coarse genetic information and is not useful when the parental species have similar genome sizes. A more generally applicable method for characterizing whether a transgenic plant is a backcross, an F_1 hybrid, or an advanced generation hybrid is to use molecular-marker-based tools, as adopted from hybridization studies in evolutionary biology. For example, one approach for determining whether hybridization has extended beyond the F_1 is to use a combination of markers with maternal and nuclear inheritance. The analysis of cytonuclear disequilibrium (Clark 1984) between maternally (or paternally) inherited and nuclear markers can reveal whether parental species are isolated or randomly mating, or whether there is limited introgression and directionality to mating events in the hybrid zone (Asmussen et al. 1987). Additional techniques employing many nuclear markers, in conjunction with other types of statistical genetic tools, also help classify individuals as F_1 or F_2 hybrids or as derived from one of several generations of backcrossing (Nason and Ellstrand 1993, Boecklen and Howard 1997, Harrison and Bogdanowicz 1997, Rieseberg et al. 1999, Miller 2000, Anderson and Thompson 2002, Vaha and Primmer 2006). The successful application of such approaches depends on the existence of crop-specific marker alleles (Linder et al. 1998), which could be difficult to find in some cases, particularly if the crop and wild population in question share recent evolutionary history. In the event that that there is indeed shared ancestry, microsatellite loci (which have higher mutation rates than many other types of markers, and thus are more likely to be differentiated between crop and related species) may be sources of crop-specific marker alleles.

Simulation together with statistical power analysis can help to establish guidelines for experimental design when the aim is to distinguish between the different possible genotypic categories into which a transgene-bearing individual may fall (e.g., F_1, F_2, or backcross generation 1, 2, and so on). Nason and Ellstrand (1993) developed a maximum-likelihood method for estimating the proportion of the sampled population that falls into each of seven classes of individuals:

the two parent species, F_1 and F_2 hybrids, backcrosses to each parent, and a catchall category of unclassified individuals. This method depends on the existence of alleles that are unique to each parental species, as well as on information about the frequencies of different alleles in the parental populations. Approaches of this type have been evaluated with respect to the number of marker loci needed to attain some minimum level of classification error. For instance, if backcrossing is unidirectional, it can be shown that backcrossed individuals may be reliably distinguished from parents and F_1 with four or five codominant markers, but distinguishing individuals derived from a more advanced generation of backcrossing may require up to several dozen markers (Boecklen and Howard 1997).

One practical problem with these approaches is that the required allele frequencies in populations of the parental species may not be easy to estimate; for example, if hybridization and backcrossing into the wild population have been ongoing for a number of generations, it may be impossible to estimate the (prehybridization) allele frequencies in the parental population of the wild species. A model-based Bayesian approach that circumvents this requirement has recently been proposed (Anderson and Thompson 2002). Unlike earlier methods, this approach does not require unique parental alleles; however, information on the number of generations over which hybridization has occurred must be available. The Bayesian approach has the advantage of assigning a posteriori probabilities that a given individual of interest, such as one possessing the transgene, belongs to a given genealogical class.

Genetic marker-based estimates of migration and selection

Using information from the marker-gene analysis to estimate the population parameters that characterize gene flow and the mating system is a highly developed area of population genetics research. Estimation often employs maximum likelihood, but other approaches, such as methods of moments or Bayesian methods (Shoemaker et al. 1999), may be useful. In the case of ongoing gene escape from transgenic plant populations, the parameters of interest include the proportion of progeny that receive pollen as a result of pollen flow from the transgenic population to the wild population (i.e., the genetically determined pollen migration rate), as well as the fitness of progeny bearing the transgene (which can be expressed as the selection coefficient of the transgene). The application of such an estimation approach would most likely require monitoring the wild population throughout several generations for changes in the frequency of the transgene and one or more marker genes.

It is important to note that simply monitoring changes in the frequency of the transgene or of a neutral marker gene alone could lead to incorrect inferences about the dynamics of transgene escape. The need to use multigenic approaches arises in part because of "genetic hitchhiking": when an allele under selection is introduced into a population by mutation or migration, the genes with which it is associated can incur an advantage or a disadvantage (Barton 2000). If the selected allele (transgene) is introduced by migration, there will be linkage disequilibrium between it and a linked marker gene (or genes). If, for example, in an effort to obtain estimates of migration from the transgenic source population into the wild one, one were to monitor changes in the frequency at one (or more) neutral marker locus (e.g., introns or intergenic regions) that is linked to the transgene, selection acting on the transgene locus might produce biased estimates of migration—selection favoring a transgene could lead to overestimation of the rate of migration, whereas selection against the transgene could lead to underestimation of the rate. Likewise, if one were to monitor changes in frequency at the transgene locus alone, ongoing migration from the transgenic source could bias the estimate of selection acting on the transgene in the wild population—ongoing migration would lead to underestimation of the selective effects of a deleterious transgene and overestimation of the selective effects of a favored transgene. Simultaneous monitoring of changes in transgene together with unlinked marker locus allele frequencies may prevent this complication (box 1).

Clinal variation and inference about forces affecting transgene frequencies

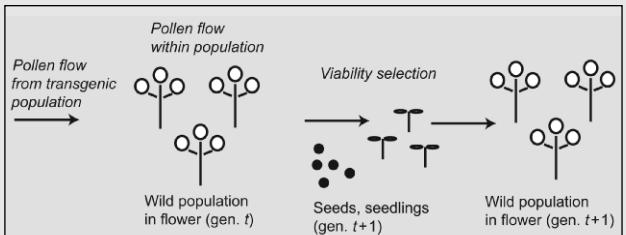
When a transgene has been present in a wild population for several generations and introgression has been detected, a cline in transgene (and other marker gene) frequencies may become established between the agricultural and the wild population. There is a large body of literature on how the characteristics of clines, such as their shape and mobility, can be used to infer properties of migration and selection (Slatkin 1973, Barton 1979, Barton and Hewitt 1985). For example, suppose that dispersal of the transgene into a wild population is ongoing, and that the variance of parent-offspring distance in one generation is σ^2. Under these conditions, a situation of dispersal-selection balance may arise, and a cline in transgene frequency may develop. It can be shown that the width of the cline should be directly related to the strength of selection acting on the transgene in the hybrid zone and to σ^2 (Barton and Hewitt 1985). Thus, if independent information on dispersal is available, and it is known how long the transgenic crop has been present in the region, the strength of selection on the transgene may be inferred. In applying this approach, it will be important to establish whether the hybrid zone is restricted by specific habitat conditions; for example, in some cases, the wild population affected by transgene flow may inhabit only roadsides, and thus the potential width of the cline may be determined by the presence or absence of roads.

When clines involving several loci (e.g., the transgene and marker loci) are measured, and information on linkage disequilibrium (D) is available for the loci in question, the strength of D can also provide information on selection. Although this approach has not yet been applied to the analysis of transgene escape, it has been applied to disentangle the effects of selection and migration influencing the fre-

Box 1. Estimating gene flow and selection of escaped transgenes.

A monitoring program that employs genetic tools for the estimation of gene flow and selection acting on a transgene would be based on population sampling conducted over two or more consecutive years, and would ask (1) what proportion of seed in the wild population arises as a result of annual gene flow with members of the agricultural population, and (2) what selective pressure is acting on the transgene in the wild populations. These questions can be answered using a model to analyze changes in frequencies of the transgene itself, along with changes in the frequency of one or more unlinked and selectively neutral marker genes.

When transgenic crops are harvested and replanted on an annual basis, and when the crop populations are sufficiently large to serve as a major source of recurrent pollen flowing into the wild population, the continental-island depiction of gene flow, in which migration effectively occurs unidirectionally from crop to wild population, may serve as an appropriate underlying model on which to base analysis (Haygood et al. 2003). Suppose we collect genotypic information from adult individuals in two successive years, denoted by the generation indices t and $t + 1$ (see the figure). Moreover, suppose that at generation t, the transgene occurs in the adults of the wild population of interest at some nonzero frequency. For simplicity, we assume that allele frequencies at the transgene and a single, unlinked marker locus are followed in a population that is outcrossing. The monitoring effort could use the allele frequency data to estimate two informative population genetic parameters: m, the proportion of seed produced by pollination with the transgenic crop, and s, the strength (and sign) of selection acting on the transgene in the wild population.



Estimation of transgene migration and selection in wild populations that hybridize and backcross with plantations of transgenic crop plants.

For instance, let locus A be the transgene locus, and locus B be an unlinked marker locus. Let A_1 and B_1 be alleles in the transgenic crop, and A_2 and B_2 be alleles in the wild population (both possibly null alleles). If we denote the frequencies of the four possible gamete types A_1B_1, A_1B_2, A_2B_1, and A_2B_2 as x_1, x_2, x_3, and x_4, respectively, the two-locus genotypes as g_{ij} (where i and j indicate the component gametes; $i \le j$ and $i, j = 1, 2, 3$, or 4), and the relative fitnesses of each genotype (i, j) as w_{ij}, then the frequencies of the two-locus genotypes after mating, g_{ij}', are simple functions of m. For example:

$$g_{11}' = m[g_{11} + (g_{12} + g_{13}) / 2 + (g_{14} + g_{23}) / 4] + (1 - m)[g_{11} + (g_{12} + g_{13}) / 2 + (g_{14} + g_{23}) / 4]^2 = mx_1 + (1 - m)x_1^2.$$

The frequencies of the two-locus genotypes after mating and selection are

$$g_{ij}'' = g_{ij}'(w_{ij} / W),$$

where W is the population mean fitness. Suppose that the transgene is detrimental to fitness and exhibits dominant gene expression; then $w_{11} = w_{12} = w_{13} = w_{14} = w_{22} = w_{23} = 1 - s$ and all other $w_{ij} = 1$. Maximum likelihood estimation of the parameters m and s would be based on the numbers of genotypes (i, j) observed at times t and $t + 1$; that is, $n_{ij(t)}$ and $n_{ij(t+1)}$. The likelihood of observing the array of genotypes $n_{ij(t+1)}$ is given by

$$L = \frac{N_{t+1}!}{\prod_{i \le j} n_{ij(t+1)}!} \prod_{i \le j} (g_{ij}'')^{n_{ij(t+1)}},$$

where N_{t+1} denotes the total sample size at generation $t + 1$, and the $n_{ij(t)}$ can be used to estimate g_{ij}. Since g_{ij}'' are functions of m and s (the latter through its relationship to w_{ij}), maximizing this likelihood yields estimates of these parameters. The approach, therefore, provides a way to distinguish how transgene frequency in the wild population is influenced separately by gene flow and selection.

quency of pesticide resistance genes in mosquito populations located adjacent to areas where ongoing pesticide applications are being conducted (Lenormand et al. 1998).

Logistical and legal issues associated with population genetics monitoring of transgene escapes

We have discussed the major types of population genetics approaches available for studying the dynamics of transgene escape. As noted, the lack of information about the particular history and ecology of hybridization between the crop and wild population, the DNA sequence of the transgenic construct, and the linkage relationships between the transgene and marker genes, as well as the availability of crop-specific markers, may limit the application of these tools. In fact, the detection of escaped transgenes could itself pose a challenge, because producers and growers of transgenic organisms are usually under no obligation to reveal the detailed location of these populations to the public and independent researchers.

Regulations concerning the growing of transgenic crops vary from country to country, depending on whether they are to be grown as field trials or sowed as commercialized seed. Thus, whether the obstacles referred to above are present depends on the commercial status of transgenic organisms and the regulatory requirements in the jurisdiction where they are grown. Examples from the United States and the European Union contrast some of the types of commercial and regulatory realities of monitoring transgenes in the wild. In the United States, there are three federal regulatory organizations with jurisdiction over transgenic plants: the USDA, the US Environmental Protection Agency (EPA), and the US Food and Drug Administration (FDA). The USDA regulates the planting and movement of transgenic plants that are potential plant pests (USDA 2006a). The EPA has authority over the uses of pesticides and associated constructs engineered into plants (see *www.epa.gov/pesticides/biopesticides/pips/pip_rule.pdf*), which are referred to as plant-incorporated protectants (PIPs). The FDA oversees the use of all food, feed, pharmaceuticals, and biologicals derived from plants, including transgenics (see *www.cfsan.fda.gov/~acrobat/fr920529.pdf*).

Before establishing experimental field test plots, transgenic crop developers must provide information to relevant regulatory organizations, including the sequences of transgenic constructs and the size, duration, and location of the proposed test plots. Such details could greatly increase the efficiency of monitoring by independent researchers, but these aspects are often deemed to be proprietary confidential business information, to which access by the general public is restricted (NRC 2002). When genes of interest are identified in nonindustry labs, the sequences are typically deposited in public databases, but this is not necessarily the case when such discoveries are made by biotech companies. Likewise, developers may see no incentive to provide researchers with plants or DNA sequence information (e.g., transgenic construct information) useful for the development of genetic markers. To proceed with commercialization of transgenic plants containing PIPs such as *Bacillus thuringiensis* (*Bt*) toxins, developers are required to register PIPs with the EPA, and in the process, developers must provide some means (enzyme-linked immunosorbent assay, polymerase chain reaction) by which independent labs can track the PIPs. These tools, however, may become available only after the field-testing phase.

During the period when their product is regulated (i.e., before commercialization of the plants), developers also must comply with a number of USDA, EPA, or FDA requirements for their field trials (note that plants grown for the production of commercial products such as pharmaceutical or certain industrial compounds are grown only under "field trial" permit regulations; the seeds of such plants are never deregulated for commercial sale [Ellstrand 2003b]). Key requirements aimed at preventing escapes include obligatory confinement of transgenic material during field testing and separation from any compatible species within prescribed buffer zones around fields of transgenic crops. Developers are also required to monitor for volunteers within test-field sites and adjacent fallow zones after the test has concluded (7 CFR 340 [2005]; USDA 2006a). It is intended that data pertaining to compliance and all deleterious effects on plants, nontarget organisms, or the environment be collected and self-reported by developers, or ascertained through USDA inspections. Documentation of noncompliance, such as an unintended release, can be made available to the public through the USDA Web site, but these notices are only of general utility for identifying geographic regions where undetected patches of wild compatible species may have been exposed to transgenic pollen or seeds. For instance, the location of the source fields where testing has been authorized typically specifies only the state (see *www.aphis.usda.gov/biotechnology/status.shtml*).

The accidental establishment of regulated transgenic plants in the wild, whether noted by government, industry, or independent monitoring, can constitute a compliance infraction, and developers can be required by the USDA to find and destroy transgenic plants outside the test plots (USDA 2006b). Such actions may substantially reduce transgene introgression and population-level effects. Independent monitoring could provide useful feedback and confirmation about the efficacy of mitigation efforts.

Once a developer has successfully petitioned for deregulation of the transgenic product and it has gone into commercial production, then neither the USDA nor the developer is required to report escapes per se. However, as a condition for registration of certain transgenic crops expressing *Bt,* the EPA has required postcommercialization monitoring for evolution of resistance to *Bt* in target pests and accumulation of the *Bt* in soil. Similar measures may hold true for future PIPs. Although postcommercialization monitoring for transgene flow out of crop fields to compatible wild populations is not yet required in the United States, reporting on adverse effects to the environment or human health is required for EPA-registered products. If new information demonstrates unanticipated effects or plant health risks, the USDA can reestablish the regulated status of the product. Even as commercialization progresses and the source fields of transgenic plants become more widely distributed, the issues of confidential business information mentioned above may still exist and impede independent marker development for tracking the transgenes' fate.

In the European Union, requests for field testing and commercialization of transgenic crops are considered on a case-by-case basis, as in the United States. When a developer wishes to conduct a field test for research on a novel transgenic variety, that developer must first submit a notification to the competent national authority of the member state where the test would be carried out. In keeping with the precautionary principle, the member state is responsible for ensuring that all appropriate measures are taken to avoid adverse effects on human health and the environment. This explicitly includes direct or indirect effects that may be brought about through gene transfer from genetically modified organisms to other organisms (Directive 2001/18/EC, part

A provisions; *www.biosafety.be/PDF/2001_18.pdf*). Notification to the competent national authority must include much of the same detailed information, as is required in the United States, about the biology of the plant, the transgenic construct, the transformation method, the test location, and the duration of the field test. Along with data on interactions between the transgenic plant and the environment, developers must also submit plans for monitoring the transgene, for responding in the case of an emergency, and for conducting an overall environmental risk assessment (Directive 2001/18/EC, part B provisions, annex III; *www.biosafety.be/PDF/2001_18.pdf*). When field testing is conducted in the European Union, substantially more of this detailed information about the experimental release of transgenic organisms is made available to the public than is released in the United States. Such information can include the particular engineered traits expressed, details about transgenic constructs, and geographic information about the field test sites, down to the level of city or global positioning system coordinates (see *http://gmoinfo.jrc.it/*).

To obtain approval for free movement and commercialization of a transgenic product after successful field testing, the developer must provide a new notification to the member state, which is intended for mandatory review by the European Commission. The notification must include (but is not limited to) information obtained from research concerning impacts on the environment, an environmental risk assessment, and a revised plan for monitoring, which may include special crop-specific stipulations. At this stage, the aim of monitoring is to check for unanticipated environmental effects resulting from the expanded scale and geographic distribution of the production sites. Approvals may be granted for a maximum of 10 years. Furthermore, as with the case of field testing, it is mandatory to share information with the public (Directive 2001/18/EC, part C provisions; *www.biosafety.be/PDF/2001_18.pdf*).

It is important to note that the developer of a transgenic organism in the European Union can request from the authority in the member state that certain confidential information be restricted from public access to protect intellectual property rights concerning the transgenic product. The competent authority makes the final decision about what information should justifiably be considered confidential (Directive 2001/18/EC, part D provisions; *www.biosafety.be/PDF/2001_18.pdf*).

Conclusions

Genetic approaches can facilitate the monitoring of escaped transgenes and, moreover, are likely to be required for studying details of the interaction between gene flow and selection, the two principal evolutionary forces that would be of interest in predicting how an escape may unfold at the population level. It is important that researchers interested in studying the fate of escaped transgenes adopt and build upon tools whose underlying assumptions, statistical characteristics, strengths, and limitations are well documented. Equally important is that researchers interested in monitoring the spread of escaped transgenes and in understanding the forces at work in their dynamics be unfettered by legal and commercial constraints that could limit access to the information required for scientific analysis and risk assessment.

As is clear from our account of population genetics–based monitoring approaches, information about the sequence and genomic position of the transgene in the crop species, and the presence of useful crop-specific genetic markers, together with information on the local history and current deployment of the transgenic species, may prove highly useful in monitoring the escape of transgenes. Thus, it is important to ask how researchers interested in studying transgene escape can acquire access to this type of information, given the legal and commercial restrictions that may surround it. The issue requires more attention in current legislation dealing with the commercial release of transgenic organisms (e.g., NRC 2002).

In closing, we note that there is a long and growing record of public involvement in research directed at biological disturbances to natural communities, much of it conducted in institutions of higher learning. Indeed, the bulk of our knowledge of the biological consequences of unintended escapes of another class of organisms—those alien species that invade and disrupt natural communities and ecosystems—stems from the work of research scientists in public institutions. Viewed from this perspective, restrictions on access to the information required to conduct independent research in the case of escaped transgenic organisms is unlikely to be in the public good.

Acknowledgments

We thank German Avila-Sakar, Louis Bernatchez, Jeremiah Busch, Anthony Ricciardi, and Allison Snow for discussions and suggestions. D. J. S. acknowledges the Natural Sciences and Engineering Research Council of Canada for continued research support. N. C. E.'s relevant research has been funded by grants from the US Department of Agriculture (Biotechnology Risk Assessment Research Grants Program #2000-33210-9801 and #2002-33210-12769) and the National Science Foundation (DEB-0409984). The information in this document has been subjected to review by the US Environmental Protection Agency's National Health and Environmental Effects Research Laboratory, Western Ecology Division, and approved for publication. Approval does not signify that the contents reflect the views of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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doi:10.1641/B580112
Include this information when citing this material.