



## Review

## Application of food and feed safety assessment principles to evaluate transgenic approaches to gene modulation in crops

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## ABSTRACT

New crop varieties containing traits such as enhanced nutritional profiles, increased yield, and tolerance to drought are being developed. In some cases, these new traits are dependent on small RNAs or regulatory proteins such as transcription factors (TF) that modify the expression of endogenous plant genes. To date, the food and feed safety of genetically modified (GM) crops has been assessed by the application of a set of internationally accepted procedures for evaluating the safety of GM crops. The goal of this paper is to review the main aspects of the current safety assessment paradigm and to recommend scientifically sound principles for conducting a safety assessment for GM crops that are developed by technologies that modify endogenous plant gene expression. Key considerations for such a safety assessment include the following:

- (1) RNA and TF are generally recognized as safe (GRAS);
- (2) Genes encoding RNAi and regulatory proteins such as TFs are an important component of the plant genome;
- (3) Crops engineered using RNAi modifications are not expected to produce heterologous proteins;
- (4) The modulation of TFs may result in quantitative differences in endogenous plant components, which can be assessed through agronomic performance and compositional analysis on a case-by-case basis.

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**Abbreviations:** dsRNA, double-stranded RNA; FAO, Food and Agriculture Organization of the United Nations; GM, genetically modified; GRAS, generally recognized as safe; hpRNA, hairpin RNA; IFBiC, ILSI International Food Biotechnology Committee; ILSI, International Life Sciences Institute; OECD, Organisation for Economic Co-operation and Development; RISC, RNA-induced silencing complex; RNAi, RNA interference; siRNAs, small interfering RNAs; TF, transcription factors; WHO, World Health Organization.

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## 1. Introduction

Following the first commercial planting of a transgenic crop in 1996, agricultural biotechnology has been rapidly adopted in many countries of the world (James, 2010). The majority of existing commercial genetically modified (GM) crops have been designed to express transgenic proteins with a limited spectrum of biological activity (e.g., insect resistance, herbicide tolerance). Accordingly, the safety assessment paradigms for existing commercial GM crops focus on the safety of the transgenic protein(s), along with an evaluation to detect any possible unintended changes in the crop plant or its derivatives. Components of the safety assessment include agronomic evaluation, compositional analyses, bioinformatic tools for allergenicity assessment, specific toxicity studies, and, on a case-by-case basis, animal feeding trials with whole GM food/feed (Codex, 2003; EFSA, 2008; Chassy et al., 2004, 2008).

To date, most GM crops exhibit traits such as insect resistance and herbicide tolerance, either as single or stacked traits. In the future, crops with improved nutritional characteristics as well as crops with important agronomic traits, such as drought and stress tolerance, will be developed. Some of these future GM crops will not express a novel transgenic protein, but rather, will express novel RNA molecules that will result in suppression of specific endogenous plant genes, which can in turn lead to the desired phenotypes. Other crops will be engineered to express novel transcription factors, or will alter expression of endogenous transcription factors, which in turn will alter the timing and magnitude of expression of specific sets of endogenous genes within the crop.

The purpose of this work is to examine if there are any novel hazards associated with this new generation of GM crops, and to evaluate the applicability of the widely accepted and currently applied safety assessment paradigm to the next generation of GM crops developed using RNAi, transcription factors, and other mechanisms for modulating endogenous plant gene expression.

## 2. Review of the current food safety assessment practices for GM crops

Transformation programs generally produce dozens to hundreds of transgenic events. Only the most promising events in terms of phenotype and genotype (i.e., the functional integrity of the transgene at the insertion site) are carried forth for further testing and development. At that point, the current food and feed safety assessment begins with a series of steps, which includes an agronomic evaluation, considers the safety of the heterologous protein, and evaluates the overall composition of the plant (Chassy et al., 2004, 2008).

### 2.1. Current practices for the agronomic evaluation of genetically modified crops

A comparative assessment of key agronomic and phenotypic characteristics of the GM crop with those of its conventional counterparts has become a required component of GM-derived product development. The morphological and agronomic characteristics of a crop plant are the result and culmination of the expression of the numerous functional genes within the crop plant that produce enzymes, plant structural components, regulatory proteins, and metabolites that create the phenotypic characteristics of the crop. Changes introduced during conventional breeding processes or by the introduction of a transgene can alter levels of endogenous components, thus influencing the final phenotype of the plant. Therefore, measuring key agronomic characteristics and comparing them to those of the non-transgenic counterpart is an established component of the safety assessment paradigm. Those GM events that demonstrate unacceptable agronomic characteristics resulting from the insertion of a transgene are not further developed or commercialized.

A major objective of agronomic evaluations of new crop varieties, whether they are the product of conventional breeding or genetic modification, is to determine whether the new variety is

morphologically and agronomically comparable to the appropriate comparator, except for intended changes. For many of the traits evaluated, there usually is extensive genetic variation among the wide array of conventional varieties within any particular crop species. If it is a GM variety that is being evaluated, the evaluation should always include the near isogenic, non-GM genotype if available; thus, the major difference between such isolines is the insertion of the transgene. In addition, due to the large inherent variability of agronomic characteristics within a crop species, agronomic evaluations of GM crop varieties often include one or more conventional reference varieties of the crop that are representative of those currently under cultivation. Data from the evaluation of these varieties help to provide a crop-specific context that can be used to evaluate the biological relevance of any observed changes between the GM crop and its near-isogenic control variety.

The important agronomic and morphological characteristics of any crop are heavily influenced by the environment, including photoperiod, soil type, temperature, moisture, previous cultivation practices, and the prevalence of insect pests and pathogens. Therefore, replicated field trials are typically conducted at several different sites representative of the geographic range where the crop is cultivated commercially in order to assess the agronomic characteristics of the crop under different environmental conditions. Accurate records of key environmental factors are compiled, as these are needed to help distinguish genotypic effects from environmental effects. Another strategy that is used to account for environmental variation is to replicate across years so as to obtain representative variation in weather patterns such as rainfall, temperature, and so on. An appropriate experimental field design is used to account for random variation and to facilitate the statistical analyses of the resulting data. For any crop being evaluated, the general agronomic characteristics measured commonly include the following broad categories: (1) germination and seedling emergence, (2) vegetative vigor, (3) time to anthesis, (4) plant height at maturity, (5) time to maturity, (6) pollen characteristics, and (7) yield. Measurements that reflect these characteristics are generally crop-specific. A consultation with an agronomist experienced with the specific crop is often useful in determining measurements and evaluations that are relevant for that crop. As an example, the agronomic parameters and assessment methods recommended for the evaluation of maize (*Zea mays* L.) are available from the US Department of Agriculture – Animal and Plant Health Inspection Service (USDA-APHIS, 2008). Other sources of general information on conducting agronomic evaluations of new crop varieties include the European Food Safety Authority (EFSA, 2006), International Life Sciences Institute (ILSI, 2003, 2007), and Information Systems for Biotechnology (ISB, 2002).

Detailed documents describing the biology of many of the important cultivated crop species have been prepared by the Organisation for Economic Co-operation and Development (OECD) (<http://www.oecd.org/home/>). In addition to maize, these documents include *Glycine max* (soybean), *Oryza sativa* (rice), *Triticum aestivum* (bread wheat), *Solanum tuberosum* (potato), and *Brassica napus* (oilseed rape), among others. These documents provide authoritative information about the biology of the species evaluated and are useful as references for evaluating the data generated in the agronomic trials. However, it should be noted that much of the information in these documents is general in nature and may not reflect specific regional information about the plant species. Therefore, the consensus documents may be supplemented with information that reflects regional conditions and contemporary varieties.

If, after comparing the data from the agronomic evaluation, the GM crop variety is determined to be agronomically equivalent to the near-isogenic control variety and/or other conventional varieties representative of the crop for the main morphological and

agronomic characteristics of the species (except for the trait conferred by the transgene), it can be concluded that the GM variety is agronomically comparable to conventional varieties that have a history of safe use and cultivation. In cases where the results of the agronomic evaluation suggest that the GM crop variety may be different from conventional varieties of the crop for one or more traits, further evaluation of the safety implications of these differences may be warranted. If by design, the objective of engineering the GM crop results in resistance to drought, salt or temperature extremes, increased yield, increased nutrient content when used for food/feed, or other such qualities, then it would be expected that the GM crop could demonstrate differences in agronomic properties from those observed for its conventional counterparts.

## 2.2. Compositional and nutritional equivalency

The safety assessment of GM crops may typically include comparisons of the composition of forage, whole grain, and/or processed fractions of the GM crop to that of conventional counterparts (Codex, 2003). Such a comparative assessment helps evaluate equivalence of the GM crop to varieties of that crop that are widely grown and consumed. The intent of composition analysis is to confirm that the key nutrient and antinutrient composition of the GM crop falls within the range of concentrations observed for conventional varieties, which serves as an indication that the GM crop is as safe as its conventional counterparts. The concept of Substantial Equivalence asserts that GM crop varieties that are compositionally equivalent to conventional varieties are considered equally safe. Furthermore, if compositional differences are observed, possible hazards can be associated only with the differences (WHO, 1991, 1995; FAO, 1996; Jonas et al., 1996; OECD, 1993, 1996, 1997). In practice, concentrations of key nutrients and anti-nutrients in a candidate GM event are compared with those of a contemporaneously grown near-isogenic line. In addition, the levels of analytes in the GM crop are compared to those observed for conventional counterparts. The potential for biological impact of any observed differences is then assessed.

Comparisons may include proximates (protein, fat, ash, carbohydrates, and moisture), fiber, minerals, amino acids, fatty acids, vitamins, anti-nutrients, endogenous allergens, and secondary metabolites (OECD, 2001a,b, 2002, 2004a,b). Studies that compare the composition of several GM crops with conventional counterparts have been published (Berberich et al., 1996; Taylor et al., 1999; Sidhu et al., 2000; Ridley et al., 2002; George et al., 2004; Hamilton et al., 2004; Herman et al., 2004, 2007a; Obert et al., 2004; McCann et al., 2005; Oberdoerfer et al., 2005; Drury et al., 2008; Lundry et al., 2008). Results emphasize that the GM and non-GM comparators are of similar composition. Examples of GM crops for which compositional equivalence to a non-GM comparator has been investigated are also presented elsewhere (AGBIOS, 2008; ILSI, 2008). A crop composition database for conventional varieties of soybean, maize, and cottonseed has been developed by ILSI (<http://www.cropcomposition.org>) (Ridley et al., 2004).

Crop composition databases have been used to establish the compositional equivalence of crops expressing transgenic proteins that confer tolerance to herbicides or resistance to insects. In some cases in which the composition has been deliberately modified, it may be more relevant to compare the composition with non-transgenic crops that contain similar compositional profiles, rather than comparing them with the parental or near-isogenic variety. For example, canola modified to enhance monounsaturated fatty acid composition is more appropriately compared with olive oil rather than with conventional canola oil (Chassy et al., 2004).

To investigate nutritional performance, feeds derived from the GM crop and from its conventional counterpart are often fed to a

fast-growing nutritionally sensitive animal species that can accommodate a large percentage of the crop of interest in its diet without deleterious effects (e.g., 42-day broiler chicken feeding study). If a GM crop is found to be compositionally equivalent to its conventional counterpart and animals grow equally well when fed the GM crop or the conventional crop, the GM crop is considered to be as nutritious as the conventional counterpart (ILSI, 2003, 2007; Chassy et al., 2004).

Recent investigations have been conducted to assess the utility of a group of technologies referred to collectively as “omics” (proteomics, metabolomics, and transcriptomics) for evaluating the compositional equivalence of GM crops relative to their conventional counterparts (Catchpole et al., 2005; Baker et al., 2006). These technologies have proven to be useful tools for research; however, application of these technologies as part of the safety assessment for GM crops is premature, as baseline data on the normal variation inherent in plant proteomes, metabolomes, and transcriptomes of conventional varieties are sparse (Dixon et al., 2006; Lay et al., 2006; Harrigan et al., 2007). In view of the observation that geography (or location), environmental conditions, and year of cultivation can produce major differences in composition, “omic” analysis cannot be considered as a compositional assessment tool until robust databases cataloguing natural variation are available. Metabolomic analyses are also limited at present because they do not provide quantitative information and are able to describe only a subset of the metabolome, often without identification of specific metabolites unless targeted analysis follows the profiling step (Harrigan et al., 2007; Fiehn et al., 2008). Given there is also no evidence that the current targeted safety assessment paradigm is inadequate, it can be anticipated that current methods of compositional analysis will continue to be the basis of comparative safety assessment for the foreseeable future.

When compositional equivalence is absent, the potential safety implications of the observed differences in composition are addressed on a case-by-case basis. For example, the composition of a crop that is engineered for pest resistance will vary from that observed for its non-GM counterpart as a result of pest feeding and pest-induced modulation of endogenous plant-defense compounds. Plants attacked by pests also undergo other compositional changes (Schwachtje and Baldwin, 2008). If there are differences between the GM and its non-GM comparator, there would be no cause for concern as long as the differences fall within an acceptable range, relative to the natural variation of the analyte level(s) in the crop (Chassy et al., 2004; Ridley et al., 2004), taking their biological relevance into account.

Likewise, when a crop is engineered to have a more desirable nutrient profile (i.e., high-lysine corn, Golden Rice, low-linolenic acid soy), differences in the nutritional composition of the GM crop and its non-GM counterpart are expected and desirable. Examples of GM crops for which compositional equivalence to a non-GM comparator has been investigated are presented elsewhere (AGBIOS, 2008; ILSI, 2008).

### 2.3. Current approaches to the assessment of heterologous protein safety

In many cases, modern plant biotechnology involves the introduction of a gene or genes into the plant genome via plant transformation technologies. When expressed *in planta*, these transgenes endow the crop with specific traits. If the transgene expression results in production of a heterologous protein, the safety assessment centers on this protein. As a class of macronutrients, proteins are an essential component of the human diet. Although the overwhelming majority of dietary proteins have not been tested for safety using the current testing regime that is applied to transgenic proteins, long-term consumption by humans and ani-

mals indicates that proteins, as a general class of macronutrients, are a safe component of the human diet. With the exception of a few well-described cases, consumption of proteins in general is not inherently associated with adverse effects.

Some proteins expressed in genetically modified crops are not present in significant quantities in food and may thus lack a clear history of safe use. Furthermore, only an exceedingly small number of proteins are known to be orally toxic or allergenic. According to AllergenOnline (<http://www.allergenonline.com>, accessed 4 December 2008), there are 1313 known or putative allergens. As a percentage of all proteins in foods, this is a very small number. Nevertheless, the current risk assessment practices recommend evaluating the safety of transgenic proteins to ensure that no potentially toxic or allergenic proteins are engineered into crops. Several national and international organizations have extensively addressed these issues (OECD, 1993; FAO/WHO, 2000; Codex, 2003; FSANZ, 2005; EFSA, 2006; Health Canada, 2006).

A number of approaches to assess the potential allergenicity of proteins have been developed over the past 20 years (Metcalf et al., 1996; FAO/WHO, 2001, 2007; Thomas et al., 2004, 2005, 2007; Goodman et al., 2005; Goodman, 2006). Although there are differences between the particular guidelines, the fundamental concept in these guidelines is to compare the amino acid sequence and physical properties of transgenic proteins with those of proteins with a documented history of allergenicity. One reason for this approach is that there are currently no validated animal models suitable for predicting potential allergenicity in humans (Helm and Burks, 2002; McClain and Bannon, 2006; Goodman et al., 2008). The most contemporary guidelines take into consideration that none of the particular comparators can by themselves identify allergenic potential. Therefore, a weight-of-evidence approach, in which the outcome of all individual analyses is considered collectively, is recommended (Codex, 2003).

The first part of the allergenicity assessment determines if there is a history of prior human exposure to the inserted transgenic protein(s) (e.g., was the protein obtained from a source with a documented history of allergenicity?). Secondly, amino acid sequence similarity of the transgenic protein to known allergenic proteins is analyzed using computational (bioinformatics) tools. *In silico* analyses are used to compare the amino acid sequences of transgenic proteins to known allergenic proteins in order to determine if they share any eight contiguous amino acids that resemble known allergen epitopes or contain more than 35% sequence identity over any 80 amino acids to a known allergen, with the latter approach considered the more relevant (Metcalf et al., 1996; FAO/WHO, 2001, 2007; Thomas et al., 2004, 2005, 2007; Goodman et al., 2005, 2008; Goodman, 2006). Goodman et al. (2008) recently stated, “Obviously, the use of short amino matching searches (6- to 8-mer) is not a useful approach for allergenicity assessment.” Instead, it has recently been demonstrated that scanning databases for matches to protein motifs that are characteristic of known allergens may be more effective at identifying potential allergens than the current sequence identity and 8-mer scanning techniques (Ivanciuc et al., 2009). In the case of three major allergen gene families (seed storage proteins, Bet v 1, and tropomyosin), motifs that overlap with known IgE epitopes have been identified (Ivanciuc et al., 2009). It can be anticipated that as new information becomes available, the allergy assessment paradigm will continue to be refined and updated.

*In vitro* studies are also conducted to determine whether the transgenic protein is sensitive to degradation by digestive enzymes, including pepsin and pancreatin (Fu et al., 2002; Okunuki et al., 2002; Thomas et al., 2004). The prevalence of the transgenic protein in food is also considered, because most proteins capable of sensitizing individuals are present at concentrations of 1% or more. In some cases, additional analyses are conducted to assess the

thermal stability of transgenic proteins (FAO/WHO, 2001). Studies using skin prick tests or sera obtained from humans with documented clinical reactions to proteins can be conducted in order to assess the potential for cross-reactivity (FAO/WHO, 2001). However, these types of studies are typically only conducted in instances where one or more of the primary indicators suggest that allergenic cross-reactivity could be a concern. Collectively, these investigations represent a comprehensive analysis of the transgenic protein for possible allergenicity, and have been used to demonstrate that the transgenic proteins expressed in GM crops do not represent a high risk for unintentionally introducing an allergenic protein into the diet.

Similarly, recommendations have recently been developed by the ILSI International Food Biotechnology Committee (IFBIC) to assess the potential for transgenic proteins to be toxic (Delaney et al., 2008). The individual components in these guidelines bear some similarity to those used in evaluating the allergenicity of transgenic proteins; however, they recommend a tiered risk assessment based on the weight of evidence at each tier. The recommendations include a first tier hazard identification that consists of assessment of the history of safe use, a bioinformatic comparison to proteins with known toxicity, and a determination of the stability to digestive enzymes using *in vitro* methods. In the event that the outcome from the first tier of testing does not support a conclusion that the protein will be innocuous, a second tier of testing that centers on further hazard characterization analyses, such as acute and repeated-dose rodent toxicity studies, and hypothesis-based testing are recommended. It is worth emphasizing that the transgenic proteins expressed in today's commercialized GM crops represent no demonstrated risk to humans or animals (Harrison et al., 1996; Martens, 2000; Wraight et al., 2000; Dale et al., 2002; El Sanhoty et al., 2004; Hammond et al., 2004; Herouet et al., 2005; Rhee et al., 2005; MacKenzie et al., 2007; Poulsen et al., 2007; Schroder et al., 2007).

Although these guidelines are based on the best scientific knowledge available, it is necessary to consider that some of the underlying principles are not universally accepted. For example, questions have been raised on the relevance of protein digestibility to prediction of protein allergenicity/toxicity. In contrast to an earlier study in which food allergens were shown to be relatively more stable to digestion than non-allergens in *in vitro* simulated gastric fluid (Astwood et al., 1996), more recent studies concluded that there was not a clear correlation between digestibility measured *in vitro* and protein allergenicity (Fu et al., 2002; Herman et al., 2007b). Despite these limitations, *in vitro* digestibility data are required currently by most, if not all, regulatory authorities as part of the safety assessment paradigm for transgenic proteins.

In summary, only a brief introduction to the issues considered in the evaluation of protein safety is presented here. All information pertinent to a potential transgenic protein should be considered and carefully weighed to make a proper hazard assessment. If concerns about the safety of a transgenic protein arise after examination of the concentration in the food or feed product, history of safe use, structural and functional characterization, and elucidation of the mode of action as well as evaluation of *in vitro* digestibility (Tier I Assessment), further *in vitro* and/or *in vivo* testing (Tier II Assessment) may be required to make a thorough risk evaluation (Delaney et al., 2008).

#### 2.4. Molecular characterization of the inserted DNA, its flanking regions, and its impact on the safety assessment

Characterization of the inserted DNA in GM crops is typically required by regulatory agencies. The key step in the molecular characterization is Southern blot analysis to determine the number of insertion sites, of both intact and fragmented copies of the trans-

gene, along with sequencing of the inserted DNA and investigations of inheritance patterns to determine if the loci are linked to each other.

A typical DNA insert contains a promoter to direct gene transcription, a coding sequence, and a 3' untranslated region that directs polyadenylation of the resulting transcript. Confirmation of intactness of the elements of the inserted DNA demonstrates that the promoter and terminator sequences are in the proper positions to regulate transcription of the inserted gene(s) such that only the intended trait protein(s) encoded by the gene(s) is/are produced from the gene cassette. Intactness of the coding sequence minimizes the chances of alternative proteins being produced. The number of copies of the intended insertion within a single integration locus may play a role in genetic and trait stability, but the value of this information to the assessment of the safety of the product is questionable and likely represents lower risk compared with conventional breeding techniques.

Information concerning the insert's flanking sequences allows assessment of possible host cell gene disruption. A transgene or transgene fragment could insert behind a promoter already in the plant, leading to altered expression patterns of both the host gene (i.e., "activation tagging") and the transgene; the production of a fusion protein; or unintended expression of a transgene fragment, in which case, it may be necessary to determine if transgene fragments are transcribed and translated. Alternatively, the transgene could be inserted near the 5' end of another gene, and read-through transcription from the transgene could lead to expression of the downstream gene, at a time when, or in tissues where, it otherwise normally would not be expressed. Although these scenarios are unlikely, the potential for these to occur may be detected by sequencing the flanking DNA as long as these regions have been properly characterized and annotated. However, meaningful perturbations would more effectively be detected by holistic weight of evidence characterization of the transgenic protein isolated from the GM crop, by use of transgenic protein expression studies, or through compositional, nutritional, and agronomic equivalency evaluations.

##### 2.4.1. Selectable marker genes

At the present time, regulatory agencies require data to determine whether or not sequences derived from the transformation vector backbone, other than those from the intended cassette, have integrated into the plant genome (EFSA, 2006). Although plant transformation is often performed using purified DNA fragments containing only the intended gene cassette, the DNA preparations can contain small levels of backbone DNA fragments derived from the plasmid used to maintain and propagate the gene cassette in bacteria. Even *Agrobacterium*-mediated transformation can include vector sequences outside the T-DNA borders (Wu et al., 2006). In addition to the gene cassette, these plasmids usually also contain a bacterial origin of replication and at least one prokaryotic antibiotic resistance marker or another form of marker. The bacterial origins of plasmid replication do not function in plants and the bacterial selectable markers are not likely to be expressed in the plant because they are typically expressed from bacterial promoter elements that generally are not recognized by the plant. However, insertion of bacterial selectable marker genes into plants has raised questions about the possibility of horizontal gene transfer of antibiotic resistance to pathogenic bacteria, even though numerous studies of this phenomenon have demonstrated that the risk of transfer is negligible (Schluter et al., 1995; Nielsen et al., 1997). A recent comparison of antibiotic-resistant bacteria in fields in which conventional or transgenic crops had been cultivated revealed no differences, which documented that horizontal transfer does not occur at a detectable frequency (Demaneche et al., 2008). Furthermore, if horizontal transfer of antibiotic resistance

genes were to occur, it would have no functional consequence because a high percentage of free-living and intestinal bacteria already possess the same antibiotic resistance genes (Bradford et al., 2005; Gay and Gillespie, 2005; Goldstein et al., 2005).

### 2.5. Summary of current safety assessment paradigm

The above is a description of the current paradigm for conducting a safety assessment of GM crops. This safety assessment paradigm is based on the state of knowledge that existed more than 20 years ago. Some aspects have been found to have a scientific basis, whereas other elements of the safety assessment have provided little relevant information on safety. Nevertheless, these elements are still performed for historical reasons or because they have been requested by different regulatory agencies around the world for their review of GM crop regulatory submissions. The current safety assessment paradigm has been applied to evaluate crops expressing introduced heterologous proteins, such as those encoded by viral genes and those conferring insect resistance (Bt traits) or herbicide tolerance. GM crops are being developed using new approaches and technologies for the modulation of endogenous gene expression. The features of these technologies that are scientifically relevant to the conduct of a valid safety assessment of GM crops developed using them are reviewed herein. Furthermore, the applicability of the current paradigm for conducting a safety assessment of GM crops developed with technologies that modulate endogenous plant gene expression is evaluated.

## 3. Technologies that modulate endogenous plant biochemical and regulatory pathways

### 3.1. Comparison of conventional breeding and genetic engineering

Most field crops cultivated today are the product of directed breeding and selection activities over thousands of years. In many cases, as a result of this breeding, these crops bear little resemblance to their progenitors. This phenomenon was documented as far back as Darwin in his *Origin of Species*. These crops possess traits, such as increased yield, tolerance to disease and stress, vigor, better taste and appearance, and other traits that are beneficial to the farmer or the consumer. Recently, *in vitro* genetic modification has been employed as an additional plant improvement tool.

Attention has been drawn to genomic disruptions associated with the production of GM crop plants, and it has been suggested that these genomic disruptions represent a potential safety issue that should require extensive molecular characterization to evaluate (Latham et al., 2006; Wilson et al., 2006). Whereas insertion of a DNA cassette *in vitro* may cause local disruptions, far more extensive disruptions and genomic alterations have now been documented to occur as a result of traditional breeding (Jacobsen and Schouten, 2007; Batista et al., 2008; Cheng et al., 2008). In fact, these naturally-occurring, or mutagenesis-induced, alterations are sources of variation that have been exploited in successful plant breeding programs (Bradford et al., 2005). Crops bred through traditional methods have a demonstrated record of safety in spite of the extensive genomic disruptions that occur during their development.

The insertion of a transgene into the genome of a crop plant by transformation is a form of insertional mutagenesis, and therefore has raised potential concerns regarding food safety (Wilson et al., 2006). However, transposable and retrotransposable elements, which mediate natural insertional mutagenesis, are extremely widespread in plants, occur in all crop species, and are a primary contributor to genome plasticity. A recent study demonstrated that one transposon resulted in approximately 50–60 new insertions

per plant per generation in a traditional Japanese rice variety, and that these insertions were taking place in individual farmers' fields (Naito et al., 2006). Another study demonstrated that of 1235 retrotransposons identified in rice, 42% had recruited genes from flanking regions that generated a large number of chimeric genes (Wang et al., 2006). Alternative transposition of the *Ac* element in a noncommercial maize variety was recently shown to generate DNA deletions that led to the creation of novel genes (Zhang et al., 2006). Because the *Ac* element preferentially transposes into genic regions, these rearrangements shuffle the coding and regulatory sequences and generate new allelic diversity. Polyploid crops undergo deletion and addition of numerous DNA sequences within a single generation following their hybrid formation, as evidenced in *Brassica* (Song et al., 1995) and wheat (Liu et al., 1998a,b). These examples are representative of a growing body of literature that illustrates the fluid and plastic nature of the plant genome.

Therefore, when the safety implications of genetic disturbances such as insertional mutagenesis, deletions, duplications, and the creation of potential chimeric genes are considered for GM crops, these assessments should consider that such genome disruptions occur naturally and frequently during plant domestication and traditional breeding, and do so without any recognized safety implications.

### 3.2. RNA interference (RNAi)

RNA interference, or RNAi, refers to a group of related natural phenomena mediated by the formation of short (approximately 21–24 bp) double-stranded RNA molecules with homology to a target gene (recently reviewed by Kusaba, 2004; Mallory and Vaucheret, 2006; Müller, 2006). Through RNAi, high levels of mRNA or aberrantly expressed mRNA are targeted for destruction or for translational suppression through formation of double-stranded RNA (dsRNA) molecules. These dsRNAs are in turn recognized by Dicer or Dicer-like enzymes, type III ribonucleases that cleave dsRNAs into approximately 21–24 nucleotide dsRNAs, called small interfering RNAs (siRNAs). These siRNAs are subsequently recognized by a multiprotein complex called the RNA-induced silencing complex (RISC). RISC activity mediates degradation of mRNA homologous to the RISC-incorporated siRNA, leading to blockage of protein production from the mRNA through mRNA degradation. The resulting phenotype is one resulting from gene suppression (Galun, 2005; Tomari and Zamore, 2005; Bonnet et al., 2006), as illustrated later.

Alternatively, inverted DNA repeats that are complementary to part of a target gene and separated by a small length of intervening DNA (known as *miR* genes), are transcribed to form a micro RNA (miRNA). miRNAs are short double-stranded RNA molecules connected by a hairpin on one end. In this case, it is the mRNA transcript of the *miR* gene that is recognized and processed by a Dicer-like enzyme to produce a functional miRNA (Bonnet et al., 2006). This functional miRNA is then incorporated into a RISC-like complex that subsequently blocks protein production, primarily through translational suppression, but also via degradation of the target mRNA.

#### 3.2.1. RNAi as a natural component of the genome

The role of miRNAs and siRNAs in animal (Ambros, 2004) and plant development has been extensively reviewed (Bonnet et al., 2006; Mallory and Vaucheret, 2006), and these molecules are known to play roles in all aspects of plant growth and development, including meristem differentiation, organ morphogenesis, flowering, stress resistance, and the maintenance of the heterochromatic chromatin state. The purpose of this work is not to review these various roles, but to point out that both siRNAs and

miRNAs are ubiquitous in plants, animals, and other eukaryotes. The exact number and prevalence of these in plant cells is largely unknown. An evaluation in the model plant, *Arabidopsis thaliana*, found over 75,000 putative siRNAs and miRNAs expressed in the plant, demonstrating the presence of multiple small RNAs per gene. *Arabidopsis* has approximately 25,000 genes, 4067 of which were found to have small RNA counterparts; some siRNAs were homologous to intergenic sequences, and the remaining siRNAs appear to have no known target. The different siRNAs were found at different steady-state levels (Lu et al., 2005; Heisel et al., 2008).

RNA interference has been used successfully to target promoter sequences as well, resulting not only in gene suppression, but also in methylation of the promoter target sequences (Cigan et al., 2005). Such RNAi-mediated methylation can stimulate methylation of homologous sequences (known as paramutation) and even be heritable (Sidorenko and Peterson, 2001), highlighting again how transcriptional gene suppression and paramutation are both naturally-occurring phenomena (Chandler and Alleman, 2008).

### 3.2.2. Examples where naturally-occurring RNAi mechanisms have played a role in plant breeding

Work in petunia established the ability of suppression mechanisms to duplicate naturally-occurring phenotypes. ‘Picotee’ is a unique pattern found naturally in many flowers, and is characterized by white edges on otherwise colored petals. Petunias genetically engineered with an extra copy of the *chs* (chalcone synthase) gene were either white or picotee (Napoli et al., 1990; van der Krol et al., 1990). Naturally-occurring picotee petunias were found to have an extra copy of the *chs* gene, whereas transgenic picotee petunias predominantly had multiple copies of the transgene (Stam et al., 1998), thus strongly implicating an RNAi mechanism in the naturally-occurring picotee petunias (Schubert et al., 2004).

Since then, the observation has been extended to edible plants, and a few crop phenotypes selected through conventional plant breeding are now known to be mediated by an RNAi mechanism, as later described. The number of phenotypes attributable to RNAi will undoubtedly increase as additional genomic information becomes available and is examined.

One of the most prominent examples of an RNAi-induced trait selected by breeders is the buff-colored seed coat of soybean, conditioned by the *I* locus (for inhibitor of color). Wild-type soybeans that are the progenitors of currently cultivated varieties have a black seed coat that is caused by the accumulation of anthocyanins. The *I* locus in soybean has been cloned, and consists of two clusters, each with three nearly identical copies of a chalcone synthase gene (*chs*). Buff-colored seed coats result from endogenous siRNAs generated against *chs*, and reversion of buff seed coats to black is associated with DNA rearrangements in one of the *chs* clusters, which interfere with its ability to form siRNA (Tuteja et al., 2004).

Low-glutelin rice is consumed by people who must restrict protein content in their diet because of reduced renal function. The phenotype of low-glutelin rice is due to an RNAi mechanism that results from the molecular structure of the *Lgc1* locus. Glutelin production is controlled by a family of genes. In the case of low-glutelin rice, a genomic deletion has brought two inverted copies of glutelin genes into close proximity, which facilitates *Lgc1* suppression through RNAi (Kusaba et al., 2003).

One locus that can be responsible for green-colored maize stalks is the *C2-Idf* (for inhibitor diffuse of color). The allele that causes green stalks came about from a triplication of a gene for chalcone synthase, resulting in RNAi-mediated suppression of the chalcone synthase mRNA (Della Vedova et al., 2005). Without this, the allele for green-colored maize stalks would normally be purple.

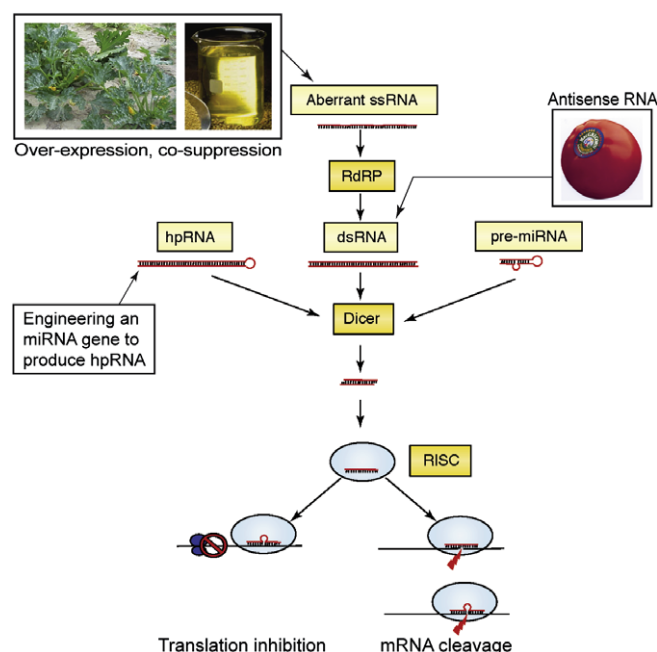
To briefly summarize, these examples show that RNAi-mediated mechanisms are a natural phenomenon that have a long history of safe use as evidenced by the role of RNAi in the domestication of major crop plants.

### 3.2.3. Role in plant defense against disease

It has long been known that inoculating a plant with a mild strain of a virus protects that plant from infection with more virulent strains of the same virus (Salaman, 1933). This provided the incentive to engineer plants expressing genes that encode virus coat protein to duplicate the same phenomenon (Abel et al., 1986). However, it soon became clear that highly resistant plants did not transcribe viral proteins, implicating an RNAi-mediated mechanism (Kusaba, 2004; Dietzgen and Mitter, 2006; Mansoor et al., 2006). Furthermore, non-transgenic plants were themselves found to use RNAi to target nucleic acids from invading viruses (Hamilton and Baulcombe, 1999) making RNAi the unifying mechanism behind both endogenous and transgenic virus resistance.

### 3.2.4. Co-suppression and antisense transformation

Transformation has also been used to obtain phenotypes resulting from gene suppression, in a manner analogous to the RNA-mediated gene suppression that has played a role in plant domestication and conventional plant breeding. Such transgenic techniques include co-suppression or sense suppression (Jorgensen, 1990, 1991) and antisense transformation (van der Krol et al., 1988, 1990). There are a number of crops produced using co-suppression or antisense transformation that have reached or are close to reaching the commercialization stage. One example is the development of a modified starch potato, developed by engineering with antisense constructs to silence the genes for starch branching enzymes A and B (Safford et al., 1998; Schwall et al., 2000). Other



**Fig. 1.** Overview of the different entry points into the RNAi pathways, and how previous transgenics based on gene-suppression are now understood to be part of the overall gene-suppression pathway. Virus-resistant squash and soybean with high oleic oil (pictured top left, respectively) were obtained by over-expressing genes, thus leading to silencing (co-suppression) of viral genes in the case of squash and of *fad-2* in soybean. In contrast, the FLAVR SAVR™ tomato (pictured right) used antisense RNA to silence the polygalacturonase gene. Diagram modified from Kusaba (2004). Photo credits: high oleic oil, American Soybean Association; squash, Seminis, Inc.; FLAVR SAVR™ tomato, Calgene.

examples include the use of antisense technology to develop the delayed ripening FLAVR SAVR™ tomato (Sanders and Hiatt, 2005; Krieger et al., 2008), high oleic soybeans, and ryegrass with hypoallergenic pollen (Petrovska et al., 2005).

Today, both co-suppression and antisense RNA are recognized as being part of the overall RNAi gene-suppression pathways (Fig. 1) (Kusaba, 2004). Co-suppression triggers the RNA-dependent RNA polymerase to make dsRNA that can be targeted by Dicer (Hamilton and Baulcombe, 1999), whereas plants engineered to produce antisense RNA can form dsRNA directly, without need for the RNA-dependent RNA polymerase.

### 3.2.5. Deregulated events obtained via co-suppression and antisense transformation technologies

As mentioned above, transgenic phenotypes previously attributed to co-suppression or antisense RNA are now demonstrated to function as part of the overall RNAi gene-suppression pathways (Fig. 1) (Kusaba, 2004; Sanders and Hiatt, 2005). Thus, transgenic crops (such as squash and papaya) that depend on RNAi mechanisms have been on the market for at least a decade, without any report of adverse effects, which helps establish a history of safe use that should be considered as part of the overall safety assessment. Table 1 presents information collected from the AGBIOS web site (<http://www.agbios.com>), and lists GM crops based on either co-suppression technology or antisense technology that have been deregulated and commercialized in at least one country. All associated regulatory documents are available at the new location of the AGBIOS web site, [www.cera-gmc.org](http://www.cera-gmc.org).

### 3.2.6. Future prospects for variety development via engineering

The realization that the foregoing examples of gene suppression were mediated by RNAi has led to a new era of gene suppression in transgenic plants. Rather than depending on stochastic factors to obtain transgenic plants with sufficient levels of mRNA to trigger the formation of corresponding siRNAs in plants engineered for co-suppression or antisense RNA production, modern vectors can be designed to produce dependable steady-state levels of siRNA, also referred to as artificial microRNAs (Ossowski et al., 2008; Warthmann et al., 2008). This has been accomplished through the construction of vectors whose transcripts generate a dsRNA substrate for Dicer. Approximately 200–400 bp of the target sequence are cloned in their sense and antisense directions, forming an inverted repeat commonly separated by an intervening sequence. The resulting RNA is able to form a double-stranded hairpin structure, and thus is known as a hairpin RNA (hpRNA) (Wesley

et al., 2001; Watson et al., 2005; Fusaro et al., 2006; Müller, 2006). Such a hpRNA is then a substrate for the RNAi pathway. From this, it is evident that any desirable trait for crop improvement that can be achieved through gene-suppression can be achieved through the use of RNAi, specifically, via hpRNA vectors (Mansoor et al., 2006).

**3.2.6.1. Pathogen and pest resistance.** As mentioned previously, one of the original uses of genetic transformation of crops was to produce virus-resistant plants, via a mechanism now understood to be mediated by RNAi. Accordingly, the current generation of virus-resistant plants is being generated using hpRNA-producing constructs. Tobacco plants engineered with hpRNA constructs against the plum pox virus P1 and HC-Pro genes were resistant to the virus (Di Nicola-Negri et al., 2005). The use of hpRNA directed against the replicase gene of barley yellow dwarf virus resulted in resistance in transgenic plants (Dennis and Peacock, 2004).

The discovery that the nematode, *Caenorhabditis elegans*, was susceptible to the RNAi-inducing effects of dsRNAs delivered via dietary administration of dsRNA-over-expressing *Escherichia coli* (Timmons and Fire, 1998; Timmons et al., 2001), suggested the possibility that parasitic nematodes could be controlled by engineering their host plants to produce dsRNAs encoding siRNAs that target essential nematode genes (Gheysen and Vanholme, 2007). Accordingly, tobacco plants were engineered to express dsRNA from either an integrase gene or a splicing gene cloned from the parasitic nematode, *Meloidogyne incognita*, resulting in significant levels of nematode resistance in the transgenic plants (Yadav et al., 2006). Steeves and colleagues engineered soybean to produce dsRNA against a gene for a major sperm protein from *C. elegans*, and achieved a measure of resistance (Steeves et al., 2006). Finally, Huang et al. (2006) engineered Arabidopsis to express dsRNA against 16D10, a gene from *M. incognita* that is directly involved in parasitism. They achieved high levels of resistance to *M. incognita*, as well as to two other species, *M. javanica* and *M. hapla* (Huang et al., 2006).

Most recently, this concept has been extended to insect pests. Cotton bollworms feeding on Arabidopsis or tobacco (*Nicotiana tabacum*) plants engineered to silence a key P450 monooxygenase, which is necessary for the detoxification of gossypol (a toxin in their diet, derived from cotton), lost their tolerance to gossypol (Mao et al., 2007). Similarly, increased mortality was observed in western corn rootworm larvae fed artificial diets containing siRNAs targeting vacuolar ATPase subunits or  $\alpha$ -tubulin. The results with

**Table 1**  
Crops approved for commercialization based on co-suppression and antisense technologies. The background and development of each trait, along with the data presented for regulatory approval, is all available from the AGBIOS web site, now at <http://www.cera-gmc.org> information is retrieved using the crop and event names provided in this table.

Crop	Event	Phenotype
<i>Co-suppression</i>		
Papaya	55-/63-1	Resistance to papaya ringspot virus through suppression of the viral coat protein gene
Potato	RBMT15-101, SEMT15-02, SEMT15-15	Resistance to potato virus Y through suppression of the viral coat protein gene
	RBMT21-129, RBMT21-350, RBMT22-082	Resistance to potato leaf roll virus through suppression of the viral replicase gene
Soybean	G94-1, G94-19, G168	High oleic acid content through suppression of <i>fad-2</i>
Squash	CZW-3	Resistance to cucumber mosaic, zucchini yellow and watermelon mosaic viruses through suppression of the corresponding viral coat protein genes
	ZW20	Resistance to zucchini yellow and watermelon mosaic viruses through suppression of the corresponding viral coat protein genes
Tomato	1345-4	Down-regulation of ethylene production by suppression 1-aminocyclopropane-1-carboxylic acid (ACC) synthase
<i>Antisense</i>		
Tobacco	Vector 21-41	Nicotine-free, through suppression of quinolinic acid phosphoribosyltransferase
Tomato	FLAVR SAVR™	Down-regulation of polygalacturonase (PG) to delay fruit softening



vacuolar ATPase were verified in transgenic corn plants (Baum et al., 2007).

**3.2.6.2. Altered nutritional profiles.** The ability of gene-suppression technologies, specifically co-suppression, to alter the nutritional profile of crops through the inhibition of individual steps in selected metabolic pathways is the basis for development of a GM high oleic acid soybean. Co-suppression has been used successfully in various species, including soybean and canola, to alter oil fatty acid profiles (Thelen and Ohlrogge, 2002), whereas antisense technology has been used to alter the oil fatty acid profile in cotton (Sunilkumar et al., 2005). Conceivably, future attempts at oil modification will rely upon RNAi. The approach has already been used successfully in Arabidopsis (Stoutjesdijk et al., 2002), and in cotton, where this technology was used to produce oil with high stearic and oleic acid content (Liu et al., 2002).

Reports of the deliberate use of RNAi technology via transformation with hpRNA-producing constructs with a crop improvement focus are currently making their way into the literature. The use of hpRNA targeted to the two potato starch branching enzymes has recreated the high-amylose phenotype previously created with antisense RNA (Andersson et al., 2006). The same approach was used to create high-amylose wheat, which improved large-bowel function when fed to rats (Regina et al., 2006).

A high-lysine maize has been achieved using RNAi technology against the *opaque-2* gene (Segal et al., 2003). The high-lysine phenotype is the same as that conferred by the naturally-occurring *opaque-2* mutation, and used in many breeding programs around the world.

Finally, RNAi-mediated suppression of the *DET-1* gene in tomato resulted in fruit with enhanced levels of carotenoids and flavonoids (Davuluri et al., 2005).

**3.2.6.3. Targeted elimination of compounds.** An example of how RNAi can be used for the elimination of specific compounds in crop plants is illustrated by the use of the technology to engineer decaffeinated coffee (*Coffea canephora*) plants (Ogita et al., 2003). Likewise, cottonseed is high in protein, but its use as a feed meal is limited by the presence of the gossypol toxin. There are cotton genotypes unable to produce gossypol, but these suffer from increased susceptibility to pests. Through the use of RNAi, it was possible to eliminate gossypol production in the seed, but not in the vegetative parts of the plant (Sunilkumar et al., 2006). Similarly, it was possible to silence production of the allergenic p34 protein in soybean (Herman, 2005).

**3.2.6.4. Altering crop growth.** As described later, transcription factors play a major role in defining plant growth and architecture. The suppression of FLC, a transcription factor that prevents early flowering, had the expected effect in Arabidopsis, with engineered plants flowering much earlier than their non-transgenic counterparts (Wesley et al., 2001). As with other traits first engineered in Arabidopsis, it is a matter of time before modifications suppressing endogenous plant transcription factors are used in transgenic crop plants.

Male sterility, which is a useful trait in breeding programs to facilitate hybrid seed production, lends itself to this technology by suppressing genes necessary for microsporogenesis. Suppression of the tapetum-specific zinc finger (TAZ1) gene in petunia (Kapoor et al., 2002) and of the orthologous *osGEN-L* gene in rice (Moritoh et al., 2005) both led to male sterility. As a final example, the elimination of petals by RNAi in oilseed rape permits more photosynthetically active radiation to reach the plant (Byzova et al., 2004).

### 3.3. Transcription factors and other gene-signaling pathway modifiers

The physiological importance of transcription factors (TFs) in plant development and stress tolerance is as great as that of RNAi; however, to date TFs have not been as widely applied in the commercial development of GM crops compared to the use of RNAi. A recent and comprehensive review by Century et al. (2008) covers the role of TFs in plant domestication and breeding, as well as future prospects for plant improvement by manipulating TFs. Consequently, it would be redundant for this article to review the same material.

Transcription factors are proteins that enhance or repress gene expression, either directly or indirectly as part of a multi-component complex, usually by binding to enhancer or promoter regions of genes, thus promoting or interfering with the binding of RNA polymerase. The use of TFs – either from the plant itself or an orthologue from another source – is designed to regulate gene expression cascades that result in an enhanced ability of a plant to survive adverse growth conditions, such as drought or temperature extremes, or obtain a desired developmental outcome, such as altered nutrient content or improved yield. In addition to modulating TFs, it is possible to achieve similar results with genes that affect plant growth regulators or signal transduction pathways. These processes often result from modulation of multiple cellular activities and functions (Zhang, 2003), and as such, are part of the endogenous system plants use to respond to varying environmental conditions by modulating suites of genes. Great insight into the function of TFs comes from evaluating the role they have played during plant domestication, as described later.

Transcription factors are generally expressed at very low levels; for example, the total number of TF mRNA transcripts ranges from 0.001 to 100 copies per cell in Arabidopsis (Czechowski et al., 2004). As a consequence, the concentration of any individual TF protein in a cell will be exceedingly low. It has been reported that the TF KAP-2 from bean (*Phaseolus vulgaris*) could be induced to levels of approximately 8 ng per gram of cell mass (Yu et al., 1993). The plant genome, however, contains numerous TF-like sequences. The Database of Rice Transcription Factors (<http://drtf.cbi.pku.edu.cn/>; accessed 15 April 2007) (Gao et al., 2006) has identified 2025 TFs in indica rice and 2384 in japonica rice, based on the *in silico* detection of the presence of DNA-binding motifs in the gene sequence. A similar database for poplar (<http://dptf.cbi.pku.edu.cn/>; accessed 15 April 2007) (Zhu et al., 2007) shows 2576 putative TFs, and one database for Arabidopsis (<http://daft.cbi.pku.edu.cn/>) has identified 1922 TFs (Guo et al., 2005). The lower number of the latter is consistent with the smaller genome of Arabidopsis, and is higher than initial estimates of approximately 1400–1500 transcription factor genes in Arabidopsis (Riechmann et al., 2000; Riechmann and Ratcliffe, 2000; Czechowski et al., 2004). A total of 1300 TFs have been identified in soybean (Tian et al., 2004), although the soybean estimate was done in the absence of a genomic sequence. Based on these data, TFs account for approximately 2–7% of genes in their corresponding genomes. Clearly, TF proteins are ubiquitous in plants, and therefore are normally present in the human diet. Although the quantitative numbers are not available, it is likely that TFs represent only a very small fraction of the total plant cell protein.

A key consideration with GM crops engineered to modulate TF activity is that TFs can control gene expression cascades within a crop. In addition to expressing heterologous or up-regulated proteins as with other types of GM crops, the expressed proteins (TFs) may alter the timing, level, and spectrum of endogenous gene expression. Many genes regulated by TFs are ordinarily differentially expressed in crops grown under different stress, environmental, or developmental conditions. Through genetic engineering, it is possible to modulate these gene expression cascades by altering

the expression of TFs or other proteins that modulate gene expression, and thus produce plants that have phenotypes such as improved stress response.

### 3.3.1. Examples of the role of alteration of TFs associated with domestication and breeding

The role of TFs during domestication is clear in tomato, maize, and rice. One TF discovered in tomato, the YABBY-like transcription factor, is responsible for the large fruit size found in many modern varieties (Cong et al., 2008).

The morphology of maize was altered during domestication, perhaps more than that of any other crop besides the brassicas. Two TFs were key to some of these morphological changes, namely, the selection for unbranched plants (Doebley et al., 1997) and for naked grains (Wang et al., 2005). The former is caused by a mutation in the *tb1* gene and the latter by a mutation in the *tga1* gene, which is a member of the SBP-domain family.

The advent of wheat and rice varieties short in stature was the basis for the Green Revolution, as the shorter plants responded well to fertilizer and did not lodge, thus leading to dramatically higher yields (Evans, 1998). For wheat, alleles at two genes for reduced height, namely *Rh-B1* and *Rh-D1*, fail to respond to gibberellins, and result in lower stature. These genes from wheat are analogous to the gibberellin-regulated transcription factor gene in Arabidopsis called Arabidopsis Gibberellin Insensitive (GAI) (Peng et al., 1999).

Lack of seed shattering is considered one of the most important domestication traits, as seed that remained on the plant was much easier to harvest than seed that fell on the ground. As such, all major crops have been selected for lack of seed shattering, but most is known about the genetic basis of shattering resistance in rice. An allele of a major gene that prevents shattering, *sh4*, is due to an amino acid substitution in a TF gene encoding a DNA-binding protein (Li et al., 2006). A single base pair substitution in another TF also contributes to non-shattering (Konishi et al., 2006).

Recently, the change from 2-row to 6-row barley, encoded by the *Vrs1* gene, has been attributed to various independently occurring mutations in a homeodomain-leucine zipper I-class homeobox transcription factor (Komatsuda et al., 2007).

### 3.3.2. Transcription factors and stress tolerance

One key function of TFs is the control of a plant's response to various types of stress. General gene expression analysis in rice suggests that transcription factors play a key role in resistance to abiotic stresses (Zhou et al., 2007). Another rice transcription factor, IDEF1, regulates the response to iron deficiency (Kobayashi et al., 2007).

In pearl millet, DREB2A (for drought-responsive element binding proteins) is a stress-inducible transcription factor, the activity of which appears to be mediated by phosphorylation, as its DNA-binding capacity is diminished when it is phosphorylated (Agarwal et al., 2007). The nuclear factor Y (NF-Y) was found to play a role in the survival of Arabidopsis under drought-stress conditions. Its orthologue from maize, *ZmNF-YB2*, led to greater drought tolerance in maize plants engineered for constitutive expression of this TF (Nelson et al., 2007).

Frost tolerance is conferred in wheat and Arabidopsis by C-repeat binding factors at the *Fr-A2* locus (Vagujfalvi et al., 2005; Miller et al., 2006) and the *ESK1* transcription factor locus, respectively. The *esk1* gene product is known to regulate 312 additional genes, many of which are also expressed during salt and osmotic stress (Xin et al., 2007).

### 3.3.3. Transcription factors and agricultural biotechnology

As of yet, no crops engineered with TFs have been commercialized. However, the diversity and number of TFs that are being

discovered in plants, along with the ability of TFs to alter a wide range of traits, from growth and development to stress tolerance and crop composition, make it likely that they will be used extensively for crop improvement in the future.

## 4. Food safety considerations for crops expressing traits derived using RNAi and transcription factors

### 4.1. The bases of food safety assessment

Safety of transgenic crops in which new traits have been introduced by engineering changes that involve RNAi or transcription factors or modulating gene-signaling pathways can be assessed by the comparative safety paradigm used for crops engineered to express transgenic proteins, sometimes called comparative safety assessment or substantial equivalence as described previously (Chassy et al., 2004; ILSI, 2008). The safety of those analytes that are both outside of the known range for that crop constituent and statistically different from contemporaneously grown non-transgenic comparators are subjected to further assessment. Gene products that do not have a history of safe consumption are subjected to an appropriate safety evaluation. These will be proteins encoded by transgenes, or other compounds whose production is catalyzed by a transgenic enzyme. Although RNAi-mediated traits do not express heterologous proteins and TF transgenic plants may only express one heterologous protein, the expression profile of endogenous proteins and/or metabolites may be altered to reflect those normally found in the plant under certain circumstances (e.g., drought). Therefore, evaluation of their comparative safety for food or feed use requires answers to three questions:

- (1) Is the inserted DNA safe to consume?
- (2) Are the products encoded by the inserted DNA (e.g., dsRNA, siRNAs, miRNA, or transcription factor) safe to consume?
- (3) Do the intended changes and any unintended changes in crop composition alter the safety of the crop for consumption?

#### 4.1.1. DNA safety

DNA is a normal component of the human diet. It has been estimated that humans typically consume between 0.1 and 1.0 g/day of DNA from dietary sources (Jonas et al., 2001). Because DNA is a ubiquitous component of the human diet and no adverse health effects have been associated with dietary DNA intake, consumption of DNA in food or feed is safe. Ingestion of transgenic DNA encoding a transcription factor or designed to produce RNAi from whole grains or processed food fractions obtained from GM crops is not an exception to this presumed safety and does not result in a substantial change in the overall amount of DNA consumed. It is also important to note that the DNA used in GM crops does not differ intrinsically or physically from the DNA that is already present in food.

DNA fragments of high-copy-number plant genes (usually from the chloroplast) can be found in some animal tissues, having been detected in the blood, muscles, and internal organs of broiler chickens (Tony et al., 2003; Aeschbacher et al., 2005), calves (Reuter et al., 2005), and pigs (Reuter and Aulrich, 2003), and in muscles (Nemeth et al., 2004) or milk and blood (Phipps et al., 2003; Nemeth et al., 2004) of cattle. A low-copy nuclear gene for zein was infrequently detected in blood samples of broiler chickens (Rossi et al., 2005). To date, transgene-derived DNA arising from transgenic feed fractions has not been detected in any vertebrate tissues (Jennings et al., 2003a,b; Bertheau et al., 2009). The sole exception is the detection of 35S DNA in leukocytes, kidney, and muscles of rainbow trout fed GM soybean. The DNA was present

in such minute quantities that ultrasensitive nested PCR was required to detect it (Chainark et al., 2006, 2008).

Nevertheless, reports that small fragments of non-transgenic DNA survive digestion, are absorbed into the circulatory system of rats, and can be found in lymphatic and placental cells, have led to claims for potential adverse effects of transgenic DNA (Schubbert et al., 1997, 1998). The validity and biological significance of observations in these reports have, however, been questioned (Beever and Kemp, 2000; Jonas et al., 2001; Goldstein et al., 2005). It is not clear that the DNA fragments are covalently bound in the nuclear DNA in the tissues in which they were identified, and in no case were the observed fragments large enough to encode a functional gene. No evidence has appeared to date that demonstrates DNA absorbed by vertebrates via their intestines can be incorporated into the vertebrate genome as whole or partial genes, nor is there any evidence for the acquisition and expression of plant genes in vertebrates (Beever and Kemp, 2000; Jonas et al., 2001; Goldstein et al., 2005). Transgenic DNA from GM crops is considered to be as safe as DNA that is a common component of foods.

#### 4.1.2. RNA safety

As with DNA, RNA is a normal component of the human and animal diet and is therefore regarded as safe to consume. The RNA content of foods and feeds is not normally reported since it contributes little to overall nutrition. RNA makes a negligible addition to energy intake and contributes small amounts of purine and pyrimidines to the diet (Carver and Walker, 1995). It has been estimated that humans consume between 0.1 and 1.0 gm/day of RNA (Jonas et al., 2001). It should be noted that the foregoing reference cites Doerfler and Schubert (1997) as a source of the average daily intake of RNA; however, this cited reference gives values only for the daily intake of DNA (Doerfler and Schubert, 1997). The net result is that there really are no direct measurements available of the average daily consumption of RNA. Amounts may be inferred from Table 1 in Jonas et al. (2001), which contains values for DNA and RNA content of commonly consumed foods and ingredients that show that the RNA and DNA content of foods are roughly comparable. The data on RNA content of foods and feeds are also in agreement with earlier work (Clifford and Story, 1976; Herbel and Montag, 1987; Lassek and Montag, 1990; Carver and Walker, 1995). Although considered safe to consume, it has been recommended that daily intake of RNA should not exceed 2 g/day, as a high intake of purines leads to increases in serum uric acid that could have adverse effect on persons with gout (Clifford and Story, 1976). Yeast and other rapidly growing microorganisms contain 6–7% RNA and it is therefore recommended that people not consume greater than about 30 g/day of yeast (UN Protein Advisory Group, 1972).

Pancreatic nucleases metabolize RNA into mono-, di-, tri- and polynucleotides which are further degraded to mononucleotides, nucleosides, and bases by the action of intestinal polynucleotidases, nucleases, phosphodiesterases, phosphatases, and nucleosidases (Carver and Walker, 1995). The resulting nucleosides and free bases are primarily absorbed in the upper portion of the small intestine. The majority of the absorbed nucleosides and bases are then catabolized in enterocytes (Carver and Walker, 1995) and around 2–5% are reincorporated into nucleotides. There are no reports of the uptake and absorption of intact RNA in vertebrates in the peer-reviewed literature. Attempts to measure RNA absorption in a neonatal pig model were unsuccessful (Baintner and Toth, 1986).

In contrast to vertebrates, invertebrates such as nematodes and insect larvae can absorb nucleic acids, and there is evidence that both have acquired functional genes by horizontal gene transfer from bacteria (Davis and Wurdack, 2004; Hotopp et al., 2007).

The nematode, *C. elegans*, also can absorb native dsRNA from ingested bacteria, a phenomenon that allows gene suppression to be exploited in the study of nematodes (Timmons and Fire, 1998; Timmons et al., 2001).

As noted previously, if humans or animals have a history of safe dietary exposure to a food or feed component, that component is assumed to be safe for consumption (Constable et al., 2007). Recent reports on the abundance of small RNAs in plants point to a history of safe consumption. Numerous small RNAs are ingested in the food supply (Ivashuta et al., 2009). For example, recent publications have documented that endogenous small RNAs are abundant in soybean seeds, corn kernels, and rice grain, plant tissues that are traditionally used in food and feed (Heisel et al., 2008; Ivashuta et al., 2009). Numerous endogenous plant small RNAs were found to have perfect complementarity or near-perfect complementarity to vital human genes as well as those of other mammals. This includes homology to such critical genes as P450 monooxygenases. The fact that small RNAs with homology to genes encoding proteins with vital functions are abundant in safely-consumed food and feed crops such as soybean, corn, and rice is a clear indication that consuming small RNAs does not pose a risk to vertebrates, regardless of homology, and establishes a history of safe consumption for dietary small RNAs (Ivashuta et al., 2009).

As discussed previously, gene-suppression technologies have been used to develop transgenic plants with phenotypes such as resistance to viruses and nematodes, delayed ripening, and altered composition (Kusaba, 2004; Dietzgen and Mitter, 2006). A number of RNA species, including dsRNA, miRNA, hpRNA, and/or siRNA, are formed as part of the RNAi mechanism. The RNAi-derived molecules represent only a modest portion of the total RNA content of the cell because ribosomal RNA alone constitutes around 80% of cellular RNA (Brown, 2002). Recently, it has been reported that approximately 0.1% to 0.2% of the RNA in a soybean is present in the small RNA (21–26 nt) fraction of the total RNA (Ivashuta et al., 2009). The ratio of the different types of RNA and the percentage of cellular RNA represented by each of these RNAi-associated RNA species in the cell are not presently known for other species and are likely to be different in each specific example. It is clear that because practically all eukaryotes employ RNAi mechanisms for control of cellular functions, humans and animals routinely consume a wide array of RNAi-associated molecules from plant and animal sources. Many of these RNAi-associated molecules from plant sources are consumed in raw foods and therefore intact and have a history of safe consumption. As noted previously, it also appears that many of these small plant-derived RNAs share sequence homology with mammalian small RNAs so that humans and animals routinely consume with no apparent adverse effect small plant-derived RNAs that could potentially target critical mammalian genes (Ivashuta et al., 2009).

The DNA inserts used in dsRNA-generating constructs are transcribed but are not translated into proteins. This part of the safety assessment process should therefore focus on the safety of the newly-introduced DNA and its RNA transcripts. In the absence of heterologous proteins, the portions of the safety assessment paradigm that evaluate the potential for protein toxicity or allergenicity cannot be applied. Additional testing can be performed to evaluate the safety and nutritional implications of any intended or unintended compositional changes.

RNA-degrading nucleases are ubiquitous and perhaps as a consequence, RNA is rapidly degraded in most environments. As noted previously, RNA is degraded to nucleotides in the human gastrointestinal (GI) system (Carver and Walker, 1995). It can be predicted that RNAi-associated RNA species will be rapidly degraded in the digestive tract, even including enhanced steady-state levels of any given dsRNA molecule, thus rendering it innocuous, regardless of any homology it may have to vertebrate genes. Although it can

be inferred that dietary small RNAs are digested in the GI tract (Ivashuta et al., 2009), the conclusion that these RNA species would be rapidly digested has apparently not been tested experimentally, perhaps owing to the well-recognized lability of RNA.

Unlike vertebrates, some lower animals can absorb nucleic acids through their GI tracts and other tissues. Feeding *E. coli* that produce dsRNA precursors of biologically active siRNAs to *C. elegans* results in effects similar to those observed upon direct micro-injection of siRNAs (Fire et al., 1998; Timmons and Fire, 1998; Timmons et al., 2001). Root-knot nematodes can absorb naked dsRNA administered by soaking in concentrated RNA-containing solutions (Tabara et al., 1998; Urwin et al., 2002).

For higher animals, direct absorption of naked RNA has been reported for pharmacological purposes using cultured animal mucosal cells and tissues, but not in dendritic cells or macrophages (Dykxhoorn et al., 2006). The difficulty in delivering dsRNA into human and animal cells *in vivo* is the major barrier to therapeutic uses of RNA interference (Behlke, 2006; Dykxhoorn et al., 2006; Crombez et al., 2007). One study reported that oral administration to rats of invasive *E. coli* containing a vector that generates short dsRNA can elicit gene suppression (Xiang et al., 2006). However, this represents an artificial situation that used expression of invasins (an enzyme that permits bacterial invasion of cells) and an RNA exporter designed to deliver the RNA to the cells. Therefore, the results of this study are not applicable to assessing oral safety of dietary dsRNAs (including siRNAs and miRNAs) from consumption of GM crops. When unmodified siRNAs targeting ApoB were injected intravenously into mice using a standard tail vein injection, a 50-mg/kg dose failed to suppress hepatic expression of ApoB mRNA or protein (Soutschek et al., 2004). This finding demonstrates that even high intravenous doses of siRNA are ineffective, further illustrating an extremely low likelihood that dietary siRNA could have oral activity.

In summary, no literature reports document GI absorption of dsRNA in higher animals. The weight of evidence indicates that it is highly unlikely that intact dsRNA and its derivatives are absorbed in the human GI system. Considering the rapid breakdown of RNA in the GI system, the lack of evidence for uptake of intact RNA in the vertebrate GI system, and the difficulty in delivery of very high doses of unmodified siRNAs into either mammalian models via intravenous injection, or into animal cell cultures as concentrated doses, absorption of plant-produced dsRNA or siRNA from food or feed by humans or animals would be an extremely infrequent event, but no direct data are available. In this regard, nematodes and insect larvae appear to be exceptional in their ability to absorb nucleic acids and are an unsuitable model for mammalian effects of siRNA. Perhaps more importantly, humans and animals have been consuming plant and animal tissues containing hundreds of thousands of RNAi-associated molecules throughout their existence with no evidence of either uptake or adverse effect. Interestingly, many of these RNAi-associated molecules have perfect or near-perfect complementarity to human and animal genes (Heisel et al., 2008; Ivashuta et al., 2009) and therefore may act on targets that are present in the vertebrate genome, an observation that reinforces the conclusion that small RNAs are not active when consumed by humans and animals in the diet.

#### 4.1.3. Safety of heterologous transcription factors

The safety of heterologous TFs can be assessed using the existing criteria for protein safety. All nucleated cells contain numerous transcription factors; thus, humans and animals have a long history of dietary exposure to transcription factors. No adverse effect has been attributed to the consumption of transcription factors, and no proteins that are known to be toxic or allergenic have been documented to be transcription factors (Delaney et al., 2008; <http://www.allergenonline.com/>). Some transcription factors that

could be used in genetic strategies for crop improvement will be derived from the crop plant itself or from another plant. The concentration and duration of expression may, however, be altered.

The primary question of a safety assessment is whether a heterologous transcription factor, or an altered level of an endogenous transcription factor in the crop, can pose risks that are not otherwise present. Considering the long history of exposure to transcription factors and the low concentration of each specific transcription factor in the diet (Kier and Petrick, 2008), it is fair to ask if further protein safety evaluation is indicated. This is particularly the case because domestication of wild plants and conventional breeding of crop plants have led to alterations in transcription factor function and expression without any evidence or suggestion of adverse effects.

In applying the existing safety assessment paradigm for proteins expressed in GM crops, the transcription factor that will be expressed in a GM crop can be screened using bioinformatics to determine whether it resembles known toxic or allergenic food proteins. Proteins that have a history of safe use, are not major proteins in the food consumed, are not similar in sequence to known allergenic or toxic proteins, and that are digestible are generally considered to be safe for humans and animals (Delaney et al., 2008).

#### 4.1.4. Safety of compositional changes

RNAi-based traits have been introduced into commercialized crop varieties to protect against viruses or to alter the endpoint of metabolic processes; for example to suppress the production of polygalacturanase or linoleic acid (Table 1). In these examples, no unintended compositional changes other than those that would normally be associated with plant breeding (Cellini et al., 2004) or modification of the target trait result from the use of RNAi as a plant protection strategy.

Expression of a heterologous TF, as well as over-expression or repression of endogenous TFs, may result in altered expression of endogenous proteins and altered plant composition. In addition, RNAi mechanisms can target regulatory proteins. For example, miRNA can act directly on a TF to modulate drought tolerance (Li et al., 2008) or time of flowering (Aukerman and Sakai, 2003). Whenever compositional changes are expected, it may be necessary to demonstrate that the compositional changes do not present an unacceptable risk to nutrition or health.

The use of RNAi may elicit compositional change(s) as an intended outcome, which may also hold true for the use of TFs. Comparative assessment with a suitable counterpart, in most cases a near-isogenic non-GM line, allows evaluation of compositional changes, if any. As described above, (Chassy et al., 2004; ILSI, 2008) the nutritional and safety consequences of any changes detected can be evaluated.

As noted previously, crop composition varies with location, environment, and cultural conditions such as climate. It is, however, also becoming increasingly clear that conventionally developed crops can show far greater heterogeneity in composition due to genetic differences than varieties of the same crop that have been produced through transgene insertion (Catchpole et al., 2005; Lehesranta et al., 2005; Shewry et al., 2007; ILSI, 2008). Moreover, it is important to recognize that compositional differences per se are not necessarily an indicator of a hazard. Unless the change is shown to cause adverse biological effects such as, for example, a specific vitamin deficiency attributable to a major decrease in vitamin content, the change does not alter safety of the food or feed. For this reason it is not recommended that an arbitrary percentage of change be set as a trigger for further risk assessment (Chassy et al., 2004; ILSI, 2008).

## 5. Conclusions and recommendations: knowledge gaps and future outlook

Transgenic technology will be used to produce crops engineered for specific alterations in composition of the edible food and feed fractions. These changes will be introduced to improve the nutritional content or healthfulness of the food or feed. High-lysine maize and soybeans with altered fatty acid oil profiles are examples. Alternatively, crops may be altered to better suit them for alternative uses, such as the altered starch structure in potato. A recent review on nutritionally enhanced crops (ILSI, 2008) describes developments in food safety assessment and applies the comparative safety paradigm to case studies of products currently being developed. The report concludes that the existing safety assessment paradigm is sufficiently robust to support the safety evaluation of crops with altered composition.

The ILSI report also concluded that the currently employed targeted analytical composition methodologies are fully capable of detecting biologically significant unintended effects. Targeted compositional analysis can be used to investigate whether or not a meaningful loss of nutrients has occurred and if an adverse increase in anti-nutrients, toxicants, or allergens has resulted from the engineering process. At this time, several “omics” technologies are emerging, but the baseline data for the proper application of these new technologies are lacking, thus precluding them from use as a tool for composition and safety assessment. The ILSI report also notes that targeted analysis has a long history of safe application to risk assessment of foods and feeds.

Introduction of new traits by RNAi and transcription factors poses no novel hazards above those attributable to other methods of genetic modification, including plant breeding. Furthermore, in view of the evidence that RNAi and alteration in transcription factors are mechanisms that have contributed to genetic change throughout the history of crop domestication and conventional breeding and consequently have a history of safe use, there is no scientific rationale to justify new or more complex safety assessments for plants modified through transgenic RNAi or TFs.

### Key conclusions

1. RNAi-mediated mechanisms have been engineered into crops that are used commercially.
2. In the near future, crops with heterologous transcription factors (TFs), or altered levels of TFs are likely to be submitted to regulators for approval.
3. Current data demonstrate that RNAi- and TF-associated mechanisms have been altered in the process of domestication and breeding of conventional crops.
4. Large numbers of small RNAs and TFs are encoded in plant and animal genomes; as a consequence, there is a long history of safe consumption by humans and animals.
5. Engineered crops depending on RNAi-type modifications will not contain heterologous proteins and so a safety assessment for a novel protein is not necessary. Engineering crops through use of TFs will introduce minute amounts of a heterologous protein. Both techniques can alter the concentration of an endogenous TF.
6. The TFs engineered into plants may come from a source organism with a history of safe use. When a history of safe use cannot be established, a Tier I protein safety assessment should be applied to assess safety of the protein.
7. The safety assessment of crops developed by the modulation of endogenous plant gene expression should

focus on a comparative assessment/compositional analysis to the non-transgenic version. In the case of a stress-tolerance trait, the comparator should also be grown under conditions that the crop has been engineered to tolerate, to ensure that no adverse compositional changes affecting safety for consumption have occurred.

8. It is concluded that engineering crops for the production of small RNAs or for the expression of TFs does not present any novel hazards. Consequently, the current safety assessment paradigm provides a high level of assurance that such engineered crops will be safe for food and feed use.

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## Conflict of interest statement

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