Roundup Ready Wheat – An Overview Based on Advancements in the Risk Assessment of Genetically Engineered Crops

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Abstract

Glyphosate tolerant wheat is being considered for commercialization ten years after the first commercial introduction of a genetically engineered (GE) crop. Substantial progress has been made in defining risks and improving risk assessment methods for GE crops during that time. However, U.S. regulatory agencies have adopted few written guidelines for determining the human and environmental safety of GE crops, leading to considerable inconsistency in safety data submitted to those agencies. Some of the important developments in GE risk assessment relevant to herbicide tolerant (HT) GE crops are reviewed and applied to glyphosate tolerant spring wheat (*Triticum aestivum* L.). The importance of using appropriate methodology to achieve reliable results is discussed using examples from the literature and GE crops to demonstrate how different methods can lead to opposite conclusions about safety. Recommendations are made for revising current practices to provide more useful data, and areas in need of additional research are identified. Topics considered include characterization of the transgene and transgenic protein, allergenicity, unintended effects, and resistance management. Using the best risk assessment methods available will be important in developing consumer confidence in this controversial new technology.

Abbreviations: GE, genetic engineering or genetically engineered; HT, herbicide tolerant; RR, Roundup Ready™; FDA, U.S. Food and Drug Administration; USDA, U.S. Department of Agriculture; EPA, U.S. Environmental Protection Agency; FAO/WHO, United Nations Food and Agriculture Organization/World Health Organization; SAP, EPA Scientific Advisory Panel; CP4 EPSPS, *Agrobacterium* CP4 enolpyruvylshikimate-3-phosphate synthase; SGD, simulated gastric digestion; JGG, jointed goat grass (*Aegilops cylindrica* Host); ALS, acetolactate synthase

Introduction

Glyphosate tolerant, or Roundup Ready™ (RR), wheat is poised to be the next big genetically engineered (GE) crop in the U.S and Canada, if current trade barriers are overcome and regulatory agencies review it favorably. Coming ten years after the first commercial genetically engineered crop, the FlavrSavr tomato, it provides an opportunity to review advances in the risk assessment of GE crops. This paper reviews several risk assessment issues pertinent to the U.S. regulatory system's risk assessment of RR wheat.

In the past ten years significant progress has been made in the methods for assessing and managing the safety of GE crops. Numerous recommendations for improving the human and environmental safety assessment of GE crops have been made by scientists working in relevant disciplines, such as allergy and immunology, agricultural sciences, ecology, and population genetics. Many of those recommendations are applicable to herbicide tolerant (HT) crops, and glyphosate tolerant wheat in particular. If implemented, many of those recommendations would give greater confidence in GE-crop risk assessments.

Those advances in risk assessment methodology could provide more accurate risk determination, or reduced cost or less time to achieve accurate results. Using the best available safety testing methods is important because tests carried out using inadequate design may have limited value, provides a false sense of assurance, and may lead to loss of public confidence if challenged.

This paper emphasizes only a few of many risk assessment issues of HT GE crops. The topics considered include aspects of the characterization of the transgene and transgenic protein, potential allergenicity of the transgenic proteins, unintended dietary effects, and resistance management of RR spring wheat. In addition, the *Agrobacterium* CP4 enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene that confers glyphosate tolerance in RR wheat has been used in previous RR crops, such as RR soybeans. Previous data on CP4 EPSPS can therefore be considered in evaluating RR wheat. Imazamox-tolerant Clearfield winter wheat, developed by non-GE means, presents some similar issues as glyphosate tolerant RR wheat, especially concerning resistance management and gene flow, and is briefly considered.

Genetically engineered crops continue to generate controversy. Even prior to commercialization, there is much concern among U.S. and Canadian farmers about possible restrictions on their ability to export wheat if RR wheat is introduced. The likelihood of contamination of conventional wheat by RR wheat, and the low tolerance for that contamination in Europe, could restrict export of all wheat. The U.S. regulatory process could help relieve, or contribute to, concerns about RR wheat, depending upon the level of confidence that the public has in the risk assessment process. It is therefore crucial that advances in risk assessment over the past ten years are carefully considered and adopted where appropriate.

Background on the U.S. Regulatory System for GE Crops

Risk assessments in the U.S. are conducted by one or more of three federal agencies charged with ensuring the food, feed, and environmental safety of GE crops. Those three agencies, the Food and Drug Administration (FDA), Department of Agriculture (USDA), and the

Environmental Protection Agency (EPA), act under laws passed prior to the advent of genetic engineering.

The dietary and animal-feed safety of the transgene, its product, and herbicide-tolerant (HT) GE plants like RR wheat are assessed by the FDA through a voluntary notification process under the Federal Food, Drug and Cosmetic Act (U.S. Food and Drug Administration, 1992). That process provides broad criteria for risk assessment, but no detailed safety testing methodologies or guidelines. FDA has conducted workshops and public meetings, but has not revised its risk assessments policies.

The USDA evaluates the environmental safety of GE HT crops under the Plant Pest Act and, more recently, the Plant Protection Act. Those crops typically go through a notification process for field testing and a more extensive permit and deregulation process prior to commercialization.

EPA regulates the human, animal, and environmental safety of pesticidal GE crops under the Federal Food Drug Cosmetic Act and the Federal Insecticide, Fungicide, and Rodenticide Act, but does not assess the safety of GE HT crops. EPA does evaluate the use of the herbicides applied to those crops. Evaluation of the safety of the herbicides used with HT wheat is not reviewed in this paper.

Currently, FDA's process for reviewing the safety of GE crops gives substantial discretion to the crop developer to determine how to conduct safety assessments, resulting in considerable variation in the data and analyses received by FDA for review (Gurian-Sherman, 2003). The U.S. National Academy of Sciences (National Research Council, 2002) has recommended the improvement of several aspects of USDA's environmental review process of GE crops. Therefore, there is a need to update and better define the data needed to assure that GE crops are safe.

Characterization of the Transgene and Transgenic Protein

Sequence data are fundamental to understanding the safety of transgenes and transgenic proteins, including those of RR wheat. Sequences are used to search for homology with deleterious proteins such as allergens or toxicants and to help determine the function of the protein. Thus, sequence data should form the foundation for any risk assessment. Sequencing of the gene from the transformed plant was recently supported as a part of GE risk assessment by a committee of the National Academy of Sciences (NRC, 2002).

Accurate sequence data is important because even small sequence variations that may occur during transformation (Kohli, et al., 1998; Pawlowski and Somers, 1998) can have structural or functional implications. Typically, GE-crop risk assessments have not included the sequence of the gene or protein from the transformed plant. Instead, the sequence of the gene prior to transformation has been submitted to U.S. regulatory agencies (Gurian-Sherman, unpublished data). Those sequences do not include changes that may occur during or after transformation. If not identified, such sequence changes could lead to inaccurate safety conclusions. Risk assessment data for RR soybeans did not include the sequence of CP4 EPSPS from the transgenic plant (Padgette et al., 1995). In any case, because unique sequence

alterations can occur with each transformation, the transgene for each new transformation, such as each RR wheat "event," should be determined.

Small sequence changes in the transgenic protein could lead to functional changes important for risk assessment. For example, only a few changed amino acids could result in new IgE allergen epitopes, which may be as short as six or eight amino acids (United Nations Food and Agriculture Organization/World Health Organization, 2001, hereafter FAO/WHO, 2001). Similarly, small changes could eliminate protease recognition sites, thereby increasing the stability and possible allergenicity of the protein. For example, a single intentional alanine substitution eliminated, and a lysine substitution decreased, trypsin sensitivity in Cry9C without substantial changes in activity on a test insect (Lambert et al., 1996). The lysine mutant was used in StarLink™ corn. Although pepsin, rather than trypsin, is used in allergen stability tests to simulate the human stomach, similar changes could increase pepsin stability. Similarly, sequence changes adding a fortuitous glycosylation recognition site, could also increase allergenicity.

Also, the analogous protein produced in bacteria is often used as a surrogate because the GE plant typically does not readily produce enough protein for safety tests. For example, CP4 EPSPS produced in *E. coli* was used for allergenicity stability tests and rodent acute toxicity tests (Harrison et al., 1996). The sequence of that bacterial protein may differ from the sequence in the GE plant. Therefore, it is important that the protein from the transformed plant is identical to the bacterial version used in safety tests (or can be shown to be functionally the same by relevant tests).[1]

Bioassays or enzyme kinetics comparing the bacterial and transgenic proteins have been used to demonstrate equivalence, but those assays might not detect changes important for human risk assessment. As noted above, several single amino acid changes reduced or eliminated trypsin protease degradation with little or no significant effect on activity against the target insect (Lambert et al., 1996). It is possible that changes may occur in properties of the protein related to safety without affecting other parameters, such as activity in a bioassay.

Similarly, post-translational modifications of CP4 EPSPS, such as glycosylation, may alter the structure and function of the protein, potentially increasing risk. The EPA's Scientific Advisory Panels (SAPs) that considered the allergenicity of Cry9C protein from GE Starlink corn were concerned about inaccurate determination of glycosylation for the bacterial-produced Cry9C. The SAPs were concerned about possible lack of biochemical equivalence between bacterial and maize versions of the protein that might affect the allergenicity assessment (U. S. Environmental Protection Agency, 2000 and 2001). Glycosylation is not a prerequisite for allergenicity but is frequently associated with it, and has been recognized as an important factor to consider in allergenicity assessments (Garcia-Casado, et al., 1996; Kimber and Dearman, 2001; U. S. Environmental Protection Agency, 2000; van Ree, et al., 2000).

In situ hybridization should be considered as a means of examining the insertion site of CP4 EPSPS in RR wheat because currently used methods may miss rearrangements. The

[1] Organisms other than bacteria may also be used to over express the protein, but all examples of transgenic crops submitted to FDA that we have examined have used bacteria. In any case, even eukaryotic expression organisms such as yeast may differ in their processing of transgenic proteins compared to the transgenic crop.

transgene insertion site may include interspersed genomic sequences that may span hundreds of kilobases and are not always readily detectable using typical restriction site mapping and Southern blots (Pawlowski and Somers, 1998; Svitashev and Somers, 2001). Such genomic rearrangements associated with simple (Makarevitch et al., 2003) as well as complex transformation insertions could affect the function of several genes with unpredictable results. And because they may form a single genetic locus, such interspersed genomic sequences will usually remain after recurrent backcrossing into agronomically desirable varieties.

Transgenes may insert into host genes, causing insertional mutations or fusion proteins. Those outcomes can be readily determined by sequencing junction DNA adjacent to the transgene(s), examining open reading frames for homology to known gene, and subjecting those sequences to expression analysis such as Northern blots. Determining potential insertional mutations and fusion proteins is recommended by European Union and Codex Alimentarius guidance documents for GE safety assessments (European Commission, 2003, Codex Alimentarius Commission, 2003).

In addition to rearrangements of the transgene that may occur during transformation, eukaryotic genes typically contain introns, and bacterial genes often have introns added to improve expression in plants (Callis, et al., 1987; Leuhrsen and Walbot, 1991). Introns are removed during mRNA processing and the protein coding regions are spliced together. Coding regions may be improperly spliced in a new organism or due to sequence changes in the intron (Brown and Simpson, 1998; Marillonet and Wessler, 1997; Sablowski and Meyerowitz, 1998), and those possibilities need to be addressed for genes containing introns. The CP4 EPSPS used in RR soybeans does not contain introns (Padgette, et al., 1995), but the CP4 EPSPS used in RR wheat is not currently available.

Stability of transgenes over several generations has often been characterized for previous GE crops, but data are often presented to FDA without appropriate statistical analysis (Gurian-Sherman, 2003). Less commonly determined is the stability of expression, which may change under various agronomic conditions, especially due to gene silencing (Al-Kaff, et al., 2000; Haslberger, 2003). Increased expression may change risk levels because most toxicological properties are dose dependent.

Human Safety

Two human safety issues pertinent to the evaluation of RR wheat that have received considerable attention are allergenicity of the transgenic protein and unintended harmful changes in the transformed plant. Concern about allergenicity arose after an allergenic protein was transferred into a food crop by genetic engineering and after StarLink corn contamination of food corn in the U.S. Concern about unintended effects comes from the recognition that many harmful substances produced in foods in small quantities can occasionally increase to harmful levels as the result of crop breeding and, by extension through GE. Several safety tests for the assessment of allergenicity and unintended effects have been recommended since the first transgenic crops. But the currently available methods are not able to predict with certainty either the allergenicity of a protein new to the food supply or harmful unintended effects.

Potential toxicity of a transgenic protein is also considered by FDA, but there are no requirements for particular toxicity tests. CP4 EPSPS acute toxicity was tested in rodents by feeding a high dose and examining test animals over a short period of time (Harrison et al., 1996). Those tests revealed no toxicity, and there have been no reports of toxicity to animals or humans since the commercialization of other RR crops. CP4 ESPSP toxicity is not considered further here. However, acute toxicity test methods and results from previous RR crops should be carefully considered to determine whether the bacterial versions of of CP4 EPSPS used in those tests are equivalent to RR wheat CP4 EPSPS, and whether other tests methods were adequate.

The safety conclusions reached in GE-crop risk assessments can be significantly affected by the particular methodology used to carry out safety tests and procedures. Therefore, consideration is given to the possible consequences of using different approaches when conducting safety tests for allergenicity and unintended effects of RR wheat.

Allergenicity

The potential allergenicity of transgenic proteins became a practical issue in 1996, when a protein transferred from Brazil nut to soybeans to improve the quality of soybean protein was found to be allergenic. The resulting GE soybeans bound IgE from sera of people allergic to Brazil nuts (Nordlee, et al., 1996). The GE soybeans were not commercialized, but that example demonstrated that an allergenic protein could be transferred into a GE food. Also, in 1998 EPA approved StarLink™ corn (containing transgenic Cry9C) only for animal feed because the risk assessment suggested possible allergenicity. When it was later found in human food, the resulting StarLink™ controversy led to the convening of several science panels by EPA and an expensive recall of the contaminated corn (U. S. Environmental Protection Agency, 2000 and 2001). The allergenicity of RR soybeans and CP4 EPSPS have been assessed and are considered here where relevant to RR wheat.

Since the FDA GE safety testing guidance in 1992, several documents and research papers have made testing recommendations concerning the evaluation of GE protein allergenicity (FAO/WHO, 2001; Metcalfe, et al., 1996)**.** Those tests have not been officially adopted by U.S. regulatory agencies, and some need further development and validation. The currently available tests cannot predict with confidence the allergenicity of proteins new to the diet. For example, although stability in a simulated gastric digestion (SGD) assay is now commonly used as part of the allergenicity risk assessment, not all food allergens are stable. Conversely, some non-allergenic food proteins are stable in the SGD assay. Using stability as the criterion for allergenicity in GE risk assessments could miss some allergens or brand some non-allergens as allergenic. Risk assessments therefore use several methods to determine potential allergenicity. But even in combination, those assays cannot reliably predict allergenicity for proteins new to the diet.

The methodology used in conducting allergenicity tests may also substantially influence the conclusions about the risk of a GE protein and crop. For example, changing the ratio of pepsin to transgenic protein used in SGD assays may change the apparent stability of the protein and thereby lead to inaccurate conclusions about possible allergenicity. Higher ratios of pepsin to test protein in SGD assays reduce the stability of some allergens (Fu, et al., 2002). Similarly, high ratios of pepsin may reduce the stability of some transgenic proteins leading to the conclusion that they are not likely to be allergenic whereas lower ratios could lead to the

opposite conclusion. Conversely, lower ratios of pepsin to test protein indicated that some non-allergens were stable (Fu, et al., 2002). Therefore, low ratios of pepsin to test protein may make some transgenic proteins appear to be stable, leading to false conclusions of possible allergenicity, contrary to higher ratios. In addition to pepsin concentration, pH and prior food processing (e.g., heating) may influence results of SGD assays (Takagi, et al., 2003).

Thus, how tests are conducted can introduce false positive or false negative results. A cautious risk assessment may use methodology that accepts more false positive results while a more permissive approach may accept more false negatives. Currently, which allergenicity test methodology is used is largely determined by the crop developers because regulatory agencies have not provided guidance on testing. The critical need to standardize both methods and interpretation of results for SGD tests has been widely recognized (Fu, 2002; Bannon, et al., 2003).

The primary tests for allergenicity of proteins, such as CP4 EPSPS, from organisms not known to be allergens and new to the food supply consist of *in vitro* SGD and database searches for homology to known allergens. The SGD assay is based on empirical observations of strong correlation between the *in* vitro stability of either whole, or large fragments of, major food allergens (Fuchs and Astwood, 1996). Although gastric stability does not perfectly correlate with food allergenicity (Bannon, et al., 2003), the assay remains an important part of allergenicity assessments and was the primary reason for concern about Cry9C in StarLink™ corn. The assay will not identify certain allergens, such as Mal D1 from apple, that are involved in oral allergy syndrome and are labile to proteases (Jensen-Jarolim et al., 1999). Some of those allergens may be identified by homology with cross-reacting respiratory or dermal allergens (Vieths, 2002; Yagami, 2002). If the transgenic protein belongs to a class that contains known allergens, immunological testing using serum from allergic individuals is recommended (FAO/WHO, 2001). That is not the case for CP4 EPSPS, which has plant homologues that are not known allergens.

Protocols establishing the SGD assay used a ~19:1 weight to weight (w/w) ratio of pepsin to test protein and pH 1.2 (Astwood, et al., 1996; Metcalfe, et al., 1996). More recently, an international consensus of experts working with World Health Organization/Food and Agriculture Organization (FAO/WHO, 2001) recommended using a ratio of ~1.3:1 w/w pepsin to test protein at pH 2.0.

The parameters used to establish the correlation between stability and allergenicity were empirically derived from *in vitro* experiments and do not necessarily reflect actual physiological conditions. In addition, recent evidence suggests that the physiological explanation for the correlation between gastric stability and allergenicity may be more complex than simply allowing exposure of intestinal immune tissue to the protein (Dearman, et al., 2002). And sensitization to a food allergen may occur through a cross-reacting respiratory (e.g. pollen) or dermal (e.g. latex) allergen exposure rather than by contact with the intestines (Vieths, 2002; Yagami, 2002). Therefore, physiological considerations, such as gastric transit time of food proteins, cannot currently be used to interpret SGD results.

Monsanto conducted SGD assays using CP4 EPSPS protein produced in *E. coli* analogous to the protein used in RR soybeans (Harrison, et al., 1996). Those assays used 1,600:1 w/w ratio of pepsin to CP4 EPSPS, or ~85-fold higher than Astwood et al. (Astwood, et al.,

1996) and ~1,230-fold higher than recommended by the FAO/WHO protocol (FAO/WHO, 2001). There have been no indications since its commercialization that CP4 EPSPS is allergenic. However, because non-GE soybeans are an important allergen, lower levels of CP4 EPSPS allergenicity could go undetected in the background of allergy to non-GE soy because there is no program to monitor possible CP4 EPSPS allergenicity. The SGD is a simple *in vitro* assay and should be repeated for RR wheat using SGD protocols recommended by FAO/WHO that are less likely than other methods to miss potential allergens.

Database searches may identify homology with known allergens (Gendel, 1998a and 1998b). Those searches should look for both overall similarity between the transprotein and database proteins and IgE epitope matches. Under FAO/WHO guidelines potential allergenicity is indicated by at least 35% homology between the GE protein and a known allergen using a window of 80 amino acids. In addition, a six-contiguous-amino-acid match may indicate an allergen IgE epitope (FAO/WHO, 2001). Risk assessment for RR soybeans containing CP4 EPSPS included overall and eight contiguous amino acid homology searches that disclosed no matches (Fuchs and Astwood, 1996, Harrison, et al., 1996). A seven contiguous amino acid match between CP4 EPSPS and the house dust mite allergen, Der p 7, was recently found (Kleter and Peijnenburg, 2002). But additional criteria for likely allergenicity suggested by the authors, that the match correspond to either a region of high antigenicity or a known IgE epitope on Der p 7, were not met. On the other hand, seven-amino-acid homology with Der p 7 would likely trigger testing of CP4 EPSPS with serum from dust mite allergic individuals under the FAO/WHO allergenicity protocols, and should therefore be carefully considered by the FDA.

Use of six contiguous amino acids could result in many false positive matches, i.e. matches to sequences that are not functional epitopes. Recognizing that possibility, the FAO/WHO recommended that six-amino-acid matches to allergens should be followed by tests using antibodies from patients allergic to the matched allergen. Such serum testing could also detect non-linear conformational epitopes for which there is little current sequence data. And, as with the SGD assay, homology searches can be performed early in the development process, avoiding significant costs if potential or actual allergenicity is found. Currently, validated serum banks are not available, and need to be developed. The feasibility of serum testing has been demonstrated in soybeans with an added Brazil nut protein (Nordlee et al., 1996). The CP4 EPSPS gene in wheat should be submitted to the updated allergenicity protocols proposed by FAO/WHO.

Unintended Adverse Effects

Concern about possible unintended adverse effects in GE crops comes from observations of such effects in conventional crop breeding, and that unexpected changes in plants following genetic engineering are common. Combining those two observations leads to the conclusion that unintended adverse health effects may occur in GE crops, although none have yet been observed with commercialized GE crops.

Several well-known cases of dangerously elevated levels of toxicants in conventionally bred potato, celery and squash are often cited to demonstrate the possibility of deleterious unintended effects (Diawara and Trumble, 1997; Kirschman and Suber, 1989; Rymal et al., 1984; Zitnak and Johnston, 1970). Levels of intrinsic allergens are also known to vary between crop varieties (Jensen-Jarolim et al., 1998, Weiss et al., 1993). As with toxins, food allergens

show a dose-dependent response and higher numbers of individuals show symptoms at higher exposure levels (Taylor and Lehrer, 1996, Moneret-Vautrin et al., 1998). Previously documented unintended adverse effects, such as higher toxicant levels, suggest that certain methods of traditional breeding, as well as GE, may warrant increased scrutiny. For example, breeding with wild relatives introduces many genes tightly linked to the desired trait that code for unknown properties. Mutation induction, used for developing some HT crops, introduces uncharacterized mutations. However, those mutations that are unlinked to the herbicide tolerance trait will be removed if recurrent backcrossing is used during variety improvement for sexually reproducing crops such as wheat.

Unintended effects in GE crops may occur due to insertional mutations of host genes by transgenes, rearrangement of genomic DNA, epigenetic effects such as gene silencing, or unexpected interactions between the transgene product and the host (Haslberger, 2003). Such changes may include increased levels of anti-nutrients, toxicants, or allergens or decreased levels of important nutrients. Unexpected changes are common in transgenic plants (Gurian-Sherman, 2003; Haslberger, 2003; Kuiper, et al., 2001), and in at least one case, unexpectedly altered (lowered) level of harmful alkaloids has occurred in non-commercialized GE potato leaves (Birch et al., 2002).

Absence of unintended effects in previous RR crops cannot be relied upon for RR wheat because unintended effects are transformation-event and crop specific. Therefore RR wheat needs to be tested for unintended effects. Those tests should examine levels of wheat allergens because wheat is an important allergenic food and several wheat allergens have been identified (Palosuo, 2003). The expression of those allergenic proteins should be compared between RR wheat and its near-isogenic progenitor and the crop. Similar comparisons should be made for amounts of celiac causing protein and known anti-nutrients and toxicants (Welch, 2002).

FDA recommends that unexpected changes in toxicants, anti-nutrients, and important nutrients be determined, but provides no guidance on which specific substances to test. The U.S. National Academy of Sciences recommended in 2000 that the primary agencies responsible for GE-crop safety develop a database of food toxicants and anti-nutrients to help developers determine proper testing (National Research Council, 2000). That database has not yet been developed. Therefore, for RR wheat, FDA should determine the substances to be tested.

In the absence of that database or specific direction from FDA, there is uncertainty concerning which toxicants and anti-nutrients should be measured. For example, in two of four examples of Bt maize submitted to the FDA for safety review, phytate, an important anti-nutrient chelator of cationic mineral nutrients, was not measured (Gurian-Sherman, 2003). Consumption of elevated levels of phytate in major food crops such as corn might have adverse effects on mineral nutrition in some circumstances (Brinch-Pedersen, et al., 2002; Manary, et al., 2002). Also, measurement of toxicants and anti-nutrients typically found only in the unconsumed portions of the non-GE crop should be considered for the consumed portions of the GE crop since ectopic expression may occur, as has been shown for other genes (Schneeberger, et al., 1995; Marillonet and Wessler, 1997).

A problem with testing for changes in specific toxicants and anti-nutrients is that not all deleterious plant substances are known. Therefore, methods are needed to assess risks that do

not depend upon our incomplete knowledge of those substances. Our incomplete knowledge of harmful substances in plants is suggested by several recent reports of potentially harmful and previously unrecognized substances in maize and wheat (Markaverich et al., 2002, MacFarlane, et al., 2003). In one case, a wheat protein has been tentatively associated with development with some cases of type 1 diabetes (MacFarlane, et al., 2003).

Methods that do not depend on prior knowledge of all harmful substances in plants are being developed. Genomic, proteomic, and "metabolomic" methods to identify changed expression of substances in transgenic crops are not ready to be applied to risk assessment (Kuiper, et al., 2001). A current difficulty with those approaches is that the safety implications of some changes in expression are not understood.

Animal feeding studies using the GE plant are occasionally used to identify adverse effects without prior identification of the harmful substance. There are significant limitations to such studies, including the difficulty in achieving a sufficient dose and the possibility that the test species will not accurately reflect human sensitivities. Further, methods have not been validated for foods that typically contain substantial amounts of harmful substances or have limited nutritional value, such as potatoes, to serve as a major constituent of the test-animal diet, as required to obtain sufficient exposure to detect problems in small groups of animals.

Some whole foods, such as grain and other staple crops, can provide adequate nutrition for some test animals when consumed as a high percentage of diet. Animal feeding studies using those crops may be important, especially because they are major constituents of the human diet. For example, previous analysis of RR soybeans included some whole-food feeding studies in several animal species where the estimated exposure was determined to be 100-fold higher than average U.S. human consumption (Hammond, et al.; 1996, Nair, et al., 2002). In some cases, other GE foods may be used in whole food toxicity tests if test animal diet is properly formulated. A 91day feeding study in rats with lyophilized Bt tomato at 10% (w/w) of diet achieved a human equivalence of 30 lbs/day consumption with no reported ill effects in the control group (Noteborn et al., 1995). In the absence of currently available alternatives, whole-food-feeding studies should be more thoroughly considered (National Research Council, 2000), including for RR wheat. Details, such as when feeding studies are appropriate, duration of tests, appropriate test animals, numbers of test animals, and analysis of test animals, need to be addressed by an expert body of scientists.

Environmental Issues

Wheat is the third most extensively grown crop in the U.S, with about 61 million acres planted in 2003, according to the USDA. Therefore, if herbicide tolerant (HT) wheat were widely planted it might yield benefits such as increased conservation tillage but also could have detrimental impacts on the environment. The predominant environmental concerns associated with RR wheat and other HT crops are the difficulty in controlling HT volunteers and more difficult control of weeds that develop resistance to the herbicides. Non-target-organism impacts from the herbicides or the crops themselves are also possible, but are not reviewed here. The possible impacts of RR volunteer wheat in the U.S. and Canada have been recently considered and are therefore only briefly mentioned (Lyon et al., 2002 and Van Acker et al., 2003). Those

authors expressed concern that loss of glyphosate for control of volunteer wheat might have detrimental impacts on crop rotation and conservation tillage.

The emergence of herbicide-resistant weeds may have substantial environmental impacts if they result in less ecologically sound agricultural practices such as increased tillage, increased herbicide use, or use of more harmful herbicides. In addition, herbicide resistance in weeds due to transgenic crop use has received little regulatory consideration. Therefore, herbicide resistance management is considered at length.

Resistance Management

Herbicide-tolerant, and in particular glyphosate-tolerant, crops have been grown for several years in the U.S. and Canada, and can serve as examples for assessment of HT wheat. The widespread adoption of RR soybeans, which now accounts for about 81% of U.S. soybeans according to the USDA, has already contributed to resistance in one important weed. Several other important weeds have developed tolerance where glyphosate has been widely used. The rapid and widespread adoption of RR soybeans and consequent weed resistance suggest that resistance by other weeds due to HT wheat will develop in the absence of effective resistance-management measures.

The development of glyphosate-resistant weeds could have important environmental and agronomic consequences. Glyphosate is generally considered to be less harmful to humans and the environment and is less persistent than several other herbicides. It is also effective against a broader spectrum of weeds than most herbicides. Imazamox, which can be used on non-GE Clearfield wheat, is also considered by EPA to be a reduced-risk pesticide (U.S. EPA, 2003), and is susceptible to resistance development. It is also important to conserve reduced-risk herbicides because fewer herbicides are being developed with new modes of action (Martinez-Ghersa, et al., 2003; Powles, et al., 1997). Emphasis on the development of HT resistant crops using current herbicides may also reduce research on herbicides with new modes of action and use of alternative cultural practices, such as biointensive integrated pest management, that reduce selection pressure for herbicide resistance (Martinez-Ghersa, et al., 2003).

a) Gene Transfer

The use of RR wheat can lead to development of resistant weeds in several ways. First, movement of the transgene to wild relatives of wheat could make those weeds resistant. Wheat has an important wild relative in the U.S., jointed goat grass (JGG, *Aegilops cylindrica* Host). JGG is a difficult-to-control weed in winter wheat because of its similar growth habit and herbicide susceptibility. Several recent studies have shown that wheat (*T. aestivum* L.) can hybridize and backcross naturally into JGG (Zemetra, et al., 1998; Morrison, et al., 2002). Although the CP4 EPSPS gene is currently being considered for commercialization only in spring wheat, where JGG is not an important weed, gene flow may occur still since cohorts of JGG can emerge and flower in or near spring wheat fields (Walenta, et al., 2002).[2] Flowering of

[2] This paper considers only RR spring wheat because it is closest to completing regulatory review. However, RR winter wheat is reported to be in development (Lyon et al., 2002), and may be proposed for commercialization. Most of the resistance issues discussed here would be exacerbated with RR winter wheat. For example, gene flow to JGG, a major weed in winter wheat, would likely occur much more quickly.

spring-emerging JGG can occur over a substantial period that may overlap with flowering in spring wheat (Walenta, et al., 2002). So hybridization and subsequent introgression of CP4 EPSPS to JGG may occur, although at reduced frequency compared to winter wheat. Initially rare JGG containing the CP4 EPSPS gene may increase through selection where glyphosate is currently used in no-till wheat fallow or as a pre-plant treatment.

Modeling JGG resistance development to imazamox (used with Clearfield winter wheat) indicates that continuous no-till use would lead to resistance in only a few years, either through gene flow or by selection for spontaneous resistance (Hanson et al., 2002). Gene flow could be relatively more important for glyphosate, where spontaneous resistance is not as frequent as with acetolactate synthase (ALS) inhibitors such as imazamox.

Although spring and winter wheat are primarily grown in separate, but adjacent, regions of the U.S. and Canada, several states grow substantial acreage of both spring and winter wheat (Table 1). The close proximity of spring and winter wheat in those states suggests that that JGG resistance to glyphosate arising in spring wheat fields would rapidly find its way into winter-wheat growing areas, where JGG is a more important weed and where glyphosate has important uses (Lyon, et al., 2002).

Hexaploid wheat and tetraploid JGG share a D genome, allowing hybridization and backcrossing to JGG. Backcrosses from hybrid wheat into JGG show increasing fertility and initial retention of chromosomes from all genomes, but with progressive loss of non-D chromosomes in subsequent crosses, as would be expected due to their lack of homology to JGG genomes (Zemetra, et al., 1998; Wang, et al., 2002). This suggests that the greatest potential for outcrossing from wheat to JGG is likely to occur from CP4 EPSPS inserted into a chromosome of the D genome. Transgenic wheat with CP4 EPSPS D genome insertions should be avoided.

Insertion even into wheat A or B genomes may not prevent outcrossing of CP4 EPSPS to JGG, because translocations have been demonstrated between genomes in wheat and might occur between wheat A or B and JGG C or D chromosomes (Wang, et al., 2000). Furthermore, it is possible for pairs of homologous A or B chromosomes to be retained in JGG, although none were observed in the experiments of Wang et al. It is not currently known whether translocations are more prevalent from particular parts of wheat A or B to JGG genomes.

b) Resistance in Weeds not Related to Wheat

A second way for herbicide-resistant weeds to develop is through selection of natural resistance alleles by the use of glyphosate. Although apparently less susceptible to resistance development than several other classes of herbicides, glyphosate resistance has developed in several important weeds such as horseweed (*Conyza canadensis* (L.) Cronq.) (VanGessel, 2001), rigid ryegrass (*Lolium rigidum* L.) (Powles, et al., 1998), and goosegrass (*Eleusine indica* (L.) Gaertn.) (Lee and Ngim, 2000). Although glyphosate-resistant weeds were not found for many years after glyphosate's introduction, the dramatic increase in use since the advent of RR crops coincides with resistant horseweed in the U.S., which developed after only three years in continuously-cropped RR soybeans in Delaware (VanGessel, 2001).

Rotation between wheat and other crops is highly desirable because it generally reduces the need for herbicides and other pesticides, facilitates conservation tillage that reduces soil

erosion, and minimizes the losses due to pests (Lyon et al., 2002, Derksen et al., 2002). However, rotation of several glyphosate-resistant crop varieties, such as wheat and canola in Canada and corn-soybeans-wheat in the U.S. Great Plains (including the northern Great Plains where spring wheat predominates), will likely result in glyphosate resistant weeds. The use of those rotations could be limited by glyphosate-resistant weeds adapted to those rotations. Canola and soybeans, which may be used in rotation with wheat, already have widely planted glyphosate-resistant varieties. RR corn is grown on limited acreage, which might increase substantially if it is found acceptable in Europe after the lifting of the *de facto* moratorium on GE crops that was imposed in 1998.

Resistance in weeds favored by conservation tillage could reduce the use of that practice. For example, horseweed may be favored in no-till spring wheat (Derksen, 2002) and no-till soybeans (VanGessel, 2001). Continuous use of RR wheat in no-till fallow systems, used especially in drier areas to conserve soil moisture, would facilitate resistance in weeds adapted to that system.

Although not strictly a resistance issue, weed-species-shifts will likely occur if glyphosate is over-relied upon. Weeds adapted to cropping systems that rely largely on glyphosate could lead to more difficult weed control (Derksen, 2002; Lyon, et al., 2002).

Resistance to glyphosate due to either method identified above may necessitate the use of more or more-harmful herbicides. Other herbicides, such as ALS inhibitors, which are considered to be reduced-risk, may not be able to replace glyphosate due to a narrower spectrum of susceptible weeds or higher cost (Van Acker, et al., 2003). Resorting to more harmful herbicides could also have negative environmental consequences. Resorting to herbicides with longer residual activity and persistence could reduce the ability to rotate to susceptible crops.

Herbicide resistant wheat could further reduce control options and in some cases discourage rotations where control of wheat volunteer plants becomes a limiting factor. Control of volunteer multiple-herbicide-tolerant canola in Canada can be limited with some rotations, for example when pulses follow canola (Beckie, et al., 2001; Van Acker, et al., 2003). While volunteer wheat can currently be controlled in rotation with canola by the use of glyphosate, control options could be limited with the introduction of glyphosate tolerant wheat (Beckie, et al., 2001).

Clearfield winter wheat, resistant to imazamox, presents a number of concerns that are similar to those of RR wheat. ALS inhibitors like imazamox are particularly susceptible to resistance-development by weeds, but are not typically as important as glyphosate for controlling volunteer wheat. Recent modeling demonstrates that resistance is likely to develop quickly without resistance management. Alternating Clearfield with non-Clearfield wheat and fallowing in those simulations prevented significant resistance (Hanson et al., 2002). Although adequate data for several parameters are not yet available, the model demonstrates both that resistance is likely and that management may prevent or delay its occurrence.

No mandatory resistance-management is currently used for HT crops. That contrasts with Bt crops where the use of non-Bt refuges are required to delay resistance development. Increasing frequencies of Bt resistance or of resistance alleles have not been reported so far in connection with Bt crops, and in particular for pink bollworm (*Pectinophora gossypiella*

Saunders) on cotton, which is closely monitored (Shelton, et al., 2002, Carriere, et al., 2003). In contrast, without resistance-management plans, resistance to glyphosate has developed in several weeds, as well as several instances of diamondback moth insect resistance to microbial Bt (Tabashnik, 1994). Given the unique atttributes of glyphosate, every effort should be made to ensure that its effectiveness is preserved. Management of imazamox resistance in Clearfield wheat should also be explored.

Leaving resistance management to pesticide company discretion has not been effective in preventing resistance, as has been seen with glyphosate. Voluntary resistance-management strategies currently used by the pesticide industry have included recommendations for rotation of mode of action on some pesticide product labels. Voluntary resistance management guidelines in the U.S. beginning in June 2001 use simple codes to identify pesticide modes of action, but have had low industry acceptance. An important reason given for low implementation is that pesticide manufacturers fear that unilateral adoption would put them at a competitive disadvantage compared to companies that did not comply (Matten, 2003). Mandatory resistance management programs might alleviate such concerns by "leveling the playing field." The lack of success of voluntary resistance management demonstrates that better polices are needed at a national level. However, resistance management must not be so onerous to farmers that they resort to less-desirable weed-control strategies to avoid it. More effort is needed to prevent resistance by using scientifically sound resistance management that reduces selective pressure on weeds rather than merely responding after resistance develops (Derksen, et al., 2002). Resistance strategies should emphasize integrated pest management including adequate crop rotations and varied planting schedules (Powles and Mathews 1996; Derksen, et al., 2002). Back-to-back planting of crops resistant to the same herbicide, or continuous use of the same herbicide mode of action, will likely lead to resistance in a short period of time in some weeds. Alternation of herbicide modes of action is a basic approach that needs to be consistently implemented (Lyon et al., 2002). Due to different crop-management constraints in different regions and at different times, several appropriate strategies need to be developed with grower input.

Conclusions

Considerable progress has been made in developing methods for GE-crop risk assessment. Reports from advisory committees convened by regulatory agencies, reports from science organizations, and the scientific literature have contributed numerous observations and recommendations to improve GE-crop risk assessment. Those recommendations, however, have not necessarily been adopted by regulatory agencies. This paper reviews and analyses some of the important developments in the risk assessment of transgenic crops that could be applied to transgenic HT wheat.

Some risk-assessment recommendations noted here follow improvements in technology, such as easier and cheaper cloning and sequencing of transgenes and small quantities of transgenic protein. Other research, such as on the correlation between digestive stability and food allergenicity, has fostered new methods for assessing risk. However, as with most risk-assessment methodology, none of the available methods can perfectly predict the hazards they are meant to assess.

The methods and recommendations that have been discussed here and by others would provide better data at little or no additional cost (Table 2). Those recommendations include the SGD protocols suggested by the FAO/WHO, sequencing the transgene from the transformed plant, and alternating herbicide modes of action. Others need considerable additional development, such as serum banks to detect certain allergens.

Although numerous advancements can improve the reliability of GE risk assessments, other important areas need additional research to develop new and more effective methods. Allergenicity assessments of RR wheat and other GE crops could be much improved by the development of an appropriate animal model (Kimber, et al., 2003) and better understanding of the properties that make food proteins allergenic. Similarly, better methods are needed to identify unintended adverse effects. Better data are also needed for the assessment of herbicide resistance, such as better understanding of the environmental and economic impacts of resistant crops, weeds, and volunteers plants, especially the broader impacts on environmentally friendly agronomic practices.

In the U.S., some inconsistencies in the risk-assessment and management of GE crops are due to regulatory authority being spread over several agencies working under different laws. That situation comes from the decision in 1986 by the U.S. federal government, called the coordinated framework, to regulate GE organisms under existing laws. Resistance management is an important area of inconsistency where Bt crops, regulated by the EPA, have mandatory requirements that appear to have forestalled resistance by insects (Shelton et al., 2002, Carriere et al., 2003), despite the presence of resistance alleles. By comparison, herbicide resistant crops, regulated for environmental impact by USDA, have not had any mandatory resistance-management requirements despite the widely acknowledged importance and unique qualities of glyphosate and other herbicides.[3] The dramatically increased use of glyphosate with the advent of RR crops has apparently led to resistance that is related to RR crops in at least one case (VanGessel, 2001), and without better management more weeds can be expected to develop glyphosate resistance as new RR crops, like RR wheat, are commercialized.

GE technology presents a dilemma for regulatory agencies because it is relatively new and developing more quickly than reliable risk-assessment methods. The combination of imperfect methods and uncertain risk make it difficult to determine the proper level of regulatory scrutiny. In addition, as recognized recently by the U.S. National Academy of Sciences (NRC, 2002), risk assessment and management go beyond science and include social issues of acceptance and perception of risk.

Determining the appropriate level of regulatory scrutiny for RR wheat and other GE crops depends upon a complex assessment of risk, science and public policy, and weighing of risks and benefits. Little systematic attention has been devoted to that important issue. The level of regulatory scrutiny can have practical implications, as in assessing the risk of allergenicity. A cautious, but not stifling, approach to assessing allergenicity of genetically engineered HT (and other) crops, such as the FAO/WHO recommendations, uses a relatively low ratio of pepsin to transgenic protein in the SGD assay and six contiguous amino acids in the allergen homology searches. Such an approach will more often conclude that a protein is likely to be allergenic than

[3] Alternatively, resistance management of the herbicide might be regulated by EPA, which regulates pesticide safety.

an approach that uses a high ratio of pepsin to transgenic protein and eight contiguous amino acids. Some of those proteins may, in fact, not be allergenic. On the other hand, the latter approach may not identify some allergenic proteins. The lack of direction by regulatory agencies on the question of regulatory scrutiny has meant that those decisions are made on an inconsistent *ad hoc* basis, often by the developers of GE crops.

Some of the issues raised by GE crops can be addressed by agencies developing detailed but flexible guidelines, with the help of expert independent scientists. So far, regulatory agencies have developed few specific guidelines for the risk assessment of GE crops. It is hoped that this paper will prompt them to establish better guidance for GE crop risk assessment as well as identify specific issues that should be addressed in the risk assessment process.

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Table 1: **Overlapping Spring and Winter Wheat** [a]

State	Counties with Greater than 20,000 Acres of Both Spring and Winter Wheat	Total Spring Wheat Acres in State	Total Winter Wheat Acres in State
Idaho	6	530,000	730,000
Montana	9	3,750,000	1,450,000
Oregon	3	150,000	800,000
South Dakota	11	1,700,000	1,300,000
Washington	11	620,000	1,800,000

[a] Source: USDA NASS Crops County Data Files
http://www.usda.gov/nass/graphics/county02/indexdata.htm

Table 2: Recommendations for Risk Assessment of Roundup Ready™ Wheat[a]

Risk Issue	Recommended Action	Primary Agency
Alteration of DNA sequence during transformation	Sequencing of transgene from GE crop	FDA
Potential structure/function differences between crop and bacterial transgenic proteins[b]	i) DNA sequence comparison between plant and bacterial transgenes, ii) determination of glycosylation, iii) sequence of mature proteins	FDA
Mutagenesis of genes at insertion site in plant genome	Sequencing and expression analysis of genomic junction of transgene	FDA
Instability of transgene	Mendelian and expression analysis for several generations, tissues, growth conditions, and crop phenology	FDA/USDA
Allergenicity of transgenic protein	SGD assay and homology with known allergens using FAO/WHO protocols	FDA
Unintended adverse effects	i) Comparison of known toxicant, anti-nutrient, and allergen levels from crop species with progenitor variety and with range for non-GE crop, ii) Whole food animal testing[c]	FDA
Weed resistance	i) CP4 EPSPS should not be located on the wheat D genome; ii) no consecutive planting of RR crops[d]	USDA

a) Many other important tests that should be included in risk assessments are not mentioned here. Absence from this table is not meant to suggest that other tests are not important; b) Where bacterial proteins are used instead of transgenic plant proteins for safety tests; c) Whole food animal testing should be performed where possible, but may be difficult for some foods; d) These are suggested minimum actions, but will require careful consideration by weed scientists. Biointensive integrated pest management methods, including crop rotations that are designed to reduce selection pressure on glyphosate, should also be strongly encouraged.