

# GENOMICS, MOLECULAR GENETICS & BIOTECHNOLOGY

## Development and Characterization of a CP4 EPSPS-Based, Glyphosate-Tolerant Corn Event

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### ABSTRACT

5-Enol-pyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. CP4 (CP4 EPSPS) confers tolerance to the nonselective herbicide glyphosate (marketed under the trade name Roundup<sup>1</sup>) when sufficiently expressed in transgenic plants. Dual CP4 EPSPS transgene cassettes were transformed into corn (*Zea mays* L.) under the transcriptional regulatory control of the rice (*Oryza sativa* L.) actin 1 (P-Ract1) and the enhanced Cauliflower mosaic virus 35S (P-e35S) promoters, respectively, to impart fully constitutive expression in corn. Resulting events were tested for lack of chlorosis and malformation injury after two sequential applications of 1.68 kg acid equivalents (a.e.) ha<sup>-1</sup> glyphosate. Agronomic parameters, male fertility, appropriate Mendelian segregation of the trait, plus characteristics of the transgene integration site were also evaluated. From this selection process, the NK603 event was chosen for commercialization as the event that embodied the most optimal profile of tolerance, agronomics, and molecular characteristics. The NK603 event exhibited high glyphosate tolerance from one transgenic locus bearing a single copy of the dual cassettes integrated into the corn genome with a minimum of target sequence disruption. Trait expression in the NK603 event has remained stable over more than eight generations as shown through tolerance testing, western blots of CP4 EPSPS accumulation, and Southern blot analysis of the transgene.

AGRICULTURAL CROPS tolerant to the herbicide glyphosate (*N*-phosphonomethyl-glycine) have been planted on increasing numbers of hectares since the introduction of the glyphosate-tolerant 40-3-2 soybean event in 1996 (Padgett et al., 1996; marketed under the Roundup Ready brand<sup>1</sup>). By 2000, transgenic glyphosate-tolerant soybean [*Glycine max* (L.) Merr.] use expanded to more than 16 000 000 ha in the USA (Carpenter and Gianessi, 2001) plus additional hectares in Argentina and other geographies. Similarly, the introduction of glyphosate-tolerant cotton (*Gossypium* spp.) and canola (*Brassica napus* L.) has met with a high level of adoption in the USA. This was attributable to a num-

ber of characteristics of the glyphosate weed management system, including nonselective postemergent weed control, crop safety, low toxicity on nontarget organisms, lack of soil mobility, flexibility of application, and cost (Franz et al., 1997). Another feature of this weed management system has been the slow development of resistance in wild plant populations despite glyphosate use for over 28 yr. Currently, only three resistant weed biotypes have been identified [*Conyza canadensis* (L.) Cronq., *Eleusine indica* L., and *Lolium rigidum* Gaud., with a recent report of *Lolium multiflorum* Lam., which is under investigation], in contrast to herbicides such as acetolactate synthase inhibitors (e.g., chlorsulfuron), where more than 70 resistant species have developed (Heap et al., 2003). The overall effectiveness of glyphosate for weed management in conjunction with herbicide tolerant crops species has also promoted environmentally sound practices such as conservation tillage for the control of soil erosion.

Two key elements needed for the development of commercially viable glyphosate-tolerant crops are a resistant target enzyme and sufficient expression of that enzyme within the transgenic plant—a multifaceted challenge that necessitates understanding quantitative, spatial, and developmental components of gene expression. 5-Enol-pyruvylshikimate-3-phosphate synthase (EPSPS) is the target enzyme for glyphosate inhibition within the aromatic amino acid biosynthetic pathway. Disruption of this pathway not only creates a deficiency in protein synthetic precursors, it also affects many other plant cell components that are derived from intermediates and derivatives of this pathway (e.g., auxins, lignans, flavonoids, anthocyanins, and quinones). Therefore, a crop plant must be engineered with a resistant enzyme to maintain flux through this pathway for uninhibited growth and development. Numerous candidate EPSPS enzymes from native and mutagenized microbial and plant sources were examined in an effort to select an enzyme with high catalytic efficiency in the presence of glyphosate (Ruff et al., 1991; Barry et al., 1992). *Agrobacterium* sp. strain CP4 EPSPS (CP4 EPSPS) was found to be an exceptional en-

<sup>1</sup>Roundup Ready and Roundup herbicide are trademarks of the Monsanto Company.

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**Abbreviations:** a.e., acid equivalents; bp, base pair; CP4 EPSPS, 5-enol-pyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. CP4; ELISA, enzyme-linked immunosorbant assay; fwt, fresh weight; kb, kilo base; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; UTR, untranslated region.

zyme during this screening process (Barry et al., 1992; Padgett et al., 1995) and is the transgenic EPSPS protein produced in glyphosate-tolerant *Roundup Ready* soybean, cotton, and canola. Transgenic expression of CP4 EPSPS within these crops provided the appropriate support to the aromatic amino acid biosynthetic pathway without negative impact on yield, compositional qualities, and nutritional value of the harvested product (Delannay et al., 1995; Hammond et al., 1995; Harrison et al., 1996; Padgett et al., 1995; Nida et al., 1996b).

The second component to successfully engineer herbicide tolerance is expression of a resistant enzyme in all cell types that receive a significant dose of the herbicide and require a functional aromatic amino acid pathway. Glyphosate is a phloem-mobile herbicide that translocates from source tissues such as mature leaves to sink tissues in much the same way that photosynthates such as sucrose are mobilized within the plant. When corn was treated with glyphosate, the herbicide was delivered to meristematic and young developing organs of the apex, roots, and inflorescences (Hetherington et al., 1999). As a result, high glyphosate concentrations occurred where a fully functional aromatic amino acid pathway was most needed. Heterologous promoters and other regulatory elements (i.e., nonnative sequences which can direct transcription and transcript accumulation) have typically been used to provide constitutive expression at stoichiometrically higher levels than the endogenous target enzymes. This ensures a broad margin of safety for the crop plant bearing the tolerance trait. Expression must also be stable under many different environmental regimes that include a variable matrix of stress and favorable growth parameters. Failure to meet these expression criteria in even a few cell types could result in morphological defects, chlorosis, sterility, and/or perceptible yield loss in the engineered crop in response to glyphosate application.

In this report, we present data on the development of the glyphosate-tolerant corn event, NK603. This represents the extension of CP4 EPSPS transgene utility demonstrated in other crops into this major agricultural species. Selection of the NK603 event was based on a combination of factors including glyphosate tolerance, agronomic characteristics, segregation, and molecular integration profile resulting in an event which meets rigorous field use requirements and grain quality standards of the harvested product (USDA, 2000, and references therein). Data used in the selection process are presented. Additional yield and tolerance trial data taken at multiple locations from the NK603 event (manuscripts in preparation) will be presented in a separate manuscript to complement the early characterization work presented here.

## MATERIALS AND METHODS

### Production of Glyphosate Tolerant Corn Events

As with glyphosate tolerant soybean (Padgett et al., 1995) and cotton (Nida et al., 1996a), transgenic cassettes were created for monocot expression from a combination of promoter, transit peptide sequence, CP4 EPSPS, and transcriptional ter-

mination sequences. For the PV-ZMGT32 plasmid used to generate the NK603 event, two cassettes were tandemly joined in a head-to-tail orientation within a pUC119-derived (Vieira and Messing, 1987) high-copy plasmid backbone bearing a kanamycin selectable marker (neomycin phosphotransferase II, *nptII*) for maintenance in bacteria. The first transgenic cassette combined the rice actin 1 promoter and intron (McElroy et al., 1990) to transcriptionally regulate a single open reading frame consisting of the *Arabidopsis thaliana* EPSPS chloroplast transit peptide (Shah et al., 1986) linked to the *Agrobacterium* sp. CP4 EPSPS sequence (Barry et al., 1992). The 3' nontranslated region of the nopaline synthase gene from *Agrobacterium tumefaciens* T-DNA borders the 3' end of the first transgenic cassette to direct appropriate processing of RNA transcripts (Fraley et al., 1983). The structure of the second transgene cassette is the same as the first, except the rice actin 1 promoter and intron have been replaced by the enhanced Cauliflower mosaic virus promoter (Kay et al., 1987) and maize hsp70 intron (Brown and Santino, 1994), respectively.

PV-ZMGT32 was digested with *MluI* to liberate an approximately 6.7-kb fragment containing both CP4 EPSPS cassettes. The vector backbone was purified away from this fragment by preparative gel electrophoresis. Precultured maize embryos (Songstad et al., 1996) from a Hi-II (A188 × B73 derivative cross, Armstrong et al., 1991) × FBLL (commercial inbred) cross were bombarded with gold particles coated with the purified *MluI* fragment of PV-ZMGT32 with a helium-driven particle accelerator. DNA was precipitated onto the gold particles using a calcium chloride-spermidine protocol, essentially as described by Klein et al. (1988). Selection of glyphosate-tolerant regenerating calli was accomplished on N6 medium containing 3 mM glyphosate (described in Howe et al., 2002). As plantlets regenerated and rooted, they were removed to soil and transferred to the greenhouse. One hundred-seven resistant callus events were produced that were regenerated into 87 plantlets for further testing under greenhouse conditions. Once in the greenhouse, plants were sprayed with 1.68 kg a.e. ha<sup>-1</sup> glyphosate (twice the typical field use rate) at approximately the V4 to V5 leaf stage (staging equivalent to seed-grown plants with four to five leaves sufficiently mature to each have an exposed ligule and flared auricles). Plants were visually scored for chlorosis and vegetative malformation 10–14 d after treatment. Forty-two events which exhibited <10 to 15% visually estimated leaf chlorosis and vegetative malformation were advanced to an out-cross with the public maize inbred, B73, to generate B73 BC0F1 testing material.

### Field Evaluations

Trials were planted in a split block design (6-m-long rows spaced 0.76 m apart; approximately 6 seeds/m) with two replications per location. Main plots received the indicated rates of glyphosate using the Roundup Ultra formulation applied in a carrier volume of approximately 94–187 L ha<sup>-1</sup>. Subplots were defined by event. Tolerance was scored 10 to 14 d after each glyphosate treatment. Glyphosate injury was evaluated as chlorosis and malformation monitored relative to untreated control plants grown under weed free conditions. Visual estimation of these agronomic characteristics at <10% of untreated controls was considered a minimum criterion for further advancement in the event selection process. Other agronomic characters were monitored at appropriate times to evaluate equivalency of the advancing events with and without glyphosate treatment.

### Immunolocalization

Anthers were dissected from immature corn tassels at the microspore mother cell stage and fixed in 4% formaldehyde overnight. Tissue was dehydrated through a graded ethanol series (35, 50, 75, 80, 95, and 100%, v/v) and then imbedded in plastic resin according to manufacturer's instructions (Immunobed Kit; Polysciences, Inc., Warrington, PA). Five-micrometer sections were cut and fixed on glass slides coated with polyglycine. General morphology was examined by staining with Toluidine Blue O. Immunolocalization of CP4 EPSPS proteins in tissue sections was performed by established conditions (Ryerse et al., 1997) with goat antiserum to CP4 EPSPS as the primary antibody (1: 100 000 dilution). Nonimmunized goat serum, also diluted 1:100 000, was used as the equivalent of a pre-immune primary antibody control. Positive detection of CP4 EPSPS was accomplished by development with biotin-conjugated rabbit anti-goat antibodies followed by peroxidase-conjugated streptavidin, and finally color development with freshly made AEC substrate (Zymed, San Francisco, CA) (Ryerse et al., 1997). Sections were examined with a Zeiss Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY) and images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

### Molecular Characterization

Corn genomic DNA was isolated (Saghai-Marooof et al., 1984) and quantified by fluorimetry. Restriction digestion, gel electrophoresis, Southern blotting, and hybridization to radiolabeled probes were completed according to standard procedures (Sambrook et al., 1989). Both short and long electrophoresis runs were done to allow optimal resolution of small and large restriction fragments, respectively, before blotting. Templates for radioactive probe synthesis were prepared from gel-purified restriction fragments of PV-ZMGT32 or generated by PCR using oligonucleotides that anneal at the ends of the respective element. Probes were labeled by means of the Klenow fragment of DNA polymerase I, random oligomers, and  $^{32}\text{P}$ -dCTP (RadPrime DNA Labeling System, Invitrogen Life Technologies, Carlsbad, CA). After hybridization, blots were washed at increasing stringency with the final wash being  $0.1\times$  SSC (150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 0.5% (w/v) sodium dodecyl sulfate (SDS) at 68°C. Multiple exposures of the blot were generated with Kodak Biomax MR film in conjunction with a Kodak Biomax MS intensifying screen (Eastman Kodak; Rochester, NY) including many-fold overexposure to permit detection of faint signals.

Genomic sequence flanking the NK603 integration site was cloned using the GenomeWalker kit (BD Biosciences Clontech, Palo Alto, CA) according to manufacturer's instructions. Two nested primer sets were designed for the 5' and 3' ends of the transgene in the P-Ract1 and nopaline synthase transcriptional terminator, respectively. The identity of cloned PCR products and their relationship to the *Mlu*I fragment of PV-ZMGT32 was confirmed by dye-terminator sequencing and alignment with vector sequences with the sequence analysis software (DNASTAR, Inc., Madison, WI). Analysis also included homology searches to investigate the nature of the cloned flanking information. The BLAST algorithm (version 2.0, Altschul et al., 1997) was used to assess homology and look for significant matches to known sequences (e.g., GenBank nonredundant nucleotide database; Benson et al., 2000), at both the nucleotide (BLASTN) and amino acid level (TBLSTX).

PCR reactions used approximately 50 ng of corn genomic DNA, 0.1  $\mu\text{M}$  of the appropriate oligonucleotide primers, 0.2  $\mu\text{M}$

deoxyribonucleotides,  $1\times$  REDTaq reaction buffer containing magnesium (1.5 mM), and 1.5 units of REDTaq thermostable DNA polymerase (Sigma, St. Louis, MO) in a 50  $\mu\text{L}$  volume. Oligonucleotide primers used were as follows: A: 5'-TGACG TATCAAAGTACCGACAAAAACATCC-3'; B: 5'-CCTTT GTTTTATTTTGGACTATCCCGACTC-3'; C: 5'-AGATTG AATCCGTGTCGGGTCTTGC-3'; D: 5'-GCGGTGTCAT CTAGTTACTAGATCGGG-3'; E: 5'-CAGCATCAGCGC TCGAAAGTTTCGTCAA-3'; F: 5'-GGCAGGGTGTGTTGTCCATTTTATGG-3'. The following cycling conditions were used: 1 cycle of 95°C for 2 min; 36 cycles of 94°C for 30s, 60°C for 30 s; 72°C for 1 min; followed by 1 cycle of 72°C for 10 min (using calculated temperature control in an MJ Research PTC-200 DNA Engine thermocycler (MJ Research, Inc., Reno, NV). PCR products were examined by agarose gel electrophoresis.

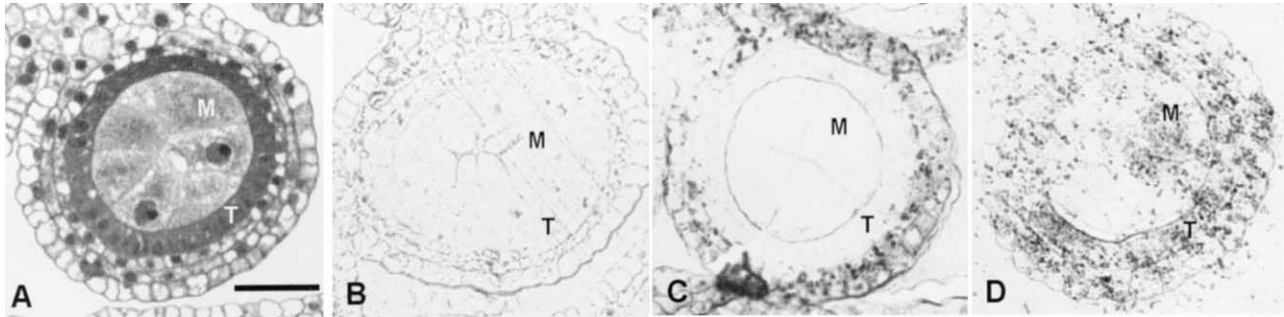
### ELISA Assays and Western Analyses for CP4 EPSPS

ELISAs to quantify CP4 EPSPS protein were done essentially as previously described (Padgette et al., 1996). For forage and leaf samples, an extraction was done at a tissue to buffer ratio of 1:50 and grain at a 1:100 tissue to buffer ratio. For western analysis, extracts were made by homogenizing 0.1 g of frozen leaf tissue in 10 mL of 10 mM  $\text{KH}_2\text{PO}_4$ , 0.1 mM  $\text{Na}_2\text{HPO}_4$ , 1.37 M NaCl, 27 mM KCl, 0.5% (v/v) Tween-20, and 0.1% (v/v) bovine serum albumin, pH 7.4 (PBST+0.1% BSA). Samples were filtered and diluted 1:2 (v/v) in PBST+0.1% BSA before adding an equal amount of  $2\times$  Laemmli sample buffer (Laemmli, 1970) and heated to 100°C for 5 min. Proteins were separated on 4 to 20% (w/v) tris-glycine gradient gels (Invitrogen Life Sciences) and transferred to a PVDF membrane (Immobilon-P, 0.45  $\mu\text{M}$ ; Millipore, Corp., Billerica, MA). A 1:5000 dilution of primary polyclonal antibodies isolated from CP4 EPSPS goat antiserum was used, followed by a 1:10 000 secondary detection antibody of rabbit anti-goat IgG conjugated to horseradish peroxidase (Sigma). For specific detection of CP4 EPSPS L214P (a form of CP4 EPSPS found in the P-e35S cassette of NK603 that was created by spontaneous mutation during transformation), rabbit antiserum was prepared by immunization with a synthetic peptide of the CP4 EPSPS region at position P214. Western analysis CP4 EPSPS L214P was performed as above, with the exception that the serum was used at a 1:1000 dilution. Immunoreactive bands were visualized on X-ray film with the Enhanced Chemiluminescent Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) according to manufacturer's instructions. Estimation of the relative contribution of CP4 EPSPS L214P to total CP4 EPSPS accumulation was determined by purifying to 90% homogeneity total CP4 EPSPSs from grain and leaf, and by determining the proportion of CP4 EPSPS L214P immunoreactive to the specific rabbit antibody relative to the total amount of CP4 EPSPSs loaded onto SDS-PAGE before transblotting.

## RESULTS AND DISCUSSION

### Development of Expression Elements for Glyphosate Tolerance in Corn

Corn transformations with the CP4 EPSPS transgene began in 1989 and over the next several years, a number of transgenic vectors were created to optimize expression of CP4 EPSPS via promoter, 5'UTR intron and synthetic coding sequence combinations to allow efficient selection during transformation and concomitant glyphosate tolerance in regenerated plants. Many vec-



**Fig. 1.** Immunolocalization of CP4 EPSPS in developing corn anthers at the microspore mother cell stage of development. Panel A: Toluidine Blue O stain of a transverse section through a developing anther showing one microsporangium. Panel B: Control section treated with pre-immune antiserum as primary antibody. Panel C: Section of a P-e35S/CP4 EPSPS event treated with anti-CP4 EPSPS polyclonal antiserum as primary antibody. Panel D: Section of P-Ract1/CP4 EPSPS event treated with anti-CP4 EPSPS polyclonal antiserum as primary antibody. Positive detection is indicated by development of dark punctate reaction product. M: microspore mother cell. T: tapetum cell layer. Size bar = 20  $\mu\text{m}$ .

tors utilized the enhanced Cauliflower mosaic virus 35S promoter (P-e35S) because of its strong and constitutive expression pattern. In some cases, secondary cassettes expressing glyphosate oxidoreductase (*GOX*, Barry and Kishore, 1995) and *N*-acyl-phosphonotransferase (*phnO*, Barry, 1999) were also used in an effort to enhance glyphosate tolerance. Experimentation showed that expression of CP4 EPSPS was sufficient to convey glyphosate tolerance in transgenic corn plants (data not shown).

From these early efforts, corn events expressing CP4 EPSPS were recovered with high vegetative tolerance to glyphosate. However, there was limited recovery of fully male-fertile transgenic events at commercial rates of glyphosate application (0.84 kg a.e. ha<sup>-1</sup>). The window of reproductive sensitivity noted in the field and histochemical analyses on greenhouse-grown material indicated that early male reproductive development was impaired in P-e35S/CP4 EPSPS transformants treated with glyphosate. Immunolocalization of CP4 EPSPS in anther sections revealed little to no accumulation in the tapetum that nourishes the developing pollen and the microspore mother cells that are antecedents to pollen, despite presence of CP4 EPSPS in surrounding anther tissue (endothecium, epidermis and connective/vascular tissue) (Fig. 1, Panel C). The P-e35 expression deficit apparently allowed glyphosate to damage critical cell types during anther development, most notably in the sporophytic tapetum and early stages of gametogenesis leading to male sterility.

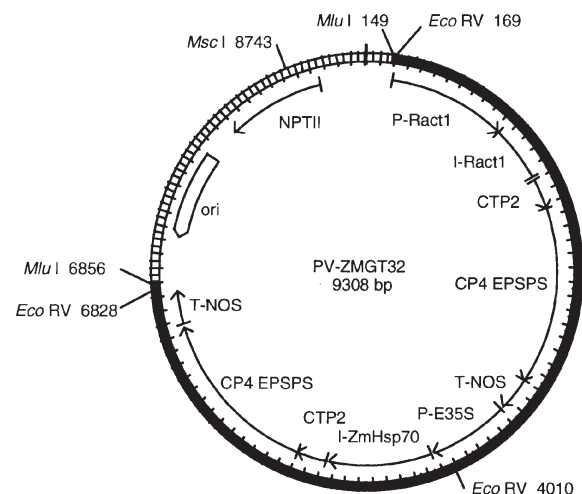
During evaluation of transgenic elements for glyphosate tolerance, it was recognized that other promoters could possibly supply the necessary male reproductive expression in corn. One such promoter was from the rice actin 1 gene (P-Ract1) (McElroy et al., 1990). In contrast to P-e35S, it directs CP4 EPSPS expression in all cell types within the corn anther (a representative P-Ract1/CP4 EPSPS event: Fig. 1, Panel D). Observations of this promoter in transgenic rice (*Oryza sativa* L., Zhang et al., 1991) in contrast to the differential expression of the P-35S promoter (Battraw and Hall, 1990; Tereda and Shimamoto, 1990) supported the potential for complementing expression. Therefore, P-Ract1 appeared to be a good candidate to enhance CP4 EPSPS expression for full glyphosate tolerance even though expression of

P-Ract1 is lower than P-e35S in mature tissues of the corn plant (data not shown).

### Selection of the NK603 event

In 1996, a number of transgenic constructs were created to complement the 35S promoter deficiency in corn and enhance overall glyphosate tolerance. Over 1300 events in total were created in this second transformation series using a variety of transgenic components and configurations. One construct tested was PV-ZMGT32 (Fig. 2) that carried two CP4 EPSPS transgene cassettes, driven by the P-Ract1 and P-e35S promoters, respectively (Table 1). It was created to encompass the regulatory capacity of both promoters and generate glyphosate-tolerant events with full vegetative and reproductive tolerance. PV-ZMGT32 transformation gave rise to a number of highly glyphosate-tolerant transgenic R0 corn events that were advanced to field evaluation.

B73 BC0F1 events derived from PV-ZMGT32 transformations were planted in the field during the summer of 1997 (event breeding schematic, Fig. 3). Two sequen-



**Fig. 2.** Plasmid map of PV-ZMGT32. *Mlu*I fragment used in transformation of NK603 is indicated by heavy darkline. Vector backbone (hatched segment) was purified away before bombardment. ori (microbial origin of replication); NPTII (neomycin phosphotransferase). Restriction enzyme sites for *Eco*RV, *Mlu*I and *Msc*I are shown.

**Table 1. Genetic elements present in PV-ZMGT32.**

Element	Size	Function
	kb	
P-Ract1	1.4	5' region of rice ( <i>Oryza sativa</i> ) actin 1 ( <i>Act1</i> ) gene containing the promoter, transcription start site and first intron (I-Ract1) (McElroy et al., 1990).
CTP2	0.2	IDNA sequence for chloroplast transit peptide, isolated from <i>Arabidopsis thaliana</i> EPSPS; transit peptide directs the CP4-EPSPS protein to the chloroplast, the site of aromatic amino acid synthesis (Shah et al., 1986).
CP4 EPSPS	1.4	The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) sequence isolated from <i>Agrobacterium</i> sp. strain CP4 which imparts tolerance to glyphosate (Barry et al., 1992).
T-NOS 3'	0.3	A 3' nontranslated region of the nopaline synthase gene from <i>Agrobacterium tumefaciens</i> T-DNA which ends transcription and directs polyadenylation of the mRNA (Fraleley et al., 1983).
P-e35S	0.6	The Cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region (Kay et al., 1987).
I-ZmHSP70	0.8	Intron from the maize ( <i>Zea mays</i> ) <i>hsp70</i> gene (heat-shock protein) present to increase the level of gene transcription (Brown and Santino, 1994).

tial applications of glyphosate were made with 1.68 kg a.e. ha<sup>-1</sup> at the V4 and V8 leaf stages. Male fertility in response to glyphosate was also examined for the first time in these transgenic events. Twenty-three events that exhibited <10 to 15% leaf chlorosis and vegetative malformation, an expected 1:1 segregation for the transgene (Table 2), and a high level of male fertility (score of 5) were advanced to field evaluations in the winter nursery of 1997. Evaluations were repeated, but a rate of 2.52 kg a.e. ha<sup>-1</sup> glyphosate applied at the V4 and V8 leaf stages was used to differentiate tolerance among the events. This application rate is three-fold higher than typically used to control weeds under field conditions. Ten events were evaluated and advanced using the same criteria in the following winter/spring nursery (early 1998), looking at both inbred and hybrid combinations in a variety of genotypes.

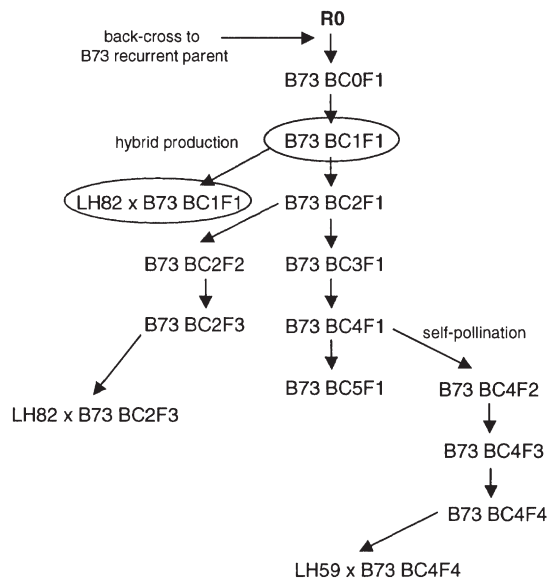
During the summer of 1998, the first small-scale field evaluations for yield were performed at two locations on hybrid material derived from the commercial inbred,

LH82, and ten of the top performing transgenic events (e.g., LH82 × NK603 B73 BC1F1, where "NK" is an event code referring to the PV-ZMGT32 transformation series and "603" is a specific event within the series). Two replications were done at each location in a split block experimental design using a rates up to 3.36 kg a.e. ha<sup>-1</sup> of glyphosate applied sequentially at V4 and V8 stages. Agronomic data from the NK603 event is shown in Table 3, comparing the treated and untreated event within the same trial. On the basis of these results, and larger trials at additional locations in 1999 and 2000, with near isogenic nontransgenic comparators (Heck et al., manuscript in preparation, and USDA, 2000 and references therein), it was concluded that NK603 had high tolerance to typical field application rates of glyphosate and that the transgenic insertion neither created nor was linked to negative agronomic characteristics which could affect glyphosate tolerance or yield performance. Separate plots of the LH82 × NK603 B73 BC1F1 generation were also grown to generate material for the compositional characterization of grain used in regulatory approval dossiers.

### Segregation and Stability of the NK603 Transgenic Insertion

Segregation data of the NK603 transgenic insertion was collected at many points in the breeding and evaluation pathways to ensure proper Mendelian inheritance for a single locus trait. Progeny were sprayed with glyphosate to evaluate presence of the transgenic locus. The resulting information corroborated molecular studies and was also used to reveal subtle fitness effects due to the transgenic integration. Segregation data for nine generations of event NK603 progeny are presented in Table 2.

All generations segregated as expected for a single active transgenic integration, except for the BC2F1 generation. The excess of positive plants in the BC2F1 generation may have resulted from gamete selection due to high application rates of glyphosate in the generation before the BC2F1 (i.e., BC1F1). Preferential selection for positive gametes in hemizygous herbicide resistant transgenic plants has been demonstrated following applications of a selective herbicide (Spencer et al., 1998;



**Fig. 3. Schematic diagram of NK603 B73 breeding lineage. Generations referenced in development of the NK603 glyphosate-tolerant corn event are shown. Material used in primary characterizations for regulatory submissions is circled. LH59, and LH82 are commercial inbreds used to make hybrid testing material.**

**Table 2. Segregation data and analysis of progeny for glyphosate-tolerant corn event NK603.**

Generation	Observed†			Expected			$\chi^2$
	Positive	Negative	Segregating	Positive	Negative	Segregating	
BC0F1‡	14	15		14.5	14.5		0.00ns
BC1F1	32	23		27.5	27.5		1.16ns
BC2F1	135	81		108.0	108.0		13.00**
BC2F2	86	26		84.0	28.0		0.12ns
BC2F3	9	16	24	12.3	12.3	24.5	2.02*
BC3F1	44	45		44.5	44.5		0.00ns
BC4F1	127	103		115.0	115.0		2.30ns
BC4F3	12	5	17	8.5	8.5	17.0	2.88*
BC5F1	26	35		30.5	30.5		1.05ns

\* Not significant at  $p = 0.05$  (chi square = 5.99, 2 df).

\*\* Significant at  $p = 0.01$  (chi square = 6.63, 1 df).

ns, not significant at  $p = 0.05$  (chi square = 3.84, 1 degree of freedom).

† Data expressed as number of positive and negative plants based on glyphosate sprays (1.68 kg a.e. ha<sup>-1</sup> or greater) except for the BC2F3 and BC4F3 generations which are number of homozygous positive ear-rows, number of homozygous negative ear-rows, and number of segregating ear-rows based on glyphosate sprays.

‡ BC0F1–BC5F1 generations (derived from crossing the R0–5th generations with the public inbred event B73); BC2F2 generation (derived from selfing individual BC2F1 plants); BC2F3 (selfed BC2F2); and BC4F3 (selfed BC4F3).

Conner, 1995). Otherwise, the Chi square segregation analyses are consistent with a single active site of integration for the PV-ZMGT32 transgene into the corn genomic DNA of event NK603. Additionally, the functional stability of the insert has been demonstrated through six generations of crossing and three generations of self-pollination.

### CP4 EPSPS Characterization in Transgenic Maize Events

The CP4 EPSPS expressed in the NK603 event was analyzed at a level of nucleotide sequence. Sequencing of the respective CP4 EPSPS coding regions from the P-Ract1 and P-e35S cassettes of the NK603 event revealed two nucleotide changes in the P-e35S CP4 EPSPS coding region. One of these changes lead to a silent codon substitution. The other converted a leucine codon to a proline codon at position 214 (CP4 EPSPS L214P) of the 455 amino acid CP4 EPSPS polypeptide. The P-Ract1 coding region remained unaltered so that two slightly different CP4 EPSPS sequences were produced in the NK603 event. Neither nucleotide change was present in the originating PV-ZMGT32 plasmid but was

assayable in archived BC0F1 material and was stably maintained in subsequent generations. MALDI-TOF (Matrix Assisted Laser Desorption and Ionization–Time of Flight) mass spectrometry of purified total CP4 EPSPS from grain also confirmed presence of both protein sequences in NK603 (George et al., manuscript in preparation). The observed change did not affect the active site of the CP4 EPSPS L214P and assays of CP4 EPSPS L214P biochemical activity show it is indistinguishable from CP4 EPSPS (George et al., manuscript in preparation). CP4 EPSPS and CP4 EPSPS L214P also exhibit a common digestibility and immunoreactivity with polyclonal antisera (antiserum raised specifically to the L214P peptide can distinguish the L214P CP4 EPSPS), thus being equivalent in the assessments performed on the NK603 event and prior studies of CP4 EPSPS (Fuchs and Astwood, 1996; Harrison et al., 1996). Corn hybrids containing event NK603 have been shown to be nutritionally equivalent to traditional corn hybrids by direct evaluations of key nutrients (Ridley et al., 2002) and by evaluation in animal feeding studies in broiler chickens (Taylor et al., 2001, 2003), swine (Fisher et al., 2002), cattle (Ipharraguerre et al., 2003), and rodents (Hammond et al., 2002).

**Table 3. Mean values of agronomic characteristics from glyphosate tolerant corn event NK603 in response to glyphosate application.**

Rate	Percent Chl V4	Percent Mal V4	Percent ELH V4	Percent Chl V8	Percent Mal V8	Percent ELH V8	P50 (DAP)	S50 (DAP)	MFR (1-5)	Plant height	Percent moisture	Yield
										m		Mg ha <sup>-1</sup>
Untreated	0.3	1.0	0.81	0.0	0.0	2.02	59	61	5.0	2.11	20.4	10.92
0.84 fb 0.84	0.0	0.0*	0.81	1.5	1.0	1.91**	60	61	5.0	2.15	19.9	11.01
1.68 fb 1.68	0.0	0.3	0.86	0.5	1.3	2.01	59	60	5.0	2.23	19.5	10.88
2.52 fb 2.52	0.0	0.3	0.87	5.0	10.0**	2.03	58	59	5.0	2.22	18.2	11.54
3.36 fb 3.36	0.3	1.0	0.86	3.0	3.0	2.02	59	61	5.0	2.11	19.0	11.44

Evaluation	Evaluation timing	Comments
Rate	V4 application followed by (fb)V8	kg a.e. ha <sup>-1</sup> glyphosate
Percent chlorosis (% Chl)	10-14 d after application (after V4 or V8 application)	visual rating based on entire row
Percent malformation (% Mal)	10-14 d after application (after V4 or V8 application)	visual rating based on entire row
Extended leaf height (ELH)	14 d after application	meters; 10 plants per plot measured
Date of 50% pollen shed (P50)	pollination	DAP when 50% of pollen is shed in plot
Date of 50% silk emergence (S50)	pollination	DAP when 50% of plants have 2.5 cm silks emerged
Male fertility rating (MFR)	pollination	1 = lack of anther development 5 = full pollen shed
Plant height (Plant Ht)	pollination	meters; 10 plants measured to tassel
Percent moisture (% moist)	harvest	mature grain moisture level (percent fresh weight)
Yield	harvest	Mg ha <sup>-1</sup> , machine harvested and weighed

\* P-value indicates probability of treated means being significantly different from untreated means at  $< 0.05$ .

\*\* P-value indicates probability of treated means being significantly different from untreated means at  $< 0.01$ .

**Table 4. CP4 EPSPS accumulation in glyphosate tolerant corn events determined by ELISA.**

Event	Genotype	Sample	Microgram per gram fresh weight†	
			Mean‡	Range§
NK541	BC1F1	V4 leaf lamina	7.30	5.92–8.27
NK543	BC1F1	V4 leaf lamina	75.4	72.5–79.0
NK600	BC1F1	V4 leaf lamina	41.2	35.3–48.0
NK603	BC1F1	V4 leaf lamina	20.8	14.9–26.8
NK603	LH82 × BC1F1	forage¶	25.9	25.7–26.1
NK603	LH82 × BC1F1	mature grain	10.9	6.9–15.6

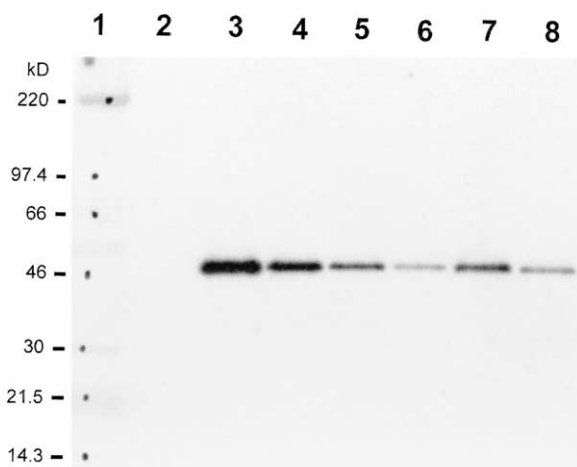
† BC1F1 introgressions using the B73 inbred. For the grain study, mature grain was harvested from hybrids made using the commercial inbred LH82.

‡ Mean of 3 greenhouse-grown individual V4 leaf lamina, pooled grain, or forage samples from 3 respective replicated field sites (Jerseyville, IL; New Holland, OH; and Claude, TX, planted in summer 1998). No CP4 EPSPS was detected in B73 or LH82 × B73 nontransgenic controls.

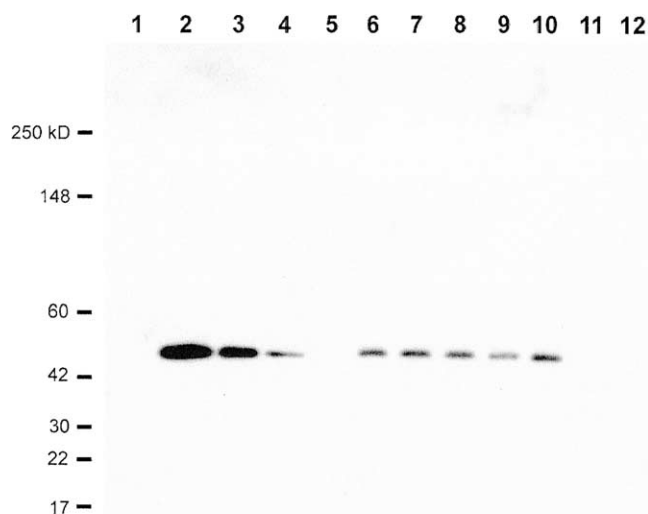
§ Range indicates highest and lowest individual sample values.

¶ Forage = homogenized whole plants minus roots taken at the soft-dough stage of grain development.

ELISA assays (Table 4) and western blot analyses (Fig. 4 and 5) were performed to evaluate accumulation of CP4 EPSPS protein in corn tissues produced by PV-ZMGT32 transformation. As illustrated in the V4 leaf sample, the NK603 event exhibited a midrange CP4 EPSPS accumulation level among four representative events advanced into field evaluations during the summer of 1998. In the NK603 event, these analyses commonly detect CP4 EPSPS and CP4 EPSPS L214P. Steady state levels of transgenic protein in the NK603 event varied by organ and developmental stage with grain accumulating less transgenic protein than leaf or forage material (Table 4). Utilizing the antibody developed for specific detection of CP4 EPSPS L214P, western blotting and subsequent densitometry suggested that CP4 EPSPS L214P accumulation was approximately 30% of the combined total CP4 EPSPS proteins in grain and leaf (Fig. 5). Overall, total accumulation of CP4 EPSPS



**Fig. 4. Western blot estimation of the amount of CP4 EPSPS L214P protein present in the isolated total CP4 EPSPS protein from NK603 grain and leaf tissue. Western blot probed with CP4 EPSPS L214P-specific antibodies. Lanes: 1) molecular weight marker; 2) 4 ng *E. coli*-expressed CP4 EPSPS; 3) 4 ng *E. coli*-expressed CP4 EPSPS L214P; 4) 2 ng *E. coli*-expressed CP4 EPSPS L214P; 5) 1 ng *E. coli*-expressed CP4 EPSPS L214P; 6) 0.5 ng *E. coli*-expressed CP4 EPSPS L214P; 7) 4 ng of total CP4 EPSPS purified from grain; 8) 4 ng of total CP4 EPSPS purified from leaves.**



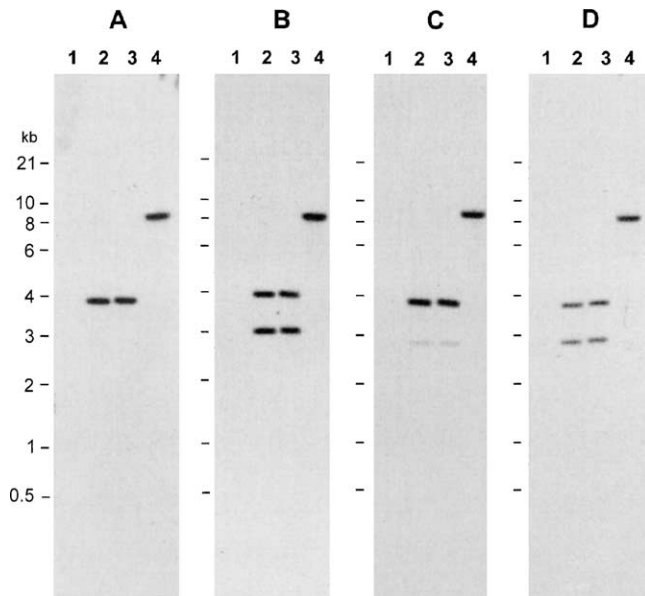
**Fig. 5. Western blot analysis of CP4 EPSPS protein expressed over five generations of NK603. Total protein was extracted from NK603 leaves. Denatured protein was separated by SDS-PAGE, blotted, and visualized by means of a chemiluminescent detection system and CP4 EPSPS antiserum. The corn inbred B73 was used as a recurrent parent in all generational material examined except for hybrid samples which utilized a cross to the LH82 inbred. Lane: 1) molecular weight markers (dye-linked markers visible on blot); 2) 5 ng of *E. coli*-expressed CP4 EPSPS; 3) 2.5 ng of *E. coli*-expressed CP4 EPSPS; 4) 1 ng of *E. coli*-expressed CP4 EPSPS; 5) buffer blank; 6) NK603 B73 BC0F1; 7) LH82 × NK603 B73 BC1F1; 8) NK603 B73 BC1F1; 9) NK603 B73 BC5F1; 10) LH82 × NK603 B73 BC2F3; 11) B73 nontransgenic inbred control; 12) LH82 × B73 nontransgenic hybrid control.**

in event NK603 was lower than that observed in the 40-3-2 glyphosate tolerant soybean event, where 415 to 443  $\mu\text{g/g}$  fwt for leaf and 201 to 288  $\mu\text{g/g}$  fwt for seed was measured (Padgett et al., 1995), although both events meet commercial tolerance specifications. CP4 EPSPS expression also remained stable in the NK603 event over multiple generations sampled (Fig. 5), consistent with glyphosate tolerance observations and Southern analyses of the transgenic insertion.

### Characterization of the NK603 Integration Site

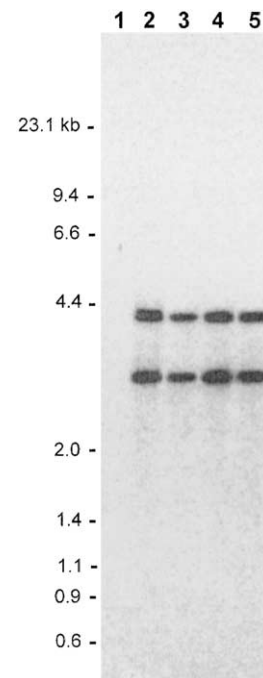
Extensive analysis of the NK603 transgenic locus has been undertaken to understand copy number, integrity of transgenic elements, and relationship of the transgene to the native corn genome. Stability of the transgene within the genome location was also assayed over generational time. A combination of Southern analysis, PCR, and DNA sequencing was used to arrive at an understanding of the integration site.

Southern analysis was performed on NK603 with probes derived from specific segments of the PV-ZMGT32 plasmid (rice actin 1 promoter, CTP/CP4 EPSPS coding region, e35S promoter, and the nopaline synthase nontranslated region, (Fig. 2). Additional probes (e.g., chloroplast transit peptide and rice actin intron, and vector backbone) were also used in the analysis and were consistent with results presented. All intended transgenic elements were present in the NK603 event



**Fig. 6.** Southern analyses of NK603 transgenic elements. Corn genomic DNA was digested with the indicated restriction enzymes, electrophoresed in replicate 0.8% (w/v) agarose gels, transferred to a nylon membrane, hybridized to  $^{32}\text{P}$ -labeled segments of PV-ZMGT32, and subjected to autoradiography. Individual lanes: 1) *EcoRV* digest of 10  $\mu\text{g}$  B73 inbred; 2) *EcoRV* digest of 10  $\mu\text{g}$  B73 inbred genomic DNA supplemented with 29  $\mu\text{g}$  of PV-ZMGT32; 3) *EcoRV* digest of 10  $\mu\text{g}$  NK603 B73 BC5S3 genomic DNA; 4) *MscI* digest of 10  $\mu\text{g}$  of B73 BC5S3 genomic DNA. Panel A, rice *Act1* promoter probe; Panel B, CTP/CP4 EPSPS probe; Panel C, e35S promoter; Panel D, NOS transcriptional termination sequence probe. Molecular weight markers are indicated on the side of each autoradiogram (kb, kilobase pairs).

and of the correct size relative to the PV-ZMGT32 plasmid (Fig. 6). Vector backbone sequences (e.g., microbial origin of replication and *nptII* gene) were not found by Southern blot hybridization using probes spanning the backbone outside the *MluI* sites (data not shown). Multiple restriction enzyme digestions (additional to the ones shown), short electrophoresis runs before blotting and overexposures of all autoradiograms were completed in addition to the results presented above to confirm that all segments of transgenic DNA, including small subfragments, were accounted for in the analysis. For instance, with this more detailed analysis, a small fragment of P-Ract1 was detectable in the Southern analysis (not visible in Fig. 6, because of its small size, and stringent washing conditions; and <39% G+C content of this 217-bp segment). Sequence determination around the integration site (see below) showed that this fragment was located at the 3' end of the integration. In addition to detecting the anticipated *EcoRV* restriction enzyme fragments (2.8 and 3.8 kb), all probes localized to a common approximately 8.5-kb *MscI* fragment. There are no *MscI* restriction enzyme sites within the PV-ZMGT32 transgene segment, therefore any fragments bearing transgenic segments are generated by digestion of surrounding maize flanking sequence DNA. This provided complementary evidence to phenotypic segregation data that only one functional transgenic locus is present in the NK603 event. Given the unit size of the PV-ZMGT32 transgene segment (approximately

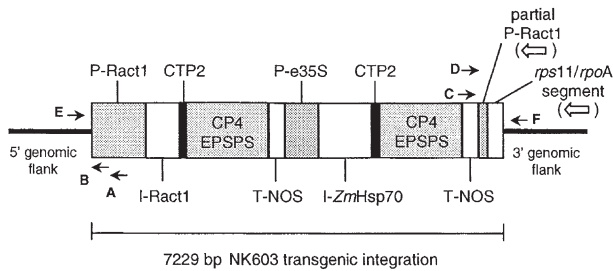


**Fig. 7.** Transgene stability at the NK603 integration site. Corn genomic DNA (10  $\mu\text{g}$ ) was digested with *EcoRV*, electrophoresed in a 0.8% (w/v) agarose gel, transferred to a nylon membrane, hybridized to a  $^{32}\text{P}$ -labeled probe from the CTP/CP4 EPSPS portion of PV-ZMGT32, and subjected to autoradiography. Individual lanes: 1, B73 nontransgenic control; 2, LH82  $\times$  NK603 B73 BC1; 3, NK603 B73 BC1F1; 4, LH82  $\times$  NK603 B73 BC2F3; 5, LH59  $\times$  NK603 B73 BC4F4. Molecular weight markers are indicated on the side of the autoradiogram (kb, kilobase pairs).

6.7 kb for both CP4 EPSPS cassettes combined), and presence of anticipated *EcoRV* fragments, only one intact copy is possible at the single locus defined by the approximately 8.5-kb *MscI* fragment.

Glyphosate tolerance segregated as anticipated for a single transgenic locus and was consistently expressed across multiple generations (Table 2). The trait stability was also assayed across generations at the DNA level by Southern analysis using genomic DNA isolated from multiple generations of the NK603 event digested with *EcoRV* (Fig. 7). Two hybridizing fragments were anticipated using a CP4 EPSPS probe (2.8 and 3.8 kb). Identical fragments were evident across this broad generational range and indicated that the transgene and surrounding genomic region was stable in the NK603 event.

Detailed structure of the NK603 transgenic integration site was determined by cloning and sequencing flanking genomic DNA bordering the 5' and 3' ends of the integration and alignment to the known transgene sequence of PV-ZMGT32. Using nested oligonucleotide primers for the 5' (oligonucleotides A and B within the P-Ract1 promoter, Fig. 8) and 3' (oligonucleotides C and D within the NOS transcriptional termination sequence, Fig. 8) ends, PCR products were generated that allowed sequence determination of 308 bp of 5' and 1019 bp of 3' sequence flanking the PV-ZMGT32 transgenic segment. While the sequence beyond the 5' end of the P-Ract1 promoter was found to be novel



**Fig. 8. Schematic diagram of NK603 transgenic locus.** The locus includes the 6711-bp *Mlu*I fragment from PV-ZMGT32 and cointegrated segments of the P-Ract1 and maize plastid genome. The sense orientation of these cointegrated sequences is indicated below each with an open arrow. Relative positions and orientation of the oligonucleotides used in flanking sequence characterization (A–F) are indicated by arrows.

corn genomic sequence, bioinformatics analysis at the 3' end revealed cointegrated segments of DNA from two sources, the PV-ZMGT32 transgene and the corn plastid genome. Neither segment was found to be native to the integration site when confirmatory sequencing of the wild-type insertion site was completed (see below). The presence of these sequences was likely the result of the complex process of double-strand break repair as the intended transgene was incorporated and sources of “filler” DNA were cointegrated at the site. Studies in a number of species have shown that it is common for various portions of the transgene and sequences derived from the host plant genome to be cointegrated whether the transformation is performed by direct DNA transfer (e.g., particle bombardment) or from *Agrobacterium* mediated methods (Kohli et al., 1998; Ohba et al., 1995; Salomon and Puchta, 1998).

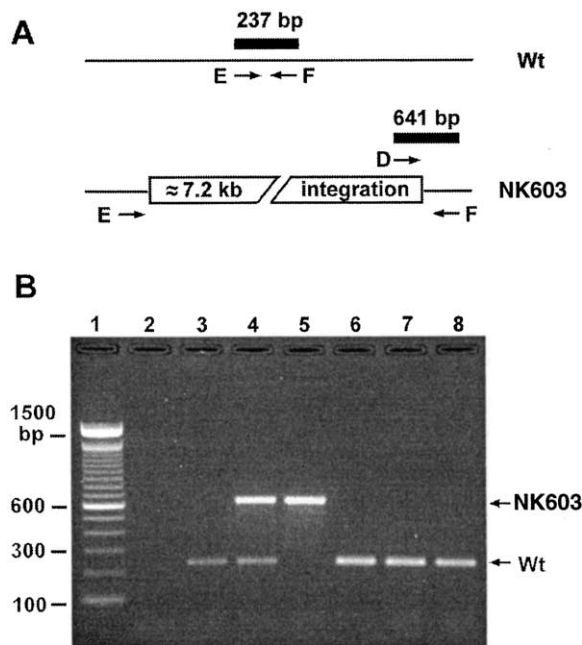
The DNA segment immediately adjacent to the 3' end of the double gene cassette in NK603 is a 217-bp inverted duplication of the 5' end of the cassette (corresponding to positions 150–366 of PV-ZMGT32). This includes polylinker sequence and the first 166 base pairs of P-Ract1 (positions –835 to –670 from the start of transcription as defined by McElroy et al., 1990). This segment of P-Ract1 does not contain the start of transcription or positive enhancers of gene expression, clarified in further experiments that progressively deleted promoter sequences or changed their orientation relative to the start of transcription (McElroy et al., 1990; Wang et al., 1992). On the basis of these findings, it is unlikely that the 166-bp portion P-Ract1 has any significant contribution to the transcriptional regulation of the CP4 EPSPS transgene or adjacent genomic sequences.

A second segment of cointegrated DNA with perfect identity to the *rpl23* gene cluster of the corn plastid genome (Rapp et al., 1992) is fused downstream of the duplicated portion of P-Ract1. The match spanned a 301-bp portion of the corn plastid open reading frames for *rps11* and *rpoA* (partial segments of each gene corresponding to bases 63–363 of GenBank accession X07810, with open reading frame orientation toward the transgene). As has been seen in a variety of plants such as wheat (*Triticum aestivum* L.), rice, barley (*Hordeum vulgare* L.), pea (*Pisum sativa* L.), beet (*Beta vulgaris* L.), and spinach (*Spinacia oleracea* L.) (Ayliffe et al., 1998;

Rice Chromosome 10 Sequencing Consortium, 2003), numerous portions of DNA of plastid origin lie within the nuclear genomes of higher plants. The sequence homology among the plastid segments can be high and many nuclear equivalents exist. Therefore, it is not possible to determine if the origin of the *rps11/rpoA* segment at the 3' end of the NK603 integration results from direct plastid genome transfer or incorporation of sequence from elsewhere in the nuclear genome during the process of transformation. As with the P-Ract1 promoter segment, the *rps11/rpoA* segment is not expected to be a significant contributor to regulation of the transgene or surrounding genomic DNA. First, it lacks the endogenous plastid promoter of the *rpl23* gene cluster and second, if cryptic regulatory sequences did exist, they would function poorly in a nuclear context (Cornelissen and Vandewiele, 1989; Scott et al., 1991).

Thus, the entirety of the NK603 transgenic integration is constituted by the *Mlu*I fragment from PV-ZMGT32, the 217 bp of duplicated 5' end of the PV-ZMGT32 transformation cassette, and 301 bp of the *rps11/rpoA* portion of the plastid *rpl23* gene cluster. Immediately downstream of the *rps11/rpoA* segment, 497 bp of true 3' flanking sequence is comprised of novel corn genomic DNA. To verify the 5' and 3' flanking sequences were correctly determined, the site of integration for the NK603 event was cloned from a nontransgenic corn genomic DNA. Using oligonucleotides flanking the transgenic integration (oligonucleotides E and F, Fig. 8), a 237-bp “wild-type” integration allele was amplified by PCR from the nontransgenic B73 inbred and sequenced. The sequence was an exact match to the flanking sequence determined on the respective sides of the integration, with the exception of 3 bp of the target site not represented in either the 5' or 3' cloned flanking genomic sequenced for the NK603 event. Thus, outside of the small deletion of the integration site, the colinearity of the corn genomic sequence surrounding the integration site and the nontransgenic genome was preserved in the NK603 event. The cloned site of integration also did not contain any native plastid sequence indicating that it was introduced during creation of the NK603 event. The understanding of the integration site was further demonstrated by the ability to perform a “zygosity test” with a multiplexed PCR reaction with oligonucleotides D, E, and F (Fig. 9). In the presence of the NK603 transgenic integration, a 641-bp NK603-specific amplicon was produced, whether a hemizygous or homozygous individual was examined. If the wild-type integration allele was present either in a nontransgenic inbred such as B73, a hemizygous NK603 individual, or a non-NK603 transgenic event, a 237-bp amplicon was evident (a long amplicon spanning the entire transgenic integration is not observed due to short amplification cycles, which bias production of smaller amplicons). All the predicted amplicons were observed when using this multiplexed assay.

In conclusion, the NK603 corn event is the product of an effort to reproduce the robust glyphosate tolerance found in other glyphosate-tolerant crops containing CP4 EPSPS. The evaluation program was developed to en-



**Fig. 9. NK603 zygosity test.** PCR based assay using three oligonucleotides D, E, and F. The schematic diagram (Panel A, not to scale) shows relative placement of oligonucleotides and the expected sizes of amplification products on non-transgenic (Wt) and NK603 templates. The assay was conducted on genomic DNA from a non-transgenic plant (Panel B, lane 3, B73 inbred), a plant hemizygous for the NK603 insertion (lane 4, B73 BC3 composition), a plant homozygous for the NK603 insertion (lane 5, B73 BC2S2 composition), or plants hemizygous for similar but independent transgenic events (Lane 6, event NK560; Lane 7, event NK561; Lane 8, event NK600). Lane 2 is a template-minus control reaction. Lane 1, 100-bp DNA ladder size standard.

sure selection of an event which surpasses tolerance requirements for weed control while embodying an excellent crop safety margin for application variability. The transgenic integration present in NK603 is a simple, single locus containing two complete CP4 EPSPS expression cassettes resulting in appropriate accumulation of CP4 EPSPS proteins. Minimal disruption of the endogenous corn genome has occurred during integration of the NK603 transgenic DNA. Additionally, NK603 has proven stable in molecular structure and trait expression over multiple generations and has proven effectual in commercial release during the 2001 through 2004 field seasons. Together with analyses of the phenotype via agronomic, compositional, and animal feedings studies, it was concluded that these molecular characterization data represent a comprehensive evaluation of Roundup Ready corn event NK603.

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