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Assessment of risk of insect-resistant transgenic crops to nontarget arthropods

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Abstract
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Comments
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An international initiative is developing a scientifically rigorous approach to evaluate the potential risks to nontarget arthropods (NTAs) posed by insect-resistant, genetically modified (IRGM) crops. It adapts the tiered approach to risk assessment that is used internationally within regulatory toxicology and environmental sciences. The approach focuses on the formulation and testing of clearly stated risk hypotheses, making maximum use of available data and using formal decision guidelines to progress between testing stages (or tiers). It is intended to provide guidance to regulatory agencies that are currently developing their own NTA risk assessment guidelines for IRGM crops and to help harmonize regulatory requirements between different countries and different regions of the world.

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IRGM crops that express Cry proteins derived from the soil bacterium *Bacillus thuringiensis* (*Bt*) have been grown in several countries on a steadily increasing acreage since their introduction in 1996. In 2006, transgenic varieties of cotton and maize that express *Bt* proteins were grown on 32.1 million hectares worldwide[1]. Several crops expressing novel insecticidal proteins are also under development and these are expected to be commercialized in the near future. (Although insecticidal traits associated with commercialized genetically modified (GM) crops have all been proteins, we recognize that future traits might not necessarily be restricted to this class of molecule.) In common with conventional agricultural pest control products (which include synthetic and organic insecticides, biological control agents and host-plant resistance developed by conventional breeding), one of the risks associated with the growing of IRGM crops is their potential to adversely affect nontarget organisms. These include a range of arthropod species that fulfill important ecological functions such as biological control, pollination and decomposition. The potential for adverse effects of IRGM crops on these NTAs has been evaluated as part of the environmental risk assessment (ERA) process that takes place before the decision to cultivate these crops commercially[2,3]. The relative novelty of IRGM crops and the complexity and sophistication of ERA procedures present regulatory authorities with a challenge when they are required to develop appropriate risk assessment methodologies. This is a particularly difficult task in the developing world, where the regulatory infrastructure is still being established.

General guidance for conducting an ERA for genetically modified (GM) plants exists[4–9]. There remains, however, a need for detailed descriptions for NTA risk assessment procedures, including selection criteria for the NTA test species and test methods that can apply to different regions if these general guidelines are to be adapted for specific crops in specific agriculture ecosystems. To address this need and to formulate the underlying rationale of the existing ERA approaches, an initiative was launched within the GM organisms working group of the West Palaearctic Regional Section (WPRS) of the International Organization for Biological and Integrated Control of Noxious Animals and Plants (IOBC) (http://www.iobc-wprs.org/)[10]. The group consists of European and North American scientists from public research institutes, regulatory agencies, the agricultural biotechnology industry and a commercial testing laboratory. The current focus of this group is the development of an IRGM-specific rationale for a tiered toxicological testing system which, when integrated with exposure, will enable poten-

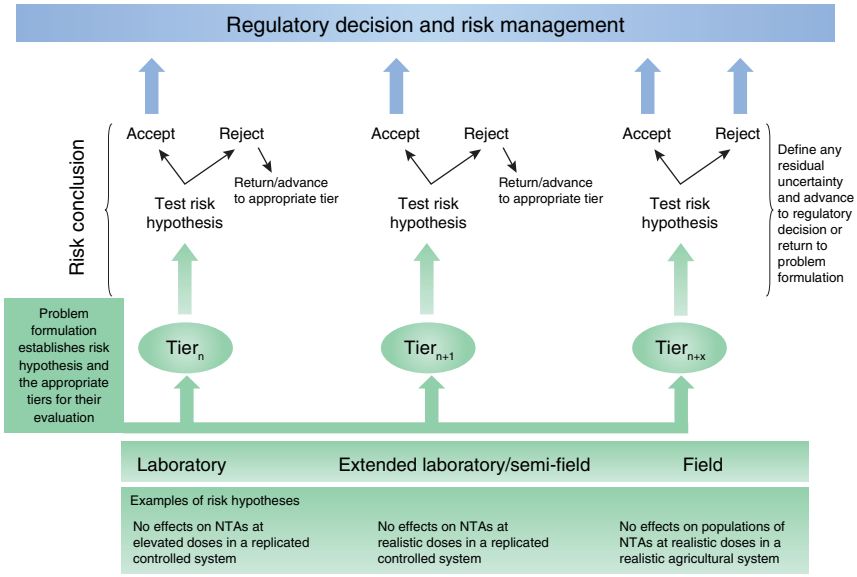

Figure 1 Assessment continuum within a tiered scheme of ecological risk assessment. The decision to reject the risk hypothesis includes consideration of residual uncertainties. With increasing tiers, the assessment becomes more complex and realistic, with conclusions that are more specific. The assessment can stop at any stage during the process as soon as sufficient information has been compiled to address the risk hypothesis. Thus collection of data irrelevant to the risk assessment is minimized. N, level of risk assessment tier; NTA, nontarget arthropod.

tial risks to NTAs to be defined and assessed. This is intended to provide regulators with a scientific rationale for the risk assessment decisions that they make. Ultimately, the risk management decision that regulators make also takes into account relevant social and political considerations[11,12].

In the following article, we outline the basic principles and rationale of the tiered approach. We also describe the ways in which this approach has been refined by scientists from public, industrial and regulatory sectors, who have extensive experience with IRGM crops.

Problem formulation

In our approach, the problem formulation stage is designed to identify the areas of greatest concern or uncertainty concerning ecological risks, and to define the scope of the risk assessment by generating testable scientific hypotheses that are subsequently addressed in the analytical phase of the risk assessment[8,13,14]. The information that is considered during problem formulation takes many forms, including published scientific literature, expert opinion, stakeholder deliberations and data developed by the registrants and submitted to the regulatory authority as part of the registration dossier. This information establishes the level of 'familiarity' (that is, the similarities in ecologically relevant characteristics) between the IRGM crop and nontransformed crop[15–17] and, together with the related concept for food of 'substantial equivalence', serves as a starting point to focus the ERA process on potential stressors of concern[18,19]. If substantial equivalence and familiarity are established, the ERA can proceed with emphasis on narrowly defined, stressor-mediated effects that arise from the expressed trait in the IRGM crop (e.g., a *Bt* protein)[14,20]. In cases where substantial differences other than those directly related to the expressed trait are detected, these characteristics become additional potential stressors that also need to be evaluated, following the same tiered approach that we outline. Thus, the greater the extent of familiarity between the IRGM crop and the nontransformed crop, the more specific and focused the risk hypotheses will be. The feedback inherent in the process described increases the efficiency of ERA for familiar crops and focuses resources on less familiar commodities as they arise.

The data commonly requested by authorities to satisfy regulatory assessments are particularly important for the establishment of familiarity and typically include a description of the host crop, the source and molecular characterization of the introduced genetic elements, the nature and stability of protein expression, the spectrum of protein activity, macro- and micronutrient composition, the content of important toxicants and antinutrients, and morphological and agronomic plant characteristics. Regulatory agencies may also request the results of field trials conducted at several locations that are representative of where the crop will be grown. In all cases, descriptions of plant characteristics, which are made with reference to plants that are generally regarded as environmentally 'acceptable', are used to identify meaningful differences that may need to be addressed in the risk assessment.[2,8,15,16,18]. A 'meaningful difference', in this context, refers to a substance or another attribute previously associated with effects that may be of environmental concern (e.g., an unintended increase in alkaloid levels in GM cotton plants modified to express a Cry protein could affect NTAs). It is evident that the degree of familiarity with a given crop and IRGM approach and its conventional comparators will increase over time and with experience.

The problem formulation may result in recommendations that a narrowly defined set of experimental evaluations should be undertaken for the risk assessment of some IRGM crops with well-known characteristics. This could result in criticism that the risk assessment is superficial and not likely to detect potential risks. However, the process explicitly considers the specifics of the stressor's mode of action, spectrum of activity and levels of exposure of NTAs to the stressor. Data to support the problem formulation may derive, for example, from the tests carried out during IRGM plant development. The pest activity spectrum is commonly explored during development by testing the activity of the insecticidal protein against a range of pest species belonging to different orders. Additional data may also exist in the literature concerning the spectrum of activity of some proteins (e.g., certain Cry proteins from *Bt*) that have been studied by public sector scientists[21,22]. The information base that is inherent to the concept of familiarity is therefore considerable; thus, we argue that risk assessment for specific applications associated with familiar crops/traits can be both thorough and accurate without requiring extensive additional testing.

Problem formulation identifies scientifically analyzable endpoints that reflect management or protection goals that are set by public policy. For example, if 'protection of biodiversity' is the management goal, a typical assessment endpoint would be the abundance and species richness of certain groups of NTAs, such as those that fulfill important ecological functions. Different regulatory agencies may define different assessment endpoints or even have different management objectives. These must all be considered explicitly in the problem formulation stage so that the risk hypotheses can generate data that address the goals of the regulator and the requirements of the policy. The problem formulation should culminate in a conceptual model and analysis plan that is consistent with the risk hypotheses and that establishes the relationship between the stressor and the ecological impacts of concern (the assessment endpoints). The conceptual model should take into account ecological considerations that might affect the nature and extent of possible environmental impacts, including the intended scale of cultivation of the IRGM crop.

The ERA framework and moving through it

Scientific assessment of risks from IRGM plants is conceptually similar to the assessment of risks from traditional synthetic insecticides even though insecticidal proteins expressed by GM plants may differ from synthetic insecticides (e.g., by mode of action, specificity, exposure route)[23]. The tiered process of toxicity testing is generally used to assess the nontarget effects posed by traditional insecticides[24] because it is suitable for assisting the decision-making process in an effective and rigorous way. We argue that it is also the most rigorous approach, from both scientific and regulatory standpoints, for determining the potential of IRGM plants to adversely affect NTAs. Versions of this approach are also in current use in established regulatory systems for GM crops[4,8].

A typical risk hypothesis that emerges from problem formulation may be that the stressor (that is, the insecticidal protein) does not harm NTAs at the concentration expressed in the field. The testing of this hypothesis frequently leads to toxicity tests on select arthropod species. These tests are conducted within experimental 'tiers' that are initiated with elevated dose exposure tests (e.g., at ten times the expected environmental exposure), often using laboratory procedures with purified protein in artificial diets and proceeding to more realistic scenarios of exposure with IRGM plants if impacts exceed certain specified threshold values (**Fig. 1**).

Examples of the risk hypotheses that are addressed at the different tiers are provided in **Figure 1**. The conceptual pathway leads from relatively simple and controllable lower tier assessments to increasingly complex higher tier assessments. The conclusion regarding risk drawn at each tier will lead either to a regulatory decision after the residual uncertainty of the assessment has been defined or to additional investigations. These need to be conducted at the appropriate tier, which could be any of the lower, current or higher tiers of evaluation. Throughout the assessment, the risk assessor needs to confirm continually that the problem being addressed is still appropriate and, if necessary, revisit the problem formulation.

Lower tier tests serve to identify potential hazards, if they exist, and are typically conducted in controlled laboratory conditions (**Supplementary Note** online). Lower-tier tests are designed to measure a specific endpoint (or set of endpoints) under controlled conditions using protein concentrations that are usually several times higher than those present in the field. Such studies provide a powerful means to detect hazards because the biological impacts of the insecticidal protein can be isolated[25]. The tests are not meant to reflect real-world exposures but to increase the likelihood that a hazard will be detected should one be present, and so provide confidence of minimal risk should no adverse effect be detected. The sequence of testing continues after the initial elevated-dose or dose-response tests if potential hazards were detected (that is, the 'no-effect' hypothesis had to be rejected) or if unacceptable uncertainties about possible hazards remain (**Fig. 1**). For example, conducting further lower tier tests in the laboratory can refine the hazard assessment by increasing the taxonomic breadth or local relevance of test species. In cases where lower tier tests detect a potential hazard (that is, the 'no-effect' hypothesis is rejected), higher tier tests, which include more complex semi-field (that is, under containment using live GM plant material) or open field tests, can then serve to confirm whether an effect can still be detected under more realistic rates and routes of exposure to the protein (**Fig. 1**). In cases where uncertainty about the risk remains after higher tier studies, one can always return to lower tiers to conduct additional studies, for example, by including additional test species (**Fig. 1**). In exceptional cases, higher-tier studies or studies

Box 1 Evaluation path for *Bt* maize expressing Cry1Ab for nontarget arthropods

In the evaluation of *Bt* maize expressing Cry1Ab, entities of concern included biological control organisms belonging to, for example, the orders of Coleoptera (lady beetles), Neuroptera (lacewings) and Hymenoptera (parasitoid wasps), as well as pollinators such as bees (also Hymenoptera), decomposers such as soil arthropods (for example, springtails) and nontarget Lepidoptera. The problem formulation identified several risk hypotheses that were subsequently addressed in the analytical phase of the risk assessment (**Fig. 2**). Because analysis of the available precursor information revealed with sufficient certainty that the only meaningful difference between *Bt* maize and its nontransformed comparators was the expression of the Cry1Ab protein, early tier (worst-case) studies were conducted using elevated doses of purified protein or plant tissue. These studies confirmed existing knowledge (precursor information) that these proteins are not likely to affect nonlepidopteran insects (risk hypotheses 1–3)[22,42]. Testing could thus be terminated at this early tier. The potential hazard to nontarget Lepidoptera (risk hypothesis 4) was recognized initially but it was concluded that the risk is negligible[43]. Additional studies under more realistic exposure conditions were triggered once a note[44] and a more comprehensive study[45] had revealed a hazard of Cry1Ab to larvae of the monarch butterfly. Studies were conducted under semi-field conditions. These studies concluded that the risk of Cry1Ab maize to monarch populations is negligible because larval exposure to Cry1Ab toxin under field conditions is low[46–49] confirming the initial risk assessment[43].

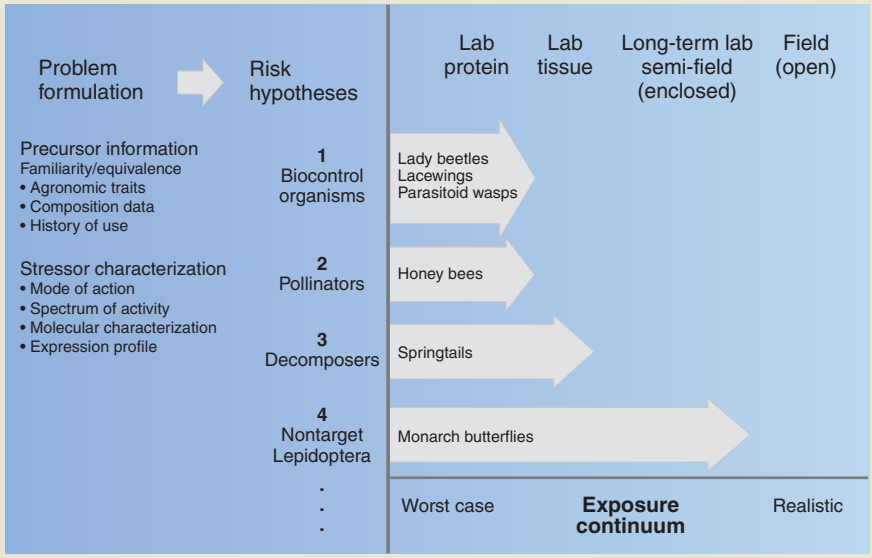

Figure 2 Reconstruction of NTA risk assessment for *Bt* maize expressing Cry1Ab showing that different risk hypotheses require different types of data and synthesis at different tiers.

using alternative designs may be conducted at the initial stage of the risk assessment process when lower tier tests are not possible. Lower tier tests are, however, extremely valuable to the ERA process because they are highly controlled, and may provide data that are broadly applicable within various risk assessments. It should be noted that field observations on *Bt* crops have failed to find any adverse NTA impacts that could not have been predicted from laboratory or small-scale field studies[22,26].

Movement between tiers takes place either because the available information is insufficient to accept the risk hypothesis of 'no effect' or because this hypothesis has been rejected. If sufficient data and experience from toxicological testing and exposure analyses are available to characterize the potential risk as being acceptable, then there is no need to undertake additional testing (**Fig. 1**). The iterative and flexible tiered testing scheme described herein is designed to provide the information to support a regulatory decision as efficiently and rigorously as possible. In cases where the risk hypothesis is rejected at the highest tier (**Fig. 1**, Tier_{n+x}), an adverse impact on NTAs can occur. As a consequence, the GM variety may either not be authorized or the regulatory agency may require monitoring or risk mitigation. Alternatively, a new problem formulation may be required in cases where a potentially adverse outcome is found.

A key principle of the tiered process is that particular studies are conducted only when they serve to reduce uncertainty in the risk assessment. Where no hazard or risk is detected, effective tiered processes prevent costly and unnecessary testing (see **Supplementary Note**; the lacewing case). Consequently, the assessment of different risk hypotheses will follow different evaluation paths (**Box 1** and **Fig. 2**). The process is designed to optimize the expenditure of resources by identifying and defining sources of potential risk, thereby minimizing the collection of data that are irrelevant to the risk assessment.

Box 2 Nontarget species selection

The number and type of NTA species that need to be tested depend on the risk hypotheses generated during the problem formulation. The species selection will also depend on how much information already exists to test the hypotheses: the level and quality of information on the plant, the specific stressor (that is, the insecticidal protein) expressed (e.g., spectrum of activity, mode of action, expression level) together with information about the feeding habits of the NTAs. Increasing the level and quality of information reduces the number of risk hypotheses that will require testing.

Example 1: novel *Bt* maize event expressing Cry1Ab. There is a high probability, based upon an extensive research history, that the protein would be active only against Lepidoptera and that harm to NTAs in other taxonomic orders would be negligible. Additionally, there is a high level of familiarity with maize, the trait and the toxin from experience with other Cry1Ab-expressing maize events. (Event indicates a unique transformation of a plant by insertion of a particular transgene into its genome). Further testing for this novel event is not scientifically justified provided that problem formulation shows the expression of the Cry1Ab protein is the only relevant difference compared with closely related, nontransformed maize varieties and that expression levels are similar to those in previously evaluated events.

Example 2: novel *Bt* maize event expressing a Cry3 protein. Because the protein is targeting corn rootworms (*Diabrotica* spp.) and has a known specificity to Coleoptera, the risk assessment should focus on nontarget coleopteran species rather than species belonging to other taxonomic groups.

Example 3: novel *Bt* maize event without toxin expression in the pollen. Honeybees are exposed only to insecticidal proteins expressed by GM maize varieties when these are present in the pollen. If the proteins are not expressed in the pollen, there is no scientific justification for conducting feeding studies with bees or other pollen-feeding arthropods.

Species selection

For practical reasons, only a small fraction of potentially exposed terrestrial arthropods can be considered for regulatory testing. It is therefore necessary to select appropriate species to serve as surrogates that can be tested effectively under laboratory conditions[27–29]. For regulatory testing of IRGM plants, surrogate species should be representatives of ecologically and economically important NTA taxa in the crop and represent different ecological functions including predation and parasitism of pest organisms, pollination and decomposition of plant material[27,30]. Key species or guilds that are representative of different functional groups are known in most systems and appropriate surrogates can therefore be selected. Despite recognized limitations[31], the application of the surrogate concept is widely applied in related fields including regulatory toxicity testing[28,29,32,33] and environmental monitoring[34–36]. In addition, the risk assessment may consider species with special aesthetic or cultural value or species classified as threatened or endangered. These species are regionally specific and can be evaluated within the ERA independent of their ecological function.

The most effective surrogate taxa, for example honeybees (*Apis mellifera*), are representatives of NTA taxa that are found in many different crops or regions. More specific, crop-associated species may be selected that represent an important genus (e.g., *Orius* spp.), and other taxa may be selected that are broadly representative of whole families (e.g., parasitic wasps of the Ichneumonidae) or orders (e.g., Coleoptera) that are known to be important. Even the nontarget pest species that are screened for their sensitivity to the insecticidal protein during product development can serve as surrogates for NTAs.

Information on the stressor (e.g., protein specificity, and the pattern and level of expression in the plant), together with information on the feeding habits of the test species, which is accumulated during problem formulation, must be considered during the selection of appropriate surrogates (**Box 2**). In general, nontarget species that are related taxonomically to the target pests are most likely to be affected by the protein; thus selection of these taxa increases the likelihood of detecting a hazard if one exists. Species that are not exposed to the insecticidal protein do not need to be tested to draw a negligible-risk conclusion. Some additional, practical considerations include the ease of working with a species, the potential for unambiguous taxonomic recognition, the ability to rear the species in captivity, the availability of permanent source colonies and validated and accepted test methods.

The purpose of using IRGM plants is the same as for any other pest management tactic; that is, to reduce pest populations below economic injury levels. As a result of applying the tactic, the abundance of pest insects should be significantly reduced and this will have corresponding implications for those organisms that exploit these pests as prey and hosts. Thus, the potential for these indirect ecological effects on biological control organisms should not be regarded as a unique ecological risk associated with the IRGM crop[8,18,22]. Large reductions, however, should be expected if the pest management strategy is effective. Because IRGM crops are often grown in vicinity with non-GM

crops to prevent resistance build-up by the target pest(s)[37], specialist antagonists can persist in these 'refuges', in other crops and in non-crop habitats and retain the potential for recolonization of the IRGM crop area. On the basis of these considerations, regulatory testing of the specialist predators and parasitoids of target pests may not be necessary.

Study design

Once the surrogate species are selected, they should be evaluated in scientifically designed and validated studies to test the risk hypotheses. Historically, protocols developed to assess the impact of pesticides on NTAs[38,39] formed the basis for the tests used to evaluate IRGM plants. These protocols were modified to account for the oral exposure pathway and the potential for extended exposure time to plant-expressed insecticidal proteins[40]. New protocols are being developed to address the specific research or regulatory needs associated with IRGM risk assessment. The new or modified tests often span a significant portion of the test insect lifespan, which is appropriate for the period of exposure to the toxin in the field, and may include a number of measurement endpoints in addition to mortality (see **Supplementary Note** for examples using these new protocols).

Conclusions

The tiered NTA testing approach presented here provides the scientific rationale for the ERA of IRGM crops to assist regulatory decision making. The framework provides a well-defined and predictable pathway for requesting, acquiring, organizing and evaluating data. It is the consensus of a diverse group of stakeholders and therefore provides a basis for improving harmonization of international risk assessment guidelines. Harmonized procedures in ERA help to facilitate risk assessment data acceptability and provide greater scope for comparison of ecological effects data internationally.

The approach presented here ensures rigorous testing of clearly stated and relevant risk hypotheses that are linked to defined assessment endpoints while optimizing data requirements. The risk hypotheses are developed from current knowledge about the biology of the crop, the introduced trait, the receiving environment and the interactions of all three. It therefore makes maximum use of the existing data and aims to minimize collection of data that are irrelevant to the risk assessment. The process is intended to be efficient and rigorous, focusing the resources to address potential risks or uncertainties and eliminating from further consideration the risks that are negligible. Potential hazards are evaluated with representative surrogate/indicator species that are selected case by case for their suitability and amenability to test relevant risk hypotheses.

The general approach we outline here has evolved based upon current events and insecticidal proteins and is flexible and adaptable to new IRGM products. Several aspects of the approach may, however, have to be further developed to take account of new traits and potential risks.

First, threshold values need to be defined that trigger the advance to higher tiers as has been done for environmental risk assessments of conventional pesticides[32,33]. If these trigger values are not exceeded, the testing stops and the regulatory decision follows. The specific triggers applied in a given case of an ERA for IRGM plants are informed by expert opinion and require deliberation among risk assessors and risk managers, who consider the problem being evaluated and the effects regarded as 'acceptable'. We recognize, however, that defining the trigger values is not solely a scientific question but also depends on whether policy-makers are concerned about under- or overestimating risks.

Second, a list of surrogate species needs to be compiled that can serve as a basis for selecting the most appropriate species for laboratory testing. The species tested should provide the most rigorous tests of the risk hypotheses for a particular IRGM plant in a specific agricultural and environmental setting.

And third, more standardized, validated test protocols for surrogate test species may need to be developed. These are needed to ensure data comparability and facilitate international regulatory acceptance.

We believe that the tiered NTA testing approach presented above minimizes the likelihood of false negatives, which could result in the release of IRGM plants with undesirable effects on NTAs and, at the same time, should reverse the trend of increased delays for introducing products that may be environmentally more benign than existing methods of pest control[41].

Note: Supplementary information is available on the Nature Biotechnology website.

DISCLAIMER

In addition to scientists that work within the public sector, the IOBC/WPRS working group includes scientists that work within regulatory agencies, the commercial biotech industry and commercial contract laboratories. Although these organizations have an interest in the final outcome of the working group program, members of the working group participate as individuals, not as representatives of these organizations. The publications of the working group reflect a consensus that has developed as a result of its open meetings and discussions and the opinions expressed by individuals or in working group publications may not necessarily, therefore, represent the policies of their organizations. Any mention of a proprietary product in meetings or publications does not constitute an endorsement or a recommendation by the working group. There is no commercial sponsorship or endorsement for the working group or its members, beyond the support provided to individual participants by their organizations to attend meetings.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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