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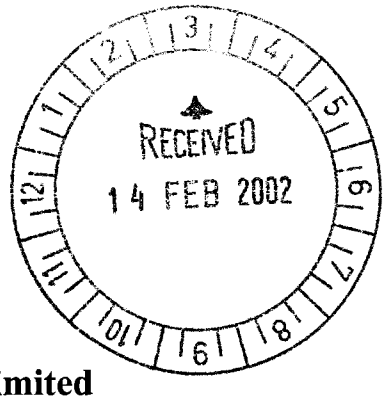

APPLICANT: **Monsanto Australia Limited**

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CP4 ESPS gene in Roundup Ready® Corn Line NK603

SUBMISSION: Application to Australia New Zealand Food Authority for the inclusion of corn containing the CP4 EPSPS gene by Monsanto in Standard A18 - Food Derived From Gene Technology

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SUPPORTING INFORMATION

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RT-PCR Analyses of the 3' End and Adjoining Genomic DNA Sequences Flanking the Insert in Roundup Ready® Maize Event NK603 – Safety Assessment

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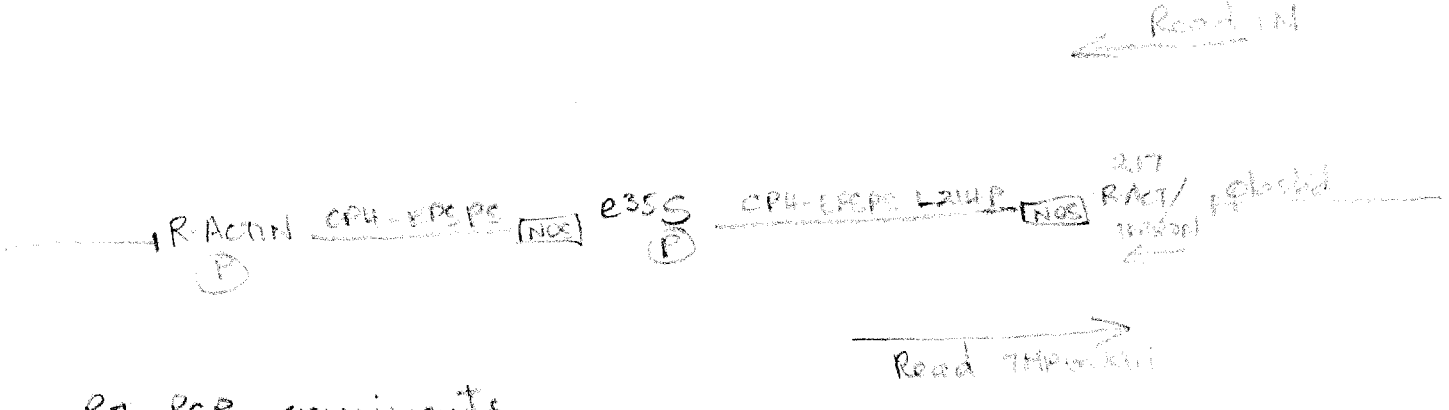
Abbreviations

~	approximately
aa	amino acid
bp, kb	base pairs, kilobase pairs
CaMV	cauliflower mosaic virus
cp4 epsps	gene encoding the 5-enol-pyruvylshikimate-3-phosphate synthase protein from *Agrobacterium sp.* strain CP4
CP4 EPSPS	5-enol-pyruvylshikimate-3-phosphate synthase protein
CP4 EPSPS L214P	The CP4 EPSPS protein produced from the e35S promoter of the NK603 insert event containing a proline instead of leucine at amino acid 214
CTP2	chloroplast transit peptide
DNA	deoxyribonucleic acid
e35S	cauliflower mosaic virus (CaMV) 35S promoter containing a duplication of the -90 to -300 bp region
FAO	Food and Agriculture Organization of the United Nations
FDA	United States Food and Drug Administration
kDa	kilodaltons
mRNA	messenger ribonucleic acid
NOS 3'	nopaline synthase 3' transcriptional termination element
ORF	open reading frame
PCR	polymerase chain reaction
P-ract1	rice actin promoter
RT-PCR	reverse transcriptase polymerase chain reaction
WHO	World Health Organization of the United Nations
*Zm*HSP70	maize (*Zea mays*) *hsp*70 gene (heat-shock protein)



RT-PCR experiments

1. Summary

The purpose of this document is to summarize the results from additional molecular characterization studies of the maize genomic DNA sequence flanking the 3' end of the insert in Roundup Ready® maize event NK603 and to assess the potential impact of these results on the food, feed and environmental safety of this product.

Roundup Ready maize event NK603 was evaluated extensively in numerous food, feed and environmental studies prior to the submission of regulatory dossiers and subsequent commercialization. This safety information, including the molecular characterization, was reviewed and this product has been approved by several regulatory agencies around the world.

Roundup Ready maize event NK603 is tolerant to the application of glyphosate, the active ingredient in Roundup herbicide, due to the expression of a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium* strain CP4 (CP4 EPSPS). The molecular characterization of Roundup Ready maize event NK603 has been previously described in detail (Deng *et al.*, 1999). This characterization demonstrated that one complete copy of the DNA segment (which contains two *cp4 epsps* gene cassettes) used for transformation was present in the genome of maize event NK603, along with a 217-bp segment containing a portion of the enhancer region of the rice actin promoter. This 217-bp segment is linked, in the inverse orientation, to the 3' end of the inserted cassette. PCR analyses have been performed to amplify the maize genomic DNA sequences flanking the 5' and 3' ends of the insert. The resulting DNA sequences of these PCR products have been previously reported (Cavato *et al.*, 2001) and are consistent with the findings from the previous molecular characterization of this event. More recently, it has also been shown that the CP4 EPSPS protein produced from the *cp4 epsps* gene under the regulation of the rice actin promoter differs from the CP4 EPSPS protein produced from the e35S promoter by a single amino acid at position 214 (Astwood *et al.*, 2001). The CP4 EPSPS protein produced under the regulation of the e35S promoter contains a proline instead of a leucine at amino acid 214 and hence is referred to as CP4 EPSPS L214P.

RT-PCR analyses were recently conducted across the 3' junction between the insert in Roundup Ready maize event NK603 and the adjacent maize genomic DNA to determine if these sequences were transcribed (Kesterson *et al.*, 2002). The RT-PCR data demonstrated that there was no detectable transcription into the NK603 insert from the maize genomic DNA sequence flanking the 3' end of the inserted DNA ("read-in" transcription). However, the results from these analyses demonstrated that a mRNA transcript was detected that initiates within the insert and continues through the NOS 3' transcriptional terminator into the genomic DNA flanking the 3' end of the insert. This transcript likely initiates at either of the two promoters (rice actin or e35S) present in the NK603 insert, proceeds through the NOS 3' transcriptional termination sequence, and terminates in the maize genomic DNA flanking the 3' end of the insert ("read-through" transcription). This result is not unexpected since the

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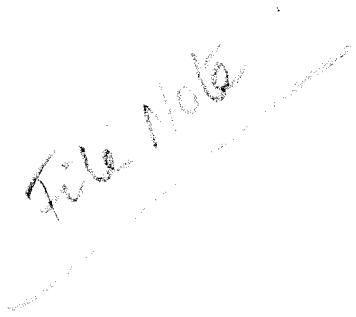
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incomplete termination or use of alternative termination sites and resulting production of multiple transcripts has been reported for endogenous genes in plants (Rothnie, 1996; Hunt, 1994; Gallie, 1993; Dean *et al.*, 1986).

Northern blot analysis was previously conducted (Mittanck and Lirette, 2000) in which the portion of the rice actin promoter that is linked, in the inverse orientation, to the 3' end of the insert in Roundup Ready maize event NK603 was used as a probe against a northern blot of poly-A^+ selected mRNA from event NK603. Based on the results of the northern analysis, no signal was detected that contains this portion of the enhancer from the rice actin promoter. The same northern blot was later stripped and probed with a segment of the *cp4 epsps* coding region. The results of this analysis demonstrated that only the expected ~1.4 kb band is observed. This signal is the expected result of transcription of the *cp4 epsps* coding regions in each of the two gene cassettes present in the insert of Roundup Ready maize event NK603. Although transcription from the insert into the maize genomic DNA flanking the 3' end of the insert was detected by RT-PCR, further evaluation by northern blot analysis did not provide any evidence of the steady state accumulation of mRNA encompassing this junction. Therefore, the level of the transcript containing this junction is at very low levels in Roundup Ready maize event NK603, in comparison to the *cp4 epsps* transcript.

Since RNA, including mRNA, is generally recognized as safe (GRAS), the presence of this longer RNA transcript resulting from transcription through the NOS 3' element raises no safety concern. The potential of this transcript to produce a protein product other than, or in addition to, the full-length CP4 EPSPS or CP4 EPSPS L214P proteins has been assessed. It was determined that the ability of this transcript to produce a translated protein other than, or in addition to, the full-length CP4 EPSPS proteins, is extremely remote. Since translation of mRNA in plants, with very rare exceptions, only produces a single polypeptide (Fütterer and Hohn, 1996), the only protein products expected to be produced from the NK603 insert are the full-length CP4 EPSPS proteins. The only CP4 EPSPS immuno-reactive proteins detected by western blots (with a limit of sensitivity of 1 ng) are the ~47 kDa full-length CP4 EPSPS proteins (Lee and Astwood, 1999). If sequences downstream from the stop codon of the *cp4 epsps* transcript were translated, a larger fusion protein containing the full-length CP4 EPSPS protein would be produced. No immuno-reactive proteins of molecular weight greater than ~47 kDa were detected in extracts of Roundup Ready maize event NK603 (Lee and Astwood, 1999; Silvanovich *et al.*, 2001).

Therefore, this further characterization of the genomic DNA sequence flanking the 3' end of the primary insert of event NK603 does not alter the previous conclusion that this event contains two functional gene cassettes which encode the ~47 kDa CP4 EPSPS and CP4 EPSPS L214P proteins. The DNA inserted into event NK603, the DNA sequences flanking this insert, and any transcripts containing the 3' flanking region were constituents in test material used to establish the food, feed and environmental safety of Roundup Ready maize event NK603. Roundup Ready maize was previously established to be as safe as conventional maize based on: the safety of the genetic elements contained on the transformation segment used to produce Roundup Ready maize event NK603; the history of safe use of the EPSPS family of proteins present in all plants, fungi and bacteria; the functionality and safety

assessment of the CP4 EPSPS and CP4 EPSPS L214P proteins; the assessment of compositional and nutritional equivalence of event NK603; a comparison of crop agronomic characteristics of event NK603 to the parental and conventional maize varieties; and a comparison of the safety and nutritional properties of event NK603 to parental and conventional maize varieties in animal feeding studies.

A review of this safety assessment confirms the conclusions reached previously that: 1) Roundup Ready maize event NK603 contains two functional *cp4 epsps* gene cassettes that are responsible for the production of the CP4 EPSPS and CP4 EPSPS L214P proteins; 2) no immuno-reactive proteins other than the expected ~47 kDa CP4 EPSPS and CP4 EPSPS L214P proteins are detected in Roundup Ready maize event NK603; 3) Roundup Ready maize event NK603 is as safe and as nutritious as conventional maize varieties and; 4) Roundup Ready maize event NK603 does not pose a plant pest risk or otherwise pose an increased risk to the environment as compared to conventional maize varieties. Any risks associated with the production and consumption of Roundup Ready maize event NK603 to human health or the environment are no different than those associated with conventional maize varieties.

2. Introduction

Roundup Ready maize event NK603 was generated through particle acceleration using an
~6.7-Kb agarose gel-isolated *Mlu* I restriction fragment from the plasmid vector PV-ZMGT32. The DNA segment used for transformation contained two gene expression cassettes: an EPSPS cassette containing the *cp4 epsps* coding sequence under the regulation of a rice actin promoter (P-ract1), a rice actin (ract1) intron, a chloroplast transit peptide (CTP2) sequence, and a nopaline synthase (NOS) 3' polyadenylation sequence; and a second EPSPS cassette containing the *cp4 epsps* L214P coding sequence under the regulation of the cauliflower mosaic virus (CaMV) enhanced 35S plant promoter (e35S), a maize heat-shock protein 70 (*Zm*HSP70) intron, CTP2, and the NOS 3' polyadenylation sequence.

Previous molecular characterization of the insert in event NK603 (Deng *et al.*, 1999) demonstrated that one complete copy of the DNA segment used for transformation is present in the genome of maize event NK603. In addition to the one complete copy, a 217 bp segment containing a portion of the enhancer region of the rice actin promoter is linked in the inverse orientation to the 3' end of the inserted transformation cassette in Roundup Ready maize event NK603. The 217 bp fragment includes polylinker sequence (50 bp) and the first 167 bp of the enhancer region of the rice actin promoter, position -835 to -669 from the start of transcription as described by McElroy *et al.* (1990). Neither the TATA box nor transcriptional initiation site is present within the segment, which suggests that this segment should not function as a promoter. This is supported by the work of Zhang *et al.* (1991) and Wang *et al.* (1992), in which the researchers clearly demonstrated that the region including -835 to -669 does not behave as a promoter. Therefore, the 217 bp segment at the 3' end of the NK603 is highly unlikely to act as a promoter.

Both of the *cp4 epsps* coding sequences in NK603 encode 455 amino acids (CTP2 + CP4 EPSPS equals 531 aa) and are terminated by tandem stop codons. It was recently shown that the CP4 EPSPS protein produced from the *cp4 epsps* gene encoded by the rice actin promoter differs from the CP4 EPSPS protein produced from the e35S promoter by a single amino acid at position 214. The CP4 EPSPS protein produced from the e35S promoter contains a proline instead of a leucine at amino acid 214 and hence is referred to as CP4 EPSPS L214P. Translation and processing of the CP4 EPSPS proteins results in the synthesis of full-length and functional ~47 kDa CP4 EPSPS proteins (5-enol-pyruvylshikimate-3-phosphate synthase) in Roundup Ready maize event NK603, as confirmed by western blotting, N-terminal sequence analysis and EPSPS activity assays (Lee and Astwood, 1999; Silvanovich *et al.*, 2001). These CP4 EPSPS proteins are structurally and functionally equivalent.

This document summarizes the additional characterization of transcriptional activity in the maize genomic DNA sequence flanking the 3' end of the insert in Roundup Ready maize event NK603. This information was evaluated within the context of what is known regarding the transcription and translation of plant genes and the potential for the production of protein products other than the full-length functional CP4 EPSPS proteins in Roundup Ready maize event NK603. These data have been assessed within the context of the established food, feed and environmental safety of this product, and lead to the conclusion that the additional molecular characterization data does not alter the previous conclusions that Roundup Ready maize event NK603 is agronomically, compositionally, and nutritionally comparable to conventional maize, except for the Roundup Ready trait.

3. Summary of Gene Transcription in Higher Plants

Gene transcription in eukaryotic organisms is a dynamic multi-stage process that leads to the accumulation of mRNA molecules. A summary of transcription termination in higher plants is provided here; however, a more detailed description of the process and the significant differences that exist between vertebrate organisms and plants is provided in Technical Appendix A.

Overall, transcription can be divided into three stages: the synthesis of the primary RNA transcript (also referred to as heterogenous nuclear RNA), post-transcriptional RNA processing to yield the mature mRNA that can be subsequently translated into protein, and mRNA degradation. The interplay and interaction between these processes is responsible for producing the measurable, steady state levels of a particular mature mRNA transcript in a given tissue. As a biochemical process, it should be noted that the observed steady-state effect of these steps represents the most favored, probabilistically and thermodynamically, condition where lower frequency processes do occur. As a consequence, all genes produce a population of transcripts, some of which are more probable and abundant than others, and these dominant gene expression products are most relevant when considering biochemical and physiological consequences.

Although many aspects of transcription are shared by all eukaryotes, subtle mechanistic differences have been identified that distinguish vertebrate and plant transcription (reviewed in Rothnie, 1996). Through sequence analysis of mRNAs from vertebrates and plants, one such mechanistic difference that has been identified in plants is the process of 3' cleavage of primary RNA transcripts. In contrast to genes from vertebrates, plants do not recognize or utilize a single highly conserved polyadenylation signal sequence to direct transcript termination and cleavage (Dean *et al.*, 1986; Hunt, 1994; Rothnie, 1996). As a result of this difference, the transcription of some plant genes leads to the accumulation of a fairly heterogeneous population of mature mRNAs that have a common coding sequence, but which differ in the sequence and length of their 3' untranslated regions exclusive of the poly-A^+ tail. These differences in the 3' untranslated region distinguish plant mRNAs from vertebrate mRNAs, such that a given plant gene displays a greater degree of heterogeneity in the length of its 3' untranslated region.

4. Transcript Analysis of the DNA Sequence Flanking the 3' End of the Insert in Roundup Ready Maize Event NK603

4.1 RT-PCR Analysis of the Junction Between the NK603 Insert and the Genomic DNA Flanking the 3' End of the Insert

To further assess the transcriptional activity of the region flanking the 3' end of the insert, RT-PCR analyses were conducted to determine if RNA transcripts containing the junction between the NK603 insert and the genomic DNA sequence adjacent to the 3' end of the insert could be detected in Roundup Ready maize event NK603 using very sensitive RT-PCR analyses (Kesterson *et al.*, 2002). Based on the results of the RT-PCR analyses, it was concluded that transcripts containing the functional *cp4 epsps* coding sequences, as well as a portion of the maize genomic DNA sequence flanking the 3' end of the insert, are produced in Roundup Ready maize event NK603. These RT-PCR experiments further demonstrated that none of these transcripts are initiated from within the genomic DNA sequence flanking the 3' end of the insert and reading into the insert. Therefore, the transcriptional activity that was detected by RT-PCR analysis is likely to initiate from either the e35S or P-ractl promoter of the NK603 insert, continue through their respective NOS 3' polyadenylation signal sequences, and proceed into the maize genomic DNA sequence flanking the 3' end of the insert.

4.2 Northern Blot Analyses of mRNA from Roundup Ready Maize Event NK603

Northern blot experiments have previously been described (Mittanck and Lirette, 2000) in which the portion of the rice actin promoter that is linked in the inverse orientation to the 3' end of the insert in Roundup Ready maize event NK603 was used as a probe against poly-A^+ selected mRNA from event NK603. Based on the results of this analysis, no signal was detected that contains this portion of the enhancer from the rice actin promoter. Additionally, the same northern blot was later stripped and probed with a segment of the *cp4 epsps* coding region. The results of this analysis demonstrated that only the expected ~1.4 kb band, resulting from transcription of the

cp4 epsps coding regions in each of the gene cassettes, is present in the insert of Roundup Ready maize event NK603. Therefore, even though RT-PCR was able to detect a product of transcription that spans from the NK603 insert into the genomic DNA flanking the 3' end of the inserted DNA, there is no evidence of any steady state accumulation of the RNA by northern blot analysis. Additionally, the level of the transcript containing this junction is at very low levels in Roundup Ready maize event NK603, in comparison to the *cp4 epsps* transcript.

4.3 Translation in Higher Plants and Relevance to Secondary Transcripts in Roundup Ready Maize Event NK603

A substantial body of published data shows that eukaryotic translational initiation occurs via a 'ribosome scanning mechanism' (Kozak, 1992). In eukaryotes, including plants, ribosomal subunits identify a unique structure at the 5' end of the mRNA called a "cap". Once bound to the 5' end of the mRNA transcript, a ribosome scans the mRNA until the first contextually correct AUG start codon is identified and translation is initiated. Translation then continues until a stop codon is encountered. A central feature of the ribosome scanning mechanism is that only one open reading frame contained within an mRNA is translated and that translation of this open reading frame is initiated at the AUG nearest the 5' end of the mRNA. Additional detail regarding the translation process in plants is provided in Technical Appendix B.

Thus, given our current knowledge of plant mRNA translation, it is predicted that if mRNA transcribed through the NOS 3' genetic element in the insert of Roundup Ready maize event NK603 were competent to be translated, the only protein that should be produced is one of the two full-length functional CP4 EPSPS proteins. All other open reading frames in the secondary transcripts would also be fully contained within the primary transcript or would be located downstream of the translational stop codon of the *cp4 epsps* coding sequences. The translation of these alternate open reading frames would require the secondary transcripts to function as polycistrons, an occurrence that is extremely rare in eukaryotic systems (Rothnie, 1996), or for the secondary transcripts to be translated via a mechanism that would be a significant and unprecedented exception to the ribosome scanning model (Kozak, 1992).

Although ribosomal scanning accounts for the vast majority of eukaryotic translation initiation events, several exceptions to this mechanism have been documented (Gallie, 1993; Fütterer and Hohn, 1996). These exceptions are related to polycistronic mRNAs that by definition contain two or more sequential and independent ORFs that are transcribed from a single transcription unit. Functional polycistronic transcripts, while fairly common among prokaryotic organisms, are exceedingly rare in eukaryotes and presently there is only one example of a non-viral polycistronic mRNA in plants. This polycistron is transcribed from the tomPRO1 locus of tomato and encodes gamma-glutamyl kinase (GK) and gamma-glutamyl phosphate reductase (GPR) (Garcia-Rios *et al.*, 1997). The mRNA encoding these two proteins is organized such that the ORFs are separated by five nucleotides. Western blot analysis of tomato tissues using either anti-GK polyclonal or monoclonal antibodies identified either a ~60 or 70 kDa polypeptide but not the expected 44 kDa GK protein. In this instance it is believed that the ribosome is engaging in '-1 translational frameshifting' or 'ribosome hopping' in order to bypass the GK stop codon and to produce a hybrid

polyprotein that displays both GK and GPR activities (Garcia-Rios *et al.*, 1997). As described below, there is no reason to believe that the *cp4 epsps* genes and sequences downstream of them would be capable of functioning as polycistrons.

4.4 Potential for Translation of Readthough Transcripts

If the transcript which extends beyond the NOS 3' polyadenylation sequence were to be viewed as a potential polycistronic mRNA transcript that is subject to translation (either through stop codon bypass or frame shifting near the stop codon), predictable changes in the apparent mobility of the CP4 EPSPS protein(s) would be observed experimentally. Inspection of the sequence immediately downstream of the CP4 EPSPS stop codons in both gene cassettes that are part of the insert in Roundup Ready maize event NK603 (the *cp4 epsps* coding sequence has tandem translational stop codons) reveals that, in the event of bypass of the tandem stop codons, the next stop codon would be encountered 13 codons downstream. The translation of these codons would add ~1500 Da to the apparent molecular weight of the CP4 EPSPS protein. Likewise, frame shifting during translation would either append 16 amino acids for a +1 shift or 37 amino acids for a -1 shift to the C-terminal of CP4 EPSPS, resulting in apparent molecular weight changes of ~1800 and ~4300 Da, respectively. No such changes in the mobility of CP4 EPSPS protein purified from Roundup Ready maize event NK603 or additional immuno-reactive protein bands have been observed (Lee and Astwood, 1999).

A second potential outcome of the translation of a polycistronic mRNA transcript is the production of two independent polypeptides. Although such translation of multiple open reading frames (ORF(s)) is at times observed (reviewed in Fütterer and Hohn, 1996), it requires that the mRNA possess two distinct structural characteristics. The first characteristic is related to the spatial organization of the translated ORFs. Of the two translated open reading frames, the upstream open reading frame must be short. Studies have demonstrated that the short upstream open reading frames can act to diminish the efficiency of translation of longer, downstream open reading frames (Fütterer and Hohn, 1996 and references therein). In the instance of a test dicistronic mRNA, a single codon upstream ORF was capable of attenuating the translation of a downstream reporter gene two-fold. A 30-codon open reading frame attenuated translation of a downstream reporter five-fold, and a 100 codon open reading frame abolished detectable translation of a downstream reporter (Fütterer and Hohn, 1992). A second characteristic of the downstream open reading frame is that it must contain a contextually optimum AUG codon to initiate translation.

Inspection of the predicted arrangement of open reading frames in the sequence downstream of the tandem CP4 EPSPS stop codons reveals that the upstream open reading frame encoding CP4 EPSPS (CP4 EPSPS + CTP2 = 531 codons) greatly exceeds the 100 codons necessary to abolish translation of any downstream open reading frames and therefore the first requirement is not met.

Inspection of all AUG trinucleotides contained in the 1757 nucleotides that are found within and contained downstream of the tandem stop codons of the Ract1-promoter-driven *cp4 epsps* cassette reveals that only two of the AUG trinucleotides could be considered contextually optimal for translational initiation. Specifically, such

trinucleotides would have to be flanked by a G at position 4 (where the A in AUG is position 1) and an A at position –3 (Gallie, 1993). The first contextually optimum start codon is located 467 nucleotides downstream of the first T of the tandem TGA CP4 EPSPS stop codons. If translation were to begin at this first contextually optimum start codon, it would yield a ~4.4 kDa, 36 amino acid polypeptide. The second contextually optimum start codon is located 728 nucleotides downstream of the first T of the tandem TGA CP4 EPSPS stop codons and, if translated, would yield a ~3.6 kDa, 31 amino acid polypeptide.

Likewise, inspection of all AUG trinucleotides contained in 1323 nucleotides that are found within and contained downstream of the tandem stop codons of the e35S-promoter-driven *cp4 epsps* cassette reveals that only two of the AUG trinucleotides could be considered contextually optimal for translational initiation. The first contextually optimum start codon is located 864 nucleotides downstream of the first T of the tandem TGA CP4 EPSPS stop codons. If translation were to begin at this first contextually optimum start codon, it would yield a ~5.3 kDa, 46 amino acid polypeptide. The second contextually optimum start codon is located 913 nucleotides downstream of the first T of the tandem TGA CP4 EPSPS stop codons and, if translated, would yield a ~7.3 kDa, 69 amino acid polypeptide.

The translation of any of the four aforementioned open reading frames would be unprecedented given their size and spatial relationship relative to the *cp4 epsps* coding sequence.

4.5 Safety Assessment of ORFs 3' of the Insert in Roundup Ready Maize Event NK603

To assess the safety of any putative polypeptides that could be translated from the region beyond the NOS 3' polyadenlyation sequence, including the maize genomic DNA adjacent to the 3'end of the insert, bioinformatics analyses have been conducted on all predicted polypeptides (ORFs) within this region. These analyses were conducted to determine whether any potential translated ORFs from the region beyond the tandem TGA stop codons display significant similarity to known toxins or allergens. No evidence exists to indicate that any of the predicted polypeptides analyzed are produced in Roundup Ready maize event NK603. This assessment indicated that the theoretical open reading frames are unlikely to produce any protein; however, if a protein were produced it would not show any sequence similarity to any known protein toxins or allergens.

These analyses represent a theoretical safety assessment that uses bioinformatic tools to predict the characteristics of a polypeptide. In order to minimize bias, no attempt is made to assess the probability that any one reading frame will be translated preferentially relative to any other reading frame. Translating the predicted polypeptides from stop codon to stop codon eliminates bias that may result from the arbitrary selection of a start codon. This bioinformatic assessment demonstrates that for the 56 predicted polypeptides of eight amino acids or greater encoded by the 1757 nucleotides of sequence that lie downstream of the tandem stop codons of the Ract1-promoter-driven *cp4 epsps* cassette, there are no significant sequence similarities to proteins identified as allergens. Moreover, the predicted polypeptides do not display

sufficient identity or similarity at the level of primary structure to indicate homology with any known toxic or pharmacologically active protein. Likewise, no significant sequence similarity was observed between any of the 42 predicted polypeptides of eight amino acids or greater encoded by the 1323 nucleotides of sequence that lie downstream of the tandem stop codons of the e35S-promoter-driven *cp4 epsps* L214P and any toxin, allergen or pharmacologically active protein. These findings are consistent with previous studies that have demonstrated the food and feed safety of Roundup Ready maize event NK603.

4.6 Conclusions on Transcript Analysis and Probable Function of Transcripts Extending Through the NOS 3' Genetic Element into Adjacent Maize Genomic DNA 3' to the Insert

As discussed above, although the junction between the 3' end of the insert in Roundup Ready maize event NK603 and the associated genomic DNA flanking the insert appears to be transcribed at some very low level based on sensitive RT-PCR analyses, there is no evidence of the steady state accumulation of this RNA by northern blot analysis. Additionally, any mRNA produced by read-through transcription of the NOS 3' transcriptional termination sequence in Roundup Ready maize event NK603 would not be expected to produce any proteins other than CP4 EPSPS or CP4 EPSPS L214P since eukaryotes, such as plants, rarely translate multiple open reading frames (ORFs) from single mRNAs. In the exceptionally rare instance where second ORFs contained on a single mRNA have been detected in plants (Garcia-Rios *et al.*, 1997; Fütterer and Hohn, 1996), a single hybrid polyprotein has been observed, rather than the synthesis of two independent proteins. The presence of multiple translational stop codons in the sequences lying downstream of the full-length *cp4 epsps* coding sequences would be expected to prevent the synthesis of a hybrid polyprotein containing elements encoded by the DNA beyond the *cp4 epsps* coding region. Western blotting confirmed that only the expected ~47 kDa CP4 EPSPS proteins are detected in Roundup Ready maize event NK603. No unexpected protein, including any large molecular weight protein potentially produced by the read-through mRNA, was detected by western blotting (Lee and Astwood, 1999).

5. Review of Food, Feed and Environmental Safety Assessment

Based on RT-PCR and western blot analyses, combined with the detailed evaluation of the sequences of the DNA flanking the 3' end of the insert in Roundup Ready maize event NK603, as well as the understanding of translation initiation and termination in plants, it is concluded that no new proteins are expected or observed to be produced which would be derived from the DNA flanking the 3' end of the insert in Roundup Ready maize event NK603. The western blotting data confirm the conclusion that Roundup Ready maize event NK603 contains two functional *cp4 epsps* coding sequences that produce the full-length ~47 kDa CP4 EPSPS proteins that confer the glyphosate tolerance trait. Furthermore, the DNA flanking the insert, and any secondary mRNA transcripts, or putative products of these transcripts, regardless of abundance, were constituents of the regulatory samples that were used in safety assessment studies that addressed the food, feed and environmental safety of Roundup Ready maize event NK603.

5.1 Applicability of Safety Data to Secondary Transcripts

A series of animal feeding studies have been completed previously using diets incorporating grain or processed grain fractions from Roundup Ready maize event NK603 (Dudek, 2001; Stanisiewski *et al.*, 2002; Taylor *et al.*, 2001). These studies address the nutritional equivalence of Roundup Ready maize event NK603 when used as animal feed, the relative safety of any expressed proteins or peptides (or any other newly produced constituent), the potential for any pleiotropic effect caused by the insertion process or site of insertion, and any other constituent that would result from the insert derived from plasmid PV-ZMGT32. These feeding studies confirmed the food and feed safety and nutritional equivalence of diets from Roundup Ready maize event NK603 to diets from the conventional maize varieties. If the presence of the secondary transcripts were to result in gene function or create potential pleiotropic effects, no measurable effects were observed. The nutritional value or wholesomeness of Roundup Ready maize event NK603, even when fed to animals at levels much higher than humans would encounter in the diet, was the same as that of conventional varieties of maize.

If a protein or peptide other than CP4 EPSPS were to be unexpectedly produced from the secondary transcripts that contain the region of maize genomic sequence adjacent to the 3' end of the insert, these proteins or peptides would be likely to contain the same amino acid sequences as the CP4 EPSPS protein. The safety of such CP4 EPSPS-containing products has previously been addressed through bioinformatics, digestive fate and acute oral toxicity studies performed with the CP4 EPSPS protein, establishing that the CP4 EPSPS protein and its proteolytic byproducts are not homologous to toxins or allergens, are rapidly digested and are non-toxic (Harrison *et al.*, 1996; Rice *et al.*, 2001).

Roundup Ready maize event NK603 has been approved for planting and/or consumption in several countries worldwide. An assessment of the food and feed safety data developed for maize containing the NK603 event established that this maize is as safe and nutritious as other commercial maize varieties. The presence of *cp4 epsps*-containing mRNA transcripts that extend beyond the NOS 3' polyadenylation sequence does not change the conclusion of this safety assessment for the following reasons: a) consumption of DNA and RNA *per se* is generally recognized as safe by food safety experts, including the FDA (US FDA, 1992) and the FAO/WHO (FAO/WHO, 1996); b) food and feed safety studies were conducted on maize that contained the secondary transcripts; c) results of compositional and nutritional analyses established that this maize is comparable to other maize varieties and is not affected by the NK603 insert itself or any other effects brought about by the insertion (McCann *et al.*, 2001; Pyla *et al.*, 2000; Ridley *et al.*, 2000); and d) a series of animal feeding studies showed that Roundup Ready maize event NK603 is as safe and as nutritious as conventional maize varieties (Dudek, 2001; Stanisiewski *et al.*, 2002; Taylor *et al.*, 2001).

6. Conclusions

RT-PCR analyses were recently conducted across the 3' junction between the insert in Roundup Ready maize event NK603 and the adjacent maize genomic DNA sequences (Kesterson *et al.*, 2002). The RT-PCR data demonstrated that there was no detectable transcription into the NK603 insert from the maize genomic DNA sequence flanking the 3' end of the inserted DNA ("read-in" transcription). The results from the RT-PCR analyses demonstrated that transcription can be detected that likely initiates from either the e35S or P-ract1 promoter of the NK603 insert, continues through the respective NOS 3' polyadenylation signal sequences, and proceeds into the maize genomic DNA sequence flanking the 3' end of the insert ("read-through" transcription). However, there does not appear to be any significant steady state accumulation of these "read-through" transcription products based on northern blot analyses utilizing probes that would have been able to detect such products. Since translation of competent mRNA transcripts in plants, with very rare exceptions, only produces a single polypeptide, the only protein products expected to be produced from the NK603 insert are the full-length CP4 EPSPS proteins. Indeed, the only CP4 EPSPS-containing proteins detected in Roundup Ready maize event NK603 are the ~47 kDa full-length CP4 EPSPS proteins.

This additional RT-PCR data of the DNA sequence flanking the 3' end of the insert in Roundup Ready maize event NK603 does not alter the previous conclusion that this event contains two functional gene cassettes encoding ~47 kDa CP4 EPSPS proteins, which are responsible for glyphosate tolerance. The DNA derived from the transformation vector used to produce event NK603, the DNA flanking the insert and any transcripts containing the 3' flanking region, or other putative products derived from it, are present as constituents in the maize test materials used to establish the food, feed and environmental safety of Roundup Ready maize event NK603.

The consequences of transcription into the genomic DNA 3' of the insert in Roundup Ready maize event NK603 have been assessed as part of the larger safety assessment. The studies led to the conclusion that Roundup Ready maize event NK603 is agronomically, compositionally and nutritionally comparable to conventional maize, except for the Roundup Ready trait. The information regarding the nature and transcriptional activity of the genomic DNA flanking the 3' end of the functional insert does not change the conclusions reached previously that: (1) Roundup Ready maize event NK603 contains two functional *cp4 epsps* gene cassettes which produce full-length CP4 EPSPS and CP4 EPSPS L214P proteins; (2) no immuno-reactive proteins other than the expected ~47 kDa CP4 EPSPS and CP4 EPSPS L214P proteins are detected in Roundup Ready maize event NK603; (3) Roundup Ready maize event NK603 is as safe and nutritious as conventional maize varieties; and (4) Roundup Ready maize event NK603 does not pose a plant pest risk or otherwise pose an increased risk to the environment relative to conventional maize varieties.

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APPENDIX A

Gene Transcription in Higher Plants and Cleavage of the 3' Ends of Primary RNA Transcripts.

Gene transcription in eukaryotic organisms is a dynamic multistage process that leads to the accumulation of mRNA molecules. Overall, transcription can be divided into three stages: the synthesis of the primary RNA transcript (also referred to as heterogenous nuclear RNA), post-transcriptional RNA processing to yield the mature mRNA which can be subsequently translated, and mRNA degradation. Each of the aforementioned stages of transcription is affected by both *cis*- and *trans*-acting factors and each stage may be further subdivided into defined events. The interplay and interaction between these processes is responsible for producing the measureable, steady-state levels of a particular mature transcript in a given tissue. As biochemical processes, it should be noted that the observed steady-state effect of these steps represents the most favored, probabilistically and thermodynamically, condition where lower frequency processes occur regularly. As a consequence, all genes produce a population of transcripts, some of which are more probable and abundant than others, and the dominant gene expression products are most relevant when considering biochemical and physiological consequences.

Although many aspects of transcription are shared by all eukaryotes, subtle mechanistic differences have been identified that distinguish vertebrate and plant transcription (reviewed in Rothnie, 1996). Through sequence analysis of mRNAs from vertebrates and plants, one such mechanistic difference that has been identified in plants is the process of 3' cleavage of primary RNA transcripts. As a result of this difference, the transcription of some plant genes leads to the accumulation of a fairly heterogeneous population of mature mRNAs that have a common coding sequence, but which differ in the sequence and length of their 3' untranslated regions exclusive of the poly-A^+ tail. These differences in the 3' untranslated region are unrelated to differential primary RNA splicing and distinguish plant mRNAs from vertebrate mRNAs, where a given gene displays a greater degree of homogeneity in the length of its 3' untranslated region.

Transcription initiation of eukaryotic genes is also a multi-step process and minimally requires the basal transcription machinery composed of RNA polymerase II and several general transcription factors. To initiate transcription, these general transcription factors, RNA polymerase II, and various cofactors must assemble at the upstream promoter DNA region of a gene to form a multi-protein transcription initiation complex. Following the formation of the transcription initiation complex, the DNA strands at the transcription start site are separated (promoter opening), RNA polymerase II initiates synthesis of the RNA transcript (transcription initiation), leaves the promoter region (promoter clearance), and translocates along the DNA template under continuous extension of the RNA chain (transcription elongation) (Singer and Berg, 1991). Primary RNA transcript elongation then proceeds to a location downstream of the coding sequence's translation stop codon, where transcriptional termination occurs.

Transcriptional termination is characterized by the cessation of RNA chain elongation coupled with the endonucleolytic cleavage of the nascent transcript and subsequent addition of a poly-A^+ tail. In vertebrates, transcriptional termination appears to be the result of RNA polymerase II pausing at a site located downstream of the highly conserved AAUAAA polyadenylation signal (Enriquez-Harriz *et al.*, 1991). The precise mechanism for RNA polymerase II pausing is not known; however, recent studies have demonstrated that two proteins associated with the RNA polymerase II complex may be responsible for transduction of the AAUAAA motif and signaling RNA polymerase to pause. This pause in transcription occurs in a gene-dependent manner some 100 to >4000 nucleotides downstream of the AAUAAA polyadenlyation motif (Proudfoot, 1989). Concomitant or prior to RNA polymerase II pausing, cleavage of the primary RNA transcript occurs at a location approximately midway between the conserved AAUAAA polyadenylation signal and U/GU-rich elements (typically 10 to 30 nucleotides downstream of the AAUAAA polyadenylation signal). This cleavage of the nascent transcript is coupled to the addition of up to 250 adenosines which comprise the "poly-A^+ tail" (Tran *et al.*, 2001). The segment of the primary RNA that is located downstream of the 3' cleavage site is degraded through the combined activities of a helicase that resolves the primary RNA-template DNA hybrid and a 5' to 3' ribonuclease (Proudfoot, 1989). Once degradation of the primary transcript has extended to the paused RNA polymerase II, RNA polymerase II detaches from the template DNA. The segment of the primary transcript that is located upstream of the cleavage site is also processed to yield the mature mRNA. In addition to 3' polyadenylation, transcript processing includes the addition of the 5' cap structure and the removal of introns through splicing. Capping and splicing may occur concomitant with primary RNA transcript elongation and prior to primary RNA cleavage, while polyadenylation is by definition a post-cleavage event.

Although less is known about transcriptional termination in plants relative to the understanding of this process in vertebrates, several key differences between vertebrates and plants have been observed. The most significant distinction is related to the configuration of elements downstream of the coding sequence translation stop codon. Specifically, plant genes do not contain *per se* a U- or GU-rich element and, more importantly, they do not utilize a single highly conserved polyadenylation signal motif to direct transcript termination/cleavage. Rather, plant genes have two less conserved elements that seem to be critical in directing transcript termination and processing of nascent transcripts. These elements are described as far upstream elements (FUEs) that enhance processing efficiency, and several AU-rich sequence motifs known as near upstream elements (NUE) that resemble the vertebrate AAUAAA polyadenylation motif (*i.e.*, AAUUAAA, AUAUAA, AAUAAU). Although cleavage and polyadenylation is observed 10 to 40 nucleotides downstream of NUEs in a manner that parallels signaling of polyadenylation in vertebrates, heterogeneity exists among the 3' ends of mRNAs for many plant genes because no single NUE is exclusively used as the 3' cleavage signal (Hunt, 1994). Thus, in plants, virtually all genes have multiple potential poly-A^+ sites and, as a result, the position of cleavage of the primary transcript is quite heterogeneous, often producing a population of mRNAs which have varied endpoints (Rothnie, 1996, and references therein). For instance, the genes encoding the small subunit of the pea ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcS*) protein and soybean small heat shock

(*Gmhsp17.5-E*) protein contain three and four 3' cleavage sites, respectively (Hunt, 1988; Czarnecka *et al.*, 1985). Likewise, an extreme example of this phenomenon is observed with a chloroplast-RNA binding protein gene in *Nicotiana plumbaginifolia* that contains fourteen 3' cleavage sites with a corresponding number of transcripts (Klahre *et al.*, 1995).

Unlike vertebrate genes that have a consensus polyadenylation signal and unique poladenylation site, plant genes have evolved to have multiple polyadenylation signals and sites. Moreover, the transcript processing machinery in plants is highly flexible and capable of identifying cryptic polyadenylation signals. Although the evolutionary driver for this flexibility in plant transcript processing is not known, it has been proposed that post-transcriptional control mechanisms are of added significance in plants due to their sessile lifestyle (Sullivan and Green, 1993). Since it is known that 3' untranslated regions and poly-A^+ tails can be determinants of mRNA stability and degradation, and the control of translation, heterogeneity among the 3' untranslated regions of mRNAs derived from a single gene may provide the added speed necessary for plants to respond to changing environmental conditions from which they are unable to flee. Likewise, transgenes that are assembled using elements such as transcriptional promoters and polyadenylation signals that are derived from plant, bacterial or viral sources, would be expected to display the same 3' untranslated region heterogeneity as endogenous plant genes.

APPENDIX B

Translation in Higher Plants

Eukaryotic translation is controlled primarily at the initiation level. The nature of the 5'- and 3'- non-coding sequences (leaders and trailers, including poly-A^+ tails) and a set of translation initiation factors are the major determinants of this control. With a limited number of exceptions (described below), the initiation of translation in eukaryotes occurs via a 5' cap dependent scanning mechanism (Kozak, 1992). Initially, components of the 40S ribosomal subunit, in combination with the poly-A^+ tail bound poly-A^+ binding protein, identify the "cap", a unique methylated, phosphorylated nucleotide located at the 5' end of a transcript. Upon identification of the cap, the transcript is scanned by the 40S subunit to find the first contextually correct AUG start codon where assembly of the ribosome complex is completed and translation is initiated. Translation then continues through the open reading frame (ORF) that is defined by this AUG as the ribosome successively translates codons with aminoacyl-tRNAs to generate the encoded polypeptide. Translation continues until the ribosome complex reaches a stop codon (UAA, UAG or UGA) for which no complementary tRNA is available. At that time, release factors associate with the ribosome, triggering the release of the nascent polypeptide and termination of translation (Fütterer and Hohn, 1996). A central feature of the ribosome scanning mechanism is that only one open reading frame contained within an mRNA is translated and that translation of this open reading frame is initiated at the AUG nearest the 5' end of the mRNA.

Study Title

RT-PCR Analysis of the DNA Sequence Flanking the 3' End of the Insert in Roundup Ready® Maize Event NK603

Authors

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Report Completed

February 11, 2002

Performing Laboratory

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Laboratory Project ID

Study 02-01-46-16
MSL-17668

Statement of No Data Confidentiality Claims

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA.10(d)(l)(A), (B), or (C).

"We submit this material to the United States Environmental Protection Agency specifically under the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by EPA strictly in accordance with FIFRA. By submitting this material to EPA in accordance with the method and format requirements contained in PR Notice 86-5, we reserve and do not waive any rights involving this material that are or can be claimed by the company notwithstanding this submission to EPA."

Company: Monsanto Company

Company Agent: _____

Title: _____

Signature: _____ Date: _____

Statement of Compliance

This study meets the requirements under GLP as specified in 40 CFR Part 160.

Submitter	Date
Linda F. Fahman	Feb 11, 2002
Sponsor Representative	Date
	Feb 11, 2002
Study Director	Date

Quality Assurance Statement

Study Title: RT-PCR Analysis of the DNA Sequence Flanking the 3' end of the Insert in Roundup Ready® Maize Event NK603

Study Number: 02-01-46-16

Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

Following is a list of reviews conducted by the Monsanto Regulatory QAU on the study reported herein.

Dates Of Inspection / Audit	Phase	Date Reported To: Study Director	Management
01/29/2002	PCR	02/08/2002	02/08/2002
02/09/2002	Raw Data Audit	02/11/2002	02/11/2002
02/11/2002	Draft Report Review	02/11/2002	02/11/2002

C. Marie Braton

Feb. 11, 2002
Date

Quality Assurance
Monsanto Regulatory, Monsanto Company

Signatures of Approval

Study Number:	02-01-46-16
Title:	RT-PCR Analysis of the DNA Sequence Flanking the 3' End of the Insert in Roundup Ready® Maize Event NK603
Facility:	Monsanto Company 700 Chesterfield Parkway North St. Louis, Missouri 63198
Sponsor Representative:	Linda K. Lahman
Study Director:	Steven E. Reiser
Contributors:	Niki K. Kesterson
Study Initiation Date:	January 28, 2002
Report Completion Date:	February 11, 2002
Records Retention:	All study specific raw data, protocol, final report, and facility records will be retained at Monsanto, St. Louis.
Sample Storage:	Any study samples that are to be retained will be stored at Monsanto, St. Louis.

Signatures of Approval:

Study Director — Feb 11, 2002 — Date

Linda K. Lahman — Feb 11, 2002

Sponsor Representative — Date

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Abbreviations

~	approximately
CaMV	cauliflower mosaic virus
cp4 epsps	5-enolpyruvylshikimate-3-phosphate synthase gene isolated from *Agrobacterium* sp. strain CP4
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase enzyme isolated from *Agrobacterium* sp. strain CP4
CP4 EPSPS L214P	The CP4 EPSPS protein produced from the e35S promoter of the NK603 insert event containing a proline instead of leucine at amino acid 214
CTP2	chloroplast transit peptide
DNA	deoxyribonucleic acid
e35S	cauliflower mosaic virus promoter with the duplicated enhancer region
MW	molecular weight
NOS 3'	nopaline synthase 3' polyadenylation sequence
P-ract1	rice actin promoter
ract1 intron	rice actin intron
*Zm*HSP70	maize (*Zea mays*) *hsp*70 gene (heat-shock protein)

I. SUMMARY

The molecular characterization of Roundup Ready® maize event NK603 has been previously reported (Deng *et al.*, 1999). This characterization, largely based on Southern blot analysis, demonstrated that one copy of the DNA restriction fragment used for transformation was present in maize event NK603, along with a 217 bp segment of DNA containing a portion of the enhancer region of the rice actin promoter inversely linked to the 3' end of the inserted transformation cassette. In the current study, strand-specific RT-PCR analyses were performed to determine whether transcripts are produced that encompass the 3' end of the insert and the adjacent genomic DNA sequence in Roundup Ready maize event NK603. Results of these analyses indicate that transcripts initiating in genomic DNA flanking the 3' end of the NK603 insert and continuing into the inserted DNA are not detected. However, a transcription product was observed that initiates from within the NK603 insert and extends beyond the NOS 3' genetic element, subsequently terminating in the genomic DNA flanking the 3' end of the insert in Roundup Ready maize event NK603. This transcription product is likely a result of incomplete termination at the NOS 3' genetic element.

II. INTRODUCTION

A. Background

Roundup Ready maize event NK603 was generated through particle acceleration using a 6.7-Kb agarose gel-isolated *Mlu* I restriction fragment from the plasmid vector PV-ZMGT32. The DNA segment used for transformation contained two gene expression cassettes: an EPSPS cassette containing the *cp4 epsps* coding sequence under the regulation of the rice actin promoter (P-ract1), a rice actin (ract1) intron, a chloroplast transit peptide (CTP2) sequence, and a nopaline synthase (NOS) 3' polyadenylation sequence; and a second EPSPS cassette containing the *cp4 epsps* coding sequence under the regulation of the cauliflower mosaic virus (CaMV) enhanced 35S plant promoter (e35S), a maize heat-shock protein 70 (*Zm*HSP70) intron, CTP2, and the NOS 3' polyadenylation sequence. Recently, it has been shown that the CP4 EPSPS protein produced from the *cp4 epsps* gene encoded by the rice actin promoter differs from the CP4 EPSPS protein produced from the e35S promoter by a single amino acid at position 214 (Astwood *et al.*, 2001). The CP4 EPSPS protein produced from the rice actin promoter contains a proline instead of a leucine at amino acid position 214 and hence is referred to as CP4 EPSPS L214P.

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Previous molecular characterization of the insert in event NK603 (Deng *et al.*, 1999) demonstrated that one complete copy of the DNA segment used for transformation is present in the genome of maize event NK603. In addition to the one complete copy, a 217 bp segment containing a portion of the enhancer region of the rice actin promoter is linked in the inverse orientation to the 3' end of the inserted transformation cassette in Roundup Ready maize event NK603 (Figure 1). The genomic DNA sequences flanking the insert were previously identified and found to be consistent with the findings from the previous molecular characterization of this event (Cavato *et al.*, 2001).

The 217 bp segment that is inversely linked to the 3' end of the inserted transformation cassette includes polylinker sequence (50 bp) and the first 167 bp of the enhancer region of the rice actin promoter, position -835 to -669 from the start of transcription as defined by McElroy *et al.* (1990). Neither the TATA box nor transcriptional initiation site is present within the segment, which suggests that this segment should not function as a promoter. This is supported by the work of Zhang *et al.* (1991) and Wang *et al.* (1992), in which the researchers clearly demonstrated that the region including -835 to -669 does not behave as a promoter. Therefore, the 217 bp segment at the 3' end of the NK603 is highly unlikely to act as a promoter. This is supported by northern blot analysis in which no transcriptional product containing this portion of the rice actin promoter was observed. Additionally, when the same northern blot was stripped and probed with a segment of the *cp4 epsps* coding region, no signal other than the expected size mRNA product from the two *cp4 epsps* gene cassettes was detected (Mittanck and Lirette, 2000).

B. Purpose

The objective of this study was to determine if transcription is occurring across the 3' end of the insert including the 217 bp segment of the enhancer region of the rice actin promoter and the adjacent genomic DNA in Roundup Ready maize event NK603 using RT-PCR as the analytical tool.

III. MATERIALS AND METHODS

A. Test Substance

The test substance was Roundup Ready maize event NK603. Leaf material for the test substance was obtained from plants grown in the greenhouse from seed material (Lot # RDR-0111-11833-S).

B. Control Substance

The control substance was non-transgenic maize line B73. Leaf material for the control substance was obtained from plants grown in the greenhouse from seed material (Lot # NTC-0111-11834-S).

C. Reference Substance

The reference substance was the 100-bp ladder molecular size marker from Invitrogen (0.1-2.1 Kb).

D. Characterization of the Test and Control Substances

The identity of the test and control substances was determined by event-specific PCR assays. The raw data are archived with Study # 00-01-46-32.

E. RNA Isolation and Poly-A^+ mRNA Selection

Isolation of total and poly-A^+ mRNA was previously performed for both the test and control substances as part of Study # 00-01-46-32. Young leaf tissue samples from Roundup Ready maize event NK603 and non-transgenic control line B73 were harvested, placed on dry ice, and stored in a -80°C freezer. These samples were then ground to a fine powder using a mortar and pestle under liquid nitrogen. Total RNA was extracted by adding 1 ml of TRIzol Reagent (Molecular Research Center Inc.) per 0.1 gram of tissue, followed by homogenization with a power homogenizer. The samples were then incubated for 5 min at room temperature followed by chloroform extraction. The tubes were shaken, incubated for 10 min at room temperature, and then centrifuged at 6,000 x *g* for 15 min at 4°C. The aqueous phase was transferred to a clean tube and the RNA was re-extracted with chloroform. The aqueous phase was transferred to a clean tube and the RNA was precipitated by adding 0.5 volumes of 100% isopropanol per initial volume of TRIzol. The samples were incubated at room temperature for 10 min, followed by centrifugation at 10,000 x *g* for 15 min at 4°C to pellet the RNA. The resulting RNA pellets were washed with cold 75% ethanol. The suspension was mixed thoroughly and the RNA was pelleted by centrifugation at 7,000 x *g* for 5 min at 4°C. The RNA pellet was dried under vacuum and resuspended in RNase-free water. Poly-A^+ mRNA was isolated from approximately 1 mg of total RNA using the Oligotex mRNA Midi Isolation System (Qiagen, Santa Clarita, CA), according to the manufacturer's instructions.

F. RNA and mRNA Quantitation

Quantitation of the amount of total RNA and mRNA in the samples was performed as part of Study # 00-01-46-32 using a Beckman DU640B spectrophotometer (Fullerton, CA) by measuring the absorbance at 260 and 280 nm.

G. Strand-Specific RT-PCR Analyses of the Junction Between the 3' End of the Insert and the Adjacent Genomic DNA Sequence in Roundup Ready Maize Event NK603

Strand-specific RT-PCR analyses were performed using the Qiagen OneStep RT-PCR system. The manufacturer's instructions were followed with the exception that the oligonucleotide primers were added sequentially to the reactions rather than at the same time. First, a specific oligonucleotide primer was added to the reaction for first strand-specific synthesis of cDNA. Next, a second primer was added to the reaction for PCR amplification of the target. The products from these reactions are shown in Figure 2. Strand-specific RT-PCR was performed using 10 ng of poly-A^+ mRNA template from both the test and control substances. An oligonucleotide primer (Primer A) specific to the genomic DNA sequence flanking the 3' end of the NK603 insert was used to reverse transcribe any transcriptional product initiating from within the insert and continuing into the DNA sequence flanking the 3' end of the insert (Figure 2). Conversely, a primer (Primer B) specific to the NOS 3' genetic element was used to reverse transcribe any transcriptional product initiating in the genomic DNA flanking the 3' end of the insert and continuing into the NK603 insert DNA (Figure 2). These oligonucleotide primers were then coupled with primers specific to either the NOS 3' genetic element (Primer B), or the genomic DNA flanking the 3' end of the NK603 insert (Primer A), respectively, in order to amplify by PCR any strand-specific cDNA products resulting from the reverse transcription reaction. Reactions using oligonucleotide primers (Primers C and D, Figure 2), designed to amplify a portion of the *cp4 epsps* coding region, were used with a range of poly-A^+ selected mRNA (10 ng, 1 ng, 0.1 ng, and 0.01 ng) isolated from Roundup Ready maize event NK603. Additionally, a negative control reaction containing no template was used to assess for contamination in the reactions. The reactions were performed under the following cycling conditions: 1 cycle at 50°C for 30 min (reverse transcription step), 1 cycle at 95°C for 15 minutes; 30 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute; 1 cycle at 72°C for 10 minutes. The products of these reactions were separated by electrophoresis using a 0.8 % agarose gel according to SOP # BR-ME-0315-01 at 140 V for 60 minutes. The PCR products were visualized by ethidium bromide staining.

H. PCR to Demonstrate Primer Compatibility and the Absence of Genomic DNA Contamination in the Poly-A^+ Selected mRNA

The compatibility of the oligonucleotide Primers A and B was demonstrated using genomic DNA template isolated from Roundup Ready maize event NK603. The PCR analyses to demonstrate the capability of the selected primer pair to generate a PCR amplicon were conducted according to SOP BR-ME-0486-01 using 10 ng of genomic DNA template in a 50 µl reaction volume. PCR was also performed directly on poly-A^+ selected mRNA (not reverse transcribed) using oligonucleotide primers specific to the 3' portion of the *cp4 epsps* coding region (Primers E and F, not highlighted in Figure 2) to determine if there was genomic DNA contamination in the poly-A^+ selected mRNA isolated from Roundup Ready maize event NK603. These PCR analyses were conducted according to SOP BR-ME-0486-01 using 10 ng of poly-A^+ selected mRNA in a 50 µl reaction volume

containing a final concentration of 1.5 mM Mg^{2+}, 0.4 µM of each primer, 200 µM each dNTP, and 2.5 unit of *Taq* DNA polymerase. The reactions were performed under the following cycling conditions: 30 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute; 1 cycle at 72°C for 10 minutes. A negative control reaction containing no template was used to assess for contamination in the reactions. The PCR products were separated by electrophoresis using a 0.8 % agarose gel according to SOP # BR-ME-0315-01 at 140 V for 60 minutes. The PCR products were visualized by ethidium bromide staining.

I. Data Rejected.

Several RT-PCR experiments were performed using the Titan One Tube RT-PCR system from Roche as outlined in the study protocol. The results from these reactions were rejected due to lower sensitivity of the system compared to the Qiagen OneStep RT-PCR system. Additionally, since the Qiagen OneStep RT-PCR system uses a heat activated *Taq* polymerase, it employs a longer incubation step to inactivate the reverse transcriptase enzymes in the reaction. These differences result in the Qiagen OneStep RT-PCR system having greater sensitivity and specificity. Additionally, some data using the Qiagen OneStep RT-PCR system were rejected due to inconsistency in the intensity of the bands observed in the serial dilutions of the mRNA from event NK603. The data that were rejected were not inconsistent with the conclusions presented in this study.

IV. RESULTS AND DISCUSSION

The results of the strand-specific RT-PCR analyses performed on poly-A^+ selected mRNA from Roundup Ready maize event NK603 and non-transgenic maize line B73 are shown in Figure 2. In these experiments, an oligonucleotide primer (Primer A) specific to the genomic DNA flanking the 3' end of the NK603 insert was used to reverse transcribe any transcriptional product that might initiate from within the insert, continue through the NOS 3' element, and into the DNA sequence flanking the 3' end of the insert. In order to detect any strand-specific cDNA that might be generated from this reverse transcription reaction, a second primer (Primer B) specific to the NOS 3' genetic element was added in order to carry out PCR. Conversely, Primer B, specific to the NOS 3' genetic element, was used to reverse transcribe any transcriptional product that may initiate in the genomic DNA flanking the 3' end of the insert and continue into the NK603 insert DNA. Primer A, specific to the genomic DNA flanking the 3' end of the NK603 insert, was then added to the reaction to amplify any strand-specific cDNA that may have been generated from the reverse transcription reaction using Primer B. The primer locations and results of these analyses are illustrated in Figure 2.

The products of the positive control RT-PCR assays using Primers C and D to amplify a portion of the *cp4 epsps* coding region are shown in Lanes 6-9. Following reverse transcription using Primer D for first strand cDNA synthesis, PCR was performed by

adding Primer C to the reactions. The reactions contained 0.01, 0.1, 1, and 10 ng, respectively, of poly-A^+ selected mRNA template from Roundup Ready maize event NK603. The expected sized product of ~443 bp was observed in each of the reactions, demonstrating that this primer set can detect RNA containing the *cp4 epsps* coding region in as little as 0.01 ng of poly-A^+ selected mRNA from Roundup Ready maize event NK603. An unexpected product of ~400 bp can be observed in Lane 9. This product is likely from nonspecific amplification of the primers in conjunction with a large amount of template mRNA in the reaction. As expected, no PCR products were observed in the negative control reactions containing no template (Lanes 10 and 13). Additionally, no signal was observed in the reaction designed to assess for possible genomic DNA contamination of the poly-A^+ selected mRNA isolated from Roundup Ready maize event NK603 that was used as a template in the RT-PCR studies (Lane 12). The presence of the ~735 bp band in Lane 11 demonstrates that Primers A and B are capable of generating a PCR product from event NK603 genomic DNA template. The results from the strand-specific RT-PCR analyses, designed to test for transcriptional initiation from within the insert and continuing into the DNA sequence flanking the 3' end of the insert of NK603, are shown in Lanes 2-3. No signal is observed in the sample in which poly-A^+ selected mRNA from the non-transgenic maize line B73 was used as a template (Lane 2). However, an ~735 bp signal is observed when poly-A^+ selected mRNA from Roundup Ready maize event NK603 is used as a template in the reaction (Lane 3). This is the predicted size of the amplicon that would be produced from Primers A and B if transcription were to initiate from within the NK603 insert and continue through the NOS 3' transcriptional termination sequence into the genomic DNA flanking the insert. The results from the strand-specific RT-PCR analyses designed to test for transcriptional initiation from the genomic DNA flanking the 3' end of the NK603 insert and continuing into the insert DNA are shown in Lanes 4-5. No signal is observed in samples containing poly-A^+ selected mRNA from either non-transgenic maize line B73 (Lane 4) or Roundup Ready maize event NK603 (Lane 5). These results demonstrate that there is no detectable transcription initiating from the genomic DNA flanking the 3' end of the NK603 insert and continuing into the DNA of the insert.

Together, these results demonstrate that RNA transcription most likely originates in either the rice actin or e35S promoter of the NK603 insert and continues through the NOS 3' transcriptional termination sequence into the adjacent genomic DNA flanking the 3' end of the insert. These results also demonstrate that no transcription is detected that originates in the genomic DNA sequence flanking the 3' end of the insert and continues into the NK603 insert DNA.

Northern blot analyses were previously conducted (Mittanck and Lirette, 2000) in which the portion of the rice actin promoter that is inversely linked to the 3' end of the insert in Roundup Ready maize event NK603 was used as a probe on a northern blot of poly-A^+ selected mRNA from event NK603. Based on the results of the northern

analysis, no signal was detected that contains this portion of the enhancer from the rice actin promoter. The same northern blot was later stripped and probed with a segment of the *cp4 epsps* coding region. The results of this analysis demonstrated that only the expected 1.4 kb band is observed. This signal is the expected size band that would result from the transcription of the *cp4 epsps* coding regions in each of the two gene cassettes present in the insert of Roundup Ready maize event NK603 (Mittanck and Lirette, 2000). Although transcription from the insert into the maize genomic DNA flanking the 3' end of the insert was detected by RT-PCR, the results of these northern blot analyses do not provide any evidence of the steady state accumulation of mRNA encompassing the junction between the NK603 insert and the genomic DNA flanking the 3' end.

The presence of longer RNA transcripts detected by the RT-PCR analyses conducted as part of this study do not raise any concerns about the safety of Roundup Ready maize event NK603. The potential of such secondary transcripts to produce a protein product other than, or in addition to, the full-length CP4 EPSPS or CP4 EPSPS L214P proteins is extremely remote. Translation of mRNA in plants, with very rare exceptions, only produces a single polypeptide (Fütterer and Hohn, 1996). Therefore, the only protein products expected to be produced from the NK603 insert are the full-length CP4 EPSPS proteins since RNA transcripts produced in plants that contain consecutive coding regions generally result in the production of only one protein. If sequences downstream from the stop codon of the CP4 EPSPS were translated, a larger fusion protein containing the full-length CP4 EPSPS protein would be produced. No immuno-reactive proteins of a molecular weight greater than the expected 47 kDa were detected by western blots (with a limit of sensitivity of 1 ng) in extracts of Roundup Ready maize event NK603 (Lee and Astwood, 1999; Silvanovich *et al.*, 2001).

V. CONCLUSIONS

The objective of this study was to determine if transcription is occurring across the 3' end of the insert including the 217 bp segment of the enhancer region of the rice actin promoter and the adjacent genomic DNA in Roundup Ready maize event NK603 using RT-PCR as the analytical tool. These analyses did not detect any transcription product that initiates in genomic DNA flanking the 3' end of the NK603 insert that continues into the inserted DNA. However, a transcriptional product was detected that initiates within the NK603 insert and extends through the NOS 3' transcriptional termination sequence into the adjacent genomic DNA flanking the 3' end of the insert in Roundup Ready maize event NK603. This is likely the result of incomplete transcriptional termination within the NOS 3' element. Since northern blot analysis did not detect the steady state accumulation of such a transcriptional product (Mittanck and Lirette, 2000), it is presumed that this RNA is rare and/or unstable. Additionally, no immuno-reactive proteins of a molecular weight greater than the expected 47 kDa were detected by western blots (with a limit of sensitivity of 1 ng) in extracts of Roundup Ready maize event NK603 (Lee and Astwood, 1999; Silvanovich *et al.*, 2001). Therefore, the finding of a minor RNA species that is the result of incomplete transcriptional termination within the NOS 3' element does not alter previous safety conclusions that Roundup Ready maize event NK603 is as safe as conventional maize varieties.

VI. REFERENCES

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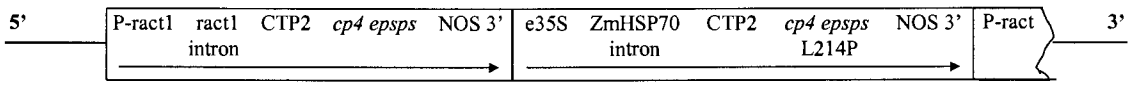

Figure 1. Schematic Representation of the Insert in Roundup Ready Maize Event NK603.

This figure depicts the predicted insert for event NK603 as presented in Deng *et al.* (1999). There is one copy of the *Mlu* I restriction fragment from plasmid PV-ZMGT32 used for transformation, which contains two *cp4 epsps* gene cassettes. Immediately 3' of the second *cp4 epsps* cassette there is a 217 bp segment of the transformation cassette that contains a portion of the enhancer region of the rice actin promoter positioned in the inverse orientation. Arrows in each of the gene cassettes represents the direction of transcription for the *cp4 epsps* coding region from each of the respective promoters.

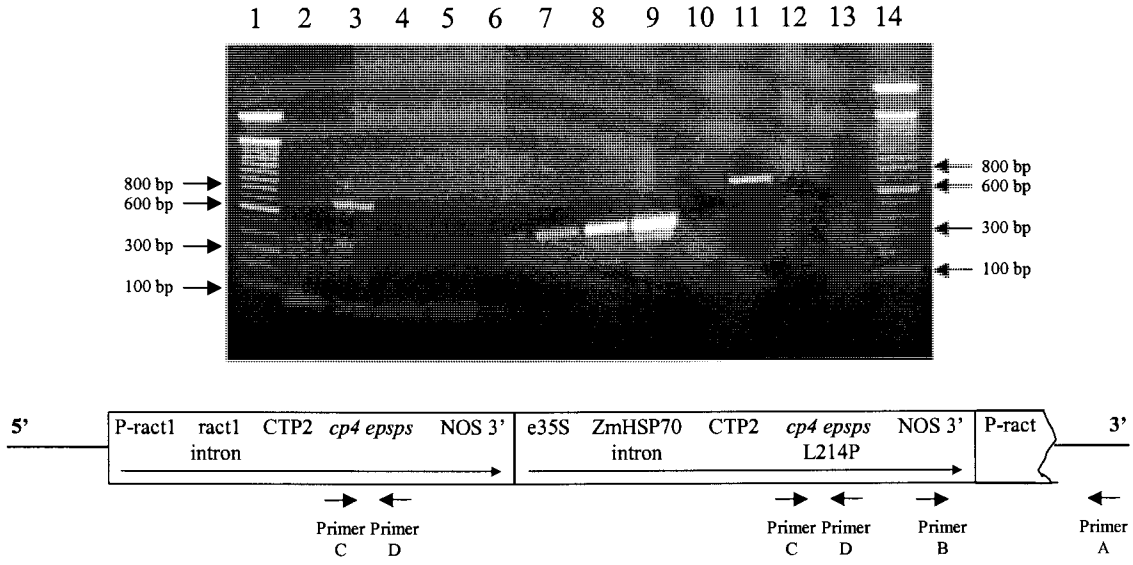

Figure 2. Strand-specific RT-PCR Analysis of the DNA Sequence Flanking the 3' End of the Insert in Roundup Ready Maize Event NK603.

Strand-specific RT-PCR was performed using a primer (Primer A) specific to the genomic DNA sequence flanking the 3' end of the NK603 insert to reverse transcribe any transcriptional product that might initiate from within the insert and continue into the DNA sequence flanking the 3' end of the insert. In order to detect the strand-specific cDNA that might be generated from the reverse transcription reaction, a second primer (Primer B) specific to the NOS 3' genetic element was added to the reaction in order to carry out PCR (Lanes 2-3, poly-A^+ mRNA template from non-transgenic maize line B73 and Roundup Ready maize event NK603, respectively). Conversely, a primer (Primer B) specific to the NOS 3' genetic element was used to reverse transcribe any transcriptional product initiating in the genomic DNA flanking the 3' end of the insert that continues into the NK603 insert DNA. Primer A, specific to the genomic DNA flanking the 3' end of the NK603 insert, was then added to the reaction to amplify any strand-specific cDNA that might have been generated from the reverse transcription reaction using Primer B (Lanes 4-5, poly-A^+ mRNA template from non-transgenic maize line B73 and Roundup Ready maize event NK603, respectively). Lanes 6-9 contain the products of RT-PCR reactions using poly-A^+ mRNA template from event NK603 in conjunction with Primers C and D which are specific to a portion of the *cp4 epsps* coding region (0.01, 0.1, 1, and 10 ng respectively). Lane 11 contains a primer compatibility reaction demonstrating the ability of the Primers A and B to generate a product from genomic DNA template isolate from Roundup Ready maize event NK603. Lane 12 is a genomic contamination control reaction using 10 ng of poly-A^+ selected mRNA template from Roundup Ready maize event NK603 using primers specific to the 3' end of the *cp4 epsps* coding region. Lanes 10 and 13 are control reactions containing no template. Lanes 1 and 14 contain Invitrogen 100 bp DNA Ladder. Thirty microliters of each PCR reaction were loaded on the gel.

⟶ Symbol denotes sizes obtained from MW markers on ethidium bromide stained gel.

Appendix 1

Study Protocol and Amendment

missing.