Safety Assessment of Roundup Ready Alfalfa Events J101 and J163

Executive Summary

Monsanto Company and Forage Genetics International have developed varieties of Roundup Ready® alfalfa that are tolerant to glyphosate, the active ingredient in Roundup® agricultural herbicides. Roundup Ready alfalfa commercialized varieties use a combination of two independent events: (J101 and J163)[1] combined (J101 × J163) through a conventional breeding process. Roundup Ready alfalfa was developed using *Agrobacterium*-mediated transformation to stably incorporate into the alfalfa genome a coding sequence (gene) producing a glyphosate-tolerant form of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). This protein is similar to naturally occurring EPSPS proteins found in other food crops, food processing aids like bakers yeast, and is the same as that found in other Roundup Ready crops on the market. This glyphosate-tolerant EPSPS was originally identified in the soil microorganism, *Agrobacterium* sp. strain CP4, and is designated CP4 EPSPS. The production of the CP4 EPSPS protein in plant tissues is the basis of the Roundup Ready trait in alfalfa varieties which contain events J101 and J163. Use of the Roundup Ready alfalfa system -- planting Roundup Ready alfalfa and subsequent application of a Roundup agricultural herbicide -- has the potential to offer significant agronomic and environmental benefits.

Alfalfa is a major forage (feed) crop in the U.S. and has been grown on over 20 million acres annually since the 1950s. Roundup Ready alfalfa was deregulated by USDA in June 2005. Available seed was planted in the US in 2005, with all products limited for domestic use. Roundup Ready alfalfa varieties enable growers to apply labeled Roundup agricultural herbicides from planting through five days before cutting, providing growers an additional tool for improved weed control, excellent crop safety and preservation of yield potential and quality. Since Roundup agricultural herbicides are highly effective against the vast majority of annual and perennial grasses and broadleaf weeds, alfalfa growers who use herbicides to control weeds will be able to reduce the number of herbicides used to control the economically destructive weeds that grow in their fields and thereby realize savings in weed control costs.

Roundup Ready alfalfa was developed for animal feed uses. However, there are minor food uses of alfalfa such as alfalfa sprouts and human consumption as dietary supplements; therefore, the safety assessment considered both food and feed uses of alfalfa. The feed and food safety assessment of Roundup Ready alfalfa utilized information on the safety and characterization of the introduced trait, the history of alfalfa as a safe feed and food source, and analyses which compared the composition of alfalfa varieties containing events J101 and J163 to control and conventional alfalfa varieties. Information and data on the introduced trait indicate that the CP4 EPSPS protein is safe

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[1] In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants," J101 has been assigned the unique identifier MON-00101-8, and J163 has been assigned the unique identifier MON-00163-7.

for consumption. The levels of key nutrients and components in Roundup ready alfalfa forage are comparable to the control and within the population of commercially available alfalfa varieties. As a result, forage from Roundup Ready alfalfa is substantially equivalent to forage of commercial varieties of alfalfa currently marketed. Collectively, these results establish that Roundup Ready alfalfa is safe for use as feed or food.

The environmental safety assessment of Roundup Ready alfalfa considered the biology and growth habits of alfalfa and determined whether the trait, the transformation and regeneration process impacted the pest or weediness potential of Roundup Ready alfalfa relative to the characteristics of the control or conventional alfalfa varieties. There were no biologically meaningful phenotypic (growth or development) differences between Roundup Ready alfalfa J101 and J163 populations and the alfalfa control or conventional reference varieties. Thus, there is no increased pest potential of Roundup Ready alfalfa populations. Other than the intentional change caused by the trait, the phenotype of alfalfa has not been changed as a result of the trait or transformation process. Based on these assessments, it was concluded that there were no unique environmental risks associated with the introduction of Roundup Ready alfalfa.

These studies establish the feed, food and environmental safety of Roundup Ready alfalfa. The feeds and foods derived from Roundup Ready alfalfa varieties are as safe and nutritious as current commercial varieties of alfalfa and the comparable feeds and foods derived from them. Introduction of Roundup Ready alfalfa varieties presents no unique risks to the environment beyond that due to use of conventional alfalfa varieties. This information has been independently reviewed by regulatory agencies in alfalfa production and in alfalfa importing countries, including reviews for environmental safety by the U.S. Department of Agriculture (http://www.aphis.usda.gov/brs/not_reg.html) and for feed and food safety by the U.S. Food and Drug Administration (http://www.cfsan.fda.gov/~lrd/biocon.html). Their reviews confirm the feed, food and environmental safety of Roundup Ready alfalfa.

Introduction

Monsanto Company and Forage Genetics International (FGI) have developed varieties of Roundup Ready alfalfa that are tolerant to glyphosate, the active ingredient in Roundup agricultural herbicides. Roundup Ready alfalfa varieties were developed using *Agrobacterium*-mediated transformation, to stably incorporate into the alfalfa genome a coding sequence that produces a glyphosate-tolerant form of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). This glyphosate-tolerant EPSPS was originally identified in the soil microorganism, *Agrobacterium* sp. strain CP4, and is designated CP4 EPSPS. The production of the CP4 EPSPS protein in plant tissues is the basis of the Roundup Ready trait in Roundup Ready alfalfa events J101 and J163. Use of the Roundup Ready alfalfa system -- planting Roundup Ready alfalfa and subsequent application of a Roundup agricultural herbicide -- has the potential to offer significant agronomic and environmental benefits.

Roundup Ready alfalfa varieties will use a combination of two different events or *cp4 epsps* loci (J101 and J163) combined (J101 × J163) through a conventional breeding process. FGI developed a conventional breeding method to rapidly introgress transgenes into alfalfa varieties. In the FGI breeding process, one copy of the *cp4 epsps* transgene is required at each of two different, independently segregating loci. The two independent gene loci are products of two separate *cp4 epsps* insertion events. Alfalfa is a perennial, autotetraploid plant with eight sets of chromosomes (x=8). Alfalfa varieties are comprised of heterogeneous populations of alfalfa breeding lines. Individual plants within each variety are phenotypically and genotypically unique. The population of alfalfa plants in commercial Roundup Ready varieties will consist of individual plants with the *cp4 epsps* gene insert copy number ranging from zero to eight, contributed by either event J101 or J163. The majority of plants will contain two copies of the *cp4 epsps* gene.

Alfalfa is a widely adapted perennial species grown for forage in most regions of the United States. Over 20 million acres of alfalfa have been grown annually in the U.S. since 1950. Roundup agricultural herbicides are used as foliar-applied, nonselective herbicides and are effective against the majority of annual and perennial grasses and broadleaf weeds. Glyphosate has no preemergence or residual soil activity (Franz et al., 1997). Furthermore, glyphosate is not prone to leaching, degrades in soil over time, and will not cause unreasonable adverse effects to mammals, birds or fish under normal use conditions (U.S. EPA, 1993; WHO, 1994; Giesy et al., 2000; Williams *et al.*, 2000). The Roundup Ready alfalfa system allows for glyphosate applications at preplant, preemergence, and/or postemergence timings in seedling and established Roundup Ready alfalfa stands, thus offering control of weeds when most appropriate. Using the Roundup Ready alfalfa system, growers can control troublesome perennial weeds such as quackgrass, Johnsongrass, nutsedge and poisonous weeds such as dodder. Many weed control products currently in use have a narrow window of application based on specific weed size or crop stage. The Roundup Ready weed control system provides growers with a simple, flexible and crop-safe weed control system producing cleaner hay and resulting in higher yields of pure alfalfa.

The following summary provides information on the methods used to develop Roundup Ready alfalfa, and an overview of the food, feed and environmental safety studies that were conducted. These include molecular characterization of the DNA inserted into Roundup Ready alfalfa events J101 and J163, an assessment of the safety of the introduced proteins, compositional analyses of the food and feed components derived from them to establish compositional equivalence to conventional alfalfa varieties, and an assessment of the environmental safety of the product. The environmental safety assessment considered the impact of the trait (herbicide tolerance) introduced into alfalfa and whether there were any unintended effects on alfalfa that might impact its potential to become a plant pest. For alfalfa, characteristics that may impact plant pest risk include: enhanced growth, vigor or stand longevity, changes in susceptibility to plant pests and diseases, increases in seed yield, and increases in seed dormancy.

These studies establish the food, feed and environmental safety of Roundup Ready alfalfa events J101 and J163. They demonstrate the safety of the CP4 EPSPS protein to humans and animals, establishing equivalent nutritional composition of Roundup Ready alfalfa varieties with contain events J101 and J163 compared to conventional alfalfa varieties, and confirming that the potential impact of Roundup Ready alfalfa on the environment is equivalent to that of conventional alfalfa varieties.

Product Development

Alfalfa (*Medicago sativa* L.) is a perennial, autotetraploid with four sets of eight chromosomes (n=8 and 4x8=32 chromosomes). Thus, alfalfa contains four copies of each chromosome (and four copies of all gene loci). Most alfalfa plants exhibit various forms of nearly complete genetic self-incompatibility or self-sterility and will not successfully self-pollinate (Viands *et al*., 1988). Alfalfa is adversely affected by inbreeding (inbreeding depression); the infrequent self-fertilized plants that occur show a dramatic reduction in forage and seed yield potential (Rumbaugh *et al*., 1988). Therefore, commercial alfalfa breeding programs are structured to avoid significant inbreeding and the resulting negative effects of inbreeding depression (Rumbaugh *et al*., 1988).

To avoid inbreeding depression, alfalfa varieties may have ten to 200 parent plants that are initially crossed in isolation from each other to form the breeder generation seed. The breeder seed of commercial alfalfa varieties is produced by open pollination of all parent plants resulting in random intercrossing. An alfalfa variety is maintained through multiple seed generations beyond breeder seed via the open pollination of their progeny in isolation from other alfalfa varieties or pollen sources. Plant varieties bred in this way are called synthetic varieties (Rumbaugh *et al*., 1988). Individual plants within a synthetic variety are genotypically and phenotypically heterogeneous; thus, no two individual plants within the variety are exactly alike. Synthetic alfalfa varieties bred in this way are closed populations that segregate, within a defined range, for most morphological traits and genetic markers. Because alfalfa varieties are segregating heterogeneous populations, alfalfa varieties are routinely described in terms appropriate to populations (defined by mean or percent trait expression).

Since alfalfa is an autotetraploid and does not "self," a strategy was needed to achieve high trait purity (i.e., tolerance to glyphosate) and minimize inbreeding depression for Roundup Ready alfalfa variety development. For that purpose, FGI developed a breeding method that identifies nonrelated plants that contain one or more copies of the same *cp4 epsps* gene at each of two independent loci (J101 × J163) for use as Syn 0 parents (see Figure 1). These J101 and J163 genotypes are called dihomogenic plants (Samac and Temple, 2004). The intercrossing of dihomogenic plants results in populations with $\geq$90% Roundup Ready trait purity in the commercial generation seed product. The FGI breeding method successfully and reliably produces high trait purity in the Syn 1, Syn 2 and Syn 3 generations; however, Syn 0 parent plants must have at least a single copy of the *cp4 epsps* gene at *both* of the two loci. The resultant population of alfalfa plants in commercial Roundup Ready varieties will consist of individual plants with the *cp4 epsps* gene copy number ranging from zero to eight, contributed by either J101 or J163.

The same Roundup Ready phenotype is accomplished whether one or more than one copy of the *cp4 epsps* gene is in the plant. This has been demonstrated in numerous Roundup Ready alfalfa varieties. Tolerance to Roundup agricultural herbicides (up to and exceeding labelled rates) in synthetic generations has been demonstrated during numerous field trials conducted prior to commercialization.

Molecular Characterization

Roundup Ready alfalfa was developed using *Agrobacterium*-mediated transformation of the alfalfa variety R2336 with plasmid PV-MSHT4 (shown in Figure 2). R2336 is a proprietary alfalfa line owned by FGI and is exclusively used for plant transformations. Line R2336 was selected from a high yielding, fall-dormant FGI alfalfa breeding population.

The molecular characterizations of events J101 and J163 show that the introduced DNA in each event is comprised of a single insert which contains a single copy of the T-DNA from plasmid PV-MSHT4. All genetic elements of the *cp4 epsps* expression cassette from the plasmid are present in each insert and are intact. Each insert consists of the following genetic elements: (1) a portion of the right border sequence derived from *Agrobacterium tumefaciens* required for transfer of the T-DNA (Depicker *et al*., 1982), (2) the 35S promoter derived from the figwort mosaic virus with a duplicated enhancer region (Richins *et al*., 1987), (3) the petunia heat shock protein 70, 5' untranslated leader sequence (Rochester *et al*., 1986), (4) the chloroplast transit peptide isolated from *Arabadopsis thaliani* EPSPS to direct the CP4 EPSPS protein to the chloroplast, the site of aromatic amino acid synthesis (Klee *et al*., 1987), (5) the *cp4 epsps* sequence coding for the CP4 EPSPS protein derived from *Agrobacterium* sp. strain CP4 (Padgette *et al*., 1996), (6) the non-translated E9 3' nontranslated region derived from the pea ribulose-1,5-bisphosphate carboxylase, small subunit (*rbc*) E9 gene (Coruzzi *et al*., 1984) and, (7) a portion of the left border sequence derived from *Agrobacterium tumefaciens* required for transfer of the T-DNA (Barker *et al*., 1983).

The genetic stability of events J101 and J163 was assessed in populations of Roundup Ready alfalfa plants produced through conventional breeding. Stability of the J101 insert and J163 insert was evaluated using Southern blot analysis, analysis of gene inheritance and tolerance to glyphosate. On the basis of Southern blot analysis and tolerance to glyphosate, it was concluded that the *cp4 epsps* gene and associated phenotype were unchanged throughout the breeding process. The glyphosate tolerance trait encoded by J101 and J163 was inherited in the expected Mendelian inheritance ratio through all of the generations included in production of a typical Roundup Ready alfalfa variety (J101 × J163). J101 and J163 have also been bred into numerous dormant and non-dormant alfalfa varieties targeted for commercial sale. Synthetic Roundup Ready alfalfa populations developed by conventional breeding show that the Roundup Ready trait appears at the expected Mendelian inheritance ratio. Continued tolerance to Roundup agricultural herbicide throughout the life of the Roundup Ready alfalfa stand was confirmed in product efficacy trials. Collectively, these data support the stability of the trait throughout the breeding process and over the life of an alfalfa stand.

CP4 EPSPS Protein Levels in Roundup Ready Alfalfa Plants

The level of the CP4 EPSPS protein in forage obtained from Roundup Ready alfalfa populations was estimated using a validated enzyme-linked immunosorbent assay (ELISA). To estimate the levels of CP4 EPSPS protein, alfalfa populations containing either event J101 or J163, as well as J101 × J163 populations, were planted at six field sites in the spring of 2001. Because alfalfa is a perennial plant that can be harvested multiple times over the growing season, the CP4 EPSPS protein level was determined at two different times during the growing season and from two different years of forage growth (2001 and 2002). Forage was harvested at all sites when plants were at the early- to late-bud growth stage, corresponding to when alfalfa is typically harvested for maximum quality (Marten *et al*., 1988).

The mean level of the CP4 EPSPS protein across two seasons and from multiple cuttings was 257 and 267 µg on a tissue fresh weight (tfw) basis for alfalfa plants that contained J101 and J163, respectively (Tab1e 1). The mean level of the CP4 EPSPS protein in the synthetic alfalfa population (J101 × J163), across two seasons and from multiple cuttings, was 252 µg/gram tfw.

Safety Assessment of the CP4 EPSPS Protein in Roundup Ready Alfalfa

The safety assessment of the CP4 EPSPS protein produced in Roundup Ready alfalfa, event J101 and J163, included protein characterization, functional and structural comparisons to ubiquitous plant and microbial EPSP syntheses with a history of safe consumption, *in vitro* digestibility in simulated gastric and intestinal fluids, acute oral toxicity in mice, and amino acid comparison to known toxins and allergens.

All of these data and information taken together demonstrate a history of safe use with respect to the family of EPSPS proteins which naturally occur in crops and microbially based processing agents and have a long history of safe consumption by humans and animals. These data also demonstrate a history of safe experience with respect to Roundup Ready crops which have been consumed in significant amounts, either directly or as processed products, by humans and animals since their initial commercialization in 1996.

CP4 EPSPS Characterization and History of Safe Use

The CP4 EPSPS proteins produced in events J101 and J163 are functionally similar to a diverse family of EPSPSs consumed in a variety of foods and animal feeds (Harrison *et al*., 1996). The *cp4 epsps* coding region has been completely sequenced and encodes a 47.6 kDa protein consisting of a single polypeptide of 455 amino acids. The CP4 EPSPS in Roundup Ready alfalfa is homologous to EPSPSs naturally present in plants, including food crops (e.g., soybean and corn), and fungal and microbial processing agents such as Baker's yeast (*Saccharomyces cerevisiae*), which have a history of safe human consumption (Padgette *et al*., 1996; Harrison *et al*., 1996). Further, the ubiquitous presence of homologous EPSPS enzymes in food crops and common microorganisms establishes that EPSPS proteins, and their enzyme activity, pose no hazards for human consumption. EPSPS synthase is part of the shikimate pathway responsible for formation of aromatic amino acids and is not present in mammals, which contributes to the very favorable toxicological profile for glyphosate (Williams *et al*., 2000). The amino acid sequence of the CP4 EPSPS produced in events J101 and J163 is identical to, or greater than 99% identical to, the CP4 EPSPSs in other Roundup Ready crops, such as soybean, corn (NK603), canola, and cotton, with a history of safe human and farm animal consumption.

Digestion of CP4 EPSPS in Simulated Gastric and Intestinal Fluids

Simulated mammalian gastric and intestinal digestive fluids were used in *in vitro* assays to assess the susceptibility of the CP4 EPSPS protein to proteolytic digestion. Rapid degradation of the protein correlates with limited exposure to the gastrointestinal tract and little likelihood that the protein can produce pharmacological, toxic or allergenic effects. Harrison *et al*. (1996) demonstrated that the CP4 EPSPS protein is rapidly degraded in simulated digestive fluids. The half-life for CP4 EPSPS was less than 15 seconds in the gastric system and less than ten minutes in the intestinal system, based on western blot analysis. Therefore, if any of the CP4 EPSPS protein were to survive in the gastric system, it would be degraded in the intestine. As a comparison, 50% of solid food has been estimated to empty from the human stomach in two hours, while 50% of liquid empties in approximately 25 minutes (Sleisenger and Fordtran, 1989). Based on this information, CP4 EPSPS protein is expected to degrade rapidly in the mammalian digestive tract.

Assessment of Acute Oral Toxicity of CP4 EPSPS in Mice

Few proteins are toxic when ingested, and those that are toxic typically act in an acute manner (Sjoblad et al., 1992). An oral toxicity study with CP4 EPSPS protein as the test material was performed in mice to directly assess any potential acute toxicity associated with the protein (Harrison et al., 1996). Since proteins that are toxic act via acute mechanisms, acute administration was considered appropriate (Pariza and Foster, 1983; Jones and Maryanski, 1991). Therefore, there were no statistically significant differences in body weight, cumulative body weight, or food consumption between the vehicle or bovine serum albumin protein control groups and CP4 EPSPS protein-treated groups. The no effect level (NOEL) for oral toxicity in mice was 572 mg/kg, the highest dose tested. This dose represents a significant (greater than 1000-fold) safety margin relative to the highest potential human consumption of CP4 EPSPS (Harrison *et al*., 1996) assuming that the protein is expressed in multiple crops. Results from this study demonstrated that the CP4 EPSPS protein is not acutely toxic to mammals. This result was expected since the CP4 EPSPS protein is readily digested in gastric and intestinal fluids *in vitro* and the protein is from a ubiquitous family of proteins with a history of safe consumption and no biologically plausible mechanism of toxicity to animals.

Assessment of CP4 EPSPS Dietary Intake from Roundup Ready Alfalfa

Food uses of alfalfa are minor, with the vast majority of alfalfa consumed by humans in the form of freshly sprouted seedlings (sprouts). Other minor uses include consumption of compressed leaf material for dietary supplements and herbal teas. Given the limited number of foods derived from alfalfa and its intended use for feed purposes, the potential for human exposure to CP4 EPSPS from Roundup Ready alfalfa is extremely limited. It is not the intention of Monsanto or FGI to introduce Roundup Ready alfalfa for sprout seed production or for any other food uses. Stewardship measures are in place to avoid this use. However, an estimate of potential CP4 EPSPS protein intake from consumption of alfalfa sprouts was made to cover the chance accidental occurrence of Roundup Ready alfalfa in food. Alfalfa consumption was based on data from the 1994-1996 and 1998 Continuing Surveys of Food Intake by Individuals (CSFII). No adjustments to intake were made for less than 100% market share or for possible losses due to processing, storage, cooking, etc. Based on these worst-case assumptions, the average chronic exposure to CP4 EPSPS protein were calculated to be 0.00015 mg/kg/day for the overall U.S. population. The upper 99.9th percentile acute exposures to CP4 EPSPS protein for the overall U.S. population was estimated to be 0.038 mg/kg/day. Using the upper acute exposure value, this level is more than 15,000-fold below the NOEL level observed in mice. Given the intentional feed only use of Roundup Ready alfalfa and minor use of alfalfa in food, there will be a minor incremental increase in exposure to CP4 EPSPS due to introduction of Roundup Ready alfalfa.

Assessment of the Allergenicity/Toxicity of CP4 EPSPS Protein

Information and data from studies support the safety of the CP4 EPSPS protein and indicate that it is unlikely to be an allergen or toxin. This is based on:

1. the source of the *cp4 epsps* coding sequence, a soil bacterium, which is not a known human or animal pathogen and for which there are no reports of allergies;

2. rapid digestion of the mature CP4 EPSPS protein;

3. the low levels of the CP4 EPSPS protein present in J101 and J163 forage, representing less than 0.6% of the total protein on a fresh weight basis;

4. lack of significant structural similarities of the CP4 EPSPS protein to known allergens, toxins or pharmacologically active proteins known to cause adverse health effects, based on bioinformatic searches of amino acid sequence databases;

5. lack of acute toxicity, based on a mouse gavage study; and the lack of any reports of allergy or adverse effects from the consumption of food products derived from other Roundup Ready crops that have been in the food supply since 1996.

Assessment of Sequence Similarity of CP4 EPSPS Protein to Known Protein Toxins

Another aspect used for the assessment of potential toxic effects of proteins introduced into plants is to compare the amino acid sequence of the protein to the sequences of known toxic proteins. Homologous proteins derived from a common ancestor have similar amino acid sequences, are structurally similar and often share common function. Therefore, it is undesirable to introduce DNA that encodes for a protein that is homologous to a protein that is toxic to animals and people. Homology is determined by comparing the degree of amino acid similarity between proteins using published criteria (Doolitte, 1990). The CP4 EPSPS protein does not show meaningful amino acid sequence similarity when compared to known protein toxins.

Assessment of Allergenic potential of CP4 EPSPS Protein

Although there are no single predictive bioassays available to assess the allergenic potential of proteins in humans (Goodman *et al*., 2005; U.S. FDA, 1992), comparing the physicochemical and human exposure profile of the protein to known protein allergens provides a basis for assessing potential allergenicity. Thus, important considerations contributing to the allergenicity of proteins ingested orally include exposure and an assessment of the factors that contribute to exposure, such as stability to digestion, prevalence in the food, and consumption pattern (amount) of the specific food (Metcalfe, et al., 1996; Kimber et al., 1999).

A key parameter contributing to the systemic allergenicity of certain food proteins appears to be stability to gastrointestinal digestion, especially stability to acid proteases like pepsin found in the stomach (Astwood et al., 1996; Astwood and Fuchs, 1996; Fuchs and Astwood, 1996; FAO, 1995; Kimber et al., 1999). Important protein allergens tend to be stable to peptic digestion and the acidic conditions of the stomach if they are to reach the intestinal mucosa where an immune response can be initiated. As noted above, the *in vitro* assessment of CP4 EPSPS digestibility indicates that the protein, like other food-derived proteins, is labile to digestion when compared to many clinically important food allergens.

Another significant factor contributing to the allergenicity of certain food proteins is their high concentration in foods (Taylor et al., 1987; Taylor, 1992; Fuchs and Astwood, 1996). Most allergens are present as major protein components in the specific food, representing from 2 to 3% up to 80% of total protein (Fuchs and Astwood, 1996). In contrast, CP4 EPSPS is present at low levels in Roundup Ready alfalfa. The CP4 EPSPS protein in Roundup Ready alfalfa represents approximately 0.6 % of the total protein in forage.

It is also important to establish that the protein does not represent a previously described allergen and does not share potentially cross-reactive amino acid sequence segments or structure with a known allergen. An efficient way to assess whether the added protein is an allergen or is likely to contain cross-reactive structures is to compare the amino acid sequence with that of all known allergens. A bioinformatic assessment of the CP4 EPSPS protein, using allergen and public domain protein sequences databases, has been performed and demonstrates the absence of sequence similarity to proteins known to pose human health risks. No immunologically relevant sequences (eight contiguous amino acid identities) were detected when the amino acid sequence of the CP4 EPSPS protein was compared to the sequences in the ALLERGEN3 public domain sequence database (Hileman et al., 2002). Together, these data demonstrate that the CP4 EPSPS protein present in Roundup Ready alfalfa does not share structurally relevant or immunologically relevant amino acid sequence similarities with allergens or gliadins. Therefore, it is highly unlikely that this protein may contain immunologically cross-reactive allergenic epitopes

The *cp4 epsps* coding sequence was obtained from a naturally occurring bacterium and has been identified by the American Type Culture Collection as an *Agrobacterium* species. Because there are no reports of allergies to *Agrobacterium* species, it can be concluded that the CP4 EPSPS protein is not from a known allergenic source.

In summary, these data and analyses support the conclusion that CP4 EPSPS does not pose a significant allergenic risk, is not derived from an allergenic source, does not possess immunologically relevant sequence similarity with known allergens, and does not possess the characteristics of known protein allergens as summarized below.

Characteristics of Known Allergenic Proteins

Characteristic	*Allergens*	CP4 EPSPS
Stable to digestion	yes	no
Stable to processing	yes	no
Similarity to known allergens	yes	no
Prevalent protein in food	yes	no

As described in Taylor (1992) and Taylor et al. (1987)

Compositional Analysis and Nutritional Assessment of Roundup Ready Alfalfa

Alfalfa and Its Uses

Alfalfa is recognized as the oldest plant grown solely for forage. According to Barnes et al., 1988) alfalfa is the most important forage crop species in the United States and Canada and is recognized as the most widely adapted agronomic crop. Alfalfa has had a long history as a superior feed source for many animal species including dairy and beef cattle (*Bos* ssp.), horses (*Equus* ssp.), swine (*Sus* ssp.), poultry, rabbits (*Oryctolagus* ssp.) and sheep (*Ovis aries*) (Van Keuren and Matches, 1988; Church, 1984). It produces more protein per acre than any crop for livestock; therefore, it has the highest feeding value of all commonly grown hay crops (Duke, 1981; Conrad and Klopfenstein, 1988). Alfalfa is highly valued for animal feed because of its large amount of readily soluble protein, high intake potential, rapid passage through the gastrointestinal tract, and excellent digestibility. Alfalfa has a relatively large percentage of highly digestible cell solubles and the lowest fraction of cell walls in comparison to other forages. Alfalfa can serve as the sole plant component in many livestock feeding programs when supplemented with the proper minerals.

Alfalfa is consumed by animals through grazing or harvested and fed as greenchop, hay, silage or dehydrated pellets. These uses are described below. Greater than 95% of alfalfa used on farms is fed as hay or silage. The majority of alfalfa purchased as feed is sold as field dried hay or dehydrated hay, both of which contain much less water by weight than fresh cut (greenchop) or ensiled (silage) alfalfa and thus are more efficient to transport.

Pre-harvest nutritive value of alfalfa changes with maturity of the crop and the age of the stand with the greatest feed quality obtained from alfalfa that is harvested near the early bud stage. As the crop matures, there is a decrease in the nutritive value, voluntary animal intake and animal performance potential of the harvested alfalfa (Marten *et al*., 1988). As the stand ages, stand decline due to disease, insect and weed infestations will generally cause a decrease in the feeding value of the harvested forage.

Ensiling is a very efficient method for nutrient preservation of all forages. Properly ensiled alfalfa forage shows minimal decreases in feeding value (Wilkins, 1988) in comparison to fresh alfalfa. The use of ensiling to preserve the nutrient content of alfalfa offers many conveniences over hay production, including more flexibility regarding harvest weather conditions (i.e., less dry-down time is required), less field loss of leaves, less labor, and it is the preferred method of nutrient preservation for on-farm use where silo space and harvesting equipment are available.

Very little loss in the nutrient content of alfalfa occurs with the practice of feeding greenchop alfalfa because alfalfa is harvested and fed fresh on-farm. However, this method is not practiced to any great extent due to its inconvenience as only a portion of the stand is harvested at each feeding. As the stand matures, subsequent harvests likely will have lower feed value making this practice difficult to manage.

A very small amount of alfalfa is sold and fed as dehydrated pellets. Dehydrated alfalfa is commonly produced from extremely high quality hay and is primarily used for export markets or for rabbit and other small animal feed. Overheating of the hay during dehydration can harm proteins in alfalfa and decrease the quality of the hay (Conrad and Klopfenstein, 1988). However, in general, dehydrated alfalfa is considered an excellent feed source equivalent to the highest quality hay.

In some regions of the U.S. a limited number of producers may directly feed pure or mixed-stand alfalfa by animal pasturing. Animals grazed on alfalfa must be carefully managed and monitored to reduce the risk of bloat, a digestive disorder that results in the accumulation of gas in the rumen.

As mentioned above, there are minor uses of alfalfa for human food. These would include the use of alfalfa seed for sprout production and the use of dried forage for dietary supplements and herbal teas.

Compositional Analysis

Compositional analyses are a critical component of the safety assessment process that integrates with the evaluation of the trait (Astwood and Fuchs, 2000). Each of the measured parameters provides an assessment of the cumulative results of numerous biochemical pathways and hence provides an assessment of a wide range of metabolic pathways within the plant. A detailed compositional assessment of the forage was conducted on the levels of 36 key nutrients and other components in forage collected from Roundup Ready events J101, J163 and J101 × J163 compared to forage collected from their parental control population and to a range of commercially available alfalfa varieties. Roundup Ready alfalfa events J101 and J163 and J101 × J163 were grown in replicated field trials during the 2001 (establishment year) and 2003 (third year) field seasons with conventional alfalfa. Alfalfa forage was harvested at the late-bud to early-bloom stage from each plot and analyzed for the levels of proximates, fibers, amino acids, minerals, and coumestrol. Constituents measured were consistent with those described in the OECD Consensus Document on Compositional Considerations for New Varieties of Alfalfa and Other Temperate Legumes: Key Feed nutrients and Secondary Plant Metabolites (2005) for the intended use of Roundup Ready alfalfa as animal feed.

In both years ground alfalfa forage samples were analyzed for proximates (ash, carbohydrates, moisture, protein, and total fat), acid detergent fiber (ADF), lignin, neutral detergent fiber (NDF), amino acids, calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc. In 2003, samples were analyzed for coumestrol.

Compositional data in this summary are limited to data derived from analysis of the forage obtained from the J101 × J163 synthetic population. Compositional data for forage derived from J101 and J163 may be found at http://www.aphis.usda.gov/brs/aphisdocs/04_11001p.pdf. Compositional data for proximates, fiber components and coumestrol are presented in Table 2. Values for individual amino acids in Roundup Ready, control and reference alfalfa are presented in Table 3, and mineral data are presented in Table 4. Results from the analyses of the combination of all field sites showed that there were no statistical differences observed between Roundup Ready alfalfa events J101 × J163 and the parental control population for 24 of the 35 analytes measured in 2001 and 31 of the 36 analytes measured in 2003. For the comparisons observed to be statistically significant ($P < 0.05$) between the Roundup Ready alfalfa and control samples, all values for Roundup Ready alfalfa were within the 99% tolerance interval of the reference alfalfa varieties, with the exception of ash, and iron in 2001. These differences were not deemed to be of biological significance because they were not observed across multiple years, were small in measure, or were skewed by artifacts associated with sample contamination with soil. A review of the literature for alfalfa forage composition is also presented. Because the majority of the literature values are actually means, and not true ranges of expected values for any given analyte, they are useful in providing confirmation that the results observed for Roundup Ready alfalfa were indeed comparable with those presented in the literature.

We conclude from these compositional data that there were no unintended effects due to the trait or plant transformation process. Hence, the composition of Roundup Ready alfalfa J101 × J163 is substantially equivalent to that of control and conventional alfalfa forage. Compositional comparisons of forage produced by J101 and J163 to control and conventional alfalfa forage lead to similar conclusions (USDA, 2005).

Environmental Assessment

Alfalfa

Alfalfa, a perennial plant that can survive winter temperatures as low as -20ºC (McKenzie et al., 1988) and can live from five to twelve years, is characterized by purple flowers and coiled pods (Quiros and Bauchan, 1988; CFIA, 2005). Depending on variety and climate, it can grow to a height of one meter and has a deep root system sometimes stretching to 4.5 meters. Alfalfa is a beneficial crop for soil health because it adds nitrogen to the soil through a symbiotic relationship formed on root nodules with nitrogen-fixing bacteria. Alfalfa (*Medicago sativa* L.) belongs in the order Fabales, family Fabaceae, tribe Trifolieae, genus *Medicago*. The genus *Medicago* is extensive, consisting of more than 60 different species; two thirds of the species are annuals and one third are perennials (Quiros and Bauchan, 1988). Cultivated alfalfa is sexually compatible with other perennial *Medicago* species; however, no perennial *Medicago* species are naturally present in the Americas, Australia, New Zealand, or South Africa (Michaud *et al*., 1988). Therefore, no risk for interspecific hybridization exists in these geographies, but cross-pollination to scattered naturalized populations of *M. sativa* would be possible.

Alfalfa, including both cultivated alfalfa and closely related subspecies, originated in Asia Minor, Transcaucasia, Turkmenistan, and Iran, and is endemic throughout the Mediterranean region, North Africa, the Middle East, most of Europe, Siberia, northern India, and China (Ivanov, 1988; Michaud et al., 1988; Quiros and Bauchan, 1988). Alfalfa has been cultivated before recorded history and can be found growing wild in Asia, Europe, and North Africa. It has become acclimatized in South Africa, Australia, New Zealand, and North and South America.

Alfalfa varieties are bred to possess different cold tolerances depending upon the intended geographic region for growth of the variety. Alfalfa undergoes biochemical changes in the fall of the year that increase tolerance to low temperature stresses. While all alfalfa tissues are capable of attaining some degree of cold tolerance, crown buds are generally the most cold tolerant. The crown, a complex structure near the soil surface, has perennial meristem activity, producing buds that develop into stems. Cold tolerance is controlled by genetic and environmental factors such as temperature, photoperiod and soil environment. The degree of winter hardiness associated with an alfalfa variety is dependent upon the source of germplasm from which it was derived, with more cold-tolerant cultivars having germplasm that originated from colder northern sources. Environmental factors such as decreased photoperiod, reduced temperature in the fall, and reduced soil moisture serve to initiate the cold-tolerance response and impact the survivability throughout the winter months. Ability to survive through the winter is strongly correlated to the varieties' reactions to shortened photoperiod. Highly fall-dormant plants dramatically reduce foliar growth in the early fall, whereas extremely non-dormant plants continue to produce growth during short winter days. Fall dormancy is measured on a scale of 1 (very fall dormant) to 11 (very non-dormant).

Alfalfa is exclusively an insect-pollinated crop that, unlike other insect-pollinated crops, is pollinated by a small number of insect species, primarily bees. Because of the nonreversible tripping mechanism within the alfalfa flower, each alfalfa bloom may be pollinated only a single time by a single pollinating insect. It does not shed pollen to the wind. After pollination, alfalfa seed requires four to six weeks of adequate growing conditions to develop and ripen.

Alfalfa seed are formed in a coiled seedpod and consist of an embryo, endosperm and testa (seed coat). Impermeable or hard seed are common in alfalfa. Seed hardness is due to the thickened outer wall of the palisade cells in the seed coat that create a moisture impermeable barrier (Bass *et al*., 1988). A minute break in this barrier causes the seed to imbibe water and initiate germination. Hard seed may remain dormant for many years.

Agronomic Performance

Agronomic evaluations are an important component of assessing the potential ecological risk of a genetically modified crop. Roundup Ready alfalfa J101, J163 and J101 × J163 have been tested in over 250 field trial locations since 1997 in the U.S. and several locations in Argentina and Mexico. Data collected prior to commercialization of Roundup Ready alfalfa show that Roundup Ready alfalfa does not differ from conventional alfalfa in agronomic characteristics such as growth and vigor, insect or disease susceptibility, response to stressors, forage yield, fall dormancy response or stand survival.

Assessment of Potential Weediness

Over the past ten years, herbicide tolerant plants have been introduced in numerous plant species, including corn *(Zea maize*), soybean (*Glycine max*), cotton (*Gossypium hirsutum*) and canola (*Brassica napus*) to name a few. In corn, cotton and canola, multiple forms of herbicide tolerance have been introduced. These herbicide-tolerant crops have achieved broad acceptance by farmers being cultivated on millions of acres (James, 2004). Introduction of herbicide-tolerance genes to these crop species have not resulted in reports of increased weediness of these species. This is because herbicide tolerance genes are considered selectively neutral (Stewart et al., 1997) providing no selective advantage in unmanaged situations and in agricultural settings. Growers easily can manage volunteer plants using alternative herbicides that remain effective against herbicide tolerant plants.

The inherent weediness of alfalfa is an important consideration in evaluating whether introduction of herbicide tolerance is likely to cause an adverse environmental impact. *M. sativa* is not listed as a serious weed in *A Geographical Atlas of World Weeds* (Holm *et al*, 1991) or as a weed in *World Weeds: Natural Histories and Distribution* (Holm *et al*. 1997), *Weeds of the North Central States* http://www.ag.uiuc.edu/~vista/html_pubs/WEEDS/list.html), *Weeds of the Northeast* (Uva *et al*., 1997), or *Weeds of the West* (Whitson *et al*., 1992 nor is it listed as a noxious weed species by the U.S. Federal Government (7 CFR Part 360).

To determine whether there was an impact on the weediness potential for Roundup Ready alfalfa, a detailed phenotypic characterization of events J101, J163 and J101 ×J163 was performed. The phenotypic characteristics of Roundup Ready alfalfa that may impact the weediness characteristics of events J101, J163 and J101×J163 were evaluated in multi-site replicated field trials. Data were collected that assess characteristics suggested by USDA-APHIS that may impact the plant pest risk of alfalfa. These phenotypic characteristics have been grouped into five general categories: 1) seed dormancy, germination and emergence; 2) vegetative growth; 3) reproductive growth and survival; 4) disease, insect, and abiotic stressor interactions; and 5) symbiotic organisms.

The phenotypic evaluation was based on both laboratory experiments and replicated, multi-site field trials conducted over five years (1999-2003) by agronomists and scientists who are familiar with the production and evaluation of alfalfa. In each of these assessments, Roundup Ready alfalfa events J101, J163 and J101 × J163 were statistically compared to an appropriate alfalfa control population. Comparisons also were made to conventional reference variety populations planted at the same location. The overall conclusions from this extensive characterization were that there were no biologically meaningful differences between Roundup Ready alfalfa populations and the alfalfa control population or alfalfa reference variety populations. Crop compositional data and agronomic data also supported the conclusion that J101, J163 and J101 × J163 alfalfa populations were not different from the alfalfa control or conventional populations. On the basis of these data, it was concluded that there were no increased pest potential of Roundup Ready alfalfa populations, and that other than the intentional change caused by the trait, the phenotype of alfalfa has not been changed as a result of the trait or transformation process.

Alfalfa is a crop that survives outside of cultivation; therefore, the safety assessment considered whether transfer of glyphosate tolerance via gene flow to feral alfalfa would cause a competitive advantage to feral alfalfa. Trait movement from cultivated Roundup Ready alfalfa to feral alfalfa is possible because alfalfa is bee-pollinated. Given this potential, the consequences of trait movement to feral alfalfa were considered. As mentioned previously, *M. sativa* has no wild relatives to which it can cross within the U.S; hence, the consequences of gene flow need only be considered for cultivated or feral *M. sativa*. While trait movement via gene flow is possible, the vast majority of alfalfa production fields are maintained to harvest prior to flowering. Alfalfa managed as forage will have little contribution to pollen-mediated gene flow under production conditions because there will be few if any open flowers in the standing canopy or mature seeds in the harvested forage. Because the trait and transformation process provide no competitive advantage to events J101, J163 and J101 × J163 relative to conventional alfalfa, movement of the *cp4 epsps* gene to feral alfalfa is considered neutral from an environmental impact perspective. Feral alfalfa populations are not typically targeted for control with herbicides, and if controlled, glyphosate is not the herbicide of choice for alfalfa (USDA, 2005). Thus, glyphosate tolerance in alfalfa provides no selective advantage and would not impact the weediness or invasiveness of feral alfalfa.

Assessment of Effects on NonTarget Organisms

The CP4 EPSPS protein produced in Roundup Ready crops, including Roundup Ready alfalfa events J101, J163 and J101 × J163, is similar to native EPSP synthases ubiquitous in plant and microbial tissues in the environment. Based on this history of occurrence, there is no *a priori* reason to suspect that EPSP synthases will possess biological activity towards other organisms in the environment. The absence of a plausible mechanism for biological activity in animals minimizes the need for extensive toxicity studies to characterize hazard. Although minimal ecological risk is predicted, several field monitoring studies have been conducted for Roundup Ready crops. Field investigations on potential adverse effects of Roundup Ready crops producing CP4 EPSPS have indicated no effects in the abundance or population dynamics of field insects (Huang *et al*., 2004; Jasinski *et al*., 2003; Jackson and Pitre, 2004; Yoshimura *et al*., 2004; Bitzer *et* *al*., 2002; Buckelew *et al*., 2000; McPherson *et al*., 2003; Rosca, 2004), or microbial populations (Siciliano and Germida, 1999; Dunfield and Germida, 2003; Kim *et al*., 2004; Shin *et al*., 2004).

In addition, laboratory studies investigating the effect of organisms exposed to Roundup Ready crops producing CP4 EPSPS have shown no adverse effects to pollinators (honey bee), soil organisms (Collembola), beneficial insects (lacewing, green cloverworm), or various pest insect species (Boongird *et al*., 2003; Jamornman *et al*., 2003; Goldstein, 2003; Michigan Farm News, 2002; Morjan and Pedigo, 2002).

Adverse effects on other field organisms are not expected in Roundup Ready alfalfa because of the ubiquitous nature of EPSPS proteins in plants, the mechanism of the EPSPS enzyme, and the extensive commercial production history of Roundup Ready crops, including the first generation Roundup Ready cotton. During field trials, Roundup Ready alfalfa was grown under agronomic and cultural practices that are typical of alfalfa production. The incidence of disease and abundance of insects and other organisms observed at any of the sites were consistent between Roundup Ready and conventional alfalfa. Additional observations during multiple years of product development trials, including impacts on symbiotic organisms, have not indicated adverse effects to other field organisms.

Potential Impact on Biodiversity

Since the naturally occurring EPSPS proteins are ubiquitous among plants and fungi in nature and nontoxic to fish, avian species, insects, mammals and other species, and exposure to these species likely is not due to their feeding preferences, the effects on wildlife from the commercialization of Roundup Ready alfalfa are not expected to be any different from those experienced in the production of conventional alfalfa. In addition, the agronomic and compositional data obtained on Roundup Ready alfalfa support the assertion that the impact on biodiversity of Roundup Ready alfalfa will be equivalent to or less than conventional alfalfa.

Monsanto is not aware of reports verifying the unaided transfer of genetic material from one alfalfa species to another species with which alfalfa cannot sexually interbreed. The DNA that was transferred into the alfalfa genome to produce Roundup Ready alfalfa does not contain microbial replication or genetic transfer functions. Additionally, Roundup Ready alfalfa does not contain an antibiotic resistance marker, and was produced by selecting plant cells, tissues, and plants with glyphosate-containing compounds.

Weed Resistance to Glyphosate

As leaders in the development and stewardship of glyphosate products for almost 30 years, Monsanto invests considerably in research to understand the proper uses and stewardship of the glyphosate molecule. This research includes an evaluation of the factors that can contribute to the development of weed resistance. Managing the potential for weed resistance is an important issue with all herbicides because it can adversely impact the utility and life cycle of products, if it is not managed properly.

As part of our product stewardship and customer service policy, Monsanto investigates cases of unsatisfactory weed control to determine the cause. Weed control failures following application of Roundup agricultural herbicides are most often the result of mismanagement and/or environmental issues and are only rarely the result of herbicide resistance. The procedures included in Monsanto's performance evaluation program provide early detection of potential resistance, field and greenhouse protocols to investigate suspected cases, and mitigation procedures to respond to confirmed cases of glyphosate resistance.

As of October 2005, some 182 herbicide-resistant species and 304 biotypes within those species have been identified (Heap, 2005). Most of these are resistant to the ALS inhibitor and photosystem II inhibitor herbicide groups; however, eight species have been confirmed as glyphosate resistant (Heap, 2005). The question has been raised as to whether the widespread introduction of crops tolerant to a specific herbicide, such as glyphosate, may increase the occurrence of weeds resistant to that particular herbicide.

It is important to recognize that weed resistance is an herbicide-related issue, not a crop-related issue. The use of a specific herbicide with an herbicide-tolerant crop is no different than the use of a selective herbicide over a conventional crop from a weed resistance standpoint. While the incidence of weed resistance is often associated with repeated applications of an herbicide product, resistance development depends very much on the chemical characteristics of the specific herbicide in question as well as the plant's ability to inactivate the characteristics. Some herbicide products are more prone to develop herbicide resistance than others. Glyphosate is a unique herbicide because it is a transition state inhibitor of EPSPS, has very limited metabolism in plants, and has a lack of soil residual activity. Extensive research has led to the hypothesis that the most effective way to minimize the potential for glyphosate weed resistance is to use a high-dose strategy. Higher herbicide doses result in higher weed mortality and less diversity of resistance genes in the surviving population (Matthews, 1994). The glyphosate rates recommended in Roundup agricultural product labelling have been evaluated for the effective control of the target weed populations, and are consistent with the high dose strategy. Glyphosate has been used extensively for three decades with relatively few cases of weed resistance development in relation to many other herbicides. In alfalfa production areas where glyphosate resistant weeds exist, recommendations are being developed for control of these weeds. Monsanto's control recommendations for specific glyphosate-resistant weeds in glyphosate-tolerant alfalfa include pre-plant tillage and use of a tank mix of glyphosate with an additional herbicide employing a different mode of action. Monsanto promotes and distributes weed-resistance management guidelines in grower presentations, technical bulletins and advertising.

Environmental Assessment Conclusions

In summary, Roundup Ready alfalfa events J101, J163 and J101 × J163 have been thoroughly characterized to determine whether the trait or transformation process impacted in any way the weediness or invasiveness of alfalfa. It was concluded that there were no meaningful differences between Roundup Ready alfalfa compared to conventional alfalfa. Further, the CP4 EPSPS protein was determined to have no impact on nontarget organisms. Given that alfalfa in not considered a weed and that the tolerance to glyphosate does not provide a selective advantage to alfalfa, the environmental impact of Roundup Ready alfalfa is considered to be no different than that due to cultivation of conventional alfalfa.

Summary

The introduction of Roundup Ready alfalfa provides alfalfa producers with a flexible, highly effective weed control system that results in production of cleaner, higher quality hay. Detailed characterization data confirm the safety of this product based on: the safety of the genetic elements contained in the transformation vector; detailed molecular characterization data confirming that only the CP4 EPSPS protein is produced from the insert present in J101 and J163; the known metabolic function and history of safe use of the EPSPS family of proteins present in all plants, fungi and bacteria; the direct assessment of the CP4 EPSPS protein for lack of allergenicity and toxicity concerns; the assessment of the compositional and nutritional equivalence of Roundup Ready alfalfa (comparing key nutrients and other components to that in control and conventional alfalfa forage); and a comparison of crop agronomics and phenotypic characteristics to the control and conventional alfalfa. These assessments confirm that Roundup Ready alfalfa is as safe and nutritious as conventional alfalfa varieties, does not pose a plant pest risk and poses no different environmental risk than conventional alfalfa varieties.

References

Astwood, J.D., J.N. Leach, and R.L. Fuchs. 1996. Stability of food allergens to digestion *in vitro*. Nature Biotechnology 14:1269-1273.

Astwood, J.D., and Fuchs, R.L. 1996. Food allergens are stable to digestion in a simple model of the gastrointestinal tract. J. Allergy Clin. Immunol. 97:241.

Astwood, J.D., and Fuchs, R.L. 2000. Status and safety of biotech crops. Pp 152-164. *In* Agrochemical Discovery-Insect, Weed and Fungal Control. Baker, D.R. and N.K. Umetsu (eds.). ACS Symposium Series 774. American Chemical Society, Washington, D.C.

Barker, R.F., K.B. Idler, D.V. Thompson, and J.D.Kemp. 1983. Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Mol. Biol. 2:335-350.

Barnes, D.K., B.P. Goplen, and J.E. Baylor. 1988. Highlights in the USA and Canada. Pp 1-24. *In* Alfalfa and Alfalfa Improvement. Hanson, A.A., D.K. Barnes and R.R. Hill, Jr. (eds.). ASA-CSSA-SSSA, Madison, Wisconsin.

Bass, L.N., C.R. Gunn, O.B. Hesterman, and E.E. Roos. 1988. Seed physiology, seedling performance, and seed sprouting. Pp 961-979. *In* Alfalfa and Alfalfa Improvement. Hanson, A.A., D.K. Barnes and R.R. Hill, Jr. (eds.). ASA-CSSA-SSSA, Madison, Wisconsin.

Bitzer, R.J., L.D. Buckelew, and L.P. Pedigo. 2002. Effects of transgenic herbicide-resistant soybean varieties and systems on surface-active springtails (*Entognatha*: Collembola). Environ. Entomology. 31:449-461.

Boongird, S., T. Seawannasri, T. Ananachaiyong, and S. Rattithumkul. 2003. Effect of Roundup Ready Corn NK603 on foraging behavior and colony development of *Apis mellifera* L. under greenhouse condition. Proc. Sixth Nat. Plant Protect. Conf. Thailand 24.

Buckelew, L.D., L.P. Pedig, H.M. Mero, M.D.K. Owen, and G.L. Tylka. 2000. Effects of weed management system on canopy insects in herbicide-resistant soybeans. J. Econ. Ent. 93:1437-1443.

CFIA (Canada Food Inspection Agency). 2005. The Biology of *Medicago sativa* L. (Alfalfa). Biology Document Bio2005-02: [http://www.inspection.gc.ca/english/plaveg/bio/dir/dir0502e.shtml]

Church, D.C. 1984. Livestock Feeds and Feeding. 2nd Edition. O&B Books. Corvallis, Oregon.

Conrad, H.R. and T.J. Klopfenstein. 1988. Role in livestock feeding – Greenchop, silage, hay, and dehy. Pp 539-550. *In* Alfalfa and Alfalfa Improvement. Hanson, A.A., D.K. Barnes and R.R. Hill, Jr. (eds.). ASA-CSSA-SSSA, Madison, Wisconsin.

Corruzzi, G., R. Broglie, C. Edwards, and N-H Chua. 1984. Tissue-specific and light-regulated expression of a pea nuclear gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. EMBO J. 3:1671-1679.

CSFII. The Continuing Survey of Food Intakes by Individuals (CSFII) and the Diet and Health Knowledge Survey (DHKS) 1989-91. [http://www.ars.usda.gov/Services/docs.htm?docid=7797#availability]

Depicker, A., S. Stachel, P.Dhaese, P. Zambryski, and H.M. Goodman. 1982. Nopaline Synthase: Transcript mapping and DNA sequence. J. Molec. Appl. Genet. 1:561-573.

Doolitte, R.F. 1990. Searching through sequence databases. Pp 99-110. *In* Molecular Evolution: Computer Analysis of Protein and Nucleic Acid Sequences. Doolittle, R.F. (ed.). Academic Press, San Diego, California.

Duke, J.A. 1981. *Medicago sativa* L. Pp 157-161. *In* Handbook of Legumes of World Economic Importance. Plenum Press, New York.

Dunfield, K.E. and J.J. Germida. 2003. Seasonal changes in the rhizosphere microbial communities associated with field-grown genetically modified canola (*Brassica napus*). Appl. Environ. Micro. 69:7310-7318.

FAO (Food and Agriculture Organization). 1995. Report of the FAO Technical Consultation on Food Allergies, Rome , Italy, November 13-14, 1995. FAO, Rome.

Franz, J.E., M.K. Mao, and J.A. Sikorski. 1997. Glyphosate: A unique global herbicide. ACS Monograph 189. American Chemical Society, Washington, D.C.

Fuchs, R.L., and J.D. Astwood. 1996. Allergenicity assessment of foods derived from genetically modified plants. Food Technol. 50:83-88.

Giesy, J.P., S. Dobson, and K.R. Solomon. 2000. Ecotoxicological risk assessment for Roundup herbicide. Rev. of Env. Contam. and Tox. 167:35-120.

Goldstein, S.M. 2003. Life history observations on three generations of *Folsoma candida* (Willem) (Collembola: Isotomidae) fed yeast and Roundup Ready Soybeans and Corn. Masters Thesis, Michigan State University, Michigan.

Goodman, R.E., S.L. Hefle, S.L.Taylor, and R. van Ree. 2005. Assessing genetically modified crops to minimize the risk of increased food allergy: a review. Int. Arch. Allergy Immunol. 137(2):153-66.

Harrison, L., M. Bailey, M. Naylor, J. Ream, B. Hammond, D. Nida, B. Burnette, T. Nickson, T. Mitsky, M. Taylor, R. Fuchs, and S. Padgette. 1996. The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested *in vitro* and is not toxic to acutely gavaged mice. J. Nutr. 126:728-740.

Heap, I. 2005. www.weedscience.com .

Hilemen, R.E., A.Silvanovich, R.E. Goodman, E.A. Rice, G. Holleschak, J.D. Astwood, and S.L. Hefle. 2002. Bioinformatic methods for allergenicity assessment using a comprehensive allergen database. Int. Arch. Allergy Immunol. 128:280-291.

Holm, L, J.V. Pancho, J.P. Herbarger, and D.L. Plucknett, D.L. 1991. A geographical atlas of weeds. Kreiger Publ. Co., Malabar, Florida.

Holm, L., J. Doll, J. Holm, J. Pancho, and J. Herberger. 1997. World weeds natural histories and distribution. John Wiley and Sons, New York.

Huang, Z.H., A.V. Hanley, W.L. Pett, M. Langenberger, and J.J. Duan. 2004. Field and semifield evaluation of impacts of transgenic canola pollen on survival and development of worker honey bees. J. Econ. Ent. 97:1517-1523.

Ivanov, A.I. 1988. Alfalfa. Amerind Publishing Co., New Delhi.

Jackson, R.E., and H.N. Pitre. 2004. Influence of Roundup Ready soybean production systems and glyphosate application on pest and beneficial insects in narrow-row soybeans. J. Ent. Sci. 39:62-70.

James, C. 2004. Executive Summary. Global status of commercialized biotech/GM crops: 2004. International Service for the Acquisition of Agri-Biotech Applications. ISAAA briefs No. 32., New York.

Jamornman, S., S. Sopa, S. Kumsri, T. Anantachaiyong, and S. Rattithumkul. 2003. Pp 28-29. Effect of Roundup Ready corn NK603 on green lacewing - *Mallada basalis* (Walker) under laboratory conditions. Proc. Sixth Nat. Plant Prot. Conf. 24-27 November 2003.

Jasinski, J.R., J.B. Eisley, C.E. Young, J. Kovach, and H. Wilson. 2003. Select nontarget arthropod abundance in transgenic and nontransgenic field crops in Ohio. Environ. Ent. 32:407-413.

Jones D.D. and J.H. Maryanski. 1991. Safety considerations in the evaluation of transgenic plants for human food. Pp 64-82. *In* Safety Considerations in the Evaluation of Transgenic Plants.

Julier, B., C. Huyghe and C. Ecalle. 2000. Within- and among-cultivar genetic variation in alfalfa: Forage quality, morphology, and yield. Crop Sci 40: 365-369.

Kim, Y.T., B.K. Park, E.I. Hwang, N.H. Yim, N.R. Kim, T.H. Kang, S.H. Lee, and S.U. Kim. 2004. Investigation of possible gene transfer to soil microorganisms for environmental risk assessment of genetically modified organisms. J. Micro. Biotech. 14:498-502.

Kimber, I., N.I. Kerkvliet, S.L. Taylor, J.D. Astwood, K. Sarlo, and R.J. Dearman. 1999. Toxicology of Protein Allergenicity: Prediction and Characterization. Toxicological Sciences 48:157-162.

Klee, H.J., Y.M. Muskopf, and C.S. Gasser. 1987. Cloning of an *arabidposis thaliana* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: Sequence analysis and manipulation to obtain glyphosate-tolerant plants. Mol. Gen. Genet. 210:437-442.

Lookhart, G.L. 1980. Analysis of coumestrol, a plant estrogen, in animal feeds by high-performance liquid chromatography. J. of Agric. and Food Chem. 28:666-667.

Marten, G.C., D.R.Buxton, and R.F. Barnes. 1988. Feeding value (forage quality). Pp 463-491. *In* Alfalfa and Alfalfa Improvement. Hanson, A.A., D.K. Barnes and R.R. Hill, Jr. (eds.). ASA-CSSA-SSSA, Madison, Wisconsin.

Matthews, J.M. 1994. Management of herbicide resistant weed populations. Pp 317-335. *In* Herbicide Resistance in Plants, Biology and Biochemistry. S.B. Powles and J.A.M. Holtum (eds.). Lewis, Ann Arbor, Michigan.

McKenzie, J.S., R. Paquin, R., and S.H. Duke. 1988. Cold and heat tolerance. Pp 259-302. *In* Alfalfa and Alfalfa Improvement. Hanson, A.A., D.K. Barnes and R.R. Hill, Jr. (eds.). ASA-CSSA-SSSA, Madison, Wisconsin.

McPherson, R.M., W.C. Johnson, B.J. Mullinix Jr., W.A. Mills III, and F.S. Peebles. 2003. Influence of herbicide tolerant soybean production systems on insect pest populations and pest-induced crop damage. J. Econ. Ent. 96:690-698.

Metcalfe, D. D., J.D. Astwood, R. Townsend, H.A. Sampson, S.L. Taylor, and R.L. Fuchs. 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. Crit. Rev. Food. Sci. Nutr. 36:S165-S186.

Michigan Farm News. 2002. MSU: Roundup Ready crops are a safe food source. Michigan Farm Bureau. 79(11):7.

Michaud, R., W.F. Lehman, and M.D. Rumbaugh. 1988. World distribution and historical development. Pp 25-91. *In* Alfalfa and Alfalfa Improvement. Hanson, A.A., D.K. Barnes and R.R. Hill, Jr. (eds.). ASA-CSSA-SSSA, Madison, Wisconsin.

Morjan, W.E. and L.P. Pedigo. 2002. Suitability of transgenic glyphosate-resistant soybeans to green cloverworm (*Lepidoptera*: *Noctuidae*). J. Econ. Ent. 95:1275-1280

Moravcová, J., T. Kleinová , and R. Loucka. 2002. The determination of coumestrol in alfalfa (*Medicago sativa)* by capillary electrophoresis. Rostlinná Vyroba. 48:224-229.

OECD. 2005. Consensus document on compositional considerations for new varieties of alfalfa and other temperate forage legumes: Key feed nutrients, anti-nutrients and secondary plant metabolites. http://appli1.oecd.org/olis/2005doc.nsf/linkto/env-jm-mono(2005)13

NRA (National Research Council). 1982. United States-Canadian Tables of Feed Composition. Pp 8-146. *In* Nutritional Data for United States and Canadian Feeds., 3rd ed. National Academy Press, Washington, DC.

Padgette, S.R., D.B. Re, G.F. Barry, D.E. Eichhlotz, X. Delannay, R.L. Fuchs, G.M. Kishore, and R.T. Fraley. 1996. New weed control opportunities: Development of soybeans with a Roundup Ready gene. Pp 53-80. *In* Herbicide-Resistant Crops: Agricultural, Environmental, Economic, Regulatory, and Technical Aspects. Duke, S.O. (ed.). CRC Press, New York.

Pariza, M.W., and E.M. Foster. 1983. Determining the safety of enzymes used in food processing. J. of Food Protec. 46:453-468.

Quiros, C.F., and G.R. Bauchan. 1988. The genus *Medicago* and the origin of the *Medicago sativa* complex. Pp 93-124. *In* Alfalfa and Alfalfa Improvement. Hanson, A.A., D.K. Barnes and R.R. Hill, Jr. (eds.). ASA-CSSA-SSSA, Madison, Wisconsin.

Richins, R., H. Scholthof, and R. Shepard. 1987. Sequence of figwort mosaic virus DNA (Caulimovirus Group). Nucleic Acids Res. 15:8451-8466.

Rochester, D.E., J.A. Winer, and D.M. Shah. 1986. The structure and expression of maize genes encoding the major heat shock protein, hsp 70. EMBO J. 5:451-458.

Rosca, I.I. 2004. Impact of genetically modified herbicide resistant maize on the arthropod fauna. IOBC Wprs Bulletin. 27:143-146.

Rumbaugh, M.D., J.L. Caddel, and D.E. Rowe. 1988. Breeding and quantitative genetics. Pp 777-808. *In* Alfalfa and Alfalfa Improvement. Hanson, A.A., D.K. Barnes and R.R. Hill, Jr. (eds.). ASA-CSSA-SSSA, Madison, Wisconsin.

Samac, D.A. and Temple, S.J. 2004. Development and Utilization of Transformation in *Medicago* species. Pp 165-202. *In* Genetically Modified Crops: Their Development, Uses, and Risks. Liang, G.H., and D.Z. Skinner, D.Z. (eds.). Haworth Press, Inc. New York.

Shin, D.W., S.H. Park, G.J. Woo, H.J. Kim, and C.S. Park. 2004. Case study for natural gene transfer from genetically modified food to food microorganisms. Food Sci. Biotechnol. 13:342-346.

Siciliano, S.D., and J.J. Germida. 1999. Taxonomic diversity of bacteria associated with the roots of field-grown transgenic *Brassica napus* cv. Quest, compared to the non-transgenic *B. napus* cv. Excel and *B. rapa* cv. Parkland. FEMS Microbiol. Ecol. 29:273-272.

Sjoblad R.D., J.T. McClintock, and R. Engler. 1992. Toxicological considerations for protein components of biological pesticide products. Regul. Toxico. and Pharmaco. 15:3-9.

Sleisenger, M.H., and J.S. Fordtran. 1989. Gastrointestinal Disease, Vol 1. Pp 685-689. *In* Pathophysiology Diagnosis Management. W.B. Saunders Company, Philadelphia.

Smith, D. 1969. Influence of temperature on the yield and chemical composition of vernal alfalfa at first flower. Agronomy J. 61(May-June):470-472.

Stewart, C.N., J.N. All, P.L. Raymer, and S. Ramachandran. 1997. Increased fitness of transgenic insecticidal rapeseed under insect selection pressure. Molec. Eco. 6:773-779.

Taylor, S.L., R.F. Lemanske Jr., R.K. Bush, and W.W. Busse. 1987. Food allergens: structure and immunologic properties. Ann. Allergy. 59:93-99.

Taylor, S.L. 1992. Chemistry and detection of food allergens. Food Technol. 46:146-152.

Townsend, M.S., J.A. Henning, D.W. Smith, I.M. Ray and C.G. Currier. 1998. Differential concentration of ten minerals among four ancestral alfalfa germplasms. Crop. Sci. 39:574-578.

U.S. EPA. 1993. Reregistration eligibility decision (RED): Glyphosate. Office of Prevention, Pesticides and Toxic Substances, Washington, D.C.

U.S. FDA. 1992. Foods Derived From New Plant Varieties. Fed. Reg. 57(104):22984-23005.

USDA. 2005 Petition for determination of nonregulated status: Roundup Ready alfalfa (*Medicago sativa* L.) events J101 and J163. http://www.aphis.usda.gov/brs/aphisdocs/04_11001p.pdf

Uva, R.H., J.C. Neal, and J.M. Ditomaso, J.M. 1997. Weeds of the Northeast. Cornell University Press, Ithaca, New York.

Van Keuren, R.W., and A.G. Matches. 1988. Pasture production and utilization. Pp 515-532. *In* Alfalfa and Alfalfa Improvement. Hanson, A.A., D.K. Barnes and R.R. Hill, Jr. (eds.). ASA-CSSA-SSSA, Madison, Wisconsin.

Viands, D.R., P. Sun, and D.K. Barnes. 1988. Pollination control: Mechanical and sterility. Pp 931-960. *In* Alfalfa and Alfalfa Improvement. Hanson, A.A., D.K. Barnes and R.R. Hill, Jr. (eds.). ASA-CSSA-SSSA, Madison, Wisconsin.

WHO (World Health Organization). 1994. Glyphosate. Environmental Health Criteria No. 159. World Health Organization, International Programme of Chemical Safety (IPCS), Geneva.

Williams, G.M., R. Kroes, and I.C. Munro. 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regul. Tox. and Pharm. 31:117-165.

Wilkins, R.J. 1988. The preservation of forages. Pp. 231-251. *In* World Animal Science; Feed Science. Orskov, E.R. (ed.). Elsevier, Amsterdam.

Whitson, T.D., L.C. Burrill, S.A. Dewwy, D.W. Cudney, B.E. Nelson, R.D. Lee, and R. Parker. 1992. Weeds of the West. The Western Society of Weed Science, Newark, California.

Yoshimura, Y., K. Matsuo, Y. Shirai, H. Ikeda, K. Itoh, K. Tsuchiya, T. Yoshida, and K. Yasuda. 2004. Effects of consecutive cultivation of glyphosate-tolerant soybean on vegetation, composition of insects and soil microorganisms in Japan. 8th Int. Symp. Biosafety Genet. Mod. Org. 2004. 26-30 Sept., Montpelier, France.

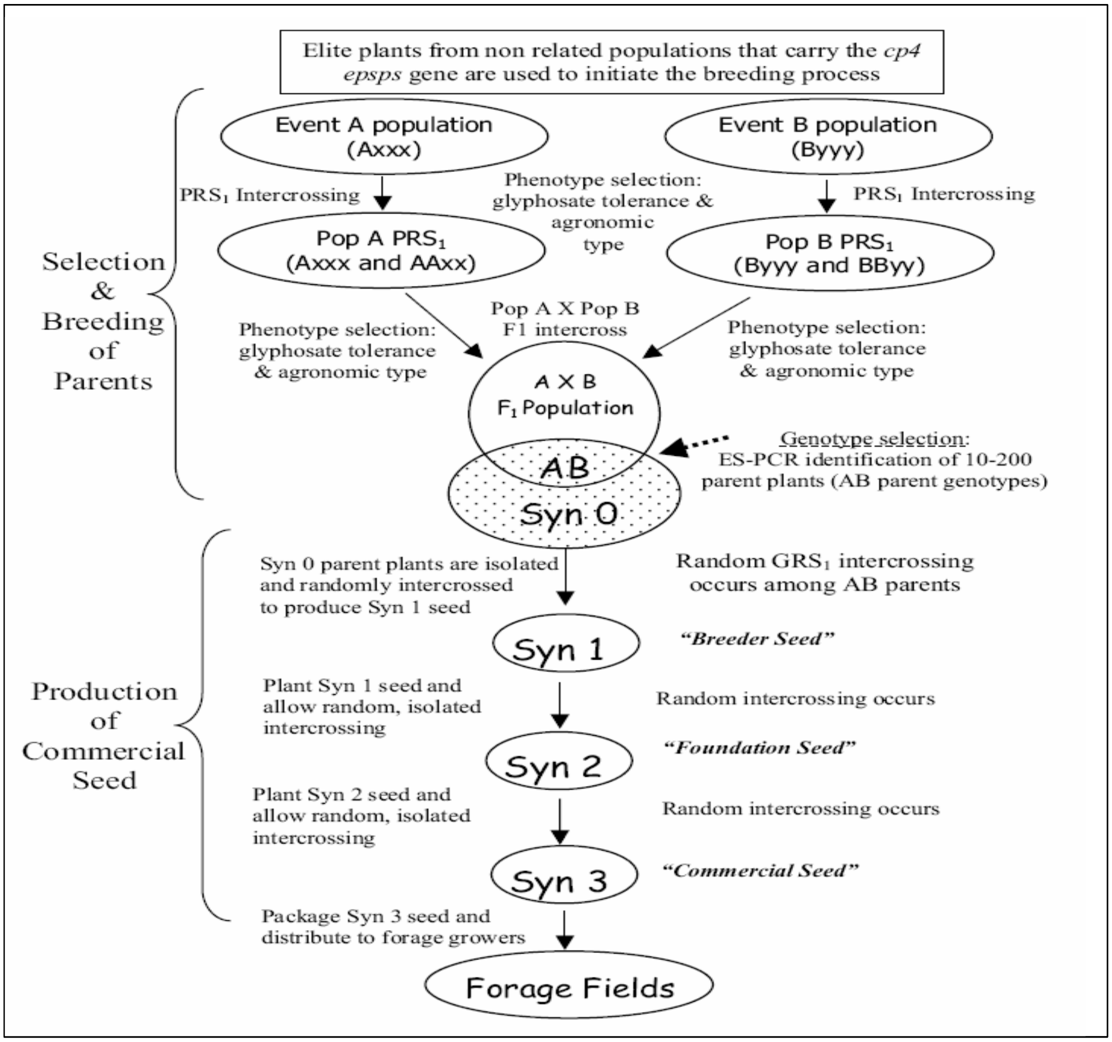

Figure 1. FGI breeding method for production of Roundup Ready alfalfa varieties. The breeding scheme for development of commercial varieties of Roundup Ready alfalfa. Population A represents plants containing the J101 allele and population B represents plants containing the J163 allele. PRS = Phenotypic Recurrent Selection (treatment of alfalfa plants with glyphosate and selection of tolerant plants). ES-PCR = Event Specific Polymerase Chain Reaction. GRS = Genotypic Recurrent Selection (selection of Syn 0 parents that contain both J101 and J163 based on ES-PCR analysis). Syn = Synthetic.

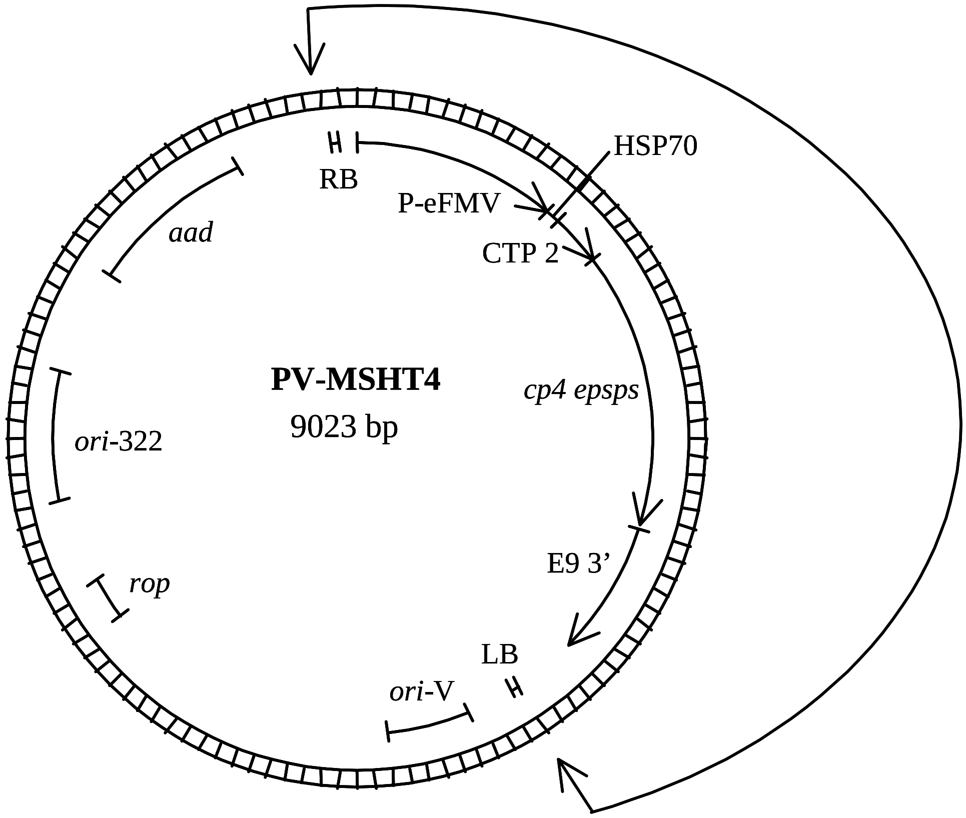

Figure 2. Plasmid Map of PV-MSHT4
A circular map of the plasmid vector PV-MSHT4 used in the development of Roundup Ready alfalfa J101 and J163 with genetic elements annotated is shown above. The genetic material transferred into the alfalfa genome begins near the right border, extends through the *cp4 epsps* coding region and ends near the left border. The transferred genetic material is enclosed within the outer arrows.

Table 1. CP4 EPSPS Levels in Roundup Ready Alfalfa J101, J163 and J101 × J163

		Alfalfa Population and Year of Forage Sampling					
		2001 Field Season			2002 Field Season		
Site	Cut #	J101	J163	J101 × J163	J101	J163	J101 × J163
California	1	270[b]	320	390	240	220	120
Illinois	1	260	320	290	270	310	200
Iowa	1	300	380	290	210	150	180
New York	1	270	290	280	220	180	140
Washington	1	220	270	330	160	140	120
Wisconsin	1	300	330	260	200	140	150
California	2	290	270	340	340	340	280
Illinois	2	230	330	270	280	290	230
Washington	2	340	290	360	220	240	310
	Mean	276	311	312	238	223	192
	Range Low	220	270	260	160	140	120
	Range High	340	380	390	340	340	310

[a] Concentration is given in micrograms/gram tissue fresh weight.
[b]Values were generated from forage collected from field plots containing alfalfa populations grown in a complete block field design with four replicates.

Table 2. Coumestrol, Fiber, and Proximate Composition of Forage From Roundup Ready Alfalfa J101 × J163

	2001 Field Season			2003 Field Season			
component[1]	J101 x J163 mean[3] ± SE (range)	Control[2] mean[3] ± SE (range)	commercial tolerance interval[5] (range)	J101 x J163 mean[4] ± SE (range)	Control[2] mean[4] ± SE (range)	commercial tolerance interval[5] (range)	literature range[1]
coumestrol	not available	not available	not available	47.42 ± 15.10 (3.07-108.00)	37.66 ± 15.05 (3.66-124.50)	0,145.77 (2.99-104.37)	10-184[n]; 133-278[o]
ADF	27.01 ± 1.62 (22.09-33.91)	25.79 ± 1.61 (18.81-33.47)	15.76,40.19 (23.12-33.39)	30.38 ± 2.42 (21.15-39.88)	30.10 ± 2.41 (23.47-36.43)	15.68,44.63 (21.26-39.25)	13-37[k]
lignin	5.31 ± 0.56 (3.48-8.16)	5.07 ± 0.56 (1.64-8.10)	0,12.92 (3.86-9.65)	6.81 ± 0.74 (2.81-9.59)	6.35 ± 0.73 (2.00-8.23)	2.10,10.61 (2.31-13.71)	7[k]; 4.5-7.6[l]
NDF	30.64[a] ± 1.38 (21.87-39.73)	28.09 ± 1.37 (22.25-32.07)	20.01,41.80 (26.53-35.72)	37.70 ± 3.18 (29.12-49.52)	37.84 ± 3.17 (26.71-51.64)	19.86,53.29 (26.85-51.09)	40-47[k]; 31-44[l]
ash	14.41[a] ± 2.46 (8.26-32.50)	11.31 ± 2.46 (8.44-15.04)	5.59,16.40 (8.58-15.25)	9.02 ± 0.60 (6.95-11.16)	9.20 ± 0.59 (7.22-11.69)	5.29,12.54 (6.86-12.79)	9.5[k]; 5.8-7.5[m]
carbohydrates	63.10[a] ± 3.01 (48.03-74.71)	65.08 ± 3.01 (55.44-73.53)	46.29,85.59 (58.03-74.38)	67.08 ± 2.00 (59.68-74.85)	66.33 ± 1.99 (58.58-71.80)	53.20,82.75 (56.63-74.80)	not available
moisture	75.78[a] ± 1.64 (70.70-83.10)	76.77 ± 1.64 (70.70-84.20)	62.91,88.67 (70.90-82.10)	76.10[a] ± 1.32 (71.90-80.90)	78.68 ± 1.32 (75.10-82.40)	66.89,88.25 (72.60-83.50)	76-77[k]
protein	20.49 ± 1.24 (15.53-27.11)	21.35 ± 1.24 (16.02-28.20)	7.98,33.81 (15.29-25.81)	21.42 ± 1.22 (17.99-25.60)	21.07 ± 1.21 (17.02-26.11)	9.20,31.10 (15.52-28.34)	17-27[k]
total fat	2.12 ± 0.17 (1.50-3.13)	2.26 ± 0.17 (1.45-3.58)	0,4.61 (1.33-3.15)	2.81[a] ± 0.35 (1.50-4.43)	3.41 ± 0.34 (1.94-4.61)	0.67,5.27 (1.47-4.49)	not available

[1]All data expressed in percent dry weight of sample, except moisture, which is percent fresh weight of sample and coumestrol, which is mg/kg dry weight of sample.
[2]Nontransgenic null-segregant control.
[3]The least squares mean of nineteen values (four replicates from each of four field sites plus three replicates from the New York field site).
[4]The least squares mean of sixteen values (four replicates from each of four field sites).
[5]T.I = Tolerance Interval, specified to contain 99% of commercial variety population with 95% confidence, negative limits set to zero.
[a]Value statistically different than the control ($P < 0.05$).
[k](National Research Council, 1982)[l]Julier *et al.*, 2000. [m](Smith, 1969). [n](Lookhart, 1980). [o](Moravcová *et al.*, 2002).

Table 3. Amino Acid Composition of Forage From Roundup Ready Alfalfa J101 × J163

component[1]	2001 Field Season J101 × J163 mean[3] ± SE (range)	2001 control[2] mean[3] ± SE (range)	2001 commercial tolerance interval[5] (range)	2003 Field Season J101 × J163 mean[3] ± SE (range)	2003 control[2] mean[3] ± SE (range)	2003 commercial tolerance interval[5] (range)	literature range[b]
Ala	6.20 ± 0.097	6.19 ± 0.097	5.55,6.80	6.07 ± 0.11	6.07 ± 0.10	5.48,6.74	0.93-1.21[l]
	(6.00-6.79)	(6.01-6.56)	(5.93-6.93)	(5.65-6.50)	(5.71-6.33)	(5.23-6.52)	
Arg	5.56 ± 0.063	5.64 ± 0.063	4.98,6.21	5.38 ± 0.18	5.51 ± 0.18	4.44,6.62	0.81[k]; 0.86-1.08[l]
	(5.10-5.99)	(5.40-6.23)	(5.40-5.90)	(4.77-5.84)	(4.78-6.01)	(4.37-6.03)	
Asp	13.16 ± 0.37	12.86 ± 0.37	9.75,16.61	15.46 ± 1.72	14.76 ± 1.71	5.21,23.47	1.97-2.15[l]
	(12.05-14.34)	(10.95-16.22)	(11.83-15.40)	(11.84-23.05)	(11.33-20.30)	(10.36-27.93)	
Cys	1.57[a] ± 0.057	1.41 ± 0.057	1.01,1.96	1.50 ± 0.10	1.56 ± 0.10	0.37,2.72	0.34[k]
	(1.41-1.84)	(1.17-1.59)	(1.23-1.76)	(1.06-1.79)	(1.05-1.93)	(0.79-2.23)	
Glu	11.03 ± 0.077	11.10 ± 0.077	10.28,11.77	10.78 ± 0.31	10.81 ± 0.31	9.03,12.68	1.88-2.40[l]
	(10.70-11.33)	(10.85-11.79)	(10.75-11.62)	(9.57-11.54)	(9.57-11.34)	(8.74-11.70)	
Gly	5.61 ± 0.044	5.56 ± 0.044	5.11,5.84	5.24 ± 0.14	5.28 ± 0.14	4.33,6.20	0.75[k]; 0.82-1.10[l]
	(5.46-6.23)	(5.39-5.97)	(5.35-5.64)	(4.68-5.64)	(4.87-5.56)	(4.29-5.58)	
His	2.70 ± 0.045	2.76 ± 0.044	2.25,3.22	2.64 ± 0.031	2.61 ± 0.030	2.45,2.79	0.38[k]; 0.48-0.60[l]
	(2.44-2.88)	(2.57-3.01)	(2.43-2.96)	(2.49-2.79)	(2.41-2.72)	(2.40-2.79)	
Ile	4.86[a] ± 0.052	4.94 ± 0.052	4.25,5.58	4.51 ± 0.14	4.68 ± 0.14	4.08,5.21	0.67[k]; 0.77-0.95[l]
	(4.64-5.14)	(4.65-5.31)	(4.60-5.20)	(3.97-4.97)	(4.29-5.06)	(3.76-5.10)	
Leu	8.55 ± 0.060	8.66 ± 0.059	8.08,9.07	8.20 ± 0.25	8.33 ± 0.25	7.02,9.78	1.19[k]; 1.35-1.62[l]
	(8.24-8.88)	(8.32-9.12)	(8.36-8.90)	(7.36-8.98)	(7.54-8.87)	(6.50-9.11)	
Lys	6.94 ± 0.098	7.05 ± 0.098	6.26,7.85	7.46 ± 0.15	7.32 ± 0.15	6.62,8.27	0.90[k]; 1.06-1.16[l]
	(6.55-7.39)	(6.62-7.34)	(6.27-7.48)	(6.74-7.97)	(6.79-8.09)	(6.25-7.96)	
Met	1.90 ± 0.031	1.89 ± 0.031	1.56,2.30	1.76 ± 0.079	1.86 ± 0.077	0.98,2.66	0.21[k]; 0.28-0.37[l]
	(1.71-2.21)	(1.57-2.16)	(1.67-2.10)	(1.41-2.04)	(1.56-2.44)	(1.12-2.36)	
Phe	5.54[a] ± 0.066	5.67 ± 0.065	4.64,6.61	5.37 ± 0.15	5.53 ± 0.15	4.58,6.55	0.78[k]; 0.87-1.08[l]
	(5.39-6.06)	(5.32-6.47)	(5.40-6.16)	(4.81-5.99)	(5.04-5.85)	(4.34-6.13)	

Table 3 (cont'd). Amino Acid Composition of Forage From Roundup Ready Alfalfa J101 × J163

Pro	5.49[a] ± 0.11	5.28 ± 0.11	4.57,6.06	4.86 ± 0.12	4.83 ± 0.12	4.24,5.57	0.65-1.26[l]
	(5.06-6.16)	(4.32-5.97)	(4.86-5.73)	(4.30-5.37)	(4.02-6.38)	(4.28-6.04)	
Ser	5.45 ± 0.11	5.36 ± 0.11	4.31,6.57	5.52[a] ± 0.059	5.34 ± 0.056	4.90,6.08	0.72[k]; 0.76-0.95[l]
	(5.05-5.92)	(4.87-5.73)	(4.92-5.91)	(5.22-5.79)	(5.10-5.74)	(5.08-5.98)	
Thr	4.59 ± 0.067	4.57 ± 0.067	3.63,5.48	4.77 ± 0.089	4.75 ± 0.088	4.31,5.29	0.66[k]; 0.78-1.11[l]
	(4.13-4.88)	(4.07-4.79)	(4.10-4.85)	(4.41-4.97)	(4.38-4.97)	(4.26-5.12)	
Trp	1.19 ± 0.057	1.22 ± 0.056	0.62,1.84	1.27 ± 0.051	1.24 ± 0.050	0.78,1.69	not available
	(0.86-1.45)	(0.81-1.48)	(0.86-1.38)	(1.02-1.43)	(1.04-1.48)	(0.76-1.83)	
Tyr	3.69[a] ± 0.046	3.83 ± 0.045	3.33,4.07	3.62 ± 0.23	3.76 ± 0.23	2.40,5.08	0.53[k]; 0.66-0.83[l]
	(3.18-3.89)	(3.46-4.51)	(3.30-3.94)	(2.76-4.19)	(3.19-4.38)	(2.57-4.32)	
Val	6.00 ± 0.052	6.01 ± 0.051	5.36,6.63	5.58 ± 0.12	5.75 ± 0.11	5.16,6.23	0.88[k]; 0.91-1.18[l]
	(5.82-6.27)	(5.58-6.41)	(5.69-6.26)	(5.02-6.02)	(5.48-6.16)	(4.95-6.12)	

[1]All data expressed in percent of total amino acids.
[2]Nontransgenic null-segregant control.
[3]The least squares mean of nineteen values (four replicates from each of four field sites plus three replicates from the New York field site).
[4]The least squares mean of sixteen values (four replicates from each of four field sites).
[5]T.I = Tolerance Interval, specified to contain 99% of commercial variety population with 95% confidence, negative limits set to zero.
[a]Value statistically different than the control ($P < 0.05$).
[b]Literature values expressed in percent dry weight.
[k](National Research Council, 1982). [l](Smith, 1969).

Table 4. Mineral Composition of Forage From Roundup Ready Alfalfa J101 × J163

component[1]	2001 Field Season			2003 Field Season			
	J101 x J163 mean[3] ± SE (range)	control[2] mean[3] ± SE (range)	commercial tolerance interval[5] (range)	J101 x J163 mean[3] ± SE (range)	control[2] mean[3] ± SE (range)	commercial tolerance interval[5] (range)	literature range[b]
calcium	1.01[a] ± 0.070 (0.81-1.38)	1.12± 0.070 (0.88-1.44)	0.48,1.89 (0.90-1.53)	1.22[a] ± 0.080 (0.90-1.75)	1.39 ± 0.079 (1.15-1.70)	0.62,2.03 (0.91-1.86)	1.39-2.30[m]
copper	8.24 ± 0.68 (6.42-12.28)	9.41 ± 0.68 (6.76-17.10)	3.12,12.64 (5.29-10.18)	6.47[a] ± 1.41 (3.36-10.68)	7.51 ± 1.41 (3.83-11.78)	0,18.56 (3.43-14.72)	10[k]; 3-4[l]; 12-52[m]
iron	730.93[a] ± 230.85 (199.10-2196.43)	410.19 ± 230.60 (184.32-764.23)	0,892.57 (235.53-1538.46)	250.27 ± 94.44 (79.83-700.00)	195.68 ± 94.13 (82.46-577.46)	0,583.85 (63.49-709.43)	66-78[l]; 204-489[m]
magnesium	0.24 ± 0.051 (0.10-0.38)	0.26 ± 0.051 (0.11-0.54)	0,0.68 (0.11-0.45)	0.26 ± 0.041 (0.15-0.45)	0.29 ± 0.041 (0.18-0.49)	0.0099,0.50 (0.13-0.39)	0.35-0.49[l]; 0.21-0.30[m]
manganese	61.83 ± 8.60 (35.90-112.95)	54.04 ± 8.57 (32.97-81.01)	0,120.37 (34.60-109.50)	33.17 ± 5.11 (16.09-45.73)	33.36 ± 5.09 (20.57-49.09)	0,66.81 (15.91-49.60)	48-60[l]; 39-46[m]
phosphorus	0.32 ± 0.027 (0.22-0.42)	0.33 ± 0.027 (0.25-0.45)	0.095,0.54 (0.22-0.45)	0.34 ± 0.032 (0.23-0.49)	0.35 ± 0.032 (0.28-0.47)	0.17,0.49 (0.23-0.46)	0.24-0.42[m]
potassium	2.96 ± 0.41 (0.85-4.32)	3.08 ± 0.41 (1.57-4.30)	0.38,5.75 (1.39-4.31)	2.78 ± 0.26 (1.41-3.66)	2.94 ± 0.26 (1.95-3.33)	1.30,4.25 (1.92-3.90)	1.34-2.35[l]; 1.48-2.78[m]
sodium	0.10 ± 0.041 (0.017-0.38)	0.079 ± 0.041 (0.018-0.23)	0,0.31 (0.017-0.21)	0.20 ± 0.099 (0.018-0.75)	0.12 ± 0.099 (0.020-0.45)	0,0.76 (0.019-0.51)	0.19[k]; 0.0024-0.19[m]
zinc	28.61 ± 2.94 (17.01-37.28)	29.58 ± 2.93 (16.70-46.15)	5.05,50.21 (18.09-35.98)	29.69 ± 2.24 (20.04-35.81)	29.15 ± 2.23 (20.90-42.96)	6.12,50.76 (15.20-43.62)	18[k]; 30-65[l]

[1]Calcium, magnesium, phosphorus, potassium and sodium expressed in percent dry weight of sample; copper, iron, manganese and zinc expressed in milligram per kilogram dry weight of sample.
[2]Nontransgenic null-segregant control.
[3]The least squares mean of nineteen values (four replicates from each of four field sites plus three replicates from the New York field site).
[4]The least squares mean of sixteen values (four replicates from each of four field sites).
[5]T.I = Tolerance Interval, specified to contain 99% of commercial variety population with 95% confidence, negative limits set to zero.
[a]Value statistically different than the control (*$P < 0.05$*).
[k](National Research Council, 1982)[l](Smith, 1969). [m](Townsend *et al.*, 1998).