Problem Formulation for the Environmental Risk Assessment of RNAi Plants

Conference Proceedings

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1. INTRODUCTION

Genetically engineered crops approved for cultivation, in the United States (U.S.) and in other countries, have been the subject of environmental risk assessments (ERAs) conducted by the regulatory agencies that are responsible for evaluating their safe use. Risk assessments of these crops systematically consider the potential adverse environmental impacts that may be associated with their cultivation and are a prerequisite to the commercial release of genetically engineered crops in all countries where they are currently cultivated. Although there are legislative and procedural differences between countries and within agencies that produce environmental risk assessments, the assessments themselves are conducted based on a set of underlying principles and practices that are outlined in international consensus documents, national laws and regulations as well as agency guidance[1]. Together, these constitute the current paradigm for ERA of genetically engineered crop plants.

The majority of approved genetically engineered crops have been transformed to express one or more novel proteins that confer useful agronomic traits such as insect resistance or herbicide tolerance[2]. The classic approach for genetic engineering has been the introduction of a transgene from one organism to another. This transgene contains a promoter, an open reading frame and a terminator which allows the gene to be transcribed and translated by the host, producing a protein which confers a new trait. There are several emerging technologies in genetic engineering that build on these earlier approaches, and one of them is the use of RNA interference (RNAi).

The term RNAi has come to refer to the effect of a common set of eukaryotic mechanisms that result in post-transcriptional gene silencing. Observations of natural phenomena that are now known to be caused by RNAi mechanisms, including some forms of virus cross protection in plants, have been known for decades, but it wasn't until the late 1990s that the molecular pathways responsible for these were discovered. Even before the molecular basis of RNAi was well understood, RNAi methods were adopted quickly by the research community because of the relative ease, specificity, and efficacy with which gene silencing could be accomplished.

The application of RNAi to produce genetically engineered crops with improved agronomic, nutritional, industrial and food-processing traits (abbreviated as "RNAi plants" in this document) is becoming increasingly common. As new products approach commercialization, it is timely to consider whether the approach currently applied to the environmental risk assessment of genetically engineered crops expressing novel proteins remains appropriate for the ERA of genetically engineered plants utilizing RNAi approaches. This question was the subject of the conference "Problem Formulation for the Environmental Risk Assessment of RNAi Plants" convened by the Center for Environmental Risk Assessment (CERA) on June 1-3, 2011. The objectives of the conference were:

1. To share information about current applications of RNAi for genetically engineered plants;
2. To use case studies to explore whether problem formulation for RNAi plants leads to new or additional risk hypotheses when compared with non-RNAi plants expressing similar traits, or if new risk assessment methodologies are necessary.

This report summarizes the proceedings of the conference, including presentations, case studies, a summary of discussions, and the points of consensus agreed by the participants.

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1 *e.g.*, OSTP. (1986). Coordinated Framework for Regulation of Biotechnology. http://usbiotechreg.nbii.gov/read_file.nbii; OECD. (1993). Safety considerations for biotechnology: scale-up of crop plants. http://www.oecd.org/dataoecd/26/26/1958527.pdf; USEPA. (1998). Guidelines for ecological risk assessment. http://oaspub.epa.gov/eims/eimscomm.getfile?p_download_id=36512; CFIA. (2001). Canada and United States 2001 Bilateral Agreement on Agricultural Biotechnology, Appendix II – Environmental Characterization Data for Transgenic Plants Intended for Unconfined Release; http://www.collectionscanada.gc.ca/webarchives/20071123101541/http://www.inspection.gc.ca/english/plaveg/bio/usda/appenannex2e.shtml.

2 For a comprehensive list of approved genetically engineered plants, see http://cera-gmc.org/index.php?action=gm_crop_database&mode=Synopsis.

2. METHODOLOGY

The conference agenda is presented in Annex 1. Invited presentations established a common basis of understanding of the molecular basis of RNAi and how RNAi is being applied to develop genetically engineered crop traits; the application of problem formulation in ERA of genetically engineered crops; and the management and protection goals that frame the ERA of genetically engineered crops by the U.S. Environmental Protection Agency (USEPA) and the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (see Section 3 below). Four case studies of RNAi plants, prepared and presented by principle investigators, were provided as practical examples to help focus, but not limit, subsequent discussions that took place in breakout groups and in the final plenary session (the case studies are provided in Annexes 2, 4, 6 and 8). For each case study, participants were asked to:

1. Discuss scenarios in which the subject plant may have adverse impact(s) on the protection and management goals as defined by USEPA and APHIS:
    a) Define plausible risk hypotheses for these scenarios, considering the potential exposure pathways and potential hazards or effects that may occur;
    b) Comment if any of the risk hypotheses identified above are specific to the RNAi mechanism used to develop the plant, or if they are applicable to the plant phenotype irrespective of how the novel trait was introduced;
2. Identify potential sources of information or data that may be available to characterize the potential risks identified in 1(b);
3. Comment if new analytical methods may be needed to enable testing of risk hypotheses for RNAi plants.

The deliberations of each working group were presented back to the plenum, which was then tasked with considering the questions imbedded in the second objective of the conference: in the context of environmental risk assessment, do RNAi plants lead to new or additional risk hypotheses when compared with non-RNAi plants expressing similar traits, and are new risk assessment methodologies necessary? In response to these questions, the plenum achieved consensus on a number of points (see Section 5 below).

3. INVITED PRESENTATIONS

3.1 INTRODUCTION TO RNAi

Vicki Vance, Ph.D., Professor of Biological Sciences, Department of Biological Sciences, University of South Carolina

This workshop is devoted to determining if there are any unique risks associated with the use of RNAi in transgenic plants that are not covered in the current risk assessment procedures. Our initial task is to ensure that the participants are all familiar with the RNAi technology. What exactly is RNAi? It stands for "RNA interference," and it refers to a set of related processes in which small regulatory RNAs direct sequence-specific repression of gene expression. Some RNAi pathways are induced by invasive nucleic acids, such as viruses or transposons, and serve to defend the host plant against such invaders (Alvarado and Scholthof 2009). Others are endogenous pathways that control an organism's own gene expression (Vazquez *et al.,* 2010). RNAi pathways are evolutionarily ancient. Various versions of these processes are found in virtually all eukaryotic organisms, and these related processes are united by shared genetic requirements and biochemical features.

Small RNA-directed regulation of gene expression was first discovered when plant scientists tried to over-express genes encoding beneficial proteins. They found that sometimes the beneficial transgenes were not expressed (Matzke *et al.,* 1989; de Carvalho *et al.,* 1992). Worse, in some cases, not only was the transgene not expressed, but even the expression of endogenous genes related to the transgene was turned off (Napoli *et al.,* 1990; van der Krol *et al.,* 1990). This latter circumstance was referred to as "co-suppression," but the more general phenomenon came to be known as "gene silencing." Two general categories of silencing were identified early on (Sijen *et al.,* 2001). Transcriptional gene silencing is due to the presence of repeated copies of the transgene promoter in the genome, which results in methylation of the promoter DNA as well as repressive chromatin structure and prevents transcription of the transgene (Matzke *et al.,* 2004). In post-transcriptional gene silencing, the transgene is transcribed, but the transcripts fail to accumulate because they are specifically targeted and destroyed (Brodersen and Voinnet 2006; Vaucheret 2006). Today it is well established that transgene-induced silencing in plants, at both the transcriptional and the post-transcriptional levels, is mediated by the RNAi pathways that defend against invasive nucleic acids. These are the inducible RNAi pathways, and their small regulatory RNAs are called short interfering RNAs (siRNAs). To date, efforts to use silencing to manipulate gene expression in plants have focused on the post-transcriptional branch of the inducible RNAi pathways. However, the endogenous RNAi pathways that use small regulatory RNAs termed micro RNAs (miRNAs) offer another powerful approach. Because the miRNA based approach also works at the post-transcriptional level, this introduction will focus on post-transcriptional silencing.

siRNA-mediated post-transcriptional silencing (inducible RNAi pathways)

The inducible RNAi pathways are triggered by double stranded RNA (dsRNA), which is not ordinarily present in eukaryotic cells. The dsRNA is cleaved by an enzyme called "dicer' that chops the dsRNA into siRNAs, which are RNA duplexes 21 to 24 nucleotides (nt) in length. The siRNAs comprise a population representing the entire region of dsRNA. They are double stranded because they derive from the long dsRNA that triggers the process. One strand of the siRNA duplex incorporates into a large protein complex called RISC (for RNA induced silencing complex). There the siRNA strand acts as a guide to find complementary RNAs that it can bind to by the base pairing rules. In post-transcriptional silencing, RISC contains a ribonuclease that cleaves the target RNA to which the siRNA guide has bound, triggering degradation of the target. In this way, targeting of RNA degradation is sequence-specific - any RNA homologous to the dsRNA trigger is found and destroyed.

siRNA-based RNAi strategies for plant biotechnology

siRNA-directed RNAi provides a powerful tool that has been widely utilized for both research and commercial purposes to manipulate gene expression in plants at the post-transcriptional level. This type of RNAi can be induced by transforming plants with transgene constructs that make dsRNA homologous to any gene of interest. Transgenes containing

regions of self complementary sequence – termed hairpin or IR (for inverted repeat) constructs - are the best inducers of silencing because their transcripts fold to produce dsRNA (Waterhouse *et al.,* 1998). Transgenes that cannot fold to make dsRNA, however, are also able to induce silencing in many cases. As yet unknown features of the transcripts of these transgenes are recognized by the cell, and the transcripts become templates for a cellular RNA-dependent RNA polymerase. This enzyme synthesizes an RNA strand complementary to the transcript, thereby producing dsRNA and triggering silencing.

Most plant viruses replicate via dsRNA intermediates or produce dsRNA at some stage in their life cycle and, therefore, induce silencing directed against the virus. Thus, silencing is an antiviral defense mechanism in plants (Ding and Voinnet 2007). An RNAi strategy that has been used successfully in research, but which is probably less well suited to commercial purposes, is to use plant viruses to silence host genes by infecting with a virus that has a portion of a host gene cloned into the viral genome. The dsRNA that is naturally produced during the virus life cycle then triggers silencing not only against the virus, but also against host transcripts homologous to the plant gene cloned into the virus.

Potential limitations of siRNA-based RNAi strategies

Because silencing is an antiviral defense mechanism, many plant viruses encode proteins that suppress silencing (Burgyan 2008; Alvarado and Scholthof 2009). Furthermore, plant viral infections are very common. As a result, it is possible that viral infection in the field might suppress transgene-induced RNAi. In addition, suppressors of silencing from unrelated viruses are structurally unrelated and employ a variety of mechanisms to block silencing, making it difficult to engineer broad-spectrum protection against this problem. A second potential limitation arises from the fact that siRNAs comprise a population of molecules representing the entire sequence of the dsRNA trigger. Although this sequence heterogeneity could make it easy to silence a family of related genes with only one construct, it also opens the door to off-target effects, in which genes with regions of homology to the intended target get silenced unintentionally. A third potential limitation stems from the fact that post-transcriptional silencing in plants is mobile. It can be induced locally and will then spread throughout the plant. Thus, siRNA-based RNAi strategies might not be suitable for some applications requiring tissue-specific silencing of genes.

miRNA-mediated silencing (endogenous RNAi pathways)

miRNAs are one type of endogenous regulatory small RNA (Xie *et al.,* 2010). miRNAs are similar in size to siRNAs and are usually 21 or 22 nt long. miRNAs, however, are encoded by endogenous genes. These genes are much larger than the miRNA itself and are designed specifically to produce miRNA. Often the same miRNA is encoded by several genes. There are hundreds of different miRNAs. Many are highly conserved, and each miRNA regulates the expression of one or more target genes. The biogenesis and functioning of miRNAs are similar to that of siRNAs in many respects. miRNA gene transcripts fold to produce a region with considerable double-stranded structure. This precursor is processed by a specific dicer enzyme, releasing the unique miRNA paired with its opposite strand (called miRNA*). The miRNA* strand does not accumulate, but is rapidly degraded. The miRNA strand incorporates into RISC and acts as a guide to bring RISC to the target message. miRNA-RISC can cause cleavage of the target message, like siRNA-RISC, but can also work by blocking translation.

Artificial miRNAs - an alternative RNAi strategy for plant biotechnology

miRNA-directed silencing using artificial miRNAs has been shown to work in plants in a research setting and has the potential to be another powerful tool in the RNAi arsenal (Ossowski *et al.,* 2008; Sablok *et al.,* 2011). The strategy uses standard cloning techniques to modify any known miRNA gene and construct a transgene that encodes a miRNA targeting the gene one wants to silence. This strategy entails designing a 21- or 22-nt miRNA homologous to the intended target gene and replacing the natural miRNA portion of the cloned miRNA gene with this new miRNA sequence. The natural miRNA* portion of the gene also has to be replaced with sequence that maintains the secondary structure of the miRNA precursor. The modified miRNA gene, under the control of an appropriate promoter, can then be introduced into plants, and transgenic plants expressing the modified miRNA gene will silence the target of the artificial miRNA.

Potential advantages and limitations of artificial miRNA-based RNAi

Artificial miRNAs provide a much more specific way to silence genes than do siRNAs because miRNAs use only a single 21- or 22-nt sequence to identify the target, whereas siRNAs comprise a population of sequences. Thus, there is a reduced chance of off-target effects with miRNA-based RNAi, and it will be easier to target individual genes in a closely related gene family. Artificial miRNAs also provide a better option for tissue-specific silencing because miRNA-directed silencing tends not to move throughout the plant. A potential limitation of artificial miRNA-based RNAi is that the silencing might not be very durable because only a single 21 or 22 nt specificity determinant is involved. Escape from miRNA-directed silencing via mutation of the target, therefore, would be easier than in siRNA-directed silencing, in which a much larger sequence is targeted. Using two (or more) different artificial miRNAs against the target is a strategy that has been used to overcome this problem.

The basic question to be addressed by this workshop

The current risk assessment procedures were designed to address potential risks posed by genetically engineered plants that encode and produce novel proteins. In the case of RNAi strategies, however, the genetically engineered plants encode and produce novel siRNAs, miRNAs, and their double-stranded RNA precursors. It is the task of this workshop to determine whether there are any unique risks posed by these novel RNAs.

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3.2 RNAi APPLICATIONS IN THE PLANT SCIENCES

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RNA-based regulation of gene expression was applied early in the development of transgenic plant science. Jorgensen and others in the early 1990s utilized sense (co-suppression) and anti-sense strategies to overexpress chalcone synthase RNA transcripts and induce gene suppression, resulting in striking color patterns and even all white flowers in an otherwise dark purple petunia parent (1). The demonstrated ability to selectively suppress plant gene expression led to rapid adoption of the technology for both basic research in plant biology and biotech improvement of crops. As a result, the first approved biotech product, Flavr Savr® tomato, was an RNA-based approach to creating a desired trait *in planta* (2).

However, understanding of the mechanistic details of this phenomenon was not known until the late 1990s when researchers in both animal and plant systems revealed that double stranded RNA (dsRNA) and small RNA derivatives (typically 21-24 base pairs in size) were at the heart of endogenous regulatory pathways for RNA transcripts (3, 4). The small RNAs serve as targeting guides for regulatory protein complexes. Variations around this rich modality of regulation (termed RNA interference or RNAi) are found in all multi-cellular organisms examined to date and are used by cells to refine native gene expression patterns via cleavage of messenger RNA transcripts or inhibition of their translation into proteins. Components of the pathways also serve to recognize and destroy invading viral RNA sequences. The utility of the system depends on the specificity of base pairing between small RNAs and target transcripts in order to direct protein machinery towards regulation of appropriate cellular targets without unintended suppression of related transcripts with lower levels of sequence identity.

Generation of small RNA triggers is a prerequisite for downstream regulatory activity (reviewed in 5). Triggers can originate from endogenous microRNA genes that generate discreet small RNAs (miRNAs) from the processing of larger, partially complementary primary transcripts. Triggers can also originate from long dsRNA precursors that arise from specific locations in the plant or a viral genome and are cleaved by "dicing" enzymes into a population of small interfering RNAs (siRNAs). miRNAs and siRNAs differ based on their biological origin, but function similarly to target transcripts. Production and mediation of small RNA activity are selectable attributes and their variation contributes to natural diversity in organisms. Agronomists have utilized this diversity by propagating mutations during the course of crop domestication that have altered dsRNA populations and as a result, modified gene activity. Modern examples of small RNA-based suppression traits include soybean yellow coat color (vs. dark colored coats of progenitors) and the low glutelin rice (vs. higher protein content grain of standard rice grain) (6, 7).

The tools of modern biotechnology permit integration of transgenes in to plants that deliberately initiate the RNAi process for a given gene transcript (reviewed in 8). Additionally, RNA-based traits offer the advantages that RNAs are Generally Recognized As Safe (GRAS), are already consumed in many forms, including small RNAs, and could fit into established procedures for evaluation of biotech products, such as environmental risk assessments (9, 10, 11, 12). Early examples of engineered suppression were typically accomplished by expression of single stranded transcripts under control of strong promoters (*e.g.*, viral promoters) with a resulting tendency to stimulate recognition of the transcripts by the cell. This recognition initiates the conversion of the single-stranded RNA transcripts to long dsRNA by RNA-dependent RNA polymerase (RdRP). The long dsRNA is, in turn, diced to small RNAs that fuel the cycle of suppression. Revelation of the central role of dsRNA in suppression has lead to the development of transgenic approaches that create small RNAs directly and more efficiently than the sense or antisense strategies. One methodology is to engineer transcripts with complementary inverted repeat sequences to the intended target so that the resulting RNA efficiently folds back upon itself to form dsRNA (a "hairpin"), obviating the need to be recognized and converted by RdRP. Strong suppression is often possible with hairpins because multiple siRNAs can be cleaved from a single precursor and simultaneously target a transcript. Creation of artificial miRNA genes offers another approach for small RNA trigger generation. Replacement of the sequence in a native miRNA precursor with 21-24 bases matching the intended target can redirect production of a single small RNA species and drive suppression of a new target. Artificial miRNAs offer highly restricted specificity but are sensitive to inactivation by sequence variation in the target transcript (*e.g.*, if an allelic variant exists of the target, mismatched base pairing can foil suppression). Utility of either inverted repeats or artificial

miRNAs transcripts can be enhanced by choice of promoters and other expression elements that align with the needs of target suppression.

From this basic toolbox of RNA-based regulation, numerous applications have arisen in crops and some have entered commerce (reviewed in 8). Engineered virus resistance was one early application. Overexpression of viral coat protein transcripts conveyed resistance and invoked small RNA production against the target virus, likely contributing to the observed long standing resistance seen in virus resistant squash and papaya released in the mid 1990's. More recent entrees to the market place have directly used efficient inverted repeats, *e.g.*, plum pox resistant plum, to confer resistance. Modification of endogenous plant gene expression for improved traits soon followed viral resistance and applications in other crops are in various states of development or de-regulated such as, modified soy oil composition, reduced alfalfa lignin content, altered potato starch content, increased corn nutritional content and reduced caffeine content in coffee (8). The spectrum of RNAi tools utilized is diverse, including inverted repeats to modify seed specific gene expression of a soy fatty acid biosynthetic gene to an artificial miRNA to reduce a catabolism of lysine and thus improve the corn grain content for this essential amino acid (8, 13).

In recent years, several examples have been published where the target of plant-produced RNAi is not a native plant gene or intracellular viral transcript, but rather a specific exogenous pest or pathogen transcript. These achievements were enabled by the initial discovery that the nematode, *Caenorhabditis elegans*, could ingest dsRNA and initiate RNAi within its cells (14). The observation has been extended to several invertebrate species leading to the effective engineering of dsRNA production *in planta* with concomitant suppression of target transcript in a pest species, *e.g.*, as in nematode (root-knot nematode), beetle (corn rootworm), and moth larvae (bollworm) (15, 16, 17). Gene suppression in a fungus (powdery mildew) and even a parasitic plant (*Triphysaria*) has been demonstrated with transgenic plants, expanding the potential realm of control to a broader spectrum of weeds and pathogens (18, 19). All of these responsive species have direct predatory, parasitic or pathogenic relationships with their host and harbor a resident faculty for internalizing plant-produced dsRNA. When the pest-targeted dsRNA is essential to the core biology of the organism or its ability to establish on the host, opportunities for plant protection are created. Because this modality of control is distinct from current biotech pest control products (*e.g.*, Bt proteins), RNAi presents the potential for insect resistance management by combining strategies.

Research continues to reveal new mechanisms and interconnectivity amongst RNA-based regulatory pathways in plants. Small RNAs not only participate in suppression of transcripts (RNAi), but can modify the chromatin environment where genes reside through sequence-based targeting of expression elements (thereby decreasing expression), and in some cases even drive up-regulation of a gene by their activity (20, 21). Further, plant cells have natural means to antagonize and regulate small RNA activity via transcripts that mimic miRNA binding sites, leading to transgenic opportunities to engineer and drive traits based on moderation of RNAi function (22). Finally, long non-coding RNAs are an additional interface with these regulatory pathways in plants and represent an emerging area of discovery in plant gene regulation (23).

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3.3 THE ROLE OF PROBLEM FORMULATION IN ERA

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Assessment of the risks posed by cultivation of transgenic crops is often said to be complex; for example, Wolfenbarger and Phifer (2000) state that "the complexity of ecological systems presents considerable challenges for experiments to assess the risks and benefits and inevitable uncertainties of genetically engineered plants". From the supposed complexity of ecological systems it is inferred that many data are needed before the risks posed by cultivation of transgenic crops can be adequately assessed, and that the more data one obtains, the better will be decisions based on those assessments.

The above analysis is wrong because scientific complexities and uncertainties are often irrelevant to the assessment of risk. Failure to distinguish between important and unimportant questions for the risk assessment, regardless of how interesting those questions may be for other activities such as fundamental scientific research, makes assessments unnecessarily complex. Problem formulation is a method by which those important questions, and simple ways to answer them, may be identified.

Risk assessment is scientific; that is, it follows the scientific method. One idea of the scientific method, known as empiricism, is that science begins with observations made without preconceptions. Once one has collected sufficient observations, one may conclude that certain things are true; for example, after observing many white swans, and no non-white swans, one may think that one has proved that all swans are white. There is, however, a flaw in this reasoning because no matter how many white swans are observed, one can never exclude the possibility that a non-white swan exists. Similarly, any number of observations of a transgenic crop cannot prove that its cultivation will be safe; that is, it will never cause harm.

In the 1930s, Karl Popper offered a solution to the logical problems of empiricism. He proposed that science begins with problems, not with observations. Once a problem is identified, we propose tentative solutions to the problem, and we make observations to identify errors in our solutions. Knowledge increases by showing that particular solutions – in science, theories and hypotheses – are false, and by making better solutions to eliminate those errors. The hypothesis that all swans are white is tested by making observations of swans. If a non-white swan is observed, that hypothesis is falsified and may be replaced by a hypothesis that explains the distribution of swan coloration. The new hypothesis represents an increase in our knowledge.

Popper's solution to the logical basis for the scientific method may be represented by a simple scheme: → initial problem [P_1] → tentative solution [TS_1] → error elimination [EE_1] → new knowledge and a new problem [P_2] →. This scheme may be applied to environmental risk assessment for the cultivation of a transgenic crop: decide what constitute harmful effects of cultivating the crop [P_1] → hypotheses that cultivation of the crop will not cause those harmful effects [TS_1] → test those hypotheses [EE_1] → increased knowledge of risk [P_2] → new hypotheses, including decision-making [TS_2] →. Under this scheme, safety of the crop could not be proved, but one may judge that cultivation of the crop is safe if the hypotheses of no harm are corroborated under rigorous testing.

Problem formulation comprises decisions about what should be regarded as harmful, production of hypotheses that cultivation of the crop will not cause harm, and a plan to test those hypotheses. Problem formulation may simplify the risk assessment at each stage:

P_1 – The task is to decide what constitute harmful effects of cultivating the crop; it is not to produce a list of all the effects that could happen. For regulatory risk assessments, decisions about harmful effects should be guided by laws, regulations and other instruments of policy. Problem formulation focuses risk assessment on the question "what is the probability that something harmful will happen if the transgenic crop is cultivated?", which is much simpler than the question "what will happen if the transgenic crop is cultivated?"

TS_1 – The task is to produce a conceptual model indicating scenarios by which cultivation of the transgenic crop could lead to the harmful effects identified in P_1: cultivation → event A → event B → event C → event D (= harm). Each step in the pathway can give a hypothesis of no harm; for example, event A will not occur, event B will occur below a certain frequency or magnitude, event C will not occur where it can cause harm, and so on. Each of these hypotheses is testable. An important property of these hypotheses is that the objective is categorisation to help decision-making, not precision for production of fundamental knowledge. This greatly simplifies the risk assessment because it is easier to test whether a value is above or below a threshold, or an event is likely to occur more frequently than it occurs currently, that it is to predict and quantify something precisely. Indeed, attempts at precise quantification are likely to confuse unless one has clear, quantitative decision-making criteria.

EE_1 – Confidence in the conclusions of a risk assessment relates to the rigour with which the hypotheses are tested. The rigour of a test comes from its ability to produce observations contrary to the predictions of the hypothesis under test. In risk assessment, where the hypotheses postulate the absence of effects, or at least effects below a threshold, tests under controlled conditions in a laboratory or glasshouse are often more rigorous than field studies; for example, if one wished to test the hypothesis that crop X does not hybridise with wild species Y, crossing X and Y in the laboratory would be a more rigorous test than a field survey of populations of Y looking for X x Y hybrids. If X and Y were found to hybridise

in the laboratory, then field surveys could be done to assess the significance of that finding; if no hybrids were produced in the laboratory, field studies would not be necessary to complete the risk assessment. Problem formulation can simplify risk assessment by identifying the most rigorous test and eliminating the need for complex, realistic tests unless the rigorous tests falsify the hypothesis.

In conclusion, environmental risk assessment for the cultivation of transgenic crops need not be complex. Good problem formulation can produce simple and effective risk assessment by focusing effort on predicting the probability of harmful effects, by formulating hypotheses that make categorical predictions about events necessary for harm to occur, and by suggesting tests of hypotheses that favour rigour over realism and complexity.

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3.4 USEPA REGULATORY FRAMEWORK AND MANAGEMENT GOALS

Chris Wozniak, Ph.D., Biotechnology Special Assistant, USEPA Office of Pesticide Programs, Washington D.C.

The U.S. Environmental Protection Agency (USEPA) regulates pesticides, including plant-incorporated protectants (PIPs) wherein the pesticidal substance is expressed *in planta*. As part of the environmental risk assessment, USEPA examines the product characterization, potential for environmental effects, and the environmental fate of the pesticide. Under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), USEPA regulates pesticides, including their sale, use and distribution. USEPA also sets either a numerical food tolerance or an exemption from the requirement of a tolerance under the Federal Food Drug and Cosmetic Act (FFDCA) for all pesticides applied to food or feed crops. All PIPs to date have received an exemption from the requirement of a tolerance.

The majority of PIPs registered to date produce proteinaceous pesticidal substances, predominantly δ-endotoxins from *Bacillus thuringiensis* (*Bt*). Data requirements for PIPs intended to examine toxicity to non-target organisms as well as environmental fate protocols utilize proteins (*e.g.*, *Bt*- δ-endotoxins) as the test substance and, in that regard, differ greatly from RNAi-based PIPs, such as those based upon viral coat protein gene sequences. The plum pox virus resistant Honeysweet C5 plum was recently registered and a significantly reduced set of data requirements was applied based primarily on the lack of a protein component to the PIP pesticidal substance.

Based upon the specificity of the gene sequence transformed into European plum for this purpose and the relatively limited exposure to the environment, many of the potential avenues for non-target effects were considered as highly unlikely to result in adverse effects. European plum (*Prunus domesticus*) does not hybridize with native American plums (*e.g.*, *P. angustifolia*, *P. americana*) due to ploidy differences, so impacts of gene flow were not a required consideration.

The environmental fate of dsRNA products derived from RNAi-based PIPs remains to be determined empirically and the USEPA is in the process of developing guidelines to indicate the type of testing which may be needed to determine a DT_{50} (*i.e.*, degradation time to 50% loss) for dsRNA. At this juncture, it is not clear if the sequence of the RNA is critical to the assessment such that a one-time assay in soil typical of the area where the PIP crop would be cultivated may be sufficient for dsRNAs in general. The influence of hairpins and other secondary structures resulting from RNA sequence mismatches is not known relative to environmental stability and will require further investigation.

All nucleic acids have previously received an exemption from the requirement of a tolerance as they are considered as being widely consumed over time with no demonstrated ill effects. This addresses issues of human dietary consumption relative to toxicity and allergenicity, however, selection of surrogate non-target species for a particular PIP will be made upon a case-by-case basis. While RNA based products are considered to have considerable specificity based upon the need for complementarity for transcriptional or translational suppression in the target pest, the degree to which this sequence similarity limits non-target effects and what insight we may gain from a bioinformatics approach to determine the probability of activity *in situ* remain to be seen.

3.5 APHIS REGULATORY FRAMEWORK AND MANAGEMENT GOALS

John Cordts, M.Sc., Supervisory Biotechnologist/Branch Chief, Environmental Risk Analysis Program, USDA/APHIS Biotechnology Regulatory Services, Riverdale, MD

USDA/APHIS has been regulating the development of genetically engineered organisms since the late 1980s using regulations found in 7 Code of Federal Regulations part 340. APHIS regulates the importation, interstate movement and environmental release of genetically engineered organisms under plant pest provisions of the Plant Protection Act of 2000. As such, the noted activities with genetically engineered organisms require authorization from APHIS. In addition to authorizing importations, movements and releases of genetically engineered organisms, APHIS inspects field trials, conducts training and workshops for customers, and conducts enforcement activities to ensure compliance with the regulations.

When a developer has generated sufficient data, they may "petition" APHIS to make a determination that the organism should no longer be regulated. A typical petition includes agronomic data collected from several years of field trials, a molecular characterization of the genetically engineered organism, a description of the crop biology and other relevant experimental data and/or publications. APHIS' primary issues in assessing a genetically engineered plant relate to potential increases in disease or pest susceptibilities, increased weediness or invasiveness of the engineered plant or wild relatives, increased damage to processed agricultural commodities, increased harm to non-target and beneficial organisms, and adverse plant pest impacts that might result from changes in cultivation practices.

Since, 1992 APHIS has deregulated at least 10 products involving gene-silencing, now known to be RNAi-mediated processes (several slow ripening tomatoes, virus resistant squash, virus resistant papaya, virus resistant potatoes, reduced nicotine tobacco, high oleic soybean, and virus resistant plum). APHIS has not identified specific issues with RNAi products that would substantively alter our current risk assessment methods and thus has not felt the need to issue guidance specific to these products.

4. DISCUSSION

Effective problem formulation (see section 3.3.) requires a determination of what constitutes an adverse effect, and this is guided by the protection or management goals defined in legal instruments or policy documents of the pertinent regulatory authorities. For the purposes of this conference, participants were asked to focus their discussions on the management goals used by USEPA and USDA/APHIS in their regulation of genetically engineered plants (see sections 3.4 and 3.5) and that are most relevant to environmental safety, as summarized below:

- No increased adverse effect to other organisms;
- No increased weediness of the crop plant;
- No gene flow to sexually compatible plants leading to increased weediness or altered exposure scenarios leading to adverse effects;
- No increased disease and pest susceptibilities;
- No increase in adverse effects due to changes in cultivation practices.

For each case study plant, scenarios by which the cultivation of the crop could potentially lead to an adverse environmental impact were identified. Plausible risk hypotheses and relevant information to test these hypotheses, including the applicability of existing information and the need for new experimentation, were also discussed and then summarized (see Annexes 3, 5, 7 and 9; the scenarios, hypotheses, and information in these tables are summaries of the problem formulation discussions).

4.1 TESTING THE RISK HYPOTHESES

In all of the cases discussed, it was determined that the testing approaches and protocols that have been used to assess currently approved genetically engineered crops can also be used to adequately assess RNAi-engineered crops. The comparative approach remains foundational to the ERA of RNAi plants: information about the biology of the host plant is critical regardless of the mechanism of genetic modification; and the results of comparative analyses of the transgenic and non-transgenic plants can be used to test hypotheses related to weediness and gene flow, and disease and pest susceptibility. Understanding current crop cultivation practices and how the genetic modification could lead to changes in these can be used to determine if these might lead to adverse environmental effects. This information is relevant to phenotype, regardless of the modification method used to develop the genetically engineered plant.

Similar tests and protocols to those used currently to assess direct adverse impacts of an insecticidal, transgenic plant on non-target arthropods and other organisms can also be used with RNAi-modified crops[3]. For currently approved products, especially *Bt* crops expressing insecticidal proteins, the exposure and potential hazard to non-target organisms is assessed using a tiered approach where early tier laboratory studies are conducted at concentrations that exceed those likely to be encountered in the environment (typically 10X the expected environmental concentration). The USEPA typically requires early tier toxicity testing of the plant incorporated protectant (PIP; see section 3.4) using a series of surrogate species representing key functional groups. Further characterization of a PIP in additional laboratory or higher-tier semi-field or field experiments is only required if effects are seen under laboratory conditions at high test substance concentrations. Activity spectrum testing is performed early in the assessment process to aid in the selection of appropriate non-target organisms. Selection criteria for test organisms for inclusion in activity spectrum testing are determined by taxonomic relatedness to the target organism, the likelihood of exposure, and amenability to toxicity testing.

3 For further information about non-target arthropod testing see: Romeis *et al*. (2011). Recommendations for the design of laboratory studies on non-target arthropods for risk assessment of genetically engineered plants. *Transgenic Research* 20: 1-22. http://cera-gmc.org/index.php?action=publications.

In the case studies where the RNAi pathway has been used to introduce insecticidal or nematicidal properties to the crop, the spectrum of activity would need to be determined. It is anticipated that these pesticidal RNAi-traits should have a narrow spectrum of activity, since they are designed to target a single or a group of closely related species. Taxonomic relatedness to the target organism would be an appropriate starting point to define the spectrum of activity, as the identity of the orthologous target sequences should decrease with taxonomic distance. For example, in the case of the insect resistant corn that incorporates an RNAi-trait to target corn rootworm, toxicity testing could potentially be limited to other coleopterans, particularly those related to the target pest as published studies have indicated a high level of insecticidal specificity for dsRNAs targeting vATPase transcripts (see references in Annex 2). In the case of the nematode resistant soybean, the potential effects on related beneficial nematodes could be tested by feeding studies with representative species. dsRNA can be synthesized *in vitro* and used in early tier tests, a situation analogous to using purified recombinant *Bt* proteins produced in microbial expression systems such as *Escherichia coli*.

4.2 DETERMINING ENVIRONMENTAL FATE

Potential routes of exposure, *i.e.*, the environmental fate of the protein or the degradation rates in different receiving environments, such as soils or water, are also considered in non-target organism assessments of PIPs. The assessments consider whether sensitive organisms will be exposed in their natural environment at levels that would be detrimental. For RNAi applications, data on expression levels of the active, small RNAs (or their precursors) in different plant tissues, as well as data on the environmental fate of these molecules in the surrounding environment, can be used to determine routes of exposure to other organisms. Data on environmental fate may not be necessary if no sensitive organisms are identified; however, the environmental fate of the RNAs that mediate RNAi has not been thoroughly studied.

Participants discussed what RNA molecules should be monitored in environmental fate studies of RNAi plants that are PIPs. The active RNA molecules in RNAi applications are the small RNAs: either the siRNAs that derive from fully double-stranded RNA precursors or the artificial microRNAs that derive from partially double-stranded RNA precursors. Each of these putative RNA samples present problems with regard to detection in environmental fate studies. The precursor molecules (either dsRNA or partially dsRNA) are rapidly processed into small RNAs *in vivo* and therefore accumulate to very low levels making their detection problematic. In addition, the small RNAs, although they accumulate to relatively high levels, are difficult to detect due to their size. Participants considered whether viroids, such as potato spindle tuber viroid, might be a suitable model for environmental fate studies because viroid RNA structure bears some similarity to microRNA precursors. After some discussion, it was accepted that viroid RNA would not be an appropriate surrogate because, unlike the small RNA precursors used in the RNAi technologies, viroid RNAs are circular molecules and would therefore display much different kinetics of degradation than the authentic RNAi molecules (which have free ends). Participants agreed that a single, well designed and comprehensive study of the environmental fate of dsRNAs, partially dsRNA or small RNAs should be generally applicable for all RNAi applications.

4.3 INFORMATION FROM BIOINFORMATIC ANALYSIS

RNAi-induced traits in genetically engineered plants, unlike protein based traits, are dependent on primary nucleic acid sequence identity. Participants considered if this requirement for sequence-specificity could be utilized to include the application of bioinformatic analyses for evaluating potential negative impacts to non-target organism. Examples where relevant information specific to the RNAi plant could be used for this purpose are highlighted using *italics* in the discussion summary tables (see Annexes 3, 5, 7 and 9).

Discussions of the insect resistant corn and nematode resistant soybean case studies focussed on the potential for adverse effects on non-target organisms (the target organisms being corn rootworm and soybean cyst nematode, respectively). In both examples, efficacy against the target pest is achieved through induction of the RNAi pathway via the production of a dsRNA that is homologous to the sequence of a specific gene in the target organism. The pesticidal phenotype of these plants is dependent on nucleic acid sequence identity, making it possible to compare the sequence of the target

gene to orthologous gene sequences from other organisms as a means of identifying the potential for adverse impacts on non-target organisms.

Extensive studies have already been performed to determine the parameters, including degree of sequence identity, that are necessary for effective RNAi activity[4]. Participants agreed that, as more sequence data becomes available, it should be possible to define the thresholds for the activity spectra based on shared sequence identity, i.e., there may be genes for which there is clearly no sequence homology between classes of organisms, and this could also reduce the number of non-target species that may need to be tested. There was some discussion about whether lack of homologous sequence in a non-target organism could be used alone to exclude further laboratory based, early tier testing. Although most agreed that this should be possible, it was considered premature to exclude early-tier testing at this time as additional validation of a bioinformatics-based approach to addressing non-target effects is required.

4 Baum, J. A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T. and Roberts, J. (2007). Control of coleopteran insect pests through RNA interference. *Nature Biotechnology* 25: 1322–1326.

5. CONCLUSIONS

Following extensive discussions by the breakout groups and in the final plenary session, the conference participants achieved the following points of consensus:

1. The paradigm currently applied to the environmental risk assessment of genetically engineered plants is adequate for the assessment of RNAi plants.
2. No plausible risk hypotheses were identified that can be considered unique to RNAi mechanisms when compared to other genetically engineered plants with similar traits.
3. The same tests and protocols that are used for evaluating other genetically engineered plants will be sufficient for testing RNAi plants, including plants expressing pesticidal traits.
4. The use of RNAi technologies allows for the use of alternative, informative tests, such as bioinformatic analyses, to address certain risk questions.
5. For plants expressing pesticidal dsRNAs, bioinformatics can be applied to characterize potential susceptibility of relevant non-target species.
6. The accumulation of bioinformatic data that defines thresholds for activity spectra based on shared sequence identity will reduce the need for non-target organism testing.
7. Baseline data about environmental fate of dsRNA will be broadly useful for future exposure analyses.

ANNEX 1 — CONFERENCE AGENDA

JUNE 1, 2011 (West Conference Room)

Time	Title	Presenter
0900	Welcome and Introductions	Morven McLean *CERA*
0910	Introduction to RNAi Q&A	Vicki Vance *University of South Carolina*
0950	RNAi Applications in the Plant Sciences Q&A	Greg Heck *Monsanto Company*
1030	**Break**	
1100	The Role of Problem Formulation in ERA Q&A	Alan Raybould *Syngenta*
1140	USEPA Regulatory Framework and Management Goals Q&A	Chris Wozniak *USEPA*
1220	APHIS BRS Regulatory Framework and Management Goals Q&A	John Cordts *USDA APHIS BRS*
1300	**Lunch**	
1400	Presentation of Case Study 1: RNAi for Insect Control Q&A	Jim Masucci *Monsanto Company*
1440	Presentation of Case Study 2: RNAi for Nematode Control Q&A	Ben Matthews *USDA ARS*
1520	Presentation of Case Study 3: RNAi for Nutritional Modification Q&A	Jennifer Anderson *Pioneer Hi-Bred*
1600	**Break**	
1630	Presentation of Case Study 4: RNAi for Reducing Endogenous Allergens Q&A	Eliot Herman *Donald Danforth Plant Science Center*
1710	Introduction for Breakout Group Activity	Karen Hokanson *University of Minnesota*
1730	**Close of Day 1**	

JUNE 2, 2011 (Breakout Rooms)

	Breakout Group Locations:	Group 1: West Conference Room A Group 2: West Conference Room B Group 3: Malaspina Room Group 4: Board Room
0830	Group 1: Case Study 1 || Group 2: Case Study 2 || Group 3: Case Study 3 || Group 4: Case Study 4	
1000	**Break**	
1030	Breakout Groups (continued)	
1200	**Lunch**	
1300	Group 1: Case Study 3 || Group 2: Case Study 4 || Group 3: Case Study 1 || Group 4: Case Study 2	
1430	**Break**	
1500	Breakout Groups (continued)	
1630	Preparation for Reports to Final Plenary Session	
1700	**Close of Day 2**	

JUNE 3, 2011 (West Conference Room)

0830	Reports from Breakout Groups (15 min per group, per case study) Case Study 1: Group 1, Group 3 Case Study 3: Group 3, Group 1	|| Case Study 2: Group 2, Group 4 Case Study 4: Group 4, Group 2
1030	**Break**	
1100	Group Discussion on Consensus Points from Breakout Group Sessions	Convener: Morven McLean
1230	**Close of Conference**	

ANNEX 2 — INSECT RESISTANT CORN (CASE STUDY 1)

James Masucci, Ph.D., Monsanto Company, Creve Coeur, MO

INTRODUCTION

Corn rootworm is an important corn pest causing significant losses to U.S. farmers. They are a highly adaptable pest complex, having developed resistance to chemical and crop rotation control strategies. Baum *et al*. (2007) demonstrated that harnessing the rootworm's RNAi pathway through a double-stranded RNA-based plant incorporated protectant (PIP) has potential for a new, highly specific tool for corn rootworm management. RNAi-based PIP products for corn rootworm control are currently in phase 2 development, on track for commercialization. In phase 2, the product concept undergoes such key activities as trait development, large-scale transformation, and preliminary regulatory data generation.

PART I: THE NON-TRANSGENIC PLANT

General Description of maize

See OECD (2003)

Reproductive Biology of maize

See OECD (2003)

Center of origin and Center(s) of genetic diversity

See OECD (2003)

Means of dispersal and establishment

See OECD (2003)

Modern-day corn cannot survive outside of cultivation (Gould and Shaw, 1968), and volunteer corn is not found growing in fence rows, ditches, and roadsides as a weed. It is difficult for corn to survive as a weed because of past selection in the evolution of corn. Seed dispersal of individual kernels does not occur naturally because of the ear structure of corn. Individual corn kernels, however, can be distributed during grain harvest and transport to storage facilities. In neither instance (natural or mechanical harvesting) does corn become a troublesome weed. Although corn from the previous crop year can overwinter and germinate the following year, it cannot persist as a weed. The appearance of corn in soybean fields following the corn crop from the previous year is a common occurrence. Measures often are taken to eliminate the plants in soybean fields, and any plants that remain and produce seed usually do not persist in the following years.

Intra-specific, inter-specific, and/or inter-generic hybridization

See OECD (2003)

Annual teosinte (*Zea mays* subsp. *mexicana*) and corn are interfertile species (Wilkes, 1972 and 1989). Corn and teosinte are genetically compatible and in areas of Mexico and Guatemala they freely hybridize when they are in proximity to each other and other conditions are favorable. Teosinte exists primarily as a weed around the margins of corn fields, and the frequency of hybrids between teosinte and corn has been studied. A frequency of one F_1 hybrid (corn × teosinte) for every 500-corn plants has been reported for the Chalco region of the Valley of Mexico (Wilkes, 1972). The F_1 hybrid of teosinte by corn is robust and fertile and is capable of backcrossing to corn. Intercrossing and gene exchange between teosinte and corn occurs freely, and, accompanied by selection, teosinte has had a significant role in the evolution of corn. Evans and Kermicle (2001) have shown that although corn can introgress into teosinte, there is incompatibility

between some corn populations and certain types of teosinte, resulting in low fitness of some hybrids that prevents a high rate of introgression.

Although corn easily crosses with teosinte, the natural distribution of teosinte is limited to the seasonally dry, subtropical zone with summer rain along the western escarpment of Mexico and Guatemala and the Central Plateau of Mexico (Wilkes, 1972; Gonzalez and Corral, 1997).

PART II: THE RECEIVING ENVIRONMENT

Cultivation of the host plant in the intended country of deployment

Corn (*Zea mays* L.), or maize, is one of the few major crop species indigenous to the Western Hemisphere. It is a member of the *Maydeae* tribe of the grass family, *Poaceae*. It is a robust monoecious annual plant, which requires the help of humans to disperse its seeds for propagation and survival. Corn is an efficient plant for capturing the energy of the sun and converting it into food, and adapts readily to different conditions of humidity, sunlight, altitude, and temperature.

Corn is grown in nearly all areas of the world and ranks third behind rice (*Oryza sativa* L.) and wheat (*Triticum* sp.) in total global production. In 2004, corn was planted globally on 146.7 million ha with a total production of 723.9 million metric tones (MMT) (FAOSTAT, 2006). The top three production countries in 2004 were: U.S.A. (299.9 MMT), China (130.4 MMT) and Brazil (41.8 MMT). In the U.S., corn is grown in almost all the states, and in 2004, it produced 11.8 billion bushels of corn grain with a market value of $24 billion (USDA-NASS, 2006).

In industrialized countries corn has two major uses: 1) as animal feed in the form of grain, forage or silage; and 2) as a raw material for wet- or dry-milled processed products such as high fructose corn syrup, oil, starch, glucose, and dextrose (Tsaftaris, 1995). These processed products are used as ingredients in many industrial applications and in human food products. In developing countries, corn is used in a variety of ways. In Latin American countries such as Mexico, one of the main uses of corn is for food. In Africa, corn is consumed as a food in the sub-Saharan region, and in Asia it is generally used to feed animals (Morris, 1998).

Presence of any sexually compatible relatives in the receiving environment

Although corn easily crosses with teosinte, teosinte is not present in the U.S. corn belt. The natural distribution of teosinte is limited to the seasonally dry, subtropical zone with summer rain along the western escarpment of Mexico and Guatemala and the Central Plateau of Mexico (Wilkes, 1972; Gonzalez and Corral, 1997).

Ecological interactions in the receiving environment

No weedy species related to corn or sexually compatible relatives have been observed in the United States.

The most relevant pests are listed below:

Emergence to Knee High (VE to V8)

Arthropods	Scientific Name	Scientific Classification
Seedcorn maggots	*Delia Platura*	Anthomyiidae : Diptera
White Grubs	*Phyllophaga* spp.	Coleoptera : Scarabaeidae
True wireworms	*Numerous* spp.	Coleoptera : Elateridae
False wireworms	*Numerous* spp.	Coleoptera : Tenebrionidae
Corn flea Beetles	*Chaetocnema pulicaria*	Coleoptera : Chysomelidae
Black cutworms	*Agrotis ipsilon*	Lepidoptera : Noctuidae
Common stalk borers	*Papaipema nebris*	Lepidoptera : Noctuidae
Grape Colaspis larva	*Colaspis brunnea*	Coleoptera : Chrysomelidae
Billbugs	*Sphenophorus* spp.	Coleoptera : Curculionidae
Thrips	*Frankliniella* spp.	Thysanoptera : Thripidae
Southern corn leaf beetles	*Myochrous denticollis*	Coleoptera : Chrysomelidae
Armyworms	*Spodoptera* spp.	Lepidoptera : Noctuidae
Chinch bug	*Blissus Leucopterus*	Hemiptera : Lygaeidae

Knee High to Tassel (V8 to VT)

Arthropods	Scientific Name	Scientific Classification
Corn rootworms (larvae and adults)	*Diabrotica* spp.	Coleoptera : Chysomelidae
Armyworms	*Spodoptera* spp.	Lepidoptera : Noctuidae
Fall Armyworms	*Spodoptera frugiperda*	Lepidoptera : Noctuidae
Corn Earworms	*Helicoverpa zea*	Lepidoptera : Noctuidae
European corn borers	*Ostrinia nubilalis*	Lepidoptera : Pyralidae
Southwestern corn borers	*Diatraea grandiosella*	Lepidoptera : Pyralidae
Grasshoppers	*Numerous* spp.	Orthoptera : Acrididae
Leafhoppers	*Dalbulus maidis*	Homoptera : Cicadellidae
Corn leaf aphids	*Rhopalosiphum maidis*	Homoptera : Aphididae

Tassel to Maturity (VT to R6)

Arthropods	Scientific Name	Scientific Classification
Corn rootworms (adults)	*Diabrotica* spp.	Coleoptera : Chysomelidae
Fall Armyworms	*Spodoptera frugiperda*	Lepidoptera : Noctuidae
Corn Earworms	*Helicoverpa zea*	Lepidoptera : Noctuidae
European corn borers	*Ostrinia nubilalis*	Lepidoptera : Pyralidae
Southwetern corn borers	*Diatraea grandiosella*	Lepidoptera : Pyralidae
Western bean cutworm	*Richia albicosta*	Lepidoptera : Noctuidae
Grasshoppers	*Numerous* spp.	Orthoptera : Acrididae
Corn leaf aphids	*Rhopalosiphum maidis*	Homoptera : Aphididae

PART III: THE TRANSGENIC PLANT

Method used to introduce the novel trait

This product was generated through *Agrobacterium*-mediated transformation of corn with the transformation vector pMON94805. The T-DNA in pMON94805 vector contains an expression cassette that generates dsRNA to a specific region of the WCR V-ATPase A coding region (Baum *et al.*, 2007).

The purpose of the transformation and mode of action

This product utilizes the RNA interference (RNAi) pathway of corn rootworm as a mechanism for control. This mode of action is distinct from the Bt toxin-based plant incorporated protectants (PIPs). Multiple modes of action ensure the long term effectiveness of plant-produced corn rootworm control through insect resistance management (IRM) prac-

tices. The development of resistant insect strains is markedly impaired when multiple modes of action are pyramided into a single product (Roush, 1998).

This product expresses a double stranded (ds)RNA that is identical to a small region of the V-ATPase gene of western corn rootworm. Upon ingestion by corn rootworm, the dsRNA enters the insect cells and induces the RNAi pathway. The dsRNA is diced into small, interfering RNAs (siRNA) specific to the V-ATPase gene causing a reduction in the levels of the endogenous V-ATPase mRNA and ultimately resulting in larval mortality.

The reduction of V-ATPase mRNA in the corn rootworm appears to be due to the sequence-specific nature of the RNAi pathway and not due to a general reduction in transcription since no effect was observed in the level of alpha-tubulin expression in corn rootworm larvae fed V-ATPase dsRNA. In addition to the sequence specificity of RNAi, only a few species, all of them invertebrates, have been shown to exhibit orally induced down regulation of gene expression through the RNAi pathway. The corn rootworm species *Diabrotica virgifera virgifera* and *D. undecimpunctata howardii*, are two examples of insects that are susceptible to orally ingested dsRNA (Baum *et al.*, 2007).

The anticipated cultivation regions

This product is intended for broad acre cultivation in any area where corn is normally grown.

Inheritance and stability of each introduced trait

During development, segregation data will be recorded to assess the heritability and stability of the expression cassette present in this product. Chi square analysis will be performed over several generations to confirm the segregation and stability of the insert. Chi square analysis is based on testing the observed segregation ratio to the expected segregation ratio according to Mendelian principles. Stability has been assessed by root protection in several generations. Root protection has been stable through five generations and no anomalies in inheritance patterns have been noted.

A summary of the introduced genetic elements in pMON94805

Genetic Element	Size	Function
RB	0.3 kb	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker *et al.*, 1982; Zambryski *et al.*, 1982)
e35S promoter	0.6 kb	Promoter and 5' UTR sequence from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell *et al.*, 1985) containing the duplicated enhancer region (Kay *et al.*, 1987) that directs transcription in plant cells
hsp70 intron	0.8 kb	Intron and flanking exon sequence of the DnaK gene from *Zea mays* encoding the heat shock protein 70 (HSP70) (Brown and Santino, 1997)
V-ATPase antisense	0.2 kb	Partial coding region from a putative vacuolar proton pump alpha subunit 2 from western corn rootworm.
Spacer		Allows for the formation of a stem-loop structure
V-ATPase sense	0.2 kb	Partial coding region from a putative vacuolar proton pump alpha subunit 2 from western corn rootworm.
Hsp17 3' end	0.2 kb	3' UTR sequence from a heat shock protein, Hsp17, of *Triticum aestivum* (wheat) (McElwain and Spiker, 1989) that directs polyadenylation of the mRNA
LB	0.4 kb	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker *et al.*, 1983)
OriV	0.4 kb	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker *et al.*, 1981)
rop	0.2 kb	Coding sequence for repressor of primer protein from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Ori322	0.6 kb	Origin of replication from plasmid pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979)
aad	0.9 kb	Bacterial promoter, coding sequence, and 3' UTR for an aminoglycoside-modifying enzyme, 3''(9)-O-nucleotidyltransferase from the transposon Tn7 (Fling *et al.*, 1985) that confers spectinomycin and streptomycin resistance

Differences in genetic and phenotypic variability from non-transgenic crop

Although no off-types have been observed during several years of field trials at several locations, specific data evaluating phenotypic differences from conventional comparators are not available. The plant characterization will follow the existing paradigm for traits derived from biotechnology and will encompass five general data categories: 1) germination, dormancy, and emergence; 2) vegetative growth; 3) reproductive growth (including pollen characteristics); 4) seed retention on the plant and lodging; and 5) environmental interactions (plant response to abiotic stress and interactions with diseases and arthropods). An overview of the characteristics assessed is presented in the table below.

The phenotypic, agronomic, and environmental interactions data will be evaluated from a basis of familiarity (Hokanson, *et al.*, 1999; OECD, 1993) and will be comprised of a combination of field and laboratory studies conducted by scientists who are familiar with the production and evaluation of maize. In each of these assessments, this product will be compared to a conventional control that has a similar genetic background but does not possess the V-ATPase A expression cassette. In addition, multiple commercial maize references will be included to provide a range of comparative values that are representative of existing commercial maize hybrids for each measured phenotypic, agronomic, and environmental interaction characteristics. The commercial references provide a range of variation for characteristics and a context for interpreting experimental results.

*Differences in modes and/or rate of reproduction from non-transgenic crop (*e.g.*, any available out crossing data)*

Reproductive data were discussed above. There is no available data at this time directly measuring modes/rates of reproduction. No obvious differences have been observed. Because maize has no wild relatives in the expected areas of U.S./Canadian cultivation no outcrossing data are necessary.

Expression levels of novel proteins in different tissues over time

The levels of V-ATPase A dsRNA expression have not yet been determined in this product. The typical methods utilized for measuring protein expression are not applicable for an RNAi-based product. V-ATPase dsRNA levels in tissues relevant to the risk assessment will be determined by a validated assay designed to quantify the dsRNA.

Differences in agronomic characteristics from non-transgenic crop

These characteristics are discussed in section 3-6. These assessments will include evaluation of five seed germination parameters, 14 plant growth and development characteristics, and two pollen characteristics. No off-types have been observed during several years of field trials at several locations.

Data category	Characteristics measured	Evaluation timing[1]	Evaluation description
Germination, dormancy, and emergence	Normal germinated[2]	Day 4 and 7 (20/30°C)	Percentage of seed producing seedlings exhibiting normal developmental characteristics
	Abnormal germinated[2]	Day 7 (20/30°C)	Percentage of seed producing seedlings that could not be classified as normal germinated
	Germinated[2]	Day 4, 7, and 12 (5, 10, 20, 30, 10/20 and 10/30°C)	Percentage of seed that had germinated normally and abnormally
	Dead	Day 4 and 7 (5, 10, 20, 30, 10/20, 10/30, and 20/30°C); Day 12 (5, 10, 20, 30, 10/20 and 10/30°C)	Percentage of seed that had visibly deteriorated and become soft to the touch (also included non-viable hard and non-viable firm-swollen seed)
	Viable hard	Day 7 (20/30°C); Day 12 (5, 10, 20, 30, 10/20 and 10/30°C)	Percentage of seed that did not imbibe water and remained hard to the touch (viability determined by a tetrazolium test[3])
	Viable firm-swollen	Day 7 (20/30°C); Day 12 (5, 10, 20, 30, 10/20 and 10/30°C)	Percentage of seed that imbibed water and were firm to the touch but did not germinate (viability determined by a tetrazolium test[3])
	Early stand count	Stage V2 - V4	Number of emerged plants in two rows, standardized to 20 ft rows
	Final stand count	Pre-harvest	Number of plants in two rows, standardized to 20 ft rows
Vegetative growth	Seedling vigor	V2 - V4	Rated on a 1-9 scale, where 1 = good and 9 = poor; a rating of 3 – 6 is normal
	Stay green	Maturity	Rated as: 1 = 90-100% green tissue, 5 = 50-59% green tissue, 9 = 0-19% green tissue
	Ear height	Maturity	Distance from the soil surface at the base of the plant to the ear attachment node
	Plant height	Maturity	Distance from the soil surface to the uppermost node on the main stem of five representative plants per plot
Reproductive growth	Days to 50% pollen shed	Pollen shed	Days from planting until 50% of the plants have begun to shed pollen
	Days to 50% silking	Silking	Days from planting until 50% of the plants have silks exposed
	Pollen viability	Tasseling	Percentage of viable pollen based on pollen grain staining characteristics
	Pollen morphology	Tasseling	Diameter of viable pollen grains
	Grain moisture	Harvest	Percentage moisture of harvested shelled grain
	Test weight	Harvest	Test weight of harvested shelled grain
	Yield	Harvest	Bushels of harvested seed per acre, adjusted to 15.5% moisture

Differences in disease and/or pest susceptibility from non-transgenic crop

These assessments are discussed in section 3-6 and will consist of observations for plant responses to abiotic stress, plant-disease and plant-arthropod interactions. No obvious differences in disease or pest susceptibility have been observed in this product compared to its conventional comparator.

Potential impact on non-target organisms in the receiving environment

Early characterization of the spectrum of activity and mode of action is critical for generating risk hypotheses in the problem formulation phase of the ecological risk assessment. Defining a narrow spectrum of activity supports the approach of testing the standard set of non-target organisms for the purpose of risk assessment.

Laboratory studies have confirmed that ingested insecticidal dsRNA demonstrates a high degree of specificity to the intended target species (Baum *et al.*, 2007; Whyard *et al.*, 2009). This narrow spectrum of insecticidal activity, and consequently low potential for off-target effects, is possible because dsRNAs have been designed to target only a single species or a group of closely related species (*e.g.*, corn rootworms).

Data category	Characteristics measured	Evaluation timing[1]	Evaluation description
Seed retention and lodging	Stalk lodged plants	Pre-harvest	Number of plants per plot broken below the ear
	Root lodged plants	Pre-harvest	Number of plants per plot leaning at the soil surface at >30°from the vertical
	Dropped ears	Pre-harvest	Number of mature ears dropped from plants
Environmental interactions	Plant response to abiotic stress	Four times per growing season	Qualitative assessment of each plot, with rating on a 0-9 scale, where 0 = no symptoms and 9 = severe symptoms
	Disease damage	Four times per growing season	Qualitative assessment of each plot, with rating on a 0-9 scale, where 0 = no symptoms and 9 = severe symptoms
	Arthropod damage	Four times during growing season	Qualitative assessment of each plot, with rating on a 0-9 scale, where 0 = no symptoms and 9 = severe symptoms
	Stalk rot disease	Harvest	Qualitative assessment of each plot (10 plants/plot), with rating on a 0-9 scale, where 0 = no symptoms and 9 = severe symptoms
	Ear and kernel rot disease	Harvest	Qualitative assessment of each plot (10 plants/plot), with rating on a 0-9 scale, where 0 = no symptoms and 9 = severe symptoms
	Corn earworm damage	R5-onset of R6 growth stage	Quantitative assessment using a plastic film grid (size of each grid = 0.5 cm2) for each ear of ten plants per plot. Counted number of grid cells matching the damaged area
	European corn borer damage	Harvest	Quantitative Assessments: Number of live larvae, number of entry and exit holes, number of feeding galleries, and total length of feeding galleries in each stalk of ten plants per plot
	Arthropod abundance	Five collection times during growing season	Quantitative Assessments: Identification and enumeration of non-target pests and beneficial arthropods abundance in sticky trap samples

1 Maize plant growth stages were determined using descriptions and guidelines outlined in Maize Growth and Development (Ritchie *et al.*, 1997).

2 For the 20/30 °C temperature regime both normal and abnormal germination measurements are taken. For all other temperature regimes germination only will be noted.

3 Viability of hard and firm-swollen seed will be determined by a tetrazolium test (AOSA, 2000).

The most developed example of high insecticidal specificity for dsRNA was recently published by Whyard *et al.* (2009). Whyard *et al.*, 2009 examined the insecticidal specificity of ingested dsRNAs through a series of bioassays with several insect orders. Ingestion of species-specific dsRNA targeting vATPase transcripts against flour beetles (*Tribolium castaneaum*), pea aphids (A*cyrthosiphon pisum*), tobacco hornworms (*Manduca sexta*) and fruit flies (*Drosophila melanogaster*) demonstrated activity against the targets. However, ingestion of the dsRNA sequences orthologous to the other tested insect genera resulted in insignificant mortality. The patterns of survival and mortality were strongly related with percent knockdown of the vATPase transcripts in the four insect species following feeding of the different vATPase dsRNAs. Furthermore, Whyard *et al.* (2009) demonstrated a high level of specificity even at the species level within the genus *Drosophila*. This data was presented and reviewed during the presentation of this case study at the meeting.

In a recent review, Huvenne and Smagghe (2010) summarized the effectiveness of orally applied dsRNA in insects. Whereas nanogram quantities of dsRNA resulted in significant effects in some Coleopteran species, microgram quantities were needed to obtain marginal effects in most other insect orders that have been tested. These data show that even within insects, not all species are susceptible to ingested dsRNA. Therefore, the activity spectrum of the dsRNA will not only depend on sequence identity to the target gene, but also on the inherent ability of the organism to respond to orally ingested dsRNA.

As previously discussed, activity spectrum testing is critical for generating risk hypotheses during the problem formulation phase of the ecological risk assessment. The ability to design the dsRNA to target only a single species, or a group of related pest species, allow for a hypothesis based approach for characterizing taxonomic specificity. Species selection for activity spectrum testing will be based upon combinations of taxonomic relatedness, sequence identity and feasibility to perform the bioassay reliably and repeatably.

Provided a high level of specificity is demonstrated within the activity spectrum assays, laboratory NTO testing will be performed with a standard battery of surrogate beneficial test species currently tested for PIPs under USEPA's assessment framework. These species represent the key functional groups that could be exposed in the field environment. The groups include pollinators (*e.g.*, honybees), predators and parasitoids (ladybird beetle, ground beetle, *Orius*, parasitic wasp), and detritivores (earthworm, *Collembola*). Additional species may be selected to support the ecological risk assessment based on the outcome of the problem formulation.

Any available non-target organism data from field studies

None available

*Any available exposure data (*e.g.*, pollen movement, protein dissipation, etc.)*

Pollen morphology, viability, movement, dissipation or other factors affecting environmental exposure are unchanged compared to conventional maize.

The potential for biodegradation of dsRNA from this product in soil will be assessed using laboratory degradation studies. Only if the potential for degradation in soil is not established from the results of laboratory studies would field dissipation studies be conducted. Methods will need to be developed to quantify the degradation of dsRNA in environmental matrices (*e.g.*, quantitative insect bioassay, sequence-specific molecular approaches).

Exposure data in soil will also be used to establish dose levels for non-target testing of soil organisms.

References for any risk assessments undertaken in other jurisdictions.

None

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ANNEX 3 — RISK SCENARIOS AND HYPOTHESES FOR CASE STUDY 1

Management Goals	Scenarios, Hypotheses, and Relevant Information	
Adverse effects to other organisms	Scenario	If there are beneficial (*e.g.*, honeybees) or other non-target insects (or other organisms) with sufficient homology to the target V-ATPase gene in corn rootworm, exposure of those insects via ingestion of maize tissues, prey- mediated transfer, or other routes such as in soil, to the RNAi-trait may have an adverse impact on those other organisms.
	Hypotheses	No beneficial or other non-target insects (or other organisms) are exposed to the RNAi. No beneficial or other non-target insects (or other organisms) are adversely impacted at realistic field exposures.
	Relevant Information	Spectrum of activity for the trait Toxicity testing on beneficial non-target organisms likely to be exposed. *Bioinformatics homology data.* *Existing studies on dsRNA specificity.* Characterizing routes of exposure and environmental fate of dsRNA. Expression patterns of dsRNA or RNAi-trait in plant.
Weediness of the crop plant	Scenario	If the RNAi-mediated corn rootworm resistance were to result in an increase in the weediness characteristics of the corn plant, corn might become a weed and thereby cause an adverse effect.
	Hypothesis	No increased weed potential or adverse environmental impact of genetically engineered plants compared to conventional control.
	Relevant Information	Comparative assessment for typical agronomic, phenotypic, and environmental interaction characteristics in the genetically engineered and the conventional corn plants. Information about the biology of corn.
Gene flow to sexually compatible plants leading to weediness or altered exposure scenarios	Scenario	If there was transfer of the RNAi-mediated corn rootworm resistance to sexually compatible plants, the recipient plants might become a weed if introduction of the transgene results in increased weediness, and/or the presence of the gene would represent novel routes of exposure by other organisms associated with the recipient plant and thereby cause an adverse effect.
	Hypothesis	There are no wild relatives of corn to which gene flow could occur in the U.S., which is the intended cultivation area.
	Relevant Information	Information about the biology of corn, including pollination biology and distribution of compatible wild relatives.
Disease and pest susceptibilities	Scenario	Because the RNAi pathway in plants is part of the natural defense mechanism against invasive nucleic acids, if the RNAi-mediated corn rootworm resistance leads to an unintended disruption of disease or pest defences via an over-loading of the RNAi pathway, there may be increased levels of disease, especially viral infection, or pest susceptibilities.[1] Susceptibility to diseases associated with corn rootworm infestation may be altered, but these are likely to decrease.
	Hypotheses	There is no difference in disease or pest susceptibility in the genetically engineered compared to the conventional corn. The RNAi-trait for the corn rootworm V-ATPase does not increase susceptibility to viral diseases compared to RNAi present due to naturally occurring virus infections, or to other diseases or pests.
	Relevant Information	Screening for susceptibility to pests and diseases, with special attention to viral diseases, and to diseases associated with corn rootworm infestation, during the normal evaluation of plant-insect and plant-disease interactions in the field. Existing studies on virus infection and dsRNA. Experience with currently approved RNAi-mediated virus resistant genetically engineered crops, *e.g.*, squash, plum, etc.
Changes in cultivation practices	Scenario	If there are changes in cultivation practices associated with the growing of the corn rootworm resistant corn, including use of chemical pesticides, these could lead to adverse effects.
	Hypotheses	There are no differences in cultivation practices between the genetically engineered and conventional corn, other than possible changed use of chemical pesticides. There are no adverse effects associated with the changed use of chemical pesticides.
	Relevant Information	Biology of corn and current cultivation practices. Typical product characterization of the genetically engineered corn.

1 Although proposed during discussions of Case Study 1, this scenario also applies to the other case studies.

ANNEX 4 — NEMATODE RESISTANT SOYBEAN (CASE STUDY 2)

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INTRODUCTION

Nematodes account for an estimated loss in soybean yield of one billion dollars annually in the U.S. Crop rotation and planting of nematode resistant varieties are two approaches used to decrease nematode damage. Chemical fumigation is environmentally and economically unfeasible. One approach to providing soybean with resistance to nematodes is to engineer genetically modified soybean that produces interference RNA (RNAi) targeted to silence essential nematode genes to impede nematode development or kill the nematode. Composite soybean plants have been tested with roots genetically transformed with vectors designed to produce RNAi to silence soybean cyst nematode (Heterodera glycines; SCN) and root-knot nematode (Meloidogyne incognita; RKN). Genetically modified plants have been constructed with a similar design, but the results are not publically available yet.

PART I: THE NON-TRANSGENIC PLANT

General Description of Soybean

See Case Study 4; and OECD (2000)

Reproductive Biology of Soybean

See Case Study 4; and OECD (2000)

Center of origin and center of genetic diversity

See Case Study 4; and OECD (2000)

Means of dispersal and establishment

See Case Study 4; and OECD (2000)

Hybridization

See Case Study 4; and OECD (2000)

PART II: THE RECEIVING ENVIRONMENT

Cultivation of the host plant in the intended country of deployment

See Case Study 4; and OECD (2000)

Presence of any sexually compatible relatives in the receiving environment

See Case Study 4; and OECD (2000)

Ecological interactions in the receiving environment

See Case study 4; and OECD (2000)

PART III: THE TRANSGENIC PLANT

Method used to introduce the novel trait

Agrobacterium rhizogenes, carrying the vector and trait of interest, was allowed to infect the wound of the stem base of seven day old soybean plantlets with the roots removed. This produced composite plants of soybean wherein the top half of the plant was not transformed. At 28-days after infection the bottom half contained transformed and untransformed roots. The untransformed roots were excised and transformed roots, identified by the presence of enhanced green fluorescent protein, were inoculated with nematodes.

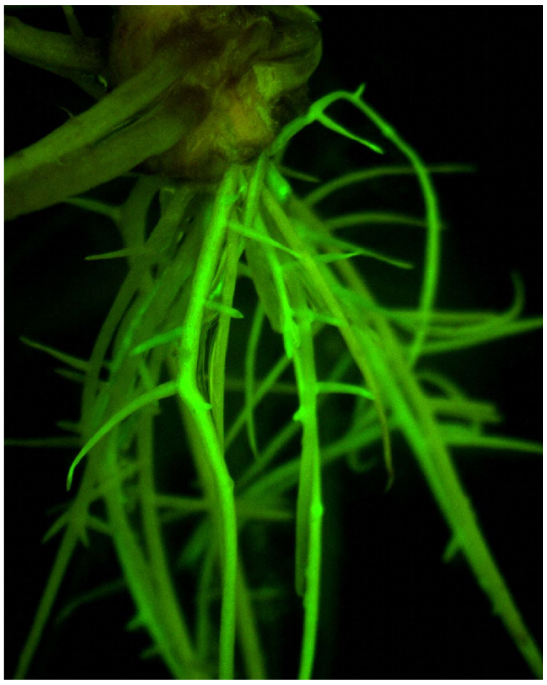

Figure 1. Soybean roots transformed and displaying the production of enhanced green fluorescent protein.

The purpose of the transformation and mode of action

Nematode resistant soybean provides a yield advantage compared to susceptible varieties in regions infested with nematodes. Reduced feeding of nematodes on soybean provides an economic advantage to the farmer. However, current soybean varieties are not resistant to all populations of nematode found in the soil. Host-mediated expression of parasite genes as interference RNA (RNAi) has been investigated as a means to disrupt development of SCN and RKN upon infection of soybean. Transgenic soybean expressing RNAi slows or stops nematode development and provides substantial resistance and economic advantage over non-transgenic soybean. Efficacy in transgenic soybean is derived from portions of genes of the target nematode through the RNAi gene silencing mechanism.

METHODOLOGY

The methods described in Klink *et al*. (2009b) were used and are given here. A Gateway®-compatible gene silencing plant transformation system was developed specifically for these experiments in *G. max*. Three steps then were taken to identify *H. glycines* candidate genes for the analysis. First, a pool of 150 highly conserved *H. glycines* homologs of genes having lethal mutant phenotypes in the free living nematode *Caenorhabditis elegans* were identified (Alkharouf *et al.,* 2007). Second, annotation of those 150 genes on the Affymetrix® soybean Genechip® allowed for the identification of a subset of 131 genes whose expression could be monitored during the parasitic phase of the *H. glycines* lifecycle (Klink *et al.,* 2009a). Third, microarray analyses identified a core set of genes that are induced during the parasitic stages of infection of the nematode. *H. glycines* genes, shown to be induced, conserved and putatively essential, were chosen for gene silencing studies. In gene silencing experiments designed using fragments of candidate *H. glycines* genes, 84-93% fewer females developed on transgenic roots containing the genes expressed as tandem inverted repeats. Those analyses compared the number of mature female cysts on the roots transformed with the tandem inverted repeat of the candidate highly conserved essential *H. glycines* gene to the number of mature female cysts on roots transformed with empty vector after 30 days. These experiments demonstrate an alternative approach to engineer resistance to *H. glycines*.

Candidate *H. glycines* genes were identified from different functional categories (*i.e.*, metabolism, cell structure). PCR primer pairs were designed from Genbank accession DNA sequences to isolate amplicons of candidate genes identified in the microarray analysis. These PCR primer pairs (Table) were designed to amplify gene fragments that were ~200-500 base pairs in length. Out of 65 genes selected in an initial analysis, 64 (98.46%) amplified the correct amplicon as determined by DNA sequencing experiments. The remaining one yielded no product. The amplicons then were purified for shuttling into the pENTR/D-TOPO® directional cloning vector. Eight colonies were selected to determine the presence of the amplicon. Colony PCR demonstrated that, typically, all eight preps contained the insert. DNA sequencing was used to identify a prep that had a perfect match in both forward and reverse sequencing reactions with the original Genbank accession. It typically required eight preps to obtain at least one amplicon with a perfect DNA sequence match to the original Genbank accession. The construct with a perfectly matching amplicon was then used for the LR reaction that shuttled the amplicon into pRAP17. Colony PCR was used to determine the presence of amplicons in the forward

and reverse orientation. This could be determined by PCR because both a pRAP17 specific and a gene specific primer is used for amplifying the forward and reverse amplicon. This results in the amplification of DNA fragments of slightly different sizes. pRAP17 constructs contained amplicons oriented in each direction 100% of the time. These colonies were then grown overnight. DNA isolated from overnight cultures of one of the positive plasmid preps for each LR reaction. A similar approach was used to identify and clone DNA fragments of RKN target genes (Ibrahim *et al.,* 2011).

The anticipated cultivation region

Nematode resistant soybean would be intended for cultivation where soybean is typically grown and where the targeted nematode is a significant problem.

A Summary of the Introduced Genetic Elements

Effect of RNAi gene silencing constructs on SCN development

Four RNAi gene silencing constructs targeted against SCN genes were independently transformed into soybean roots resulting in composite plants. Gene fragments were derived from the SCN 40S ribosomal protein gene homolog (*rps-3a*), 40S ribosomal protein gene homolog (*rps-4*), SR spliceosomal protein (*spk-1*), and synaptobrevin-1 (*snb-1*). Non-transformed roots were excised and the roots each were inoculated with 2000 J2 SCN, respectively. Roots transformed with empty pRAP17 vector served as controls. The number of mature cysts were counted after 30 days. A reduction in the number of mature SCN cysts of more than 80% was achieved (Klink *et al.,* 2009b).

Summary of introduced gene segments of soybean cyst nematode in soybean. (Klink *et al.*, 2009b)

H. glycines **homolog**	**Description**	**Number female cysts**
Hg-rps-3a	40S ribosomal protein S3a	16 N(4)
Hg-rps-4	40S ribosomal protein S4	28 (N2
Hg-spk-1	Spliceosomal SR	17
Hg-snb-1	synaptobrevin	11
Control	Empty pRAP17	149

Summary of introduced gene segments of root-knot nematode in soybean. (Ibrahim *et al.,* 2011)

M. incognita **homolog**	**Description**	**No of galls/ plant root**	**Nematode diameter (um)**
Mi-Tp	Tyrosine phosphatase	1.5	16
Mi-msp70	Mitochondrial stress-70 protein	2.3	13
Mi-ATPs	ATP synthase	10.3	32
Mi-ldh	Lactate dehydrogenase	12,2	20
Control		28.5	85

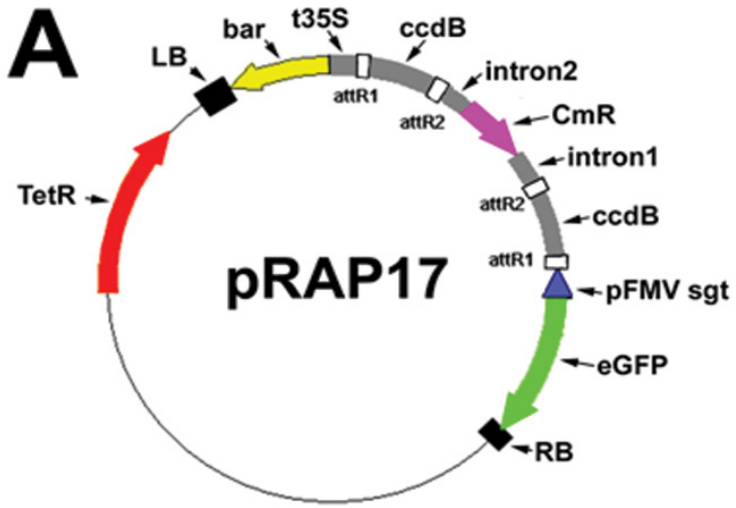

Figure 2. Diagram and features of the pRAP17 vector used for transformation of soybean roots (Klink *et al.,* 2009b). The vector contained a gene encoding tetracycline resistance (TetR), the left border (LB) of the tumor inducing (Ti) plasmid, the gene encoding BASTA® resistance (bar) controlled by the CaMV 35S promoter 9t35S), Invitrogen Gateway ® cloning sites (attR1, attR2) and selection marker (ccdB) for inserting selected DNA sequence in one direction, intron spacer (intron2), chloramphenicol resistance (cmR), continuation of intron spacer (intron1), Gateway®

Effect of RNAi gene silencing constructs on root-knot nematode (RKN) development

Four RNAi gene silencing constructs targeted to RKN genes were independently transformed into soybean roots resulting in composite plants. Gene targets were tyrosine phosphatase (TP), mitochondrial stress-70 protein precursor (MSP), ATP synthase, and lactate dehydrogenase. Non-transformed roots were excised and the roots were inoculated with 3000 RKN eggs per plant. Roots transformed with empty pRAP17 vector served as controls. The number of galls were counted after 30 days and the nematode diameter in the gall was determined. Gene constructs targeting TP and MSP genes each decreased the number of galls formed on transformed roots by over 90%.

Elements of vector used for transformation

Transformed soybean plants have not been evaluated beyond the tests presented above.

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ANNEX 5 — RISK SCENARIOS AND HYPOTHESES FOR CASE STUDY 2

Management Goals	**Scenarios, Hypotheses, and Relevant Information**	
Adverse effects to other organisms	Scenario	If there is homology between the target gene in the parasitic nematode and the genes of beneficial nematodes, or of other non-target organisms with a route of exposure, the RNAi-trait may have an adverse impact on those other organisms. Because the resistance is expressed in the roots, harm to soil dwelling organisms and routes of exposure such as root exudates and decomposing plant tissue, among others, should be considered.
	Hypotheses	No beneficial nematodes or other non-target organisms are adversely impacted at field exposure levels. Beneficial nematodes will not be exposed to the dsRNA through feeding on soybean. Beneficial nematodes, or other non-target soil-dwelling or multitrophic organisms, will not be exposed to the dsRNA for nematode resistance through root exudates, decomposing plant tissue, or prey-mediated transfer.
	Relevant Information	Information on biology of beneficial and other nematodes (abundance, species diversity, association with crop species, impacts from nematicides). Toxicity testing on organisms likely to be exposed. Spectrum of activity for the trait. *Bioinformatics homology data.* *Existing studies on dsRNA specificity.* Characterizing routes of exposure and environmental fate of dsRNA.
Weediness of the crop plant	Scenario	If the RNAi-mediated nematode resistance were to result in an increase in the weediness characteristics of the genetically engineered soybean plant, it might become a weed.
	Hypotheses	No increased weed potential or adverse environmental impact of genetically engineered soybean plants compared to conventional soybean plants.
	Relevant Information	Comparative assessment for typical agronomic, phenotypic, and environmental interaction characteristics in the genetically engineered and the conventional soybean plants. Information about the biology of soybean.
Gene flow to sexually compatible plants leading to weediness or altered exposure scenarios	Scenario	If there was transfer of RNAi-mediated nematode resistance to sexually compatible plants, the recipient plants might become weeds if introduction of the transgene increases weediness, and/or the presence of the gene would represent novel routes of exposure by other organisms associated with the recipient plant.
	Hypotheses	There are no wild relatives of soybean to which gene flow could occur in the U.S., which is the intended cultivation area.
	Relevant Information	Information about the biology of soybean, including pollination biology and distribution of compatible wild relatives.
Disease and pest susceptibilities	Scenario	Since nematode infestation can result in increased susceptibility to diseases in soybean, nematode resistance might result in a change in susceptibility to diseases (although it is likely to be reduced rather than increased, as with conventional nematode resistant soybean varieties).
	Hypotheses	There is no difference between the genetically engineered and conventional soybean, and other conventional nematode resistant soybeans, in disease or insect susceptibility.
	Relevant Information	Screening for susceptibility to pests and diseases during the normal evaluation of plant-insect and plant-disease interactions in the field. Information about the disease susceptibility in conventional nematode resistant soybean varieties.
Changes in cultivation practices	Scenario	If there are changes in cultivation practices associated with the growing of nematode resistant soybean varieties, including crop rotation patterns, these could lead to adverse effects.
	Hypotheses	There are no differences in cultivation practices between the genetically engineered and conventional soybeans or other conventional nematode resistant varieties, other than possible changes in crop rotation patterns.
	Relevant Information	Biology of soybean and current cultivation practices Typical product characterization of the genetically engineered soybean. Information about the cultivation practices in soybean varieties, including crop rotation patterns in nematode resistant varieties

ANNEX 6 — REDUCED PHYTATE SORGHUM (CASE STUDY 3)

Jennifer Anderson, Ph.D., Pioneer Hi-Bred Intl. Inc., Ankeny, IA

INTRODUCTION

Because sorghum can be cultivated in semi-arid environments and in marginal soils, millions of people in Africa rely on a sorghum-based diet for calories and nutrients. Traditional sorghum varieties contain low levels of pro-vitamin A, poor protein digestibility and low bioavailability of iron and zinc. While sorghum does contain adequate levels of zinc and iron, these cations are largely bound by phytate, a negatively-charged phosphorous storage complex. Silencing the myo-inositol kinase gene via RNA interference has been shown to effectively decrease phytate biosynthesis and increase the bioavailability of zinc and iron. To date, greenhouse trials have been conducted with constructs in Iowa and Kenya. Confined field trials have been conducted in Iowa and Hawaii and are planned for Nigeria and Kenya in 2011. Products will likely be commercialized in 2017 or 2018.

PART I: THE NON-TRANSGENIC PLANT

For further information, refer to the OECD Consensus Document on Compositional Considerations for New Varieties of Grain Sorghum (2010).

General sorghum plant biology

Within the genus Sorghum, there are over 30 species of perennial cereal grasses native to Africa. *Sorghum bicolor* (L.) is cultivated as an annual plant in many regions of the world (1) and is a staple in the diets of many food insecure populations of Africa and Asia (2, 3, 4). Next to maize, wheat, rice and barley, sorghum is the fifth most cultivated cereal crop globally (3, 5), and is second only to maize in Africa (1).

Typically, sorghum plants resemble short-stature maize plants, but some cultivars can grow as tall as maize. They contain a long "canelike" stalk, which supports 14-18 alternating "maize-like" leaves and is topped with a panicle (multiple flowers). The panicle contains the seeds, which can be densely packed or loosely bunched (6). Sorghum seeds range in color from pale yellow to dark brown and are 3-4mm in diameter (6). A diverse fibrous root network, which can extend over five feet deep into soil, supports the plant (7).

Like maize plants, sorghum undergoes C4 photosynthesis, as is typical of tropical grasses (8). Being tolerant to drought and intermittent water-logging (5), sorghum is ideal for cultivation in arid, semi-arid, and tropical climates. Sorghum grasses are adapted to many different soil types, soil pH ranges, drought, water-logging, and high temperatures, however are sensitive to cold and frost (7). Typically, sorghum requires 60-70 days to flower and approximately 120 days to reach full grain maturity (9).

Reproductive biology of sorghum

Sorghum can be self-fertilized and cross-pollinate readily (9, 13). For typical sorghums, pollen shed can last for 6-9 days (13).

Center of origin and genetic diversity of sorghum

Sorghum is native to Africa, and the center of origin is thought to be in Ethiopia and Sudan (7). Africa remains the center of biodiversity for wild species (7). There are five cultivated races (bicolour, kafir, guinea, durra, and caudatum) that are adapted for different areas of Africa (reviewed by 5). Races differ in panicle and grain shape.

Means of sorghum establishment and dispersal

Cultivated sorghum plants are annual grasses ($2n$ = 2x = 20) that are sexually compatible with other wild sorghum and wild relatives (*e.g.*, Johnsongrass, shattercane). Cultivated sorghum plants are not rhizomatous and dispersal may occur through seeds (9). On the other hand, Johnsongrass (*Sorghum halapense*) is a perennial grass that will persist and disperse through rhizomes and seed dispersal, making it one of the "world's most noxious weeds" (12).

PART II: THE RECEIVING ENVIRONMENT

Cultivation and Management practices for sorghum

In the United States and other developed countries, sorghum is commercially cultivated for livestock feed (5), silage (13) and ethanol production. In Africa, sorghum is primarily produced by subsistence farmers, and is a dietary staple for over 500 million Africans.

Typically, subsistence farmers in Africa do not irrigate fields, and both pesticide and fertilizer use may be limited (1). Likewise, due to limited availability, African farmers rarely spray herbicides to control weed populations in sorghum crops, relying on manual weeding for weed control (5). Because herbicide use is limited, wild relatives of sorghum are commonly found within cultivated fields. Depending on the preparation of the seed bed, volunteer sorghum is likely to occur. Wild sorghum was reported by 96% of African farmers in one district (14), indicating that feral sorghum populations are persistent within and adjacent to cultivated fields. As a management practice to control gene flow, farmers tend to plant at different times to decrease flowering overlap, separate sorghum fields and remove feral weeds prior to flowering (14). In rural regions, many seeds are traded between farmers or at the market, which may make segregation of seeds and grain products difficult (15).

Potential for out-crossing to weedy relatives

Because Sorghum can be self-fertilized and cross-pollinate readily (13, 9), gene flow between sorghum crops and weedy populations is likely (4) and can lead to contamination of non-transgenic varieties in the U.S. and in Africa (2). Wild-relatives of sorghum are common within and adjacent to sorghum cultivated fields (2). For example, sorghum crops in Niger and Ethiopia were surveyed to characterize the prevalence of wild sorghum (16). This study reported that wild relatives were present within 7-56% of sorghum crops, and 9-70% of cropped fields had wild relatives in adjacent fields (cropped, abandoned or fallow) or crop field margins (16). Likely these wild relatives could be pollinated by the sorghum crop. Sorghum hybridization with its wild relative *Sorghum halepense* (Johnsongrass) occurs at distances ranging from 0.5 to 100 m (reviewed by 17); however, using mathematical models, it was estimated that gene flow could occur up to 200-700m (18). Temporal overlap of pollen shed between the sorghum crop and the weedy relatives is likely to occur. In Niger and Ethiopia, pollen shed in the sorghum crop and the neighboring wild relatives overlapped in 31-100% of surveyed fields (16). Sorghum can be self-pollinating, but pollen movement by wind and insects also contributes to outcrossing to wild-relatives. Additionally, seed dispersal could occur between the sorghum field and the adjacent fields (16).

Because the transgenic sorghum plants will be modified for improved nutrition, rather than agronomic traits, introgression of transgenes into the feral populations may not correlate with a fitness advantage (14). Nevertheless, because gene flow is likely, the impacts from gene flow into wild populations and races on the genetic biodiversity of sorghum native to Africa or on the biodiversity of other flora if wild sorghum becomes invasive (4), should be considered in the risk assessment.

Ecological Interactions in the receiving environment

Several diseases are common to sorghum, including anthracnose, leaf blight, charcoal rot, smut, and the parasitic weed *Striga hermonthica* (9). In addition to disease, crop pests include sorghum midge (*Contarinia sorghicola*), and birds (*e.g.*, African weaver bird, *Quelea quelea*) (9).

Method used to introduce novel trait

Sorghum bicolor (L.) has been modified through *Agrobacterium*-mediated transformation to produce decreased levels of phytate in the grain (23).

Purpose of transformation and mode of action

Phytate (also known as phytic acid or myo-inositol hexakisphosphate; $C_6H_{18}O_{24}P_6$) serves as a phosphorous storage complex common in bran, seeds, beans and tubers (19). A critical enzyme in the phytate biosynthetic pathway is myo-inositol kinase (MIK), which catalyzes the phosphorylation of myo-inositol to myo-inositol-1-phosphate (20, 21). The precursor to phytate, myo-inositol-1-phosphate, undergoes successive phosphorylation steps to become myo-inositol hexakisphosphate. Phytate will readily bind zinc and iron and form an insoluble complex which cannot be digested in human digestive tracts (22), thereby decreasing the bioavailability of these trace elements.

RNA interference (RNAi) is used to silence the myo-inositol kinase gene, which encodes for myo-inositol kinase, as described above. The maize embryo-preferred 16KD oleosin promoter, is used to drive expression of an RNAi cassette consisting of two copies of a fragment of the sorghum myo-inositol kinase gene coding sequence arranged in an inverted repeat, with the two fragments separated by a small intron. The transcript of the inverted myo-inositol kinase fragments forms a long double-stranded RNA (dsRNA) molecule. The long dsRNA is processed by dicer (an endoribonuclease) into 21-25 nucleotide double-stranded RNA fragments, termed small interfering RNAs (siRNA). The binding of these siRNAs to the endogenous MIK transcript triggers the cleavage of the transcript and significantly reduces the amount of transcript available to be translated into the MIK enzyme, resulting in reduced biosynthesis of phytate. Reduced synthesis of phytate effectively increases the bioavailability of zinc and iron in sorghum grain.

Figure 1. Field of *Sorghum bicolor* (6)

Native cultivars of sorghum are typically suited for hot, dry climates and can be grown where maize, fruits, and other vegetables cannot due to inadequate rainfall (7). Because sorghum is one of the only cereal crops that can grow in drought-prone, marginal soils common to many areas of Africa, a large proportion of African diets are primarily sorghum-based. Sorghum grains are typically milled and used to make bread (2), beer, porridge, and fermented foods (5). While the process of fermenting can increase the bioavailability of nutrients, cooking can decrease digestibility of the protein by half (3). Sorghum stover may also be used for fodder for farm animals (9).

Sorghum shares a similar nutritional profile with maize (reviewed by 3). Like many other cereal grains, the nutritional composition of sorghum is low in essential amino acid content, vitamin A, vitamin E (3) and protein (approximately 9% protein content). Additionally, sorghum seeds contain phytic acid (phytate), which decreases the bioavailability of zinc and iron (10) and decreases protein digestibility (11). While Africans have long relied on sorghum as a staple in their diets, sorghum alone provides inadequate levels of essential nutrients, which can have significant health impacts. Approximately 60% of blindness in Africa, India and China is a result of diets low in vitamin A (5). Likewise, many children in Africa do not receive the required levels of iron and zinc from their diet, which can result in anemia, poor growth and development, and in general poor health. While conventional breeding has been effective in increasing yield, it has not resulted in an improved nutritional profile of sorghum.

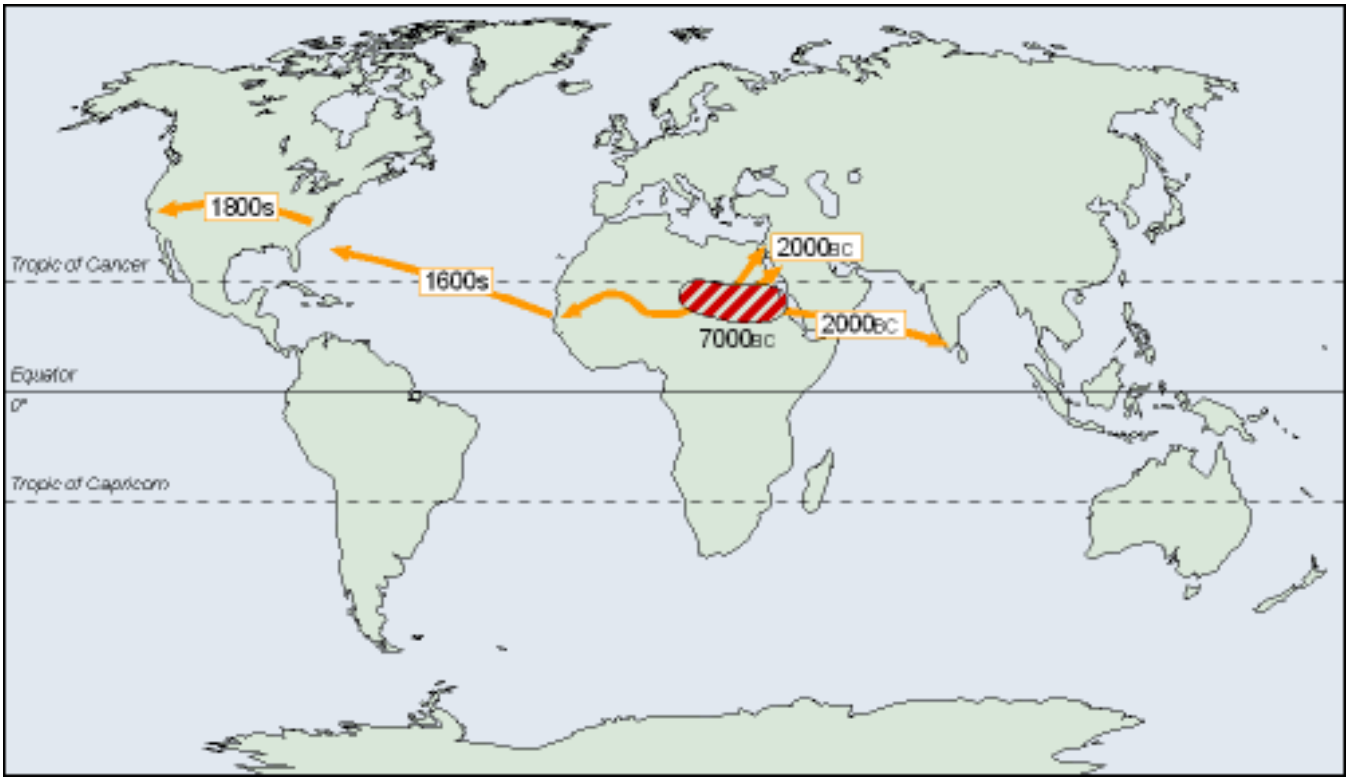

Figure 2. Pattern of domestication of Sorghum (26). Sorghum is indigenous to Ethiopia and Sudan regions of Africa. There is no consensus on when sorghum first originated in Africa; however it could be as early as 7000BC. Sorghum was brought to North America during the slave trade and disseminated to Asia and India through shipping and trade (26).

In the future, reduced-phytate sorghum may be stacked with additional nutrient enhancing traits, including traits that will enhance protein digestibility (through kafirin reduction) and traits that will enhance vitamin A. Kafirin reduction could be engineered in sorghum using RNAi technology to reduce accumulation of the family of kafirin seed storage proteins. Vitamin A enhancement on the other hand could result from overexpression of the phytoene synthase gene from Zea mays and/or the carotenoid reductase gene from the soil microbe *Erwinia uredovora*. Both genes catalyze steps in the carotenoid biosynthetic pathway.

Anticipated cultivation region

Sorghum is cultivated by subsistence farmers across Africa. A change in cultivation area is not expected.

Inheritance and stability of introduced trait

Information not yet available.

Differences in modes and/or rate of reproduction from non-transgenic crop

Reproductive differences between conventional and reduced-phytate sorghum have not been observed to date. Phytate reduction has been observed to decrease germination rate and/or seed weight in other crops, so reproduction rate continues to be monitored.

Expression levels of novel proteins in different tissues over time

Information not yet available.

Differences in agronomic characteristics from non-transgenic crop

No agronomic differences have been observed. Compositional differences between traditional sorghum and reduced-phytate sorghum are expected. Reduced-phytate sorghum is engineered to have improved nutrition and increased bio-availability of zinc and iron.

Differences in disease and/or pest susceptibility from non-transgenic crop

No differences in the susceptibility to pests or diseases have been reported. It has however been noted that indirect effects of nutritional changes on characteristics such as bird preference or fungal disease susceptibility should be considered (4).

Potential impact on non-target organisms in the receiving environment

Information not yet available.

Available exposure data

Information not yet available.

References to risk assessments in any other jurisdiction

To date, few exposure assessments, gene flow assessments, and overall risk assessments have been conducted to determine the potential effects of cultivating genetically engineered Sorghum in the U.S. or in Africa.

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ANNEX 7 — RISK SCENARIOS AND HYPOTHESES FOR CASE STUDY 3

Management Goals	Scenarios, Hypotheses, and Relevant Information	
Adverse effects to other organisms	Scenario	If there is homology between the target MIK gene in sorghum and genes in other non-target/beneficial organisms feeding on sorghum seed (*e.g.*, birds), or through other routes of exposure, the RNAi-trait may have an impact on these organisms.
	Hypotheses	There is no adverse effect to exposed non-target organisms from consuming the reduced phytate sorghum seed.
	Relevant Information	Data collected for food/feed safety assessment. Spectrum of activity for the trait. *Bioinformatics homology data.* *Existing studies on dsRNA specificity.* Characterizing routes of exposure and environmental fate of dsRNA.
Weediness of the crop plant	Scenario	If the RNAi-mediated reduced phytate in the seed results in increased weediness characteristics of the sorghum plant, sorghum might become a weed.
	Hypothesis	No increased weed potential or adverse environmental impact of genetically engineered sorghum compared to conventional sorghum.
	Relevant Information	Comparative assessment for typical agronomic, phenotypic, and environmental interaction characteristics in the genetically engineered and the conventional sorghum plants, including seed-specific characteristics such as germination and dormancy that might be dependent on phytate levels in the seed. Information about the biology of sorghum.
Gene flow to sexually compatible plants leading to weediness or altered exposure scenarios	Scenario	Because the transfer of the transgene to weedy wild relatives (*e.g.*, 'Johnsongrass' and 'Shattercane' in the U.S.) can occur, if the RNAi-trait functions in the recipient plant, and if the RNAi-mediated reduced phytate results in increased weediness, the recipient plants might become more weedy, or there might be altered routes of exposure by other organisms associated with the recipient plant (see above).
	Hypothesis	There are no more weediness characteristics in the genetically engineered than the conventional sorghum plants. Reduced phytate in the seed does not increase the weediness of sorghum.
	Relevant Information	Comparative assessment for typical agronomic and other characteristics related to weediness in the genetically engineered and the conventional sorghum plants, including seed-specific characteristics such as germination and dormancy. Information about the biology of sorghum, including pollination biology, distribution of wild relatives, and weediness. Information about characteristics related to weediness in sorghum. *Bioinformatics homology data.*
Disease and pest susceptibilities	Scenario	Reduced phytate in the sorghum seed could increase the susceptibility of sorghum to seed-specific diseases or increase feeding preference by pests, such as insects, or birds and rodents.
	Hypotheses	There is no difference between the genetically engineered and conventional sorghum in disease and pest susceptibility, including feeding by birds or rodents.
	Relevant Information	Screening for susceptibility to pests and diseases, with special attention to pests associated with the seed, during the normal evaluation of the plant for performance in the field.
Changes in cultivation practices	Scenario	If there are changes in cultivation practices associated with the growing of the reduced phytate sorghum, these could lead to adverse effects.
	Hypotheses	There are no differences in cultivation practices between the genetically engineered and conventional sorghum.
	Relevant Information	Biology of sorghum and current cultivation practices Typical product characterization of the genetically engineered sorghum.

ANNEX 8 — REDUCED ALLERGEN SOYBEAN (CASE STUDY 4)

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INTRODUCTION

Legume seed allergy is a wide-spread and growing problem. When an individual becomes sensitive and cross-reactive to one legume protein there is a tendency for sensitivity to spread and intensify by acquiring sensitivity to homologous proteins in other legumes. An approach that might benefit a large portion of individuals destined to become sensitized is to silence the initially sensitizing dominant allergen that may then impede acquiring the added sensitization to other allergens. The seed protein P34/Gly m Bd 30k is the immunodominant allergen of soybean sensitive infants in the U.S. often fed soy-based formula. RNAi has been used to silence P34/Gly m Bd 30k to create low-allergen content seeds. Transgenic nulls for P34/Gly m Bd 30k have been created in an agronomic cultivar.

PART I: THE NON-TRANSGENIC PLANT

General description of soybean

Soybean (*Glycine max*) is a member of legume family that has become one of the world's great crops. Soybean seeds are rich in high quality protein and oil and often serve dual use with seeds being crushed for oil, both food and fuel, and the proteins isolated from the remnant material used as protein concentrate for animal feed and as components of processed food. Soybean is an erect annual plant yielding ave approx 80 bushels/acre (U.S.).

See OECD (2000)

Reproductive biology of soybean

Soybean is a self-fertilized seed bearing plant.

See OECD (2000)

Center of origin and center(s) of genetic diversity

Soybean is Asian in origin. Ancestral and related Glycine species are found in South Asia, Northern tropical Australia, and Papau New Guinea. Many ancestral species are perennial vines. Soybean domestication appears to have occurred in China > 2,000 yr BP. *Glycine max* immediate ancestor appears to be *G. soja* which is cross-fertile with *G. max*. The center of genetic diversity is China with additional diversity from other Asian adopter countries including Korea and Japan. Among national collections is that of the U.S. Deptartment of Agriculture which consists of > 20,000 accessions that are freely available. The U.S. collection contains accessions acquired throughout Asia extending back well over one hundred years ago. Assessing Asian collections has often proved problematic for foreign researchers.

See OECD (2000)

Means of dispersal and establishment

Soybean is dispersed by seed. Soybean is not grown as hybrid so seeds from one growth season can readily be saved for another continuing the same genetic line. Soybean as a domesticated crop has very limited seed shatter and without human intervention its seeds would be dispersed only in the immediate vicinity of its parent plant.

See OECD (2000)

Intra-specific, inter-specific and/or inter-generic hybridization

Soybean can be easily crossed to its immediate ancestor *G. soja* and with some difficulty to other Glycine species. Reported crosses are limited to related species that share the Asian origin of soybean. Soybean has not been crossed to any other legume.

G. max x *G soja* crosses are viable and fertile. Other crosses have been limited and to some extent also are viable.

See OECD (2000)

PART II: THE RECEIVING ENVIRONMENT

Cultivation of the host plant in the intended country of deployment

Soybean is one of the great crops of the U.S. with large internal consumption and export. The primary center of production is the swath of farm belt in the Midwest of the U.S. but it is grown in a majority of the U.S. Soybean breeding has produced lines optimized for being grown from the Canadian border to the Gulf coast. Most soybean cultivation in the U.S. is east of the Rocky Mountains. Soybean needs relatively warm summer weather likely inherited from its tropical origins. Soybean is water stress sensitive and requires a well watered environment.

See OECD (2000)

Presence of any sexually compatible relatives in the receiving environment

Soybean has no naturally occurring relatives in the Americas that are capable of being partners in crosses even with assistance of breeders.

See OECD (2000)

Ecological interactions in the receiving environment

Soybean farming has the ecological impact that any farming has as a large acreage monoculture. Current methods using round-up resistant soy for most of the crop has resulted in the implementation of no-till methods that are far less destructive to soil. Recent publications suggest that no-till methods have arrested soil erosion problems and will maintain soil depth and quality far into the future.

Soybean is subject to several different pests that include bacterial disease, fungal disease, and notably cyst nematodes that result in considerable loss and economic damage annually. Research to improve pest resistance is an active area with a large number of academic, industrial, and government scientists pursuing solutions with approaches ranging from conventional breeding, to gene identification, and production of transgenic lines.

See OECD (2000)

PART III: THE TRANSGENIC PLANT

Method used to introduce the novel trait(s)

Biolistic transformation of silencing sequence contained within a seed-specific expression cassette containing Kan selection marker resistance was used.

The purpose of the transformation and mode of action

Legume seed allergy is a wide-spread and growing problem. Sensitization occurs primarily in infants and young children to one of the legumes with soybean, peanut, and chickpea among the more significant foods. The allergenic proteins

of legumes and indeed other seeds have considerable sequence and therefore epitope homology. When an individual

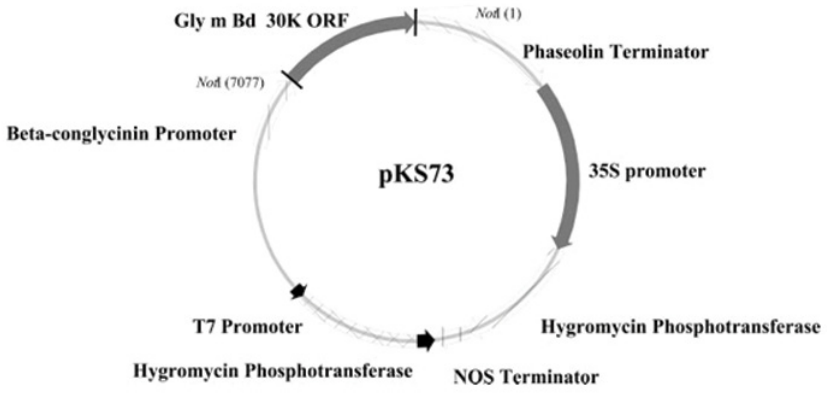
becomes sensitive and cross-reactive to one legume protein there is a tendency for sensitivity to spread and intensify by acquiring sensitivity to homologous proteins in other legumes. For most seeds there are both dominant and minor allergens. It is likely, but unproven, that a dominant allergen sets the stage for acquisition of allergy, and once sensitized the allergic individual then acquires additional sensitization to other allergens from the same and related species. This suggests an approach that might benefit a large portion of individuals destined to become sensitized which is to silence the initially sensitizing dominant allergen that may then impede acquiring the added sensitization to other allergens.

The seed protein P34/Gly m Bd 30k is the immunodominant allergen of soybean sensitive infants in the U.S. often fed soy-based formula. Although a minor protein (1-2% total) it binds a majority of the anti-soy IgE from those soy sensitive infants. Immunological analysis has shown there are at least 10-15 different IgE binding epitopes on P34/Gly m Bd 30k indicating small sequence variations will not eliminate its IgE binding. By eliminating P34/Gly m Bd 30k it is hoped that the initial dominant allergen's absence will result in decreased probability of the other soybean proteins sensitizing an infant, and subsequently to lower the risk of acquiring sensitivity to other legumes such as peanut. The transgenic vector was designed to silence the immunodominant allergen to create low-allergen content seeds. Silencing proved to be very effective with the resulting soybeans containing much less than 1% of the protein found in wild type. The objective is to create soybean infant formula that will not elicit food allergy response that can spread to other foods creating potentially life-threatening and life-long health problems.

The current status of this project is that we have transgenic and conventional nulls for P34/Gly m Bd 30k, the later acquired by screening 20,000 lines in the U.S. national soybean collection. The transgenic is already in an agronomic cultivar while the naturally occurring null will require years of breeding to introgress the trait into agronomic germplasm. To establish the utility of the approach to silence allergens and its role in possibly impeding the acquisition and spreading of allergenic responses, animal tests are needed. To conduct these tests either new support will be needed to test with a mouse model or an expansion of support and tests are needed with a swine model. A multi-institutional group (Purdue, NCSU, Danforth Center, U Ark) is producing an inbred population of swine that is highly prone to acquire food allergy. Now in its third generation allergen sensitivity is breeding true and the swine will be used to evaluate the immunological sensitizing differences between normal and low allergen content soybeans. Such efficacy tests are essential to approach FDA to make a regulatory case for deployment. The FALPA law (food and allergen labeling and protection act) did not anticipate and provides no guidance for reduced allergen food. To deploy this technology this will need to be addressed either at FDA or through supplemental legislation.

The anticipated cultivation region

Small scale identity preserved production for specific end-use is the short-term goal.



Map of Gly m Bd 30 K-silencing construct. Features indicated are the Gly m Bd 30K-coding region, the a-conglycinin promoter,and the bean phaseolin termination region. Also shown are the transcriptional units for bacterial (T7) and plant (35S) hygromycin selectable marker genes.

A summary of the introduced genetic elements

Inheritance and stability of each introduced trait

Biolistic introduced traits through somatic embryo transformation have proven stable. In this particular case the trait was stable through several generations.

Differences in genetic and phenotypic variability from non-transgenic crop

None observed, except for P34 trait.

Differences in modes and/or rate of reproduction from non-transgenic crop

None.

Expression levels of novel proteins in different tissues over time

As part of the assessment of the transgenic plants, detailed proteomic analysis of the P34/Gly m Bd 30k knockdown was compared to wild-type with the result that the only demonstrated difference between the transgenic and wild-type was the suppression of the targeted gene product (see Herman *et al* 2003).

Differences in agronomic characteristics from non-transgenic crop

No observed differences in greenhouse grown plants, field test not conducted.

Differences in disease and/or pest susceptibility from non-transgenic crop

Not assessed but of significant interest. Data indicates the potential that P34/Gly m Bd 30k is related to *Pseudomonas* resistance. The gene maps to *Pseudomonas* resistance site and the protein binds a syringolide elicitor from the bacteria. In a survey of the entire soybean germplasm collection only a single accession was isolated that was null indicating that P34/Gly m Bd 30k is conserved. Critical evaluation of *Pseudomonas* resistance of P34 nulls compared to wild-type has not yet been conducted.

Potential impact on non-target organisms in the receiving environment

None

Any available exposure data

None

References for any risk assessments undertaken in other jurisdictions

None

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ANNEX 9 — RISK SCENARIOS AND HYPOTHESES FOR CASE STUDY 4

Management Goals	Scenarios, Hypotheses, and Relevant Information	
Adverse effects to other organisms	Scenario	P34 is a similar protein to cysteine proteases (CPs), and CPs are involved in enzymatic digestion in the guts of some insects (mainly coleopterans). If the P34 RNAi-trait has homology to CPs in insects (or their predators) feeding on developing seed where the RNAi-trait is expressed, this could potentially lead to an adverse effect on those insects. Participants indentified stinkbug as one pest of soybean that is known to feed on the developing seed.
	Hypotheses	No beneficial/non-target organisms are adversely impacted at realistic exposure levels.
	Relevant Information	Information on known insect pests or pest predators of soybean. Toxicity testing on organisms likely to be exposed. *Bioinformatics homology data.* Characterization of routes of exposure and environmental fate of dsRNA.
Weediness of the crop plant	Scenario	If the RNAi-mediated reduced allergenicity were to result in an increase in the weediness characteristics of the soybean plant, soybean might become a weed and thereby cause an adverse effect.
	Hypothesis	No increased weed potential or adverse environmental impact of genetically engineered soybean compared to conventional soybean.
	Relevant Information	Comparative assessment for typical agronomic, phenotypic, and environmental interaction characteristics in the genetically engineered and the conventional soybean plants. Information about the biology of soybean.
Gene flow to sexually compatible plants leading to weediness or altered exposure scenarios	Scenario	If there was transfer of the RNAi-mediated reduced allergenicity to sexually compatible plants, the recipient plants might become a weed if the transgene results in increased weediness, or there might be new routes of exposure by other organisms associated with the recipient plant.
	Hypothesis	There are no wild relatives of soybean to which gene flow could occur in the U.S., which is the intended cultivation area.
	Relevant Information	Information about the biology of soybean, including pollination biology and distribution of compatible wild relatives.
Disease and pest susceptibilities	Scenario	There is some evidence that P34 is associated with resistance to *Pseudomonas*. If P34 is associated in some way with protection against *Pseudomonas*, the P34 RNAi-trait could increase susceptibility in the soybean to this disease. P34 is a similar protein to cysteine proteases, and some cysteine proteases have insecticidal activity. If P34 has insecticidal activity and if there is exposure to insects feeding on the developing seed where P34 is expressed (such as stinkbugs), the P34 RNAi-trait might increase susceptibility to some insect pests.
	Hypotheses	There is no difference between the genetically engineered and conventional soybean in disease or insect susceptibility
	Relevant Information	Screening for susceptibility to pests and diseases, with special attention to *Pseudomonas* and insect pests, during normal evaluation of the plant performance in the field.
Changes in cultivation practices	Scenario	If there are changes in cultivation practices associated with the growing of the reduced allergen soybean, these could lead to adverse effects.
	Hypotheses	There are no differences in cultivation practices between the genetically engineered and conventional soybean. There are no plant pest effects associated with the small scale identity preserved production of the reduced allergen soybean.
	Relevant Information	Biology of soybean and current cultivation practices. Typical product characterization of the genetically engineered soybean.

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