Application to Food Standards Australia New Zealand for the Inclusion of Maize MON 94804 in *Standard 1.5.2 – Food produced using Gene Technology*

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ABBREVIATIONS AND DEFINITIONS

AA	Amino Acid
aadA	Bacterial promoter, coding sequence, and 3′ UTR for an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase from the transposon Tn*7*
ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
AFSI CCDB	Agriculture and Food Systems Institute Crop Composition Database
APHIS	Animal and Plant Health Inspection Service
BIO	Biotechnology Innovation Organization
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
CPS	*ent*-copalyl diphosphate synthase
DNA	Deoxyribonucleic Acid
dsRNA	Double stranded Ribonucleic Acid
dw	Dry Weight
DWCF	Dry Weight Conversion Factor
E. coli	*Escherichia coli*
ent-CDP	*ent*copalyl diphosphate
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
ETS	Excellence Through Stewardship
FA	Fatty Acid
FDA	Food and Drug Administration
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act (US)
FFDCA	Federal Food, Drug and Cosmetic Act
FOIA	Freedom of Information Act
fw	Fresh weight
GA	Gibberellic acid/gibberellin
GA20ox	Gibberellic acid 20 oxidase
GE	Genetically engineered
GGDP	geranylgeranyl diphosphate
GLP	Good Laboratory Practices
GM	Genetically Modified
GRAS	Generally Recognized as Safe
HMW	High Molecular Weight
HSP70	Heat shock protein 70
ILSI	International Life Sciences Institute
KAO	*ent*-kaurenoic acid oxidase
kb	Kilobase
KO	*ent*-kaurene oxidase
KS	*ent*kaurene synthase
LMW	Low Molecular Weight

LOQ	Limit of Quantitation
mRNA	Messenger RNA
miRNA	Micro RNA
MOA	Mechanism of Action
NDF	Neutral Detergent Fiber
NGS	Next Generation Sequencing
nos	nopaline synthase
nt	nucleotide
OECD	Organization for Economic Cooperation and Development
ORF	Open Reading Frame
OSL	Over Season Leaf
OSR	Over Season Root
PBN	Premarket Biotechnology Notice
PCR	Polymerase Chain Reaction
Pdc3	pyruvate decarboxylase
PIP	Plant-Incorporated Protectant
RNA	Ribonucleic Acid
RNAi	RNA Interference
RSR	Regulatory Status Review
RTBV	Rice tungro bacilliform virus
SD	Standard Deviation
SE	Standard Error
siRNA	Small interfering RNA
sRNA	Small RNA
TDF	Total Dietary Fiber
T-DNA	Transfer-DNA
U.S.	United States
USDA	United States Department of Agriculture
UTR	Untranslated Region
v/v	Volume to volume ratio
w/v	Weight to volume ratio

PART 1 GENERAL INFORMATION

B Applicant details

(a) Applicant's name/s	Bayer CropScience Pty Ltd
(b) Name of contact person	[redacted]
(c) Address (street and postal)	Level 9, 109 Burwood Road, Hawthorn, Victoria 3122
(d) Telephone number	[redacted]
(e) Email address	[redacted]
(f) Nature of applicant's business	Technology provider to the agricultural and food industries
(g) Details of other individuals, companies or organisations associated with the application	

C Purpose of the application

This application is submitted to Food Standards Australia New Zealand by Bayer CropScience Pty Ltd on behalf of the Bayer Group.

The purpose of this submission is to make an application to vary **Standard 1.5.2** – *Food Produced Using Gene Technology* of the *Australia New Zealand Food Standards Code* to seek the addition of maize line MON 94804 and products containing maize line MON 94804 (hereafter referred to as MON 94804) to Schedule 26-3 Food produced using gene technology of plant origin (see below).

Commodity	Food derived from:
Corn	short stature corn line MON 94804

D Justification for the application

(a) The need for the proposed change

Bayer has developed short stature maize MON 94804 that contains a suppression cassette expressing an inverted repeat sequence designed to target endogenous maize *gibberellic acid 20 oxidase* (*GA20ox*) genes, *ZmGA20ox3* and *ZmGA20ox5*. The expressed inverted repeat transcript is recognized by the endogenous RNA interference (RNAi) machinery, resulting in downregulation of the targeted *GA20ox* gene expression. This suppression results in the reduction of gibberellic acid/gibberellin (GA) levels in the stalk, leading to a reduction of internode length and consequently reduced overall plant height compared to the conventional control maize.

(b) The advantages of the proposed change over the status quo, taking into account any disadvantages

Short stature MON 94804 maize is substantially equivalent to other conventional corns except the intended change of reduced plant height (short stature). Short stature wheat and rice were a cornerstone of the Green Revolution having a tremendous impact on worldwide food production, socioeconomic conditions, and environmental sustainability (Khush, 2001). Short stature maize MON 94804 was developed to enable growers to harness these benefits in maize (Paciorek *et al.*, 2022). MON 94804 can provide agronomic and environmental benefits, including reduced lodging and green snap, season-long crop access using standard ground equipment, and potential for improved environmental sustainability with more precise, well timed, and "as needed" mid to late season application of agrochemicals (*e.g.*, fungicide) and/or key nutrients (*e.g.*, nitrogen). This product is intended for cultivation in North America, South America, and potentially other key maize markets.

MON 94804 maize will be combined with other authorized biotechnology derived traits through traditional breeding methods to provide growers with products that offer protection against maize pests, herbicide tolerance, other traits offering broader grower choice, and the potential for improved yield protection.

D.1 Regulatory Impact Information

D.1.1 Costs and benefits of the application

If the proposed variation to permit the sale and use of food produced from MON 94804 is approved, possible affected parties may include consumers, industry sectors and government. The consumers who may be affected are those that consume food containing ingredients derived from maize. Industry sectors affected may be food importers and exporters, distributors andretailers, processors and manufacturers. Lastly, government enforcement agencies may be affected.

A cost/benefit analysis quantified in monetary terms is difficult to determine. In fact, most of the impacts that need to be considered cannot be assigned a dollar value. Criteria would need to be deliberately limited to those involving broad areas such as trade, consumer information and compliance. If the proposed variation is approved:

(a) The cost and benefits to the consumers e.g. health benefits:

- There would be benefits in the broader availability of maize products.
- There is unlikely to be any significant increase in the prices of foods if manufacturers are able to use comingled maize products.
- Consumers wishing to do so will be able to avoid GM maize products as a result of labeling requirements and marketing activities.

(b) The costs and benefits to industry and business in general, noting any specific effects on small businesses e.g. savings in production costs:

- Sellers of processed foods containing maize derivatives would benefit as foods derived from maize MON 94804 would be compliant with the Code, allowing broader market access and increased choice in raw materials. Retailers may be able to offer a broader range of maize products or imported foods manufactured using maize derivatives.
- Possible cost to food industry as some food ingredients derived from maize MON 94804 would be required to be labelled.

(c) the costs and benefits to government e.g. increased regulatory costs:

- Benefit that if maize MON 94804 was detected in food products, approval would ensure compliance of those products with the Code. This approval would ensure no potential for trade disruption on regulatory grounds.
- Approval of maize MON 94804 would ensure no potential conflict with WTO responsibilities.
- In the case of approved GM foods, monitoring is required to ensure compliance with the labeling requirements, and in the case of GM foods that have not been approved, monitoring is required to ensure they are not illegally entering the food supply. The costs of monitoring are thus expected to be comparable, whether a GM food is approved or not.

D.1.2 Impact on international trade

If the proposed variation to permit the sale and use of food produced from MON 94804 was rejected it would result in the requirement for segregation of any maize derived products containing MON 94804 from those containing approved maize or conventional maize, which would be likely to increase the costs of imported maize-derived foods.

It is important to note that if the proposed variation is approved, maize MON 94804 will not have a mandatory introduction. The consumer will always have the right to choose not to use/consume this product.

E Information to support the application

See Part 2.

F Assessment procedure

Bayer is submitting this application in anticipation that it will fall within the General Procedure category.

G Confidential commercial information (CCI)

Any CCI information has been identified as CCI and has been treated according to the FSANZ Application Handbook 2019.

H Other confidential information

Any non CCI information that we want to be treated as confidential has been treated according to the FSANZ Application Handbook 2019.

I Exclusive capturable commercial benefit

This application is likely to result in an amendment to the Code that provides exclusive benefits and therefore Bayer intends to pay the full cost of processing the application.

J International and other national standards

J.1 International standards

Bayer makes all efforts to ensure that safety assessments are aligned, as closely as possible, with relevant international standards such as the Codex Alimentarius Commission's *Principles for the Risk Analysis of Foods Derived from Modern Biotechnology* and supporting *Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants* (Codex Alimentarius, 2009).

In addition, the composition analysis is conducted in accordance with OECD guidelines and includes the measurement of OECD-defined maize nutrients and anti-nutrients based on conventional commercial maize varieties (OECD, 2002a).

J.2 Other national standards or regulations

Bayer has submitted a food and feed safety and nutritional assessment summary for MON 94804 to the United States Food and Drug Administration (FDA) and has also requested a Regulatory Status review (RSR) for a determination of plant pest risk potential of MON 94804, including all progenies derived from crosses between MON 94804 and conventional maize, or deregulated biotechnology-derived maize by the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA).

Consistent with our commitments to the Excellence Through Stewardship® (ETS) Program[1], regulatory submissions have been or will be made to countries that import significant maize or food and feed products derived from North and South America maize and have functional regulatory review processes in place.

[1] Excellence Through Stewardship is a registered trademark of Excellence Through Stewardship, Washington, DC. (http://www.excellencethroughstewardship.org)

K Statutory declaration

See Part 3.

L Checklist

The checklists can be found on page viii

PART 2 SPECIFIC DATA REQUIREMENTS FOR FOODS PRODUCED USING GENE TECHNOLOGY

A. TECHNICAL INFORMATION ON THE FOOD PRODUCED USING GENE TECHNOLOGY

A.1 Nature and Identity of the Genetically Modified Food

A.1(a) A description of the GM organism from which the new GM food is derived

Bayer has developed short stature maize MON 94804 that contains a suppression cassette expressing an inverted repeat sequence designed to target endogenous maize *gibberellic acid 20 oxidase* (*GA20ox*) genes, *ZmGA20ox3* and *ZmGA20ox5*. The expressed inverted repeat transcript is recognized by the endogenous RNA interference (RNAi) machinery, resulting in downregulation of the targeted GA20ox gene expression. This suppression results in the reduction of gibberellic acid/gibberellin (GA) levels in the stalk, leading to a reduction of internode length and consequently reduced overall plant height compared to the conventional control maize.

A.1(b) Name, line number and OECD Unique Identifier of each of the new lines or strains of GM organism from which the food is derived

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants" MON 94804 has been assigned the unique identifier MON-948Ø4-4.

A.1(c) The name the food will be marketed under (if known)

Maize containing the transformation event MON 94804 will be produced in North America, South America, and potentially other key maize markets. There are currently no plans to produce this product in Australia or New Zealand. A commercial trade name for the product has not been determined at the time of this submission and will be available prior to commercial launch of the product in North America.

A.2 History of Use of the Host and Donor Organisms

A.2(a) For the donor organism(s) from which the genetic elements are derived

A.2(a)(i) Any known pathogenicity, toxicity or allergenicity of relevance to the food

The sequence present in the *GA20ox_SUP* suppression cassette in MON 94804 maize contains a miRNA coding suppression cassette, *GA20ox_SUP*, that was designed with sequences from rice and maize to provide an inverted repeat sequence derived from coding sequences of *ZmGA20ox3* and *ZmGA20ox5* genes from *Zea mays* (maize). Considering the long history of safe consumption as food and detailed scientific assessment of maize, the pathogenic, toxic or allergenic risk of maize to humans is likely to be very low. There have been no reports on the productivity of toxic substances which have had a bad influence on human health in maize. Maize contains anti-nutrients such as phytic acid and other harmful substances such as raffinose (OECD, 2002a). Although trypsin inhibitor is also contained in maize, it is supposed that its content is low and will not become a problem nutritionally (OECD, 2002a). Maize is not considered a common allergenic food and there have been few reports of allergenic reactions to the consumption of maize products (OECD, 2002a). The rare studies reporting maize allergenicity generally involve patients with multiple allergies which complicate diagnosis due to potentially weak and irrelevant cross-reactivity exhibited by skin prick tests and *in-vitro* IgE binding evaluations (Pastorello *et al.*, 2000). Therefore maize is not regarded as a major allergy-inducing food (Tanaka *et al.*, 2001), and it is not listed as an allergenic food in any national and international food allergy labeling regulation (Allen *et al.*, 2014; Gendel, 2012; U.S. FDA, 2004).

The *GA20ox_SUP* suppression cassette in MON 94804 maize also contains sequences from *Oryza sativa* (rice) to provide the backbone structure of the initial transcript. Generally, rice is considered to be a safe source of food and is not considered to be a common allergic food. There are very few compounds in rice which are considered unfavourable for humans, and these compounds have not been observed to exist at levels in rice-based foods that would be a concern for food or feed safety (OECD, 2016). Considering the long history of safe use of rice as a food and feed (Khush, 1997), it would not pose pathogenic, toxic, or allergenic risks to humans.

The *GA20ox_SUP* DNA sequence in MON 94804 maize is under the regulation of the RTBV1 promoter and leader from the rice tungro bacilliform virus (RTBV) (Yin and Beachy, 1995), *Hsp70* intron and flanking exon sequence from *Zea mays* (maize) of the *hsp70* gene encoding the heat shock protein 70 (HSP70) (Rochester *et al.*, 1986). The *GA20ox_SUP* suppression cassette also utilizes the GST43 3′ UTR sequence developed from multiple 3′ UTR sequences from *Zea mays* (maize) (To *et al.*, 2021). RTBV is not known to caues pathogenic, toxic, or allergenic effects on humans.

The *cp4 epsps* coding sequence is under the regulation of the promoter, 5' UTR, and intron of the *OsRact1* gene from *Oryza sativa* (rice) encoding rice actin protein (Jeon *et al.*, 2000) and the *nos* 3′ UTR sequence from of the *nopaline synthase* (*nos*) gene from *Agrobacterium tumefaciens* pTi encoding NOS (Bevan *et al.*, 1983; Fraley *et al.*, 1983). The *cp4 epsps* expression cassette, which is flanked by *loxP* sites, was excised from progeny plants using the Cre/*lox* recombination system for marker removal as described in Section A.3 (Hare and Chua, 2002; Zhang *et al.*, 2003). *Agrobacterium* species are not known for human or animal pathogenicity and are not commonly allergenic (FAO-WHO, 1991; Mehrotra and Goyal, 2012; Nester, 2015). The history of safe use of the CP4 EPSPS protein from *Agrobacterium* sp. strain

CP4 has been previously reviewed regarding Roundup Ready® events of soybean, canola, maize, sugar beet, alfalfa, and cotton.

A.2(a)(ii) History of use of the organism in food supply or history of human exposure to the organism through other than intended food use (e.g. as a normal contaminant)

Maize, *Zea mays* is a crop plant with a long history of use as food and feed. It is one of the most important crops in the world serving as a primary food and feed source. For the 2021/2022 market year, values for U.S. domestic maize usage were 38% for feed and residual uses; 36% for fuel ethanol; 10% for food, seed, and industrial uses other than ethanol for fuel; and 17% for exports (McConnell *et al.*, 2022). Global demand for maize has increased due to greater meat consumption in emerging economic countries, including China, and biofuels production (Edgerton, 2009).

Maize is used extensively as a livestock feed for reasons that include its palatability, digestibility, and metabolizable energy (Loy and Lundy, 2019) and its relatively low cost (OECD, 2002a). Maize grain may be fed whole (Watson, 1988), but in many cases it is ground and mixed with other ingredients to provide a balanced ration (Leath and Hill, 1987). As reviewed by Loy and Lundy (2019), animal feed products from the wet milling process include maize gluten feed and maize gluten meal. Animal feed products from the dry milling process include hominy feed (Loy and Lundy, 2019). Ethanol production from dry milled maize provides distillers grains, another source of animal feed (Loy and Lundy, 2019). Maize can also be fed as a whole plant silage.

As described in A.2(a)(i), *Oryza sativa* (rice) (Maeda *et al.*, 2019) is a crop plant with a long history of use as food and feed. It is one of the most important crops in the world serving as a primary food source for more than one third of the world's population (Khush, 1997). Rice is consumed as milled rice, rice flour, and cooking oil (OECD, 2016). Rice is cultivated in more than 80 countries around the world (USDA-FAS, 2023)[2], being one of three major staple crops after maize and with a total production similar to wheat.

A.2(b) For the host organism into which the genes were transferred

A.2(b)(i) Its history of safe use for food

The host organism of MON 94804 is maize (*Zea mays*). Please see Section A.2(a)(ii) for the history of safe use for consumption of maize as food.

A.2(b)(ii) The part of the organism typically used as food

Foods derived from maize include whole grain, sweet corn, flour, starch, kibble, oil, corn flakes and syrup which are described in the next section.

A.2(b)(iii) The types of products likely to include the food or food ingredient

Food uses of maize include processed products from field maize and direct consumption of sweet maize and popcorn. Food products derived from the wet milling process include starch and sweetener products (e.g., high fructose maize syrup) (May, 1987). Food products derived from the dry milling process include maize grits, maize meal, and maize flour (Watson, 1988).

[2] https://apps.fas.usda.gov/psdonline/downloads/psd_grains_pulses_csv.zip

Maize oil may be derived from either milling process (Watson, 1988). The types of food derived from processed maize are described in detail in the next section.

A.2(b)(iv) Whether special processing is required to render food safe to eat

Maize grain processing has been reviewed by Watson (1988). On a dry weight basis, the main components of maize kernels are endosperm (83%), germ (11%), and pericarp (bran) (5%) (Watson, 1988). Milling separates the grain into these components, with subsequent products dependent on the milling type (Watson, 1988).

Products from wet milling: As reviewed by Raush *et al*. (2019), the products of the wet milling process include starch and sweeteners used in foodstuffs. Native or modified maize starch is used in a wide range of foods, including bakery products, puddings and custards, snack foods, salad dressings, meat products, prepared soups, and many others (Rausch *et al.*, 2019). Starch is also converted into a variety of sweetener products including high fructose maize syrup (Watson, 1988). The various sweeteners are also used in a wide range of foods, including bakery products, breakfast foods, desserts, prepared soups, canned fruits and juices and many others (Rausch *et al.*, 2019). In addition to starch and sweeteners, oil is obtained from the germ fraction that is separated during the wet milling process (Rausch *et al.*, 2019).

Products from dry milling: The products of the dry milling process include maize grits, maize meal, and maize flours, each of which is derived from the endosperm (Watson, 1988). The food uses of these products have been reviewed by Rooney and Serna-Saldiver (2003). Maize grits have the largest particles and have less than 1% oil content (Rooney and Serna-Saldivar, 2003). Grits are used in making breakfast cereals and snacks (Rooney and Serna-Saldivar, 2003), and are eaten in the U.S. as side dish (Watson, 1988). Maize meal has smaller particles than maize grits (Rooney and Serna-Saldivar, 2003). It is used in baked products like maize bread and muffins, and may be enriched with vitamins and minerals like thiamine, riboflavin, niacin, and iron (Rooney and Serna-Saldivar, 2003). Maize flour is made up of fine endosperm particles and has many uses as a food ingredient (e.g., in ready to eat snacks or pancake mixes) or binder (e.g., in processed meats) (Rooney and Serna-Saldivar, 2003). In addition to endosperm products, oil is obtained from the germ faction that is separated during the dry milling process (Watson, 1988).

Products from fermentation: Products from the wet and dry milling processes (e.g., corn syrups, grits) can also be used in producing distilled beverages through fermentation (Rooney and Serna-Saldivar, 2003; Watson, 1988).

A.3 The Nature of the Genetic Modification

Characterization of the genetic modification in MON 94804 maize was conducted using a combination of sequencing, PCR, and bioinformatics. The results of this characterization demonstrate that MON 94804 maize contains a single copy of the intended T-DNA containing the *GA20ox_SUP* suppression cassette that is stably integrated at a single locus and is inherited according to Mendelian principles of inheritance over multiple generations. These conclusions are based on the following lines of evidence:

- Molecular characterization of MON 94804 maize by NGS demonstrated that MON 94804 maize contained a single DNA insert. These whole genome sequence analyses provided a comprehensive assessment of MON 94804 maize to determine the presence of sequences derived from PV-ZMAP527892

transformation plasmid vector and demonstrated that MON 94804 maize contains a single DNA insert with no detectable backbone sequences from PV-ZMAP527892 or *cp4 epsps* selectable marker sequence or any sequences from the *cre* genecontaining transformation plasmid vector PV-ZMOO513642 (Section A.3(c)(ii)).

- Directed sequencing (locus-specific PCR, DNA sequencing and analyses) of MON 94804 maize, which characterized the complete sequence of the single DNA insert from PV-ZMAP527892, the adjacent flanking DNA, and the 5' and 3' insert-to-flank junctions. This analysis confirmed that the sequence and organization of the DNA insert is identical to the corresponding region in the PV-ZMAP527892 T-DNA. Directed sequencing also confirmed that the *cp4 epsps* selectable marker cassette which was excised, along with one *loxP* site, by Cre recombinase, is not present in MON 94804 maize. Furthermore, the genomic organization at the insertion site was assessed by comparing the sequences flanking the T-DNA insert in MON 94804 maize to the sequence of the insertion site in conventional maize. This analysis determined that no major DNA rearrangement occurred at the insertion site in MON 94804 maize upon DNA integration, although there was a 41 bp deletion that likely occurred upon T-DNA integration in MON 94804 maize (Section A.3(c)(iii)).

- Generational stability analysis by NGS demonstrated that the single PV-ZMAP527892 T-DNA insert in MON 94804 maize has been maintained through five breeding generations, thereby confirming the stability of the T-DNA in MON 94804 maize (Section A.3(e)(i)).

- Segregation data confirm that the inserted T-DNA segregated following Mendelian principles of inheritance, which corroborates the insert stability demonstrated by NGS and independently establishes the nature of the T-DNA at a single chromosomal locus (Section A.3(e)(ii)).

Taken together, the characterization of the genetic modification in MON 94804 maize demonstrates that a single copy of the T-DNA was stably integrated at a single locus of the maize genome and that no PV-ZMAP527892 plasmid vector backbone, *cp4 epsps* selectable marker, or any sequences from the *cre* gene-containing transformation plasmid vector PV-ZMOO513642 are present in MON 94804 maize.

A.3(a) A description of the method used to transform the host organism

MON 94804 maize was developed through *Agrobacterium tumefaciens*-mediated transformation of HCL301 maize mature seed embryo explants based on the method described by Ye *et al*. (2022) utilizing plasmid vector PV-ZMAP527892. Mature embryos were excised from a postpollinated maize ear of HCL301. After coculturing the excised mature embryos with *Agrobacterium tumefaciens* carrying the transformation plasmid vector PV-ZMAP527892, the embryo explants were placed on the first bud induction medium for a period of one week before transfer to the second bud induction medium containing glyphosate to inhibit the growth of untransformed plant cells. Explants after the second bud induction for two weeks were subsequently transferred to hormone free medium for shoot regeneration and rooting. The rooted plants (R0) with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

Leaf samples were collected from R0 plants. Genomic DNA was extracted from the leaf samples and used as templates for screening for the presence of T-DNA and absence of plasmid vector backbone sequences by realtime polymerase chain reaction (PCR) assays. The R0 plants carrying one copy of T-DNA were self-pollinated to produce R1 seed. Subsequently, the R1 population was screened for the presence of T-DNA and absence of plasmid vector backbone sequences by realtime PCR assays. Only plants that were homozygous positive for T-DNA and negative for plasmid vector backbone were selected for further development and their progenies were subjected to further molecular and phenotypic assessments. As is typical of a commercial event production and selection process, hundreds of different transformation events (regenerants) were generated in the laboratory using the transformation plasmid vector PV-ZMAP527892. Selected R2 events were crossed with a line expressing the Cre recombinase protein and screened for the absence of *cp4 epsps* and the *cre* genes using realtime PCR assays. After careful selection and evaluation of these events in the laboratory, greenhouse and field, MON 94804 maize was selected as the commercial event based on superior trait efficacy, agronomic, phenotypic, and molecular characteristics according to the general process described in Prado *et al*. (2014). Studies on MON 94804 maize were conducted to further characterize the genetic insertion and the expressed product, and to establish the food safety relative to conventional maize. The major steps involved in the development of MON 94804 maize are depicted in Figure 1. The result of this process was the production of MON 94804 maize with the *GA20ox_SUP* suppression cassette.

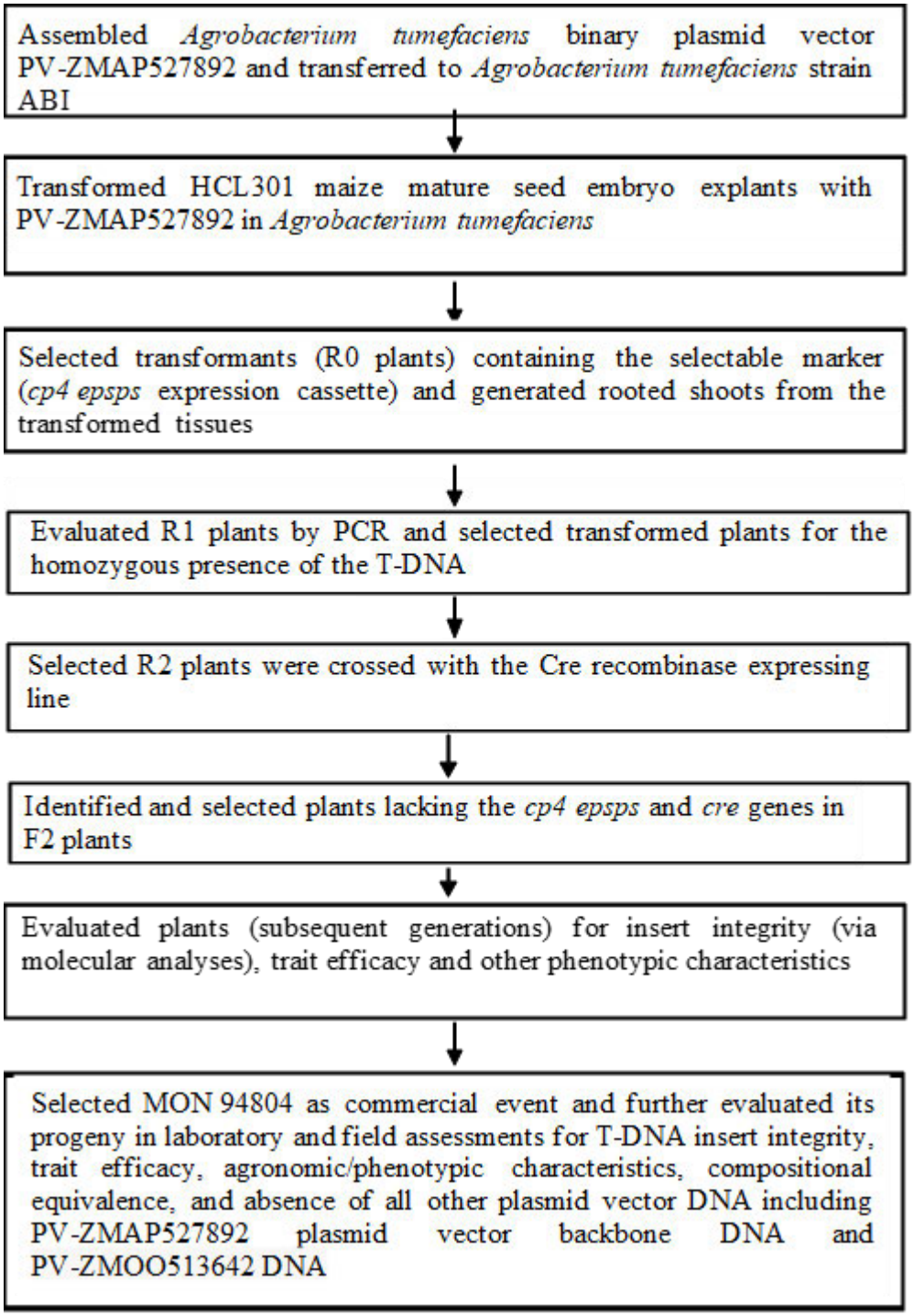


Figure 1. Schematic of the Development of MON 94804

A.3(b) A description of the construct and the transformation vectors used, including:

A.3(b)(i) The size, source and function of all the genetic components including marker genes, regulatory and other elements

Plasmid vector PV-ZMAP527892 was used for the transformation of conventional maize to produce MON 94804 maize and its plasmid vector map is shown in A.3(b)(ii). A description of the genetic elements and their prefixes (*e.g.*, B, P, I, TS, CS, T, and OR) in PV-ZMAP527892 is provided in Table 1. PV-ZMAP527892 is approximately 10.1 kb and contains a single T-DNA that is delineated by Left and Right Border regions. The T-DNA contains the *GA20ox_SUP* suppression cassette and the *cp4 epsps* selectable marker cassette. During transformation, the T-DNA was inserted into the maize genome. Following transformation, Cre/*lox* recombination through crossing with a Cre line, segregation, molecular screening and selection were used to isolate those plants that contained the *GA20ox_SUP* suppression cassette and did not contain the backbone sequences from the transformation plasmid vector PV-ZMAP527892, the *cp4 epsps* selectable marker cassette or any sequence from the *cre* gene containing plasmid vector PV-ZMOO513642.

The *GA20ox_SUP* DNA sequence in MON 94804 maize is under the regulation of the RTBV1 promoter and leader from the rice tungro bacilliform virus (RTBV) (Yin and Beachy, 1995) that directs transcription in plant cells. Additionally, the sequence is regulated by the *Hsp70* intron and flanking exon sequence from *Zea mays* (maize) of the *hsp70* gene encoding the heat shock protein 70 (HSP70) (Rochester *et al.*, 1986) and is involved in regulating gene expression (Brown and Santino, 1997). The *GA20ox_SUP* suppression cassette also utilizes the GST43 3′ UTR sequence developed from multiple 3′ UTR sequences from *Zea mays* (maize) (To *et al.*, 2021).

The *cp4 epsps* selectable marker cassette is also part of the originally inserted T-DNA in MON 94804 maize. The *cp4 epsps* coding sequence is under the regulation of the promoter, 5’ UTR, and intron of the *OsRact1* gene from *Oryza sativa* (rice) encoding rice actin protein (Jeon *et al.*, 2000) and the *nos* 3′ UTR sequence from of the *nopaline synthase* (*nos*) gene from *Agrobacterium tumefaciens* pTi encoding NOS (Bevan *et al.*, 1983; Fraley *et al.*, 1983) that direct transcription in plant cells. The *cp4 epsps* expression cassette, which is flanked by *loxP* sites, was excised from progeny plants using the Cre/*lox* recombination system for marker removal (Hare and Chua, 2002; Zhang *et al.*, 2003).

The backbone region of the transformation plasmid vector PV-ZMAP527892, located outside of the T-DNA, contains two origins of replication for maintenance of the plasmid vector in bacteria (*ori V*, *oripBR322*) and a bacterial selectable marker gene (*aadA*).

A.3(b)(i)(i) Marker Removal Through Cre/*lox* Recombination System

The use of the Cre/*lox* recombination system for marker removal has been previously described (Hare and Chua, 2002; Russell *et al.*, 1992; Zhang *et al.*, 2003). The Cre/*lox* recombination system is derived from the bacteriophage P1 and consists of the Cre recombinase and a stretch of DNA flanked by two copies of the *loxP* sites. The *loxP* site is 34bp in length and consists of two 13bp inverted repeats and an asymmetrical 8bp spacer. The 13bp inverted repeats are the Cre recombinase binding sequence, and the 8bp spacer is essential for the recombination reaction. Cre recombinase binds to the inverted repeat sequence in the *loxP* site, catalyzing a crossover in the 8bp spacer regions of the two *loxP* sites. The results of this crossover are twofold: one is the excision of the DNA fragment flanked by the two half *loxP* sites (in

MON 94804 maize, this is the *cp4 epsps* selectable marker cassette) forming a circular extragenomic DNA fragment, the other is the recombination of linear DNA between the remaining two half *loxP* sites within the maize genome (Gilbertson, 2003).

As reviewed by Gilbertson (2003), one of the advantages of the Cre/*lox* recombination system is the specificity of the enzyme for the wildtype *loxP* 34bp recognition sequence. The frequency of Cre recombinase-mediated DNA recombination can be significantly reduced with even a single nucleotide change in specific regions of the *loxP* sequence (Hartung and Kisters-Woike, 1998; Hoess *et al.*, 1986; Lee and Saito, 1998). Therefore, neither the specific DNA insert, nor the usage of the Cre/*lox* recombination system was expected to negatively influence the stability of the T-DNA in MON 94804 maize across breeding generations, which has been confirmed, and is described in Section A.3 of this submission. This technology was previously reviewed by FSANZ as part of the Food and Feed Safety Assessment for LY038 maize (A549, 2006), and most recently as part of the review of Lepidopteran Protected Maize MON 95379 (A1226, 2022) and Coleoptreran-Protected Maize MON 95275 (A1262).

A maize line expressing Cre recombinase (developed with the *cre* genecontaining plasmid vector PV-ZMOO513642) was crossed with lines transformed with the transformation plasmid vector PV-ZMAP527892. In the resulting hybrid plants, the *cp4 epsps* selectable marker cassette that was flanked by the *loxP* sites was excised. The excised *cp4 epsps* selectable marker cassette (circular extragenomic DNA) was subsequently not maintained during cell division. The *cre* gene and associated genetic elements were subsequently segregated away from the *GAox20_SUP* suppression cassette by conventional breeding to produce the MON 94804 maize product lacking the *cp4 epsps* selectable marker cassette. The absence of both the *cp4 epsps* selectable marker cassette and *cre* gene were confirmed in the generation prior to the one used to initiate commercial breeding of MON 94804 (Figure 2). Since the *cp4 epsps* selectable marker cassette and sequence derived from the *cre* genecontaining plasmid vector PV-ZMOO513642 were eliminated through conventional breeding, the resulting progeny only contain the gene of interest (*GA20ox_SUP*) but not the genes used for selection or marker excision.

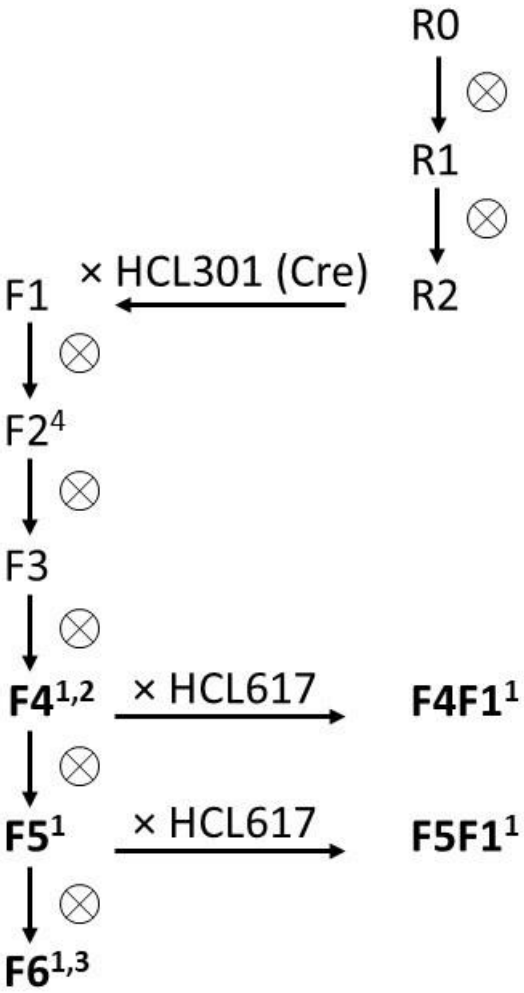


Figure 2. Breeding History of MON 94804 Maize

The generations used for molecular characterization and insert stability analyses are indicated in bold text. R0 corresponds to the transformed plant, ⊗ designates selfpollination.

[1] Generations used to confirm insert stability
[2] Generation used for molecular characterization
[3] Generation used for commercial development of MON 94804 maize
[4] The F2 generation was screened for plants absent of the *cre* gene. Only those plants absent of the *cre* gene were self-pollinated to create a *cre* free F3 generation

Table 1. Summary of Genetic Elements in PV-ZMAP527892

Genetic Element	Location in Plasmid Vector	Function (Reference)
T-DNA		
B[1]-Left Border Region	1-442	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker *et al.*, 1983)
Intervening sequence	443-509	Sequence used in DNA cloning
***lox*P**	510-543	Sequence from Bacteriophage P1 for the *loxP* recombination site recognized by the Cre recombinase (Russell *et al.*, 1992)
Intervening sequence	544-557	Sequence used in DNA cloning
P[2]-*Ract1*	558-1478	Promoter and leader of the *act1* gene from *Oryza sativa* (rice) encoding the rice Actin 1 protein (McElroy *et al.*, 1990) that directs transcription in plant cells
I[3]-*Ract1*	1479-1956	Intron and flanking UTR sequence of the *act1* gene from *Oryza sativa* (rice) encoding rice Actin 1 protein (McElroy *et al.*, 1990) that is involved in regulating gene expression
Intervening sequence	1957-1965	Sequence used in DNA cloning
TS[4]-*CTP2*	1966-2193	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann, 1995; Klee *et al.*, 1987)
CS[5]-*cp4 epsps*	2194-3561	Coding sequence of the *aroA* gene from *Agrobacterium tumefaciens* sp. strain CP4 encoding the CP4 EPSPS protein that provides herbicide tolerance (Barry *et al.*, 2001; Padgette *et al.*, 1996)
Intervening sequence	3562-3576	Sequence used in DNA cloning
T[6]-*nos*	3577-3829	3' UTR sequence of the *nopaline synthase (nos)* gene from *Agrobacterium tumefaciens* pTi encoding NOS that directs polyadenylation (Bevan *et al.*, 1983; Fraley *et al.*, 1983)
Intervening sequence	3830-3850	Sequence used in DNA cloning
loxP	3851-3884	Sequence from Bacteriophage P1 for the *loxP* recombination site recognized by the Cre recombinase (Russell *et al.*, 1992)
Intervening sequence	3885-4039	Sequence used in DNA cloning
P-*RTBV1*	4040-4805	Promoter and leader from the rice tungro bacilliform virus (RTBV) (Yin and Beachy, 1995) that directs transcription in plant cells
Intervening sequence	4806-4825	Sequence used in DNA cloning

Genetic Element	Location in Plasmid Vector	Function (Reference)
I-*Hsp70*	4826-5629	Intron and flanking exon sequence of the *hsp70* gene from *Zea mays* (maize) encoding the heat shock protein 70 (HSP70) (Rochester *et al.*, 1986) that is involved in regulating gene expression (Brown and Santino, 1997)
GA20ox_SUP	5630-6037	Sequence composed of an inverted repeat (Plasmid location: 57425762 and 57975817) derived from coding sequence of *ZmGA20ox3* and *ZmGA20ox5* genes from *Zea mays* (maize) that encodes the gibberellic acid 20 oxidase 3 and 5 proteins (Song *et al.*, 2011), flanked and separated by three OsamiR1425 fragments (Plasmid location: 56305741, 57635796, 58186037) from *Oryza sativa* (rice) (Lacombe *et al.*, 2008)
Intervening sequence	6038-6069	Sequence used in DNA cloning
T-*GST43*	6070-6369	A 3′ UTR that has been developed from multiple 3′ UTR sequences from *Zea mays* (maize) (To *et al.*, 2021) that directs polyadenylation of the mRNA
Intervening sequence	6370-6486	Sequence used in DNA cloning
B-Right Border Region	6487-6817	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T–DNA (Depicker *et al.*, 1982)
Plasmid Vector Backbone		
Intervening sequence	6818-6961	Sequence used in DNA cloning
aadA	6962-7850	Bacterial promoter, coding sequence, and 3′ UTR for an aminoglycosidemodifying enzyme, 3"(9)–*O*–nucleotidyltransferase from the transposon Tn*7* (Fling *et al.*, 1985) that confers spectinomycin and streptomycin resistance
Intervening sequence	7851-8384	Sequence used in DNA cloning
OR[7]-ori-pBR322	8385-8973	Origin of replication from plasmid pBR322 for maintenance of plasmid in *Escherichia coli* (*E. coli*) (Sutcliffe, 1979)
Intervening sequence	8974-9595	Sequence used in DNA cloning
OR-*ori V*	9596-9992	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium tumefaciens* (Stalker *et al.*, 1981)
Intervening sequence	9993-1007	Sequence used in DNA cloning

[1] B, Border
[2] P, Promoter
[3] I, Intron
[4] TS, Targeting Sequence
[5] CS, Coding Sequence
[6] T, Transcription Termination Sequence
[7] OR, Origin of Replication

A.3(b)(i)(ii) The *GA20ox_SUP* Coding Sequence

The sequence present in the *GA20ox_SUP* suppression cassette in MON 94804 maize expresses an miRNA coding sequence, *GA20ox_SUP*, that was designed with sequences from rice to provide the backbone structure of the initial transcript and sequences from maize to provide an inverted repeat sequence derived from coding sequences of *ZmGA20ox3* and *ZmGA20ox5* genes. The expressed inverted repeat transcript is recognized by the endogenous RNAi machinery, resulting in downregulation of the endogenous GA biosynthetic genes, *ZmGA20ox3* and *ZmGA20ox5* (Paciorek *et al.*, 2022). This suppression results in the reduction of bioactive GA levels in the stalk, leading to a reduction of internode length and consequently reduced overall plant height compared to the conventional control maize.

A.3(b)(i)(iii) Regulatory Sequences

The T-DNA contains the *GA20ox_SUP* suppression cassette and initially contained the *cp4 epsps* selectable marker cassette, each with their own regulatory sequences. The regulatory sequences (promoters, introns, transcription terminators) associated with these cassettes are described in Section A.3(b)(i) and Table 1.

A.3(b)(i)(iv) T-DNA Border Regions

PV-ZMAP527892 contains Left and Right Border regions (Figure 3 and Table 1) that were derived from *Agrobacterium tumefaciens* plasmid. The border regions each contain a nick site that is the site of DNA exchange during transformation (Barker *et al.*, 1983; Depicker *et al.*, 1982; Zambryski *et al.*, 1982). The border regions separate the T-DNA from the plasmid vector backbone region and are involved in the efficient transfer of T-DNA into the maize genome.

A.3(b)(i)(v) Genetic Elements Outside the T-DNA Border Regions

Genetic elements that exist outside of the T-DNA border regions are those that are essential for the maintenance or selection of PV-ZMAP527892 in bacteria and are referred to as plasmid vector backbone. The origin of replication, *ori V*, is required for the maintenance of the plasmid vector in *Agrobacterium tumefaciens* and is derived from the broad host range plasmid RK2 (Stalker *et al.*, 1981). The origin of replication, *oripBR322*, is required for the maintenance of the plasmid vector in *Escherichia coli (E. coli)* and is derived from the plasmid pBR322 (Sutcliffe, 1979). The selectable marker *aadA* is the coding sequence for an aminoglycosidemodifying enzyme, 3"(9)–O–nucleotidyltransferase from the transposon Tn7 (Fling *et al.*, 1985) that confers spectinomycin and streptomycin resistance in *E. coli* and *Agrobacterium tumefaciens* during molecular cloning. Because these elements are outside the border regions, they are not expected to be transferred into the maize genome. The absence of the backbone and other unintended plasmid vector sequence in MON 94804 maize was confirmed by sequencing and bioinformatic analyses (Section A.3(b)(i)).

A.3(b)(ii) A detailed map of the location and orientation of all genetic elements contained within the construct and vector, including the location of relevant restriction sites

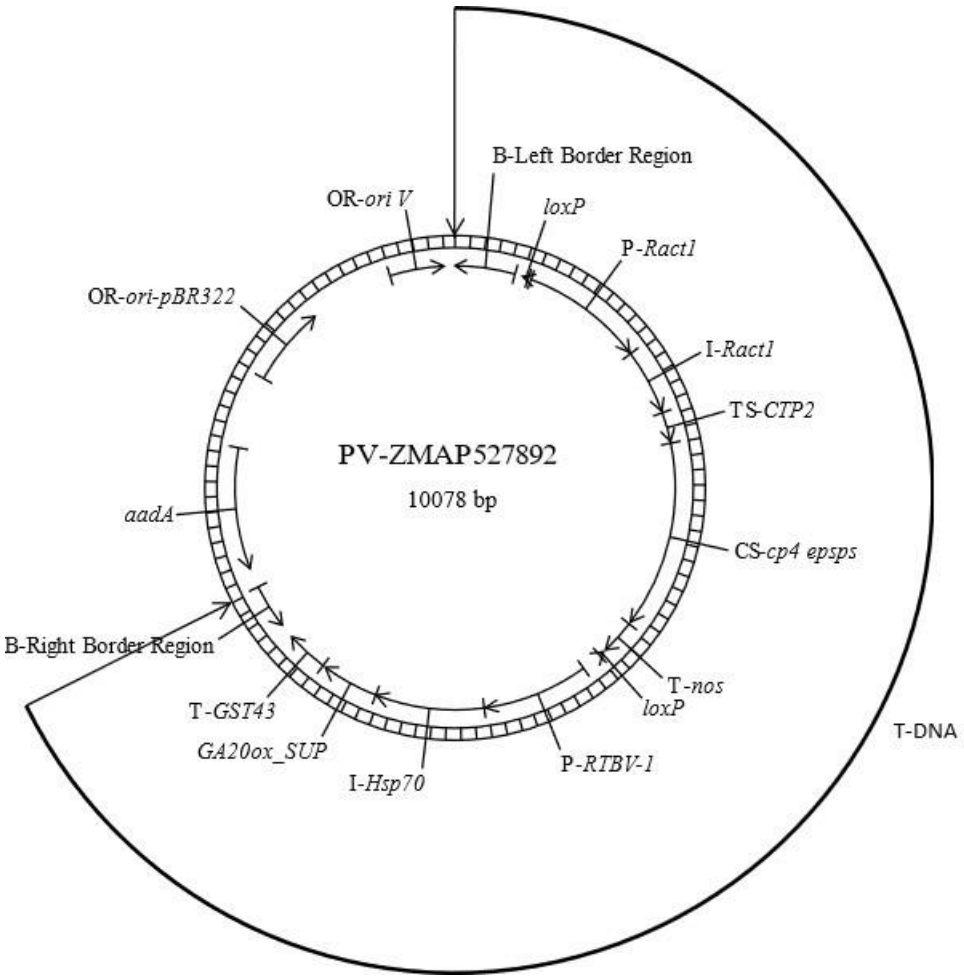


Figure 3. Circular Map of PV-ZMAP527892

A circular map of PV-ZMAP527892 used to develop MON 94804 maize is shown. PV-ZMAP527892 contains one T–DNA. Genetic elements are shown on the exterior of the map.

A.3(c) A full molecular characterisation of the genetic modification in the new organism, including:

A.3(c)(i) Identification of all transferred genetic material and whether it has undergone any rearrangements

This section describes the methods and results of a comprehensive molecular characterization of the genetic modification present in MON 94804 maize. It provides information on the DNA insertion(s) into the plant genome of MON 94804 maize, and additional information regarding the arrangement and stability of the introduced genetic material. The information provided in this section addresses the relevant factors in the Codex Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants, Section 4, paragraphs 30, 31, 32, and 33 (Codex Alimentarius, 2009).

Description of Methodology Used to Characterize MON 94804 Maize

A schematic representation of the Next Generation Sequencing (NGS) methodology and the basis of the characterization using NGS and PCR sequencing are illustrated in Figure 4 below.

For details, please refer to Appendix 1.

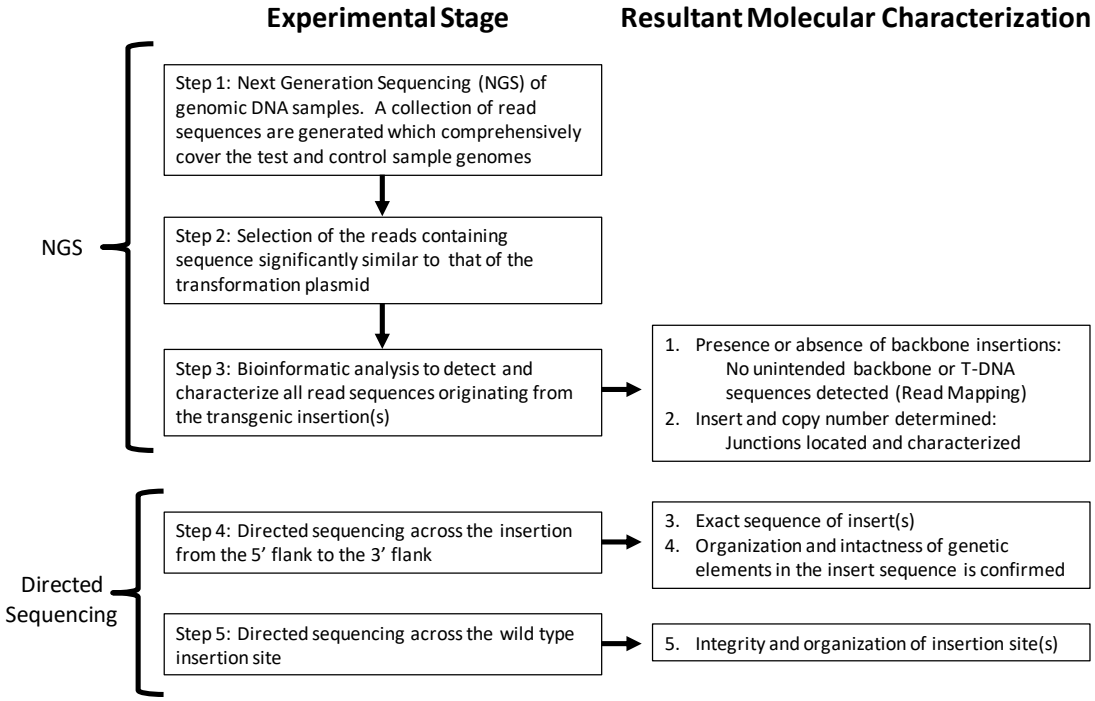


Figure 4. Molecular Characterization Using Sequencing and Bioinformatics

Genomic DNA from the MON 94804 maize and the conventional control maize was sequenced using technology that produces a set of short, randomly distributed sequence reads that comprehensively cover test and control genomes (Step 1). Utilizing these genomic sequence reads, bioinformatics searches are conducted to identify all sequence reads that are significantly similar to the transformation plasmid vector (Step 2). These captured reads are then mapped and analyzed to determine the presence/absence of transformation plasmid vector backbone sequences, identify insert junctions, and to determine the insert and copy number (Step 3). Overlapping PCR products are also produced which span any insert and their wild type locus (Step 4 and Step 5, respectively); these overlapping PCR products are sequenced to allow for detailed characterization of the inserted DNA and insertion site.

The whole genome sequencing and bioinformatic strategy used to determine the number of insertion loci and the number of copies of the integrated plasmid vector DNA was designed to ensure that all transgenic segments present in the genome were identified. Genomic DNA from MON 94804 maize and the conventional controls were used to generate short (~150 bp), randomly distributed, sequence fragments (sequencing reads) in sufficient numbers to yield comprehensive coverage of the maize genomes. The depth of coverage (the mean number of times each base of the genome is independently sequenced) was ≥75× for each generation that was evaluated. It has previously been reported that 75× genome coverage is adequate to provide comprehensive coverage and detection of all inserted DNA (Kovalic *et al.*, 2012). In a comprehensive analysis of NGS, as a characterization method, it has been shown that at levels of coverage as low as 11× it is possible to detect both intended transgenes as well as unintended inserted fragments as small as 25 bp in length (Cade *et al.*, 2018). This makes 75× coverage a robust level of sequencing for the accurate characterization of both homozygous and

hemizygous transgenes, and well in excess of the levels which have been demonstrated as capable of identifying unintended inserted fragments. The level of sensitivity achieved in this study was sufficient to detect 100% of the plasmid vector sequence when sampled at both a 1 genome equivalent and a $1/10^{th}$ of a copy per genome equivalent.

The number of DNA inserts of MON 94804 maize was determined by mapping of sequencing reads relative to the transformation plasmid vector and identifying junctions and unpaired read mappings adjacent to the junctions. Examples of five types of NGS reads are shown in Figure 5. The junctions of the DNA insert and the flanking DNA are unique for each insertion (Kovalic *et al.*, 2012). Therefore, insertion sites can be recognized by analyzing for sequence reads containing such junctions.

Directed sequencing (locus-specific PCR and DNA sequencing analyses, Figure 4, Step 4) complements the NGS method. Sequencing of the insert and flanking genomic DNA determined the complete sequence of the insert and flanks by evaluating if the sequence of the insert was identical to the corresponding sequence from the T-DNA in PV-ZMAP527892, and if each genetic element in the insert was intact. It also characterizes the flank sequence beyond the insert corresponding to the genomic DNA of the transformed maize. Results are described in Section A.3(c)(ii) and A.3(c)(iii).

For details, please refer to Appendix 1.

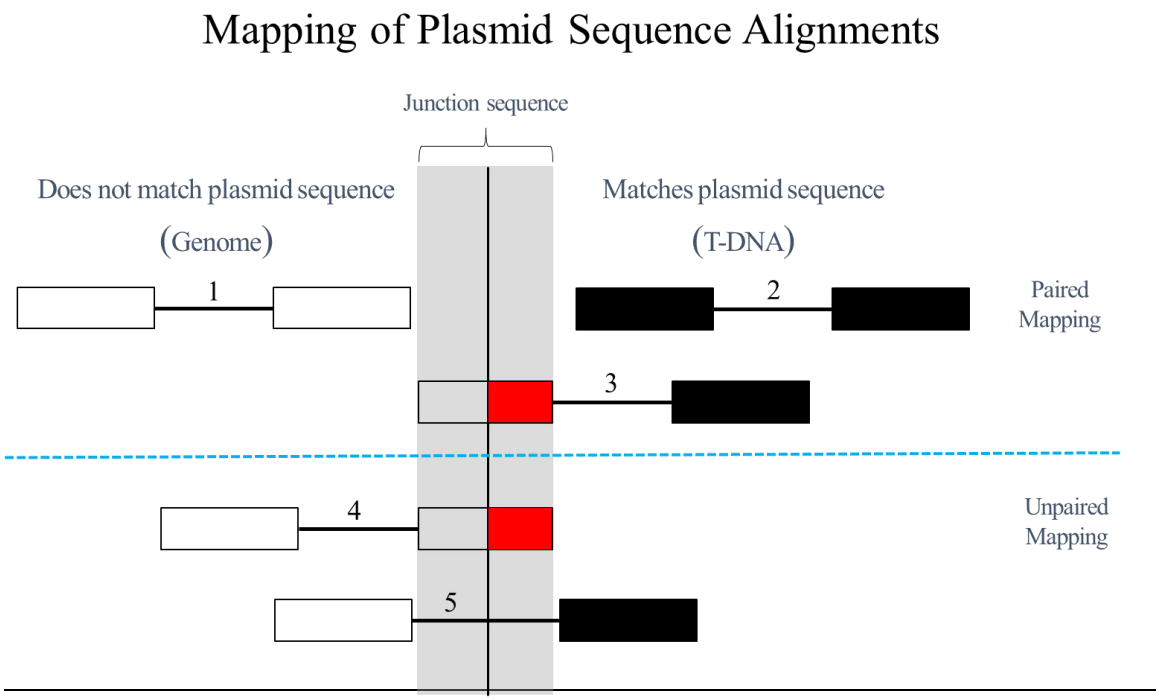


Figure 5. Five Types of NGS Reads

Next Generation Sequencing (NGS) yields data in the form of read pairs where sequence from each end of a size selected DNA fragment is returned. Depicted above are five types of sequencing reads/read pairs generated by NGS sequencing which can be found spanning or outside of junction points. Sequence boxes are color filled if it matches with plasmid vector sequence, and empty if it matches with genomic sequence. Grey highlighting indicates sequence reads spanning the junction. Junctions are detected by examining the NGS data for reads having portions of plasmid vector sequences that span less than the full read, as well as reads mapping adjacent to the junction points where their mate pair does not map to the plasmid vector sequence. The five types of sequencing reads/read pairs being (1) Paired and unpaired reads mapping to genomic sequence outside of the insert, greater than 99.999% of collected reads fall into this category and are not evaluated in this analysis, (2) Paired reads mapping entirely to the transformation plasmid vector sequence, such reads reveal the presence of transformation sequence in planta, (3) Paired reads where one read maps entirely within the inserted DNA and the other read maps partially to the insert (indicating a junction point), (4) Single read mapping partially to the transformation plasmid vector DNA sequence (indicating a junction point) where its mate maps entirely to the genomic flanking sequence and (5) Single read mapping entirely to the transformation plasmid vector DNA sequence where its mate maps entirely to genomic flanking sequence, such reads are part of the junction signature.

A.3(c)(ii) A determination of number of insertion sites, and the number of copies at each insertion site

The number of inserted DNA sequences from the transformation plasmid vector PV-ZMAP527892 in MON 94804 maize was assessed by generating a comprehensive

collection of reads via NGS of MON 94804 maize genomic DNA using the F4 generation (Figure 2). A plasmid vector map of PV-ZMAP527892 is depicted in Figure 3 and Table 2shows elements present in MON 94804 maize. A schematic representation of the insert and flanking sequences in MON 94804 maize is shown in Figure 6. For full details on materials and methods see Appendix 1.

A.3(c)(ii)(i) Next Generation Sequencing for MON 94804 Maize and Conventional Control Genomic DNA

Genomic DNA from five breeding generations of MON 94804 maize (Figure 2) and conventional controls were isolated from seed and prepared for sequencing. For material and method details see Appendix 1. These genomic DNA libraries were used to generate short (~150 bp) randomly distributed sequencing reads of the maize genome (Figure 4, Step 1).

To demonstrate sufficient sequence coverage the ~150 bp sequence reads were analyzed by mapping all reads to a known single copy endogenous gene (*Zea mays* pyruvate decarboxylase (*pdc3*), GenBank Accession: AF370006.2) in each of the five breeding generations. The analysis of sequence coverage plots showed that the depth of coverage (*i.e.*, the mean number of times any base of the genome is expected to be independently sequenced) was 75× or greater for the five generations of MON 94804 maize (F4, F4F1, F5, F5F1, F6) and the conventional control (Appendix 1). It has been previously demonstrated that whole genome sequencing at 75× depth of coverage provides comprehensive coverage and ensures detection of inserted DNA (Cade *et al.*, 2018; Kovalic *et al.*, 2012).

To demonstrate the method's ability to detect any sequences derived from the PV-ZMAP527892 transformation plasmid vector or the *cre* gene-containing transformation plasmid vector PV-ZMOO513642, samples of PV-ZMAP527892 or PV-ZMOO513642 were sequenced by NGS following the same processes outlined for all samples. The resulting reads were randomly selected to achieve a depth of one genome equivalent (relative to the mean coverage of the HCL301 conventional control), and 100% coverage of the known PV-ZMAP527892 and PV-ZMOO513642 sequences was observed. In addition, the PV-ZMAP527892 and PV-ZMOO513642 (majority of samplings for PV-ZMOO513642) plasmid sequences are shown to be amenable to sequencing and display no holes in coverage even when sampled at $1/10^{th}$ of a genomic equivalent (Appendix 1). This result demonstrates that all nucleotides of PV-ZMAP527892 and PV-ZMOO513642 sequences are detectable by the sequencing and bioinformatic assessments performed and that a detection level of at least $1/10^{th}$ genome equivalent was achieved for the plasmid vector DNA sequence assessment.

Table 2. Summary of Genetic Elements in MON 94804 Maize

Genetic Element	Location in Sequence	Function (Reference)
5′ Flanking DNA	1-1000	DNA sequence flanking the 5’ end of the insert
B[1]-Left Border Region	1001-1040	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T–DNA (Barker *et al.*, 1983)
Intervening sequence	1041-1107	Sequence used in DNA cloning
loxP	1108-1141	Sequence from Bacteriophage P1 for the *loxP* recombination site recognized by the Cre recombinase (Russell *et al.*, 1992)
Intervening sequence	1142-1296	Sequence used in DNA cloning
P[2]-*RTBV-1*	1297-2062	Promoter and leader from the rice tungro bacilliform virus (RTBV) (Yin and Beachy, 1995) that directs transcription in plant cells
Intervening sequence	2063-2082	Sequence used in DNA cloning
I[3]-*Hsp70*	2083-2886	Intron and flanking exon sequence of the *hsp70* gene from *Zea mays* (maize) encoding the heat shock protein 70 (HSP70) (Rochester *et al.*, 1986) that is involved in regulating gene expression (Brown and Santino, 1997)
GA20ox_SUP	2887-3294	Sequence composed of an inverted repeat (Plasmid location: 5742-5762 and 5797-5817) derived from coding sequence of *ZmGA20ox3* and *ZmGA20ox5* genes from *Zea mays* (maize) that encodes the gibberellic acid 20 oxidase 3 and 5 proteins (Song *et al.*, 2011) , flanked and separated by three Osa-miR1425 fragments (Plasmid location: 5630-5741, 5763-5796, 5818-6037) from *Oryza sativa* (rice) (Lacombe *et al.*, 2008)
Intervening sequence	3295-3326	Sequence used in DNA cloning
T[4]-*GST43*	3327-3626	A 3′ UTR that has been developed from multiple 3′ UTR sequences from *Zea mays* (maize) (To *et al.*, 2021) that directs polyadenylation of the mRNA
Intervening sequence	3627-3733	Sequence used in DNA cloning
3′ Flanking DNA	3734-4733	DNA sequence flanking the 3’ end of the insert

[1] B, Border
[2] P, Promoter
[3] I, Intron
[4] T, Transcription Termination Sequence

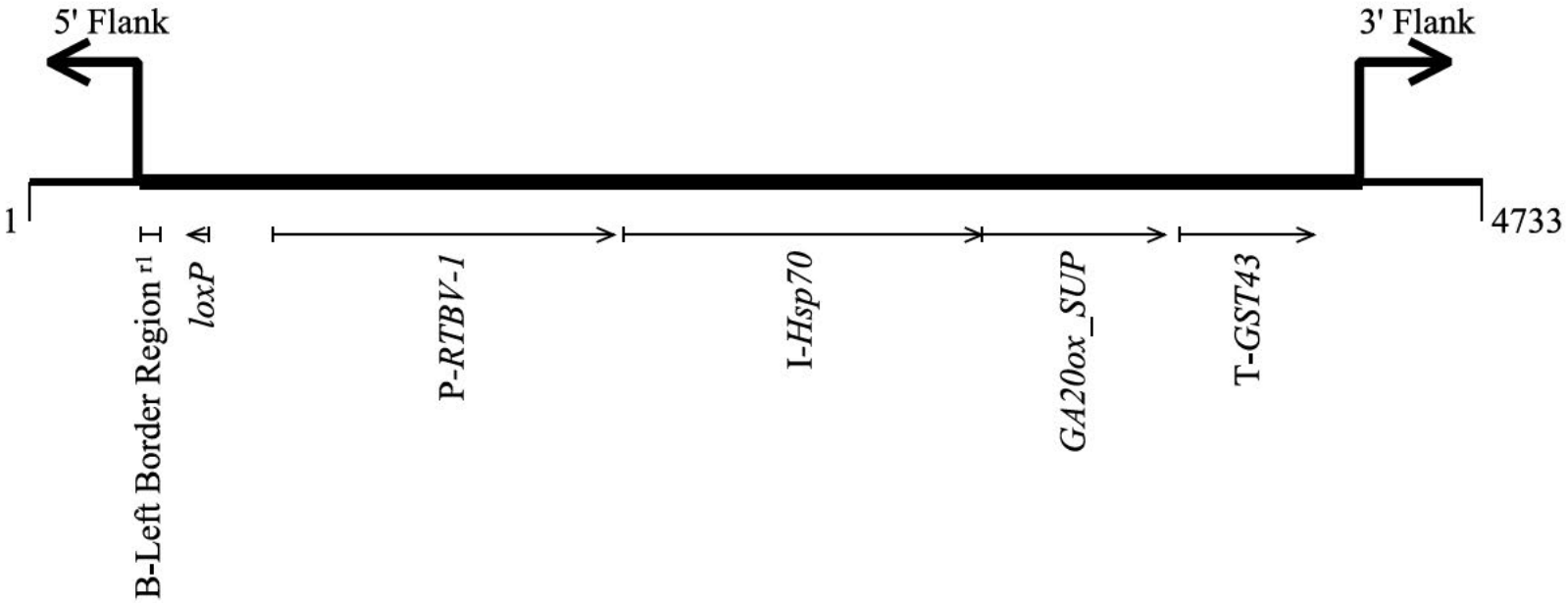


Figure 6. Schematic Representation of the Insert and Flanking Sequences in MON 94804 Maize

DNA derived from T-DNA of PV-ZMAP527892 integrated in MON 94804 maize. Right-angled arrows indicate the ends of the integrated T-DNA and the beginning of the flanking sequence. Identified on the map are genetic elements within the insert. This schematic diagram may not be drawn to scale.

R1 Superscript in the Left Border Region indicates that the sequence in MON 94804 maize was truncated compared to the sequences in PV-ZMAP527892.

A.3(c)(ii)(ii) Selection of Sequence Reads Containing Sequence of the PV-ZMAP527892 and PV-ZMOO513642

PV-ZMAP527892 was transformed into the parental inbred HCL301 to produce MON 94804 maize. Consequently, any DNA inserted into MON 94804 maize will consist of sequences that are similar to the PV-ZMAP527892 DNA sequence. Therefore, to fully characterize the DNA from PV-ZMAP527892 inserted in MON 94804 maize, it is sufficient to completely analyze only the sequence reads that have similarity to PV-ZMAP527892 (Figure 4, Step 2). Similarly, to confirm the absence of the Cre-containing transformation plasmid vector PV-ZMOO513642 or any sequences from this plasmid in MON 94804, it is sufficient to completely analyze only the sequence reads that have similarity to PV-ZMOO513642.

Using established criteria (described in the materials and methods, Appendix 1), sequence reads similar to PV-ZMAP527892 or PV-ZMOO513642 were selected from MON 94804 maize sequence datasets (PV-ZMOO513642 mapping was only conducted on the F4 generation) and were then used as input data for bioinformatic junction sequence analysis. PV-ZMAP527892 and PV-ZMOO513642 sequences were also compared against the conventional control sequence dataset.

A.3(c)(ii)(iii) Determination of T-DNA Copy Number and Presence or Absence of Selectable Marker Cassette and Plasmid Vector Backbone

Mapping sequence reads relative to the transformation plasmid vector allows for the identification of junction signatures, the presence or absence of plasmid vector backbone sequence and the number of T-DNA insertions. For a single copy T-DNA insert sequence at a single genomic locus and the complete absence of plasmid vector backbone, a single junction signature pair and few if any reads aligning with the transformation plasmid vector backbone sequences are expected.

When reads from the control HCL301 dataset were aligned with the transformation plasmid vector sequence, large numbers of reads mapped to the PV-ZMAP527892 transformation plasmid vector sequence elements P-*Ract1* and I-*Hsp70* sequence Figure 7). The alignment of these sequence reads is the result of endogenous maize sequences that are homologous to the PV-ZMAP527892 transformation plasmid vector elements P-*Ract1* and I-*Hsp70* sequence, which is expected.

When reads from the MON 94804 maize (F4) dataset were aligned with the transformation plasmid vector sequence, large numbers of reads mapped to the intended T-DNA sequence (Figure 8), demonstrating that the expected T-DNA is inserted in MON 94804 maize.

Additionally, as a result of the homology of P-*Ract1* and I-*Hsp70* to endogenous maize sequences, additional alignments of reads, over these elements, were observed in MON 94804 (Figure 8), similar to what was seen in the control HCL301 dataset. There is also a relatively small number of reads that map to the B-Left border region just upstream of the majority of the mapped reads. These reads are present at a very low coverage, are of varying quality, observed in a single library preparation replication, and do not possess the attributes associated with an inserted T-DNA such as a common termination point which might be indicative of a junction. Therefore, these low coverage reads do not indicate the presence of a secondary T-DNA in MON 94804 maize. The sporadic low-level detection of plasmid sequences such as OR-*ori-pBR322* has previously been described and reported (see Supplemental Figure S1 in

Yang *et al.* (2013)) and is likely due to the presence of environmental bacteria in tissue samples used in the preparation of genomic DNA used for library construction. As such, the incidence of this sequence from environmental bacteria does not indicate the presence of backbone sequence in MON 94804 maize. This analysis indicates that MON 94804 maize does not contain inserted sequence from the transformation plasmid vector backbone.

Following transformation of HCL301 maize mature seed embryo explants, plants from selected transformation events were self-pollinated to increase seed supplies. A Cre recombination system was used to remove the *cp4 epsps* selectable marker starting with selected events at the R2 generation. The selected transformant was crossed with a Cre recombinase expressing line. The Cre*/lox* recombination system enables the removal of DNA sequence positioned between two excision targeting sequences called *loxP* sites. In this instance, the Cre recombinase enzyme is responsible for the excision of the selectable marker cassette (P-*Ract1*, I-*Ract1*, TS-*CTP2*, CS-*cp4 epsps*, and T-*nos*) which was inserted during the transformation as part of the T-DNA insertion. After excision of the selectable marker cassette, a single *loxP* site remained. A plant homozygous for the intended T-DNA and lacking the *cp4 epsps* and *cre* genes was selected and designated MON 94804. The successful excision of the selectable marker cassette will be visualized in the MON 94804 data set as an absence of mapped reads, over the removed region. This gap in mapped reads is present and illustrated in Figure 8. Although there are a few reads that map to P-*Ract1*, as discussed above, a similar small pile-up of reads is present in the control HCL301 dataset and thus, do not indicate the presence of the selectable marker cassette in MON 94804 maize.

To determine the copy number in MON 94804 maize, selected reads mapping to T-DNA as described above were analyzed to identify junctions. A bioinformatic analysis was used to find and classify partially matched reads characteristic of the ends of insertions. The number of unique junctions determined by this analysis are shown in Table 3.

Table 3. Unique Junction Sequence Results for MON 94804 Maize Versus PV-ZMAP527895

Sample	Junctions Detected
MON 94804	2
HCL301	0

Detailed mapping information of the junction sequences is shown in Figure 8. The location and orientation of the junction sequences relative to the DNA insert in MON 94804 maize are shown in Figure 8 Panels 1, 2 and 3. As shown in Figure 8, there are two junctions identified in MON 94804 maize. Both junctions contain the T-DNA border sequence joined to flanking genomic sequence, indicating that they represent the sequences at the junctions of the intended T-DNA insert and the maize genome. As described earlier, no junctions were detected in any of the conventional maize control samples.

Considered together, the absence of plasmid vector backbone and the presence of two junctions (joining T-DNA borders and flanking sequences) indicate a single intended T-DNA insertion at a single locus in the genome of MON 94804 maize. Both junctions originate from the same locus of the MON 94804 maize genome and are linked by contiguous, known and expected DNA sequence (with the exception of the selectable marker cassette which was excised as described earlier). This is demonstrated by complete coverage of the sequenced reads spanning

the interval between the junctions and the directed sequencing of overlapping PCR products described in Section A.3(c)(iii).

Based on the comprehensive NGS and junction identification it is concluded that MON 94804 maize contains one copy of the T-DNA inserted into a single locus. This conclusion is confirmed by the sequencing and analysis of overlapping PCR products from this locus as described Section A.3(c)(iii).

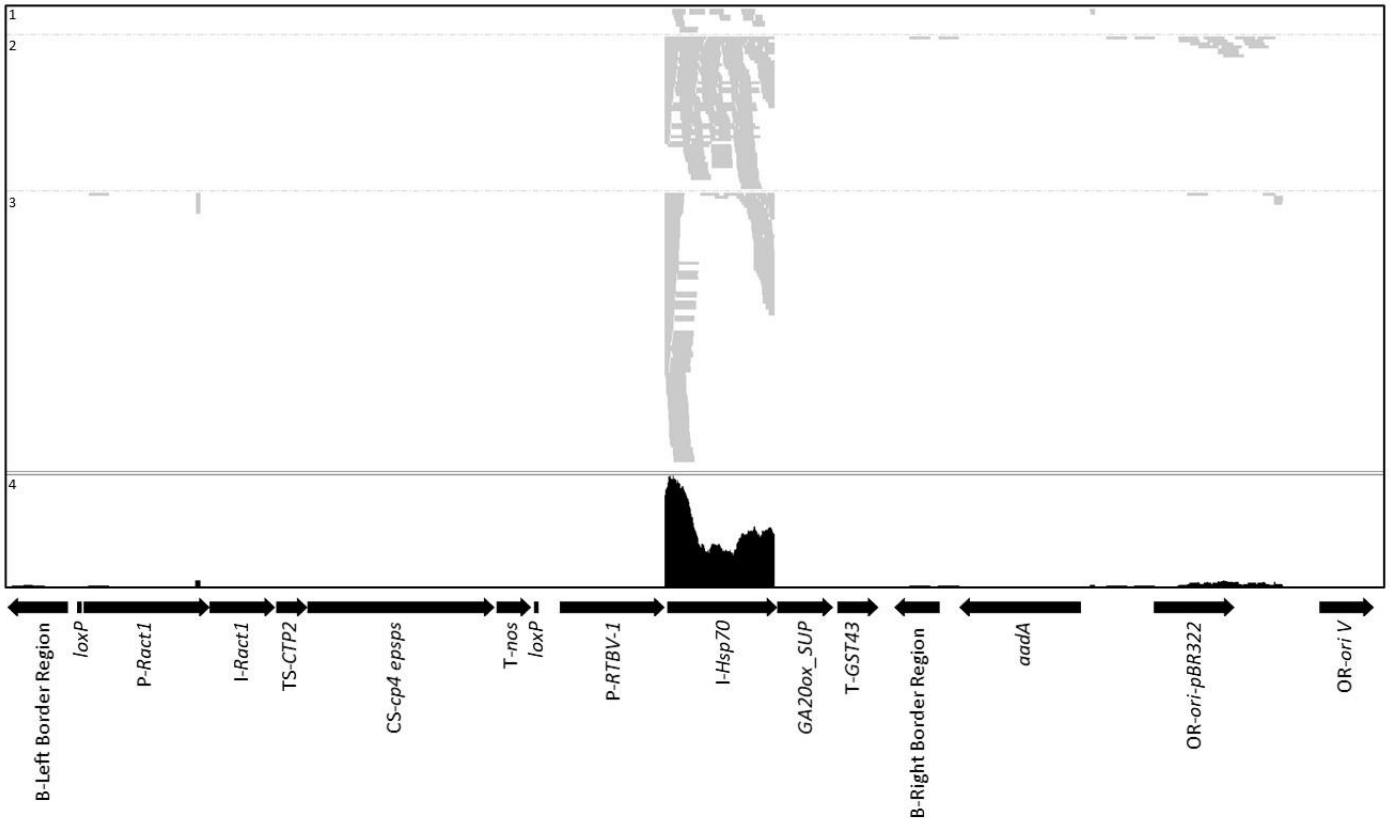


Figure 7. Read Mapping of Conventional Maize HCL301 Versus PV-ZMAP527892

Panel 1 shows the location of right to left oriented paired reads. Panel 2 shows the location of left to right oriented paired reads. Panel 3 shows unpaired reads and Panel 4 is a representation of combined read depth for unpaired and paired reads with a read depth range from 0 to 119.

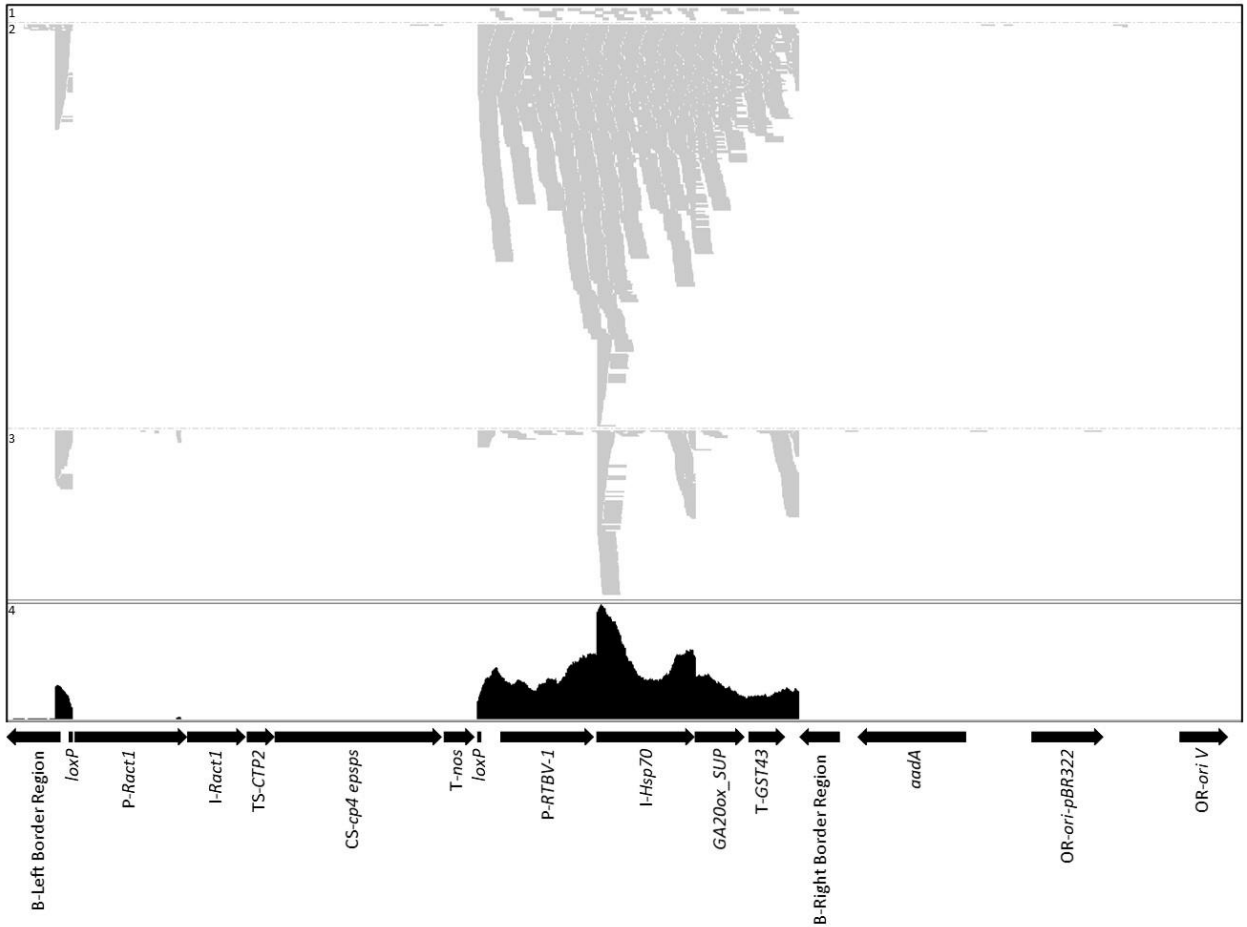


Figure 8. Read Mapping of MON 94804 Maize (F4) Versus PV-ZMAP527892

Panel 1 shows the location of right to left oriented paired reads. Panel 2 shows the location of left to right oriented paired reads. Panel 3 shows unpaired reads and panel 4 is a representation of combined read depth for unpaired and paired reads with a read depth range from 0 to 352.

A.3(c)(ii)(iv) Determination of Absence of the *cre* Gene-Containing Plasmid Vector PV-ZMOO513642

At the R2 generation (Figure 2), the selected transformant was crossed with a Cre recombinase expressing line. MON 94804 maize was crossed with a Cre recombinase expressing line. The Cre/*lox* recombination system enables the removal of the selectable marker cassette (P-*Ract1*, I-*Ract1*, TS-*CTP2*, CS-*cp4 epsps*, and T-*nos*) which is flanked by *loxP* sites. This selection cassette was inserted during transformation as part of the T-DNA insertion that also included the *GA20ox_SUP* suppression cassette. The resulting F1 progenies were self-pollinated and plants from the F2 generation were screened for the absence of the c*re* gene and any sequences from plasmid vector PV-ZMOO513642, allowing for selection of lines lacking the Cre recombinase cassette that were used as the progenitor of subsequent generations and the final product. A plant homozygous for the transfer DNA (T-DNA) and lacking the *cp4 epsps* and *cre* genes was selected and designated MON 94804 maize.

To confirm the absence of the Cre recombinase cassette and any part of the c*re* gene-containing plasmid vector, MON 94804 maize was assessed by generating a comprehensive collection of reads via NGS of MON 94804 maize genomic DNA and subsequently mapping it to the c*re* gene-containing plasmid vector (PV-ZMOO513642) sequence (Figure 9). In the absence of any PV-ZMOO513642 insertions there should be zero junction signature pairs and limited reads aligning with the PV-ZMOO513642 sequences.

When reads from the MON 94804 maize (F4) dataset were aligned with the PV-ZMOO513642 sequence, a number of reads aligned to the left border region (B-Left Border Region). Only a small number of reads mapping to the promoter element *Ract1* mapped to T-DNA, and a few reads mapping to origins of replication *ori-pBR322* and sequences used during DNA cloning were identified which aligned to the PV-ZMOO513642 backbone (Figure 9).

Table 4. Unique Junction Sequence Results for MON 94804 Maize Versus PV-ZMOO513642

Sample	Junctions Detected
MON 94804	0
HCL301	0

The mapping of a number of reads from the MON 94804 maize (F4) dataset to the left border region (B-Left Border Region) is expected since PV-ZMOO513642 and PV-ZMAP527892 share the same left border region sequence and PV-ZMAP527892's left border region is present in MON 94804 maize (Figure 8). The small number of reads mapping to the promoter element *Ract1* are also present in the HCL301 control background. This is fully consistent with the presence of a homologous sequence being present in the HCL301 control background (Figure 10) and does not indicate the presence of PV-ZMOO513642 T-DNA in MON 94804 maize. Additionally, a relatively few number of reads were found to align with OR-*ori-pBR322* sequences (Figure 9). The sporadic low level detection of plasmid vector sequences such as OR-*ori-pBR322* has previously been reported (see Supplemental Figure S1 in Yang *et al.* (2013)), and is due to the presence of environmental bacteria in tissue samples used in the preparation of genomic DNA used for library construction. As such, the incidence of this sequence from environmental bacteria does not indicate the presence of backbone sequence

from PV-ZMOO513642 in MON 94804 maize. Considered together, the limited reads aligning with the PV-ZMOO513642 sequences and the absence of junctions (joining T-DNA borders and flanking sequences) (Table 4) indicate that MON 94804 maize (F4), and subsequent generations, do not contain inserted sequence from the *cre* gene-containing plasmid vector, PV-ZMOO513642.

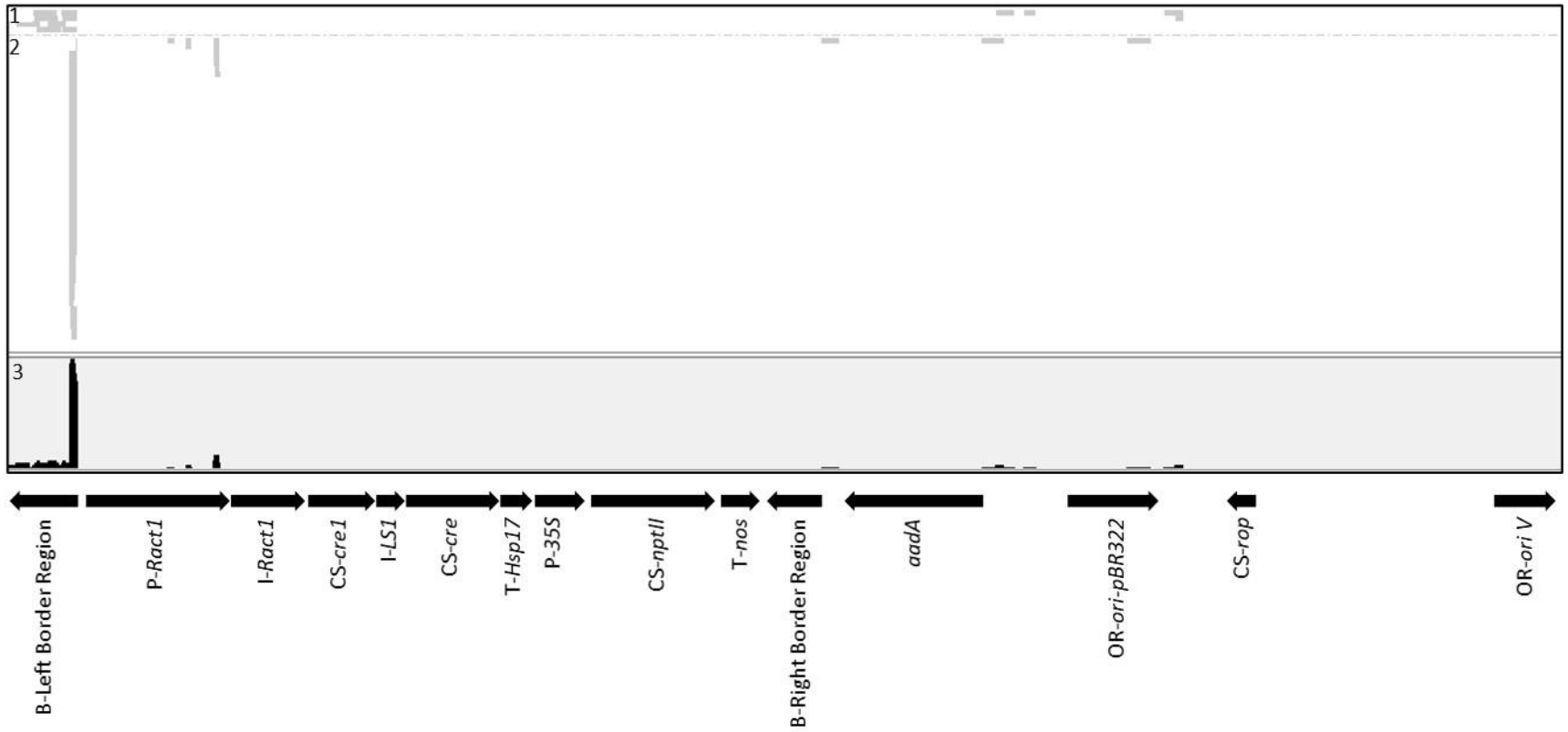


Figure 9. **Read Mapping of MON 94804 Maize (F4) Versus PV-ZMOO513642**

Panel 1 shows the location of left to right paired mapped reads. Panel 2 shows unpaired mapped reads and Panel 3 shows a representation of combined read depth for paired and unpaired reads with a read depth range from 0 to 57.

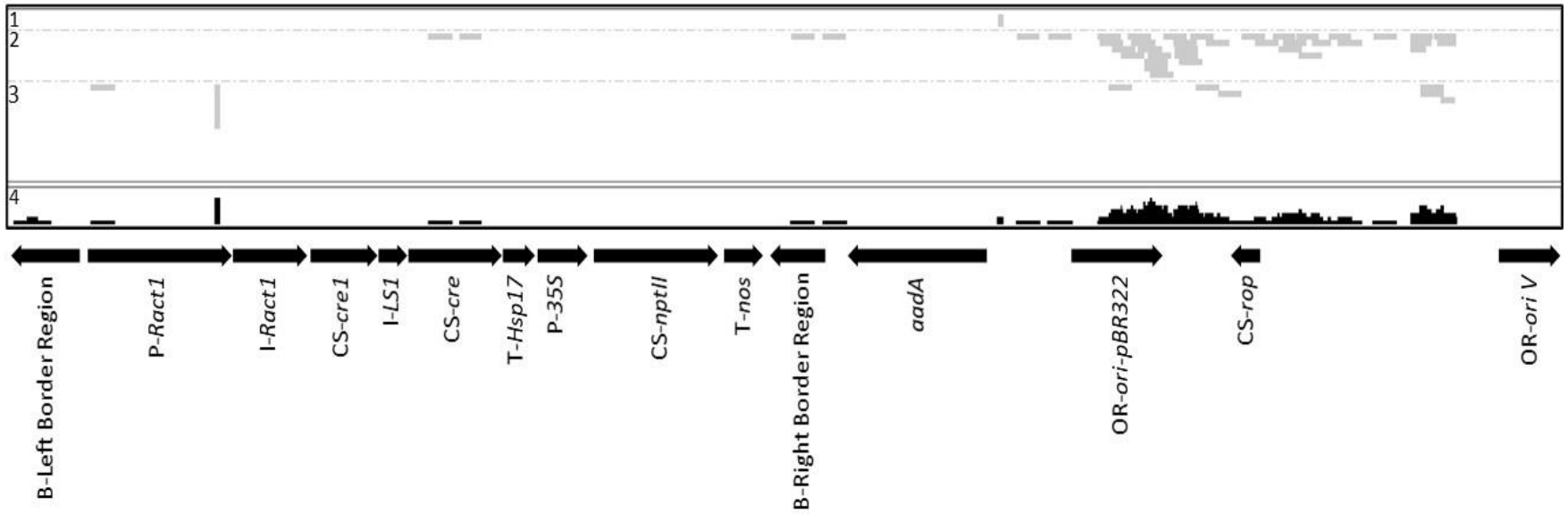


Figure 10. **Read Mapping of Conventional Maize HCL301 Versus PV-ZMOO513642**

Panel 1 shows the location of right to left oriented paired reads. Panel 2 shows the location of left to right oriented paired reads. Panel 3 shows unpaired reads and panel 4 is a representation of combined read depth for unpaired and paired reads with a read depth range from 0 to 10.

A.3(c)(iii) Full DNA sequence of each insertion site, including junction regions with the host DNA

A.3(c)(iii)(i) Organization and Sequence of the Insert and Adjacent DNA in MON 94804 Maize

The organization of the elements within the DNA insert and the adjacent genomic DNA was assessed using directed DNA sequence analysis (refer to Figure 4, Step 4). PCR primers were designed to amplify two overlapping regions of the MON 94804 maize genomic DNA that span the entire length of the insert and the adjacent DNA flanking the insert (Figure 11). The amplified PCR products were subjected to DNA sequencing analyses. The results of this analysis confirm that the MON 94804 maize insert is 2,733 bp and that each genetic element within the *GA20ox_SUP* cassette present in the T-DNA is intact compared to PV-ZMAP527892, with the exception of the border regions. The right border region was absent and the left border region contained small terminal deletions with the remainder of the inserted border region being identical to the sequence in PV-ZMAP527892. The sequence and organization of the insert was also shown to be identical to the corresponding T-DNA of PV-ZMAP527892 as intended. As noted, in Section A.3(b)(i), the selectable marker cassette (P-*Ract1*, I-*Ract1*, TS-*CTP2*, CS-*cp4 epsps*, and T-*nos*) and one *loxP* site (bases 544 through 3,884 of the PV-ZMAP527892 sequence) were excised by Cre recombinase, and as expected, are not present in the MON 94804 maize insert sequence. This analysis also shows that only T-DNA elements (described in Table 2) were present. In addition, 1000 bp flanking the 5′ end of the MON 94804 maize insert (Table 2, bases 1-1000) and 1000 bp flanking the 3′ end of the MON 94804 maize insert (Table 2, bases 3,734-4,733) were determined.

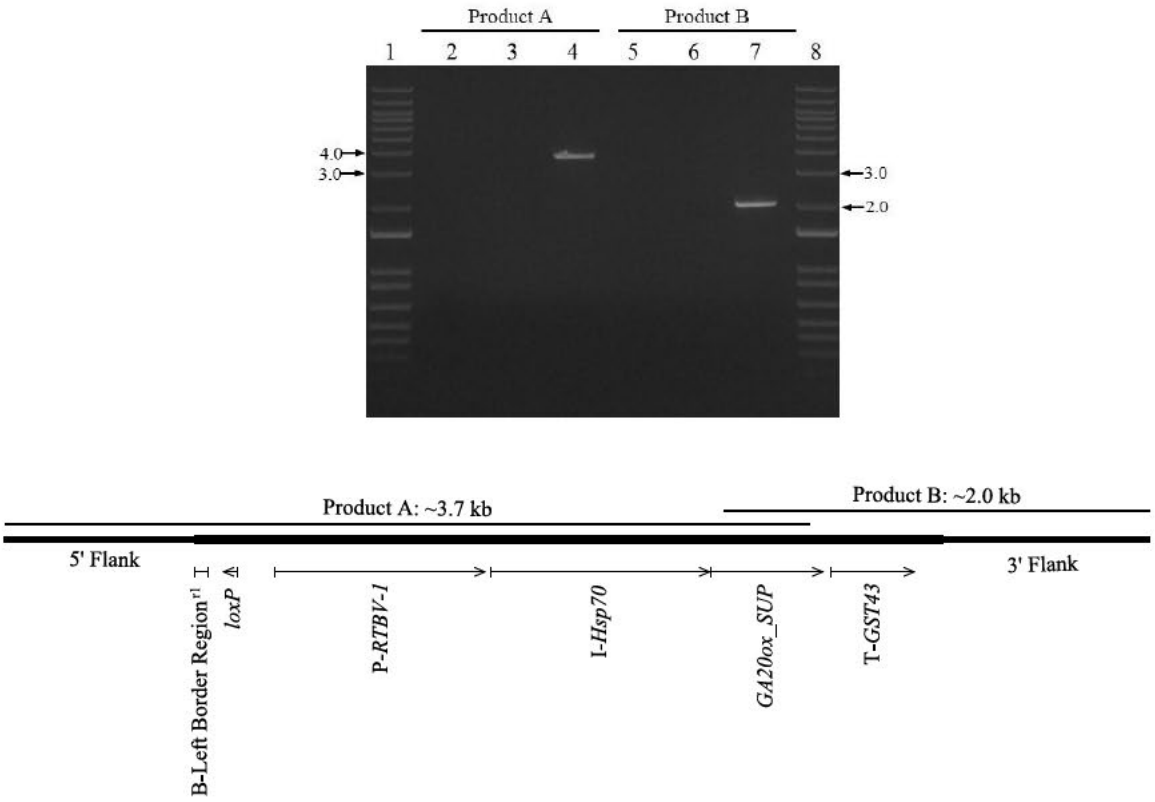


Figure 11. Overlapping PCR Analysis Across the Insert in MON 94804 Maize

PCR was performed on both conventional control genomic DNA and genomic DNA of the F4 generation of MON 94804 maize using two pairs of primers to generate overlapping PCR fragments from MON 94804 maize for sequencing analysis. To verify the PCR products, 2 µl of each of the PCR reactions was loaded on the gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 94804 maize that appears at the bottom of the figure. This figure is a representative of the data generated in the study. Lane designations are as follows:

Lane	
1	1 Kb Plus DNA Ladder
2	No template control
3	HCL301 Conventional Control
4	MON 94804 maize
5	No template control
6	HCL301 Conventional Control
7	MON 94804 maize
8	1 Kb Plus DNA Ladder

Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb Plus DNA Ladder (Invitrogen) on the ethidium bromide-stained gel.

r1 Superscript in the Left Border Region indicates that the sequence in MON 94804 was truncated compared to the sequences in PV-ZMAP527892.

A.3(c)(iii)(ii) Sequencing of the MON 94804 Maize Insertion Site

PCR and sequence analysis were performed on genomic DNA extracted from the conventional control to examine the insertion site in conventional maize (see Figure 4, Step 5). The PCR was performed with one primer specific to the genomic DNA sequence flanking the 5′ end of the MON 94804 maize insert paired with a second primer specific to the genomic DNA sequence flanking the 3′ end of the insert (Figure 12). A sequence comparison between the PCR product generated from the conventional control and the sequence generated from the 5′ and 3′ flanking sequences of MON 94804 maize revealed a 41 bp of maize genomic DNA deletion that likely occurred upon T-DNA integration in MON 94804. Such changes are common during plant transformation (Anderson *et al.*, 2016) and these changes presumably resulted from double stranded break repair mechanisms in the plant during A*grobacterium tumefaciens*-mediated transformation process (Salomon and Puchta, 1998). The remainder of the maize genomic DNA sequences flanking the insert in MON 94804 maize are identical to the conventional control.

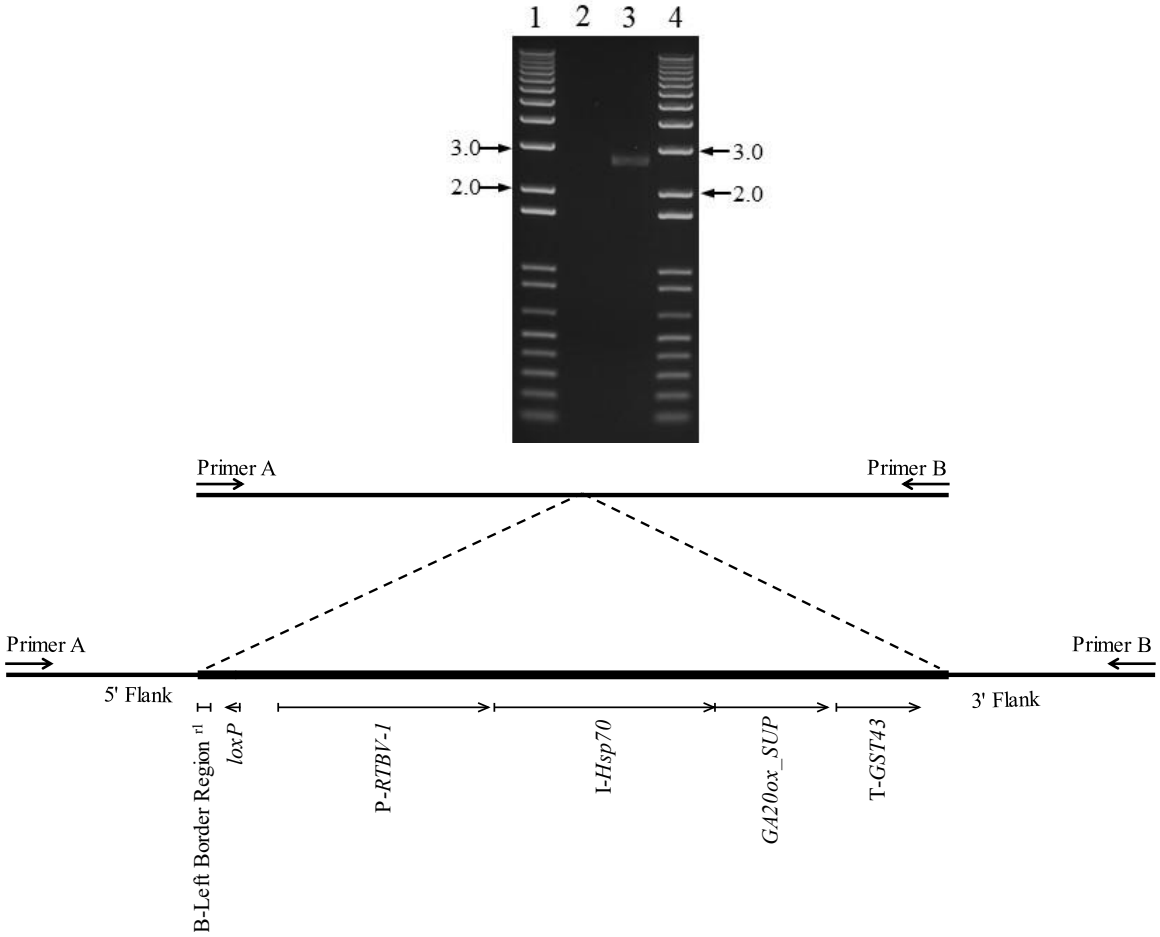


Figure 12. PCR Amplification of the MON 94804 Maize Insertion Site

PCR analysis was performed to evaluate the insertion site. PCR was performed on conventional control DNA using Primer A, specific to the 5′ flanking sequence, and Primer B, specific to the 3′ flanking sequence of the insert in MON 94804 maize. The DNA generated from the conventional control PCR was used for sequencing analysis. This illustration depicts the MON 94804 maize insertion site in the conventional control (upper panel) and the MON 94804 maize insert (lower panel). Approximately 1 µl of each of the PCR reactions were loaded on the gel. This figure is representative of the data generated in the study. Lane designations are as follows:

Lane	
1	1 Kb Plus DNA Ladder
2	No template control
3	HCL301 Conventional Control
4	1 Kb Plus DNA Ladder

Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb Plus DNA Ladder (Invitrogen) on the ethidium bromide-stained gel.

[r1] Superscript in Left Border Region indicates that the sequence in MON 94804 maize was truncated compared to the sequences in PV-ZMAP527892.

A.3(c)(iv) A map depicting the organization of the inserted genetic material at each insertion site

PCR and DNA sequence analyses performed on MON 94804 and the conventional control determined the organisation of the genetic elements within the insert as given in Figure 12.

A.3(c)(v) Details of an analysis of the insert and junction regions for the occurrence of any open reading frames (ORFs)

The 2009 Codex Alimentarius Commission guidelines for the safety assessment of food derived from modern biotechnology (Codex Alimentarius, 2009) includes an assessment element on the identification and evaluation of "*open reading frames within the inserted DNA or created by the insertions with contiguous plant genomic DNA*." These assessments examine the potential homology of any putative polypeptides or proteins that could be produced from open reading frames (ORFs) in the insert or at the plant-insert junction to known toxins or allergens. These analyses are conducted even if there is no evidence that such alternative reading frames in the insert or such ORFs at the plant-insert junction are capable of being transcribed or translated into a protein. Bioinformatic analyses were performed on the MON 94804 insert and flanking genomic DNA sequences to assess the potential for allergenicity, toxicity, or biological activity of putative polypeptides encoded by all six reading frames present in the MON 94804 insert DNA, as well as ORFs spanning the 5' and 3' insert DNA-flanking sequence junctions. The results from these bioinformatics analyses demonstrate that any putative polypeptides encoded by the MON 94804 event sequence are unlikely to exhibit allergenic, toxic or otherwise biologically adverse properties.

These various bioinformatic evaluations are depicted in Figure 13. ORFs spanning the 5' and 3' maize genomic DNA-inserted DNA junctions were translated from stop codon to stop codon in all six reading frames (three forward reading frames and three reading frames in reverse orientation)[3]. Polypeptides of eight amino acids or greater from each reading frame were then compared to toxin, allergen and all proteins databases using bioinformatic tools. Similarly, the entire T-DNA sequence was translated in all six reading frames and the resulting deduced amino acid sequence was subjected to bioinformatic analyses. The data generated from these analyses confirm that even in the highly unlikely occurrence that a translation product was derived from frames one to six of the insert DNA or the ORFs spanning the insert junctions, they would not share a sufficient degree of sequence similarity with other proteins to indicate they would be potentially allergenic, toxic, or have other safety implications. Therefore, there is no evidence for concern regarding the relatedness of the putative polypeptides for MON 94804 to known toxins, allergens, or biologically active putative peptides.

[3] An evaluation of sequence translated from stop codon to stop codon represents the most conservative approach possible for flank junction analysis as it does not take into consideration that a start codon is necessary for the production of a protein sequence.

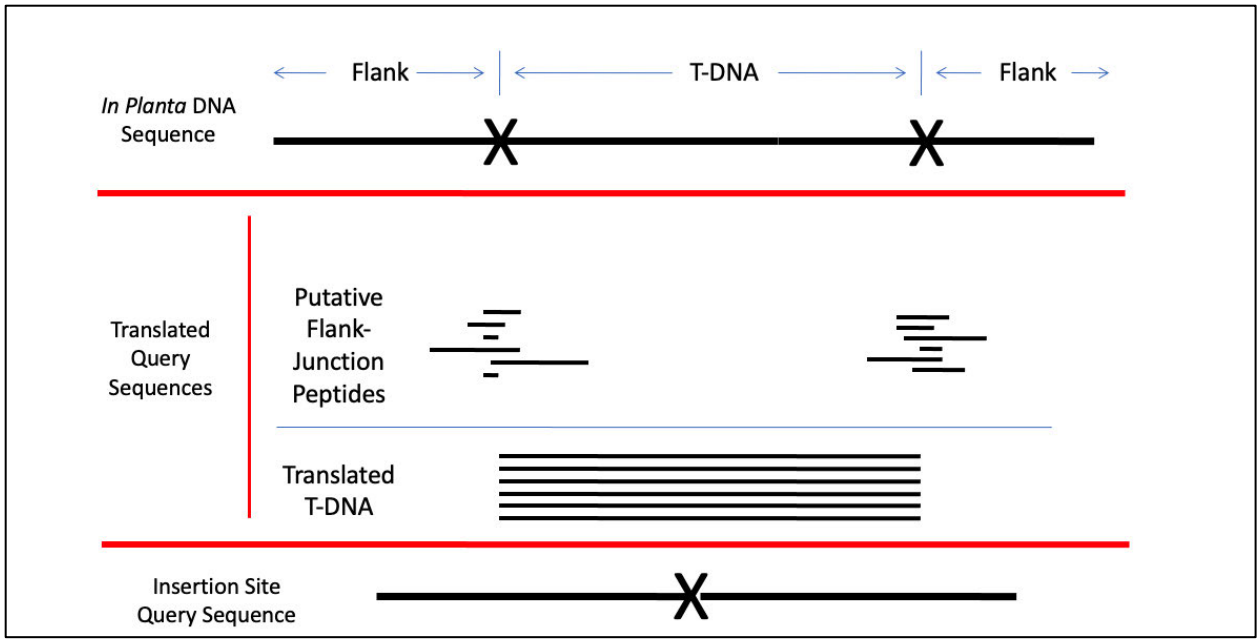


Figure 13. Schematic Summary of Queries used in Bioinformatic Analyses

Translated query sequences from the Putative Flank-Junction peptides and Translated T-DNA were used in the analysis of MON 94804.

(Note: The figure has been modified as the protein of interest (POI) translation was not used as there is no expressed protein of interest in MON 94804.)

A.3(c)(v)(i) Bioinformatics Evaluation of the T-DNA Insert in MON 94804

Bioinformatic analyses were performed to assess the potential of toxicity, allergenicity or biological activity of any putative peptides encoded by translation of reading frames 1 through 6 of the inserted T-DNA in MON 94804 (Figure 13).

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and any protein sequences in the AD_2022, TOX_2022, and PRT_2022 databases. Structural similarities shared between each putative polypeptide with each sequence in the databases were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignments, and their calculated percent identity and alignment lengths to ascertain if they exceeded Codex (Codex Alimentarius, 2009) thresholds for FASTA searches of their respective databases. Alignments having an *E*-score-less than 1×10^{-5} are deemed significant because they may reflect shared structure and function among sequences.

In addition to the FASTA comparison of the query sequence to the allergen database (AD_2022) to assess overall structural similarity, an eight amino acid sliding window search was performed to identify whether a linearly contiguous match of eight amino acids existed between the query sequence and sequences within the allergen database (AD_2022). This program compares the query sequence to each protein sequence in the allergen database using a sliding window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window.

A final assessment of potential allergenicity was also applied to the query sequences wherein sequences are identified as potentially cross-reactive if linear identity is greater than 35% in an ≥80 amino acid overlap (Codex Alimentarius, 2009). For this search, the translated T-DNA frame query sequences were split into overlapping 80 amino acid query sequences; that is, with a 79 amino acids overlap with the previous window. Resulting alignments were screened to assess if any query yielded an alignment containing 29 or more identities, the number required to surpass the threshold of 35% identity thought to indicate a potential for allergenic cross-reactivity (Codex Alimentarius, 2009).

The results of these data indicate that no biologically relevant sequence similarities were observed between the six reading frames translated from the T-DNA and allergens, toxins, or biologically active proteins associated with adverse effects for human or animal health. A single short match (8-mer) polypeptide match was observed from frame 6 to a sequence in the allergen database, described as "Allergen collagen alpha-2(I) chain precursor" from *Bos taurus.* However, the putative peptide containing this 8-mer lacks a contextually appropriate methionine start codon to initiate translation and consequently there is no evidence to suggest that the putative peptide containing the single 8-mer match to NP_776945.1 is produced *in planta*.

Together, the results of these bioinformatic analyses indicate that in the unlikely occurrence that any of the sequences analyzed herein is produced *in planta*, none would share significant similarity or identity to known allergens, toxins, or other biologically active proteins that could affect human or animal health.

For details, please refer to Appendix 2.

A.3(c)(v)(ii) Bioinformatics Evaluation of the DNA Sequences Flanking the 5′ and 3′ Junctions of the MON 94804 Insert: Assessment of Putative Peptides

Analyses of putative polypeptides encoded by DNA spanning the 5' and 3' genomic junctions of the MON 94804 inserted DNA were performed using a bioinformatic comparison strategy. The purpose of the assessment is to evaluate whether the novel open reading frames (ORFs) have homology to known allergens, toxins, or proteins that display adverse biological activity. Sequences spanning the 5' and 3' genomic DNA-insert DNA junctions, (Figure 13) were translated from stop codon (TGA, TAG, TAA) to stop codon in all six reading frames. Putative polypeptides from each reading frame, that were eight amino acids or greater in length, were compared to AD_2022, TOX_2022, and PRT_2022 databases using FASTA and to the AD_2022 database using an eight amino acid sliding window search. A total of 12 putative peptides were compared to allergen (AD_2022), toxin (TOX_2022), and all protein (PRT_2022) databases using bioinformatic tools.

The FASTA sequence alignment tool was used to assess the relatedness between the query sequences and any protein sequence in the AD_2022, TOX_2022, and PRT_2022 databases. Similarities shared between the query sequence with each sequence in the database were examined. The extent of relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent identity, and the *E*-score. Alignments having *E*-scores of ≤1e-5 (1×10^{-5}) are deemed significant because they may reflect shared structure and function among sequences. In addition to sequence similarity, sequences were screened for short peptide matches of eight contiguous and identical amino acids, as well as a linear short match search looking for greater than 35% in an ≥80 amino acid overlap which might indicate immunological relevance.

Notably, the bioinformatic analysis performed using the putative peptide sequences translated from junctions is theoretical as there is no reason to suspect, or evidence to indicate, the presence of transcripts spanning the flank junctions.

The results of these bioinformatic analyses indicate that no structurally relevant sequence similarities were observed between the putative polypeptides derived from the flank junction sequences and allergens, toxins, or biologically active proteins. Furthermore, no FASTA alignments met or exceeded the Codex threshold of greater than 35% over 80 amino acids, and no eight amino acid matches were identified against the allergen database. As a result, in the unlikely occurrence that any of the peptides analyzed herein are produced *in planta*, none would share significant similarity or identity to known allergens, toxins, or other biologically active proteins that could affect human or animal health.

For details, please refer to Appendix 3.

A.3(d) A description of how the line or strain from which food is derived was obtained from the original tranformant (i.e. provide a family tree or describe the breeding process) including which generations have been used for each study

The transformation to generate the MON 94804 maize was conducted with maize (*Zea mays* L. subsp. mays) inbred line HCL301. HCL301 is a Bayer proprietary yellow dent maize line in the Stiff Stalk heterotic group that is best adapted to the central regions of the U.S. corn belt.

Following transformation of HCL301 maize mature seed embryo explants, the mature embryos were placed on selection medium containing glyphosate in order to inhibit the growth of untransformed plant cells, then plants from selected transformation events were self-pollinated to increase seed supplies. A Cre recombination system was used to remove the *cp4 epsps* selectable marker starting with selected events at the R2 generation. A plant homozygous for the transfer DNA (T-DNA) and lacking the *cp4 epsps* and *cre* genes was selected and designated MON 94804. This event was subsequently evaluated in product testing, safety assessment studies, and commercial hybrid development. The non-transformed line, HCL301, was used as a conventional maize comparator (hereafter referred to as conventional control) in the safety assessment of MON 94804 maize..

For more details, see MON 94804 breeding history, Figure 2.

A.3(e) Evidence of the stability of the genetic changes, including:

A.3(e)(i) The pattern of inheritance of the transferred gene(s) and the number of generations over which this has been monitored

In order to demonstrate the stability of the T-DNA present in MON 94804 maize through multiple breeding generations, NGS was performed using DNA obtained from five breeding generations of MON 94804 maize. The breeding history of MON 94804 maize is presented in Figure 2 and the specific generations tested are indicated in the figure legend. The MON 94804 maize F4 generation was used for the molecular characterization analyses discussed in A.3(c)(ii) and A.3(c)(iii) and shown in Figure 4. To assess stability, four additional

generations were evaluated by NGS and compared to the fully characterized F4 generation. The conventional controls used for the generational stability analysis included HCL301, with similar genetic background to the F4, F5, and F6 generations and represents the original transformation line; and HCL301+HCL617, a hybrid with similar genetic background to the F4F1 and F5F1 hybrids. Genomic DNA isolated from each of the selected generations of MON 94804 maize and conventional control was used for NGS mapping, and subsequent junction identification (Table 5).

Table 5. Junction Sequence Detected

Sample	Junction Sequence Detected
MON 94804 (F4)	2
MON 94804 (F4F1)	2
MON 94804 (F5)	2
MON 94804 (F5F1)	2
MON 94804 (F6)	2
HCL301	0
HCL301+HCL617	0

As shown by alignment to the full flank/insert sequence obtained from directed sequencing, a single conserved pair of junctions linked by contiguous known and expected DNA sequence is present in MON 94804 maize (F4). Two identical junctions are found in each of the breeding generations (F4F1, F5, F5F1 and F6), confirming the insertion of a single copy of PV-ZMAP527892 T-DNA at a single locus in the genome of MON 94804 maize, and the consistency of these junctions in the mapping data across all generations tested demonstrates that this single locus is stably maintained throughout the MON 94804 maize breeding process.

These results demonstrate that the single locus of integration characterized in the F4 generation of MON 94804 maize is found in five breeding generations of MON 94804 maize, confirming the stability of the insert. This comprehensive NGS and bioinformatic analysis of NGS data from multiple generations supports the conclusion that MON 94804 maize contains a single, stable insert T-DNA.

For details, please refer to Appendix 1.

A.3(e)(ii) The pattern of inheritance and expression of the phenotype over several generations and, where appropriate, across different environments

A.3(e)(ii)(i) Inheritance of the Genetic Insert in MON 94804 Maize

The MON 94804 maize T-DNA resides at a single locus within the MON 94804 maize genome and therefore should be inherited according to Mendelian principles of inheritance. During development of lines containing MON 94804 maize, genotypic segregation data were recorded to assess the inheritance and stability of the MON 94804 maize T-DNA using Chi square ($\chi 2$)

analysis over several generations. The $\chi 2$ analysis is based on comparing the observed segregation ratio to the expected segregation ratio according to Mendelian principles of inheritance.

The MON 94804 maize breeding path for generating segregation data is described in Figure 14. The transformed R0 plant was self-pollinated to generate R1 seed. An individual plant homozygous for the MON 94804 maize T-DNA (homozygous positive) was identified in the R1 segregating population via a RealTime-TaqMan® PCR assay.

At the R2 generation, plants were crossed with a Cre recombinase expressing line. After excision of the *cp4 epsps* selectable marker cassette, a single *loxP* site remains in the F1 generation. The resulting F1 progeny were self-pollinated and the F2 generation was screened for lines that contained the *GA20ox_SUP* suppression cassette and did not contain *cre*, thus successfully removing the Cre recombinase cassette from subsequent generations. The *cre*-free F2 plants were self-pollinated to produce F3 seed. The *GA20ox_SUP* suppression cassette homozygous positive F3 plants were self-pollinated to give rise to F4 seed. The *GA20ox_SUP* suppression cassette homozygous positive F4 plants were crossed via traditional breeding techniques to a Bayer CropScience LP proprietary elite inbred parent that does not contain the *GA20ox_SUP* suppression cassette to produce hemizygous F4F1 seed. The hemizygous F4F1 plants were self-pollinated to produce F4F2 seed. Hemizygous positive F4F2 plants were self-pollinated to produce F4F3 seed and hemizygous positive F4F3 plants were self-pollinated to produce F4F4 seed.

The inheritance of the MON 94804 maize T-DNA was assessed in the F4F2, F4F3, and F4F4 generations using a qualitative endpoint TaqMan® PCR assay for *GA20ox_SUP* coding sequence. At the F4F2, F4F3 and F4F4 generations, the MON 94804 maize T-DNA was predicted to segregate at a 1:2:1 ratio (homozygous positive: hemizygous positive: homozygous negative) according to Mendelian principles of inheritance.

A Pearson's Chi square (χ^2) analysis was used to compare the observed segregation ratios of the MON 94804 maize T-DNA coding sequence to the expected ratios.

The Chi square was calculated as:

$$\chi 2 = \sum [(| o - e |)2 / e]$$

where o = observed frequency of the genotype or phenotype and e = expected frequency of the genotype or phenotype. The level of statistical significance was predetermined to be 5% ($\alpha = 0.05$).

The results of the χ^2 analysis of the segregating progeny of MON 94804 maize are presented in Table 6. The χ^2 value in the F4F2, F4F3 and F4F4 generations indicated no statistically significant difference between the observed and expected segregation ratios of MON 94804 maize T-DNA. These results support the conclusion that the MON 94804 maize T-DNA resides at a single locus within the maize genome and is inherited according to Mendelian principles of inheritance. These results are also consistent with the molecular characterization data indicating that MON 94804 maize contains a single intact copy of the T-DNA inserted at a single locus in the maize genome.

For details, please refer to Appendix 4.

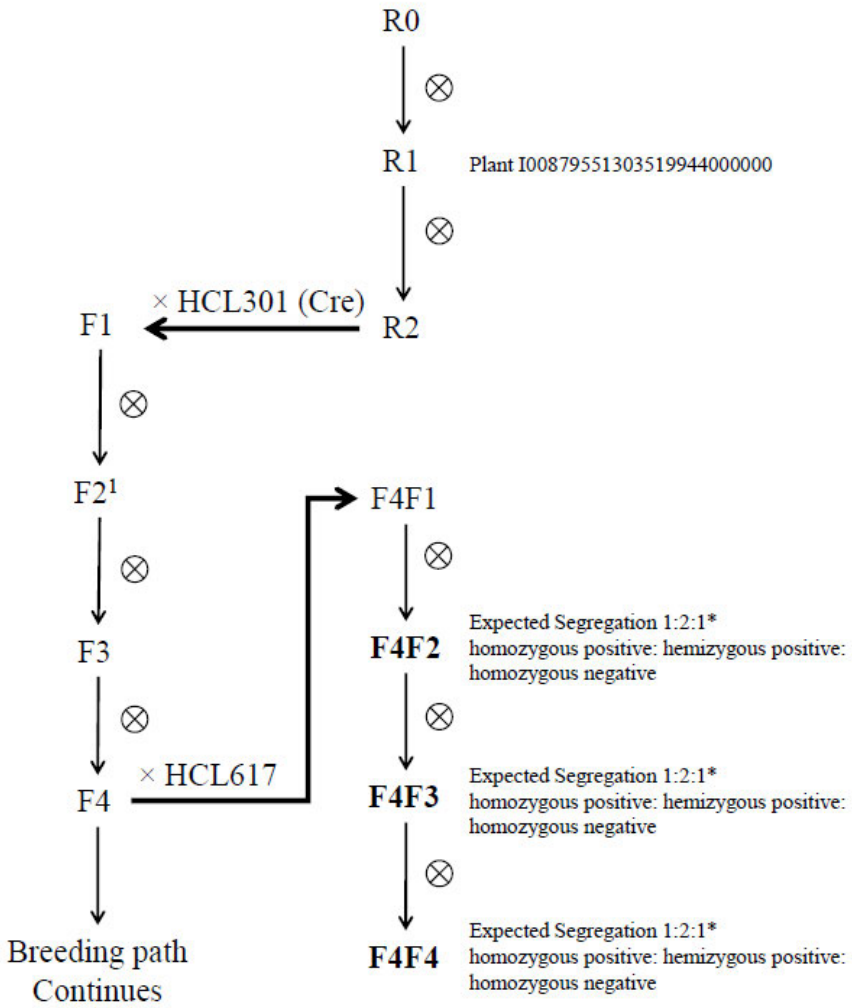


Figure 14. Breeding Path for Generating Segregation Data for MON 94804 Maize

*Chi-square analysis was conducted on segregation data from F4F2, F4F3, and F4F4 generations (bolded text).
U: Self-Pollinated
[1] The F2 generation was screened for plants absent of *cre*. Only those plants containing the *GA20ox_SUP* suppression cassette and lacking *cre* gene cassette were self-pollinated to create the F3 generation that lacked the *cre* gene cassette.

Table 6. Segregation of the *GA20ox_SUP* Suppression Cassette During the Development of MON 94804 Maize

					1:2:1 Segregation				
Generation	Total Plants	Observed # Plant Homozygous Positive	Observed # Plant Hemizygous Positive	Observed # Plant Homozygous Negative	Expected # Plant Homozygous Positive	Expected # Plant Hemizygous Positive	Expected # Plant Homozygous Negative	χ^2	Probability
F4F2	336	65	180	91	84.00	168.00	84.00	5.74	0.057
F4F3	244	73	110	61	61.00	122.00	61.00	3.54	0.170
F4F4	364	98	174	92	91.00	182.00	91.00	0.90	0.637

A.3(e)(ii)(ii) Expression of the Genetic Insert

It was determined by NGS that the single PV-ZMAP527892 T-DNA insert in MON 94804 maize has been maintained through five breeding generations. To likewise demonstrate the generational stability of the transcripts expressed from the *GA20ox_SUP* suppression cassette, northern blot analyses were conducted on leaf tissue from five breeding generations. Because the GA20ox_SUP transcripts can exist in a range of sizes as unprocessed, partially processed transcripts or as fully processed small RNA (miRNA) transcripts, MON 94804 maize was analyzed by both high molecular weight (HMW) and low moleular weight (LMW) northern blot analyses for the presence of the unprocessed or partially processed transcripts and mature miRNA transcripts, respectively.

MON 94804 that contains a suppression cassette expressing an inverted repeat sequence designed to target endogenous maize gibberellic acid 20 oxidase (GA20ox) genes, *ZmGA20ox3* and *ZmGA20ox5*. The expressed inverted repeat transcript is recognized by the endogenous RNA interference (RNAi), resulting in down-regulation of the targeted GA20ox gene expression. Specifically, miRNA-encoding genes, such as *GA20ox_SUP* in MON 94804, are transcribed by Polymerase II, and folded into stem loop structures called primary miRNA (pri-miRNA). In plants, Dicer Like 1 (DCL1) processes the RNA initially into a duplex precursor miRNA (pre-miRNA), from which the mature miRNA (~21 nt) is incorporated into the functional Argonaute (AGO). AGO facilitates target sequence recognition and messenger RNA (mRNA) cleavage which leads to specific suppression of the target mRNA (Borges and Martienssen, 2015; Siomi and Siomi, 2009). Two types of northern blot analyses, HMW and LMW, were performed to determine the presence or absence of the transcripts from *GA20ox_SUP* suppression cassette in leaf tissue collected from five generations of MON 94804.

A map of PV-ZMAP527892 annotated with the two DIG-labled probes used in the HMW northern analysis is presented in Figure 15. A DIG-labeled 21 nt oligo probe designed to target the mature miRNA sequence in MON 94804 was used for the LMW analysis. The generations used for the northern blot analyses are depicted in the breeding history diagram shown in Figure 16. Total RNA extracted from MON 94804 maize and control maize leaf tissue were subjected to northern blot analyses. By hybridizing with the GA20ox_SUP probes generated from the *GA20ox_SUP* suppression cassette, any RNA molecules that contain the GA20ox_SUP sequence will produce a hybridization signal on at least one of the two blots, medium to large transcripts (~0.2 kb to 6.0 kb) on the HMW blot and small transcripts (~15 nucleotides to 1.0 kb) on the LMW blot.

The details of the materials and methods for the generational stability of GA20ox_SUP miRNA transcripts are described in Appendix 5.

HMW Northern Blot Analysis of GA20ox_SUP miRNA in MON 94804 Maize Leaf Tissue

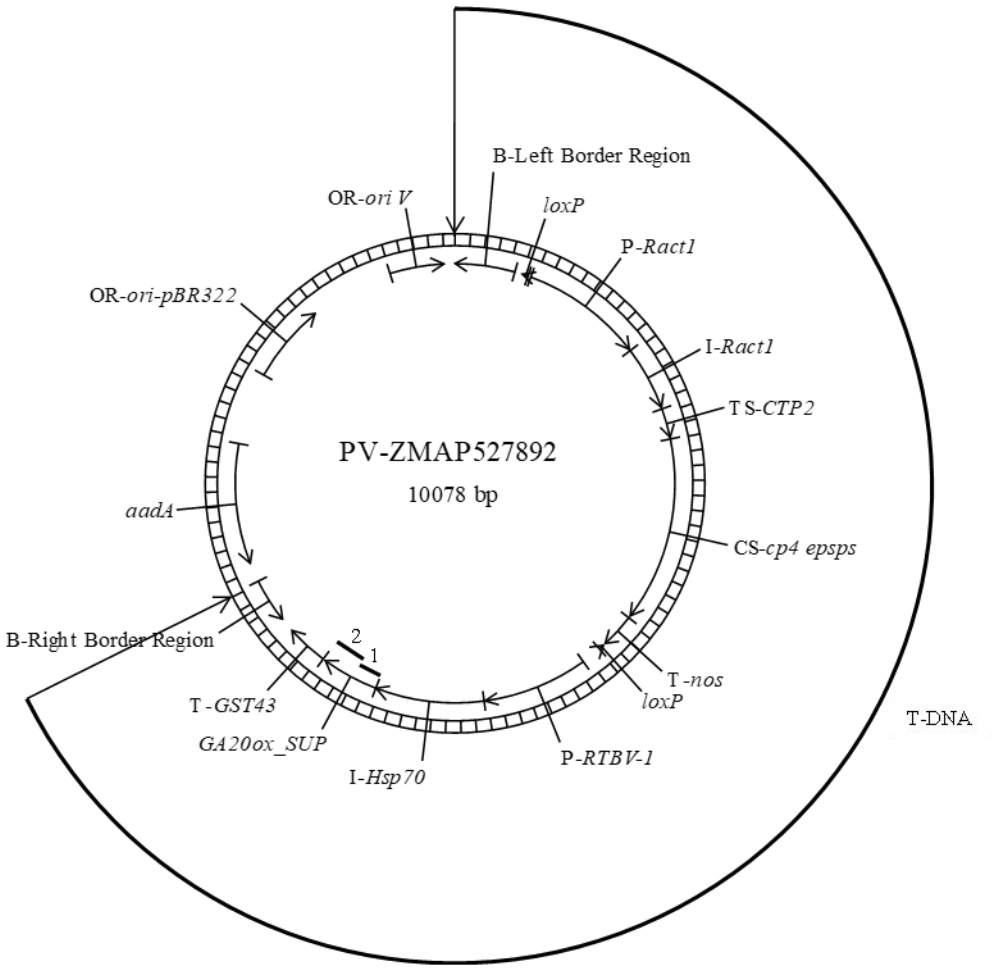
Approximately 10 µg of total RNA extracted from conventional or MON 94804 maize leaf tissue were resolved on a 1.0% agarose/~7% formaldehyde gel. After the transfer of the RNA onto a nylon membrane, the blot was hybridized with DIG-labeled GA20ox_SUP probes (Figure 17A). As the positive hybridization controls in the HMW northern blot, 2 pg of each probe template was spiked into a conventional maize RNA sample. Both probe template controls showed the expected hybridization signals (Figure 17A, Lanes 8 and 9). Detection of the positive controls indicates that the probes hybridized to the target DNA sequences. The RiboRuler High Range RNA ladder serves as a broad reference for the approximate positions of the northern signals from RNA lanes and it is not applicable to the DNA probe template lanes. Total RNA isolated from the leaf tissue of the conventional control showed no detectable GA20ox_SUP hybridization bands as expected Figure 17A, Lanes 1 and 2). Total RNA isolated from leaf tissue from five generations of the MON 94804 produced a hybridization band at ~0.9 kb (Figure 17A, Lanes 3-7), which is the expected size of the primary miRNA (pri-miRNA) transcript in MON 94804. No precursor miRNA (pre-miRNA) band was detected likely due to the rapid processing of this RNA. Ethidium bromide staining of the agarose/formaldehyde gel indicated that the total RNA from conventional and the five generations of MON 94804 plants were of similar intensity, suggesting equal loading of the MON 94804 and control RNA samples (Figure 17B).

LMW Northern Blot Analysis of GA20ox_SUP miRNA in MON 94804 Leaf Tissue

Approximately 10 µg of total RNA extracted from conventional or MON 94804 leaf tissue were resolved on a 15% TBE-Urea gel. After the transfer of the RNA onto a nylon membrane, the blot was hybridized with DIG-labeled oligo probe which was designed to target the mature miRNA sequence in MON 94804 (Figure 18A). Total RNA isolated from the leaf tissue of the conventional controls showed no detectable hybridization bands as expected (Figure 18A, Lanes 1 and 2). Total RNA isolated from leaf tissue from five generations of the MON 94804 produced a hybridization band at ~21 nt (Figure 18A, Lanes 3-7), which is the expected size of the mature miRNA transcript in MON 94804 from LMW northern analysis. Ethidium bromide staining of the TBE-Urea gel indicated that the total RNA from conventional and the five generations of MON 94804 plants were of similar intensity, suggesting equal loading of the MON 94804 and control RNA samples (Figure 18B).

GA20ox_SUP miRNA Transcript Generational Stability Conclusion

Leaf tissue samples from five generations of MON 94804 maize were collected and analyzed by HMW and LMW northern blot analyses for the presence of GA20ox_SUP miRNA transcripts. The northern blot analyses demonstrated that two stable populations of transcripts from the *GA20ox_SUP* suppression cassette, pri-miRNA and mature miRNA, have been maintained through five generations of breeding.



Probe	Start Position	End Position	Total Length (bp)
1	5630	5783	154
2	5784	6037	254

Figure 15. Map of PV-ZMAP527892

A circular map of plasmid vector PV-ZMAP527892 used to develop MON 94804 is shown. PV-ZMAP527892 contains one T-DNA. Genetic elements are shown on the exterior of the map. The probes used in the HMW northern analysis are shown on the interior of the map and listed in the accompanying table. The probes are not drawn to scale.

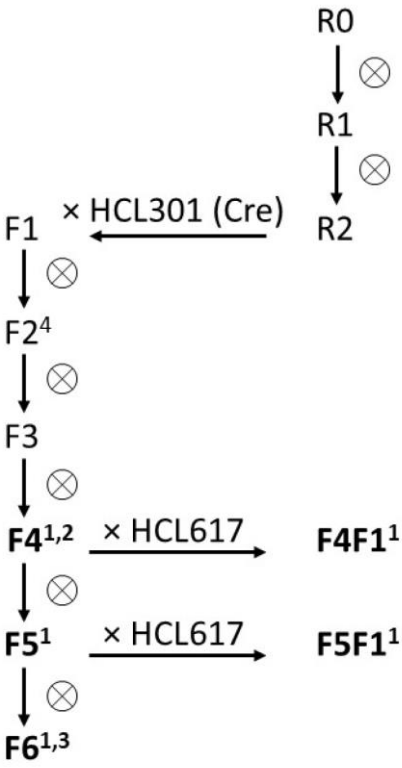


Figure 16. Breeding History Diagram MON 94804 Maize

The generations used for northern blot analysis of GA20ox_SUP miRNA are indicated in bold text. R0 corresponds to the transformed plant, ⊗ designates self-pollination.
[1] Generations used to confirm insert stability
[2] Generation used for molecular characterization
[3] Generation used for commercial development of MON 94804 maize
[4] The F2 generation was screened for plants absent of the *cre* gene. Only those plants absent of the *cre* gene were self-pollinated to create a *cre* free F3 generation

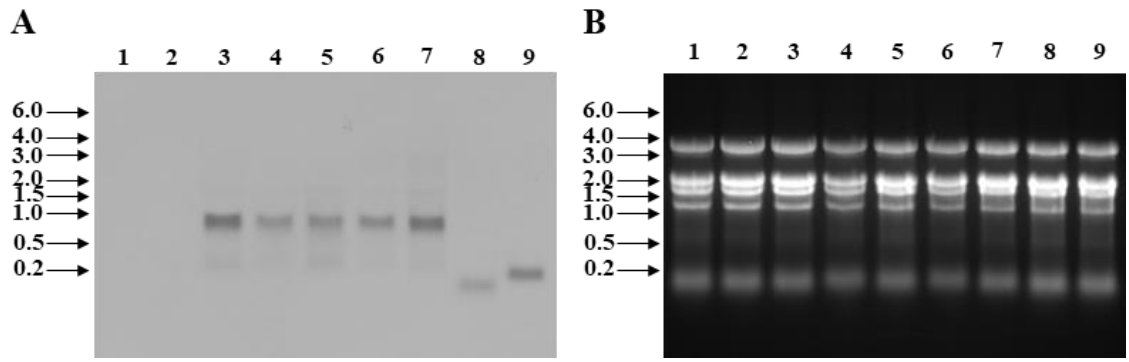


Figure 17. Northern Blot Analysis of HMW Transcript from *GA20ox_SUP* Suppression Cassette in MON 94804 Leaf Tissue

Approximately 10 µg of total RNA from leaf tissue of conventional control and MON 94804 were resolved on a 1.0% agarose/~7% formaldehyde gel. Panel A is the northern blot hybridized with DIG labeled GA20ox_SUP probes. Panel B is the fluorescent image of the ethidium bromide stained formaldehyde/agarose gel prior to transfer onto a nylon membrane. Lane designations are as follows:

Lane

1	Conventional Control (HCL301+HCL617 hybrid)
2	Conventional Control (HCL301 inbred)
3	MON 94804 (F4 inbred)
4	MON 94804 (F4F1 hybrid)
5	MON 94804 (F5 inbred)
6	MON 94804 (F5F1 hybrid)
7	MON 94804 (F6 inbred)
8	Conventional Control (HCL301+HCL617 hybrid) + GA20ox_SUP probe template-1 (2pg)
9	Conventional Control (HCL301+HCL617 hybrid) + GA20ox_SUP probe template-2 (2pg)

Arrows denote the size of the RNA, in kilobases, obtained from the RiboRuler High Range RNA Ladder (Thermo Scientific) on the ethidium bromide stained gel.

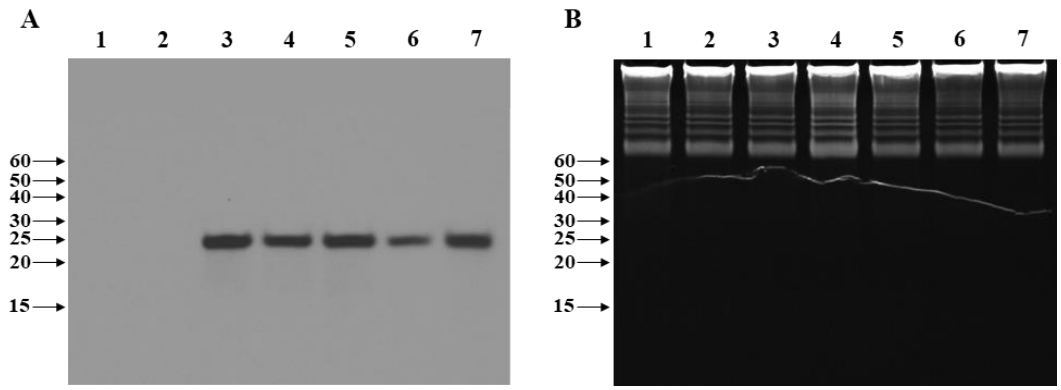


Figure 18. Northern Blot Analysis of LMW Transcript from *GA20ox_SUP* Suppression Cassette in MON 94804 Leaf Tissue

Approximately 10 µg of total RNA from leaf tissue of conventional control and MON 94804 were resolved on a 15% TBE-Urea gel. Panel A is the northern blot hybridized with the DIG-labeled oligo probe. Panel B is the fluorescent image of the ethidium bromide stained TBE-Urea gel prior to transfer onto a nylon membrane. Lane designations are as follows:

Lane

1 Conventional Control (HCL301+HCL617 hybrid)
2 Conventional Control (HCL301 inbred)
3 MON 94804 (F4 inbred)
4 MON 94804 (F4F1 hybrid)
5 MON 94804 (F5 inbred)
6 MON 94804 (F5F1 hybrid)
7 MON 94804 (F6 inbred)

Arrows denote the size of the RNA, in nucleotides, obtained from the 10/60 Ladder[1] (IDT) on the ethidium bromide stained gel.

[1] The 10 nt oligo standard was not visible on the TBE-Urea Gel

A.3(g) An analysis of the expressed RNA transcripts, where RNA interference has been used

The sequence present in the *GA20ox_SUP* suppression cassette in MON 94804 maize expresses an miRNA coding sequence, GA20ox_SUP, that was designed with sequences from rice to provide the backbone structure of the initial transcript and sequences from maize to provide an inverted repeat sequence derived from coding sequences of *ZmGA20ox3* and *ZmGA20ox5* genes. The expressed inverted repeat transcript is recognized by the endogenous RNAi machinery, resulting in down-regulation of the endogenous GA biosynthetic genes, *ZmGA20ox3* and *ZmGA20ox5* (Paciorek *et al.*, 2022). This suppression results in the reduction of bioactive GA levels in the stalk, leading to a reduction of internode length and consequently reduced overall plant height compared to the conventional control maize. The detailed result of characterization of *GA20ox_SUP* in MON 94804 are described in Section B.3B.3.

B. CHARACTERISATION AND SAFETY ASSESSMENT OF NEW SUBSTANCES

B.1 Characterisation and Safety Assessment of New Substances

B.1(a) Full description of the biochemical and phenotypic effects of all new substances (e.g. a protein or an untranslated RNA) that are expressed in the new GM organism, including their levels and site of accumulation, particularly in edible portions

The GA20ox_SUP miRNA was expressed under transcriptional control of the rice tungro bacilliform virus (RTBV) promoter which showed the highest expression level within internodes in maize and low activity in reproductive tissues, which makes it a desired tool for targeted suppression of GA in vegetative tissues (Paciorek *et al.,* 2022).

GA20ox_SUP miRNA is specific to the targeted transcripts of endogenous *ZmGA20ox3* and *ZmGA20ox5* genes (Paciorek *et al.*, 2022). The effect of suppression of ZmGA20ox3 and ZmGA20ox5 gene expression on endogenous bioactive GA levels was also assessed. Levels of bioactive GAs, GA_1 and GA_4, were reduced significantly in the vegetative tissues of stalk internode and leaf with no significant reduction of GA levels being detected in reproductive tissues (Paciorek *et al.*, 2022). Therefore, suppression of both targeted genes resulted in the reduction of bioactive GA levels in the stalk internode, leading to a reduction of stalk internode length, which consequently reduced overall plant height without affecting the reproductive potential when compared to the control maize (Paciorek *et al.*, 2022).

The detailed result of characterization of *GA20ox_SUP* in MON 94804 are described in section B.3.

B.1(b) Information about prior history of human consumption of the new substances, if any, or their similarity to substances previously consumed in food.

The detailed information about history of safe consumption of RNA are described in section B.3(b)

B.1(c) Information on whether any new protein has undergone any unexpected post-translational modification in the new host

It is not applicable since MON 94804 does not produce a protein.

B.1(d) Where any ORFs have been identified, bioinformatics analysis to indicate the potential for allergenicty and toxicity of the ORFs

As described in section A.3(c)(v), a conservative bioinformatics assessment of potential allergenicity, toxicity and adverse biological activity for putative polypeptides derived from different reading frames of the entire insert MON 94804 or that span the 5' and 3' insert junctions was conducted. There are no analytical data that indicate any putative polypeptides

subjected to bioinformatics evaluation are produced by MON 94804. Moreover, the data generated from these analyses confirm that even in the highly unlikely occurrence that a translation product was derived from frames 1 to 6 of the insert DNA, or the ORFs spanning the insert junctions; they would not share a sufficient degree of sequence similarity with other proteins to indicate they would be potentially allergenic, toxic, or have other safety implications. Therefore, there is no evidence for concern regarding the putative polypeptides for MON 94804 relatedness to known toxins, allergens, or biologically active putative peptides.

B.2 New Proteins

Not relevant for this product since MON 94804 does not produce a protein.

B.2(a) Information on the potential toxicity of any new proteins, including:

B.2(a)(i) A bioinformatic comparison of the amino acid sequence of each of the new proteins to know protein toxins and anti-nutrients (e.g. protease inhibitors, lectins)

Not relevant for this product since MON 94804 does not produce a protein.

B.2(a)(ii) Information on the stability of the proteins to proteolysis in appropriate gastrointestinal model systems

Not relevant for this product since MON 94804 does not produce a protein.

B.2(a)(iii) An animal toxicity study if the bioinformatic comparison and biochemical studies indicate either a relationship with known protein toxins/anti-nutrients or resistance to proteolysis

Not relevant for this product since MON 94804 does not produce a protein.

B.2(b) Information on the potential allergenicity of any new proteins, including:

B.2(b)(i) Source of the new proteins

Not relevant for this product since MON 94804 does not produce a protein.

B.2(b)(ii) A bioinformatics comparison of the amino acid sequence to known allergens

Not relevant for this product since MON 94804 does not produce a protein.

B.2(b)(iii) The new protein's structural properties, including, but not limited to, its susceptibility to enzymatic degregation (e.g. proteolysis), heat and/or acid stability

Not relevant for this product since MON 94804 does not produce a protein.

B.2(b)(iv) Specific serum screening where a new protein is derived from a source known to be allergenic or has sequence homology with a know allergen

Not relevant for this product since MON 94804 does not produce a protein.

B.2(b)(v) Information on whether the new protein(s) have a role in the elicitation of gluten-sensitive enteropathy, in cases where the introduced genetic material is obtained from wheat, rye, barley, oats, or related cereal grains

Not relevant for this product since MON 94804 does not produce a protein.

B.3 Other (non-protein) New Substances

MON 94804 contains a suppression cassette expressing an inverted repeat sequence designed to target endogenous maize *gibberellic acid 20 oxidase* (*GA20ox*) genes, *ZmGA20ox3* and *ZmGA20ox5*. The expressed inverted repeat transcript is recognized by the endogenous RNA interference (RNAi), resulting in down-regulation of the targeted GA20ox gene expression. Specifically, miRNA-encoding genes, such as *GA20ox_SUP* in MON 94804, are transcribed by Polymerase II, and folded into stem loop structures called primary miRNA (pri-miRNA). In plants, Dicer Like 1 (DCL1) processes the RNA initially into a duplex precursor miRNA (pre-miRNA), from which the mature miRNA (~21 nt) is incorporated into the functional Argonaute (AGO). AGO facilitates target sequence recognition and messenger RNA (mRNA) cleavage which leads to specific suppression of the target mRNA (Borges and Martienssen, 2015; Siomi and Siomi, 2009).

B.3(a) The identity and biological function of the substance

The GA20ox_SUP miRNA was expressed under transcriptional control of the rice tungro bacilliform virus (RTBV) promoter which showed the highest expression level within internodes in maize and low activity in reproductive tissues, which makes it a desired tool for targeted suppression of GA in vegetative tissues (Paciorek *et al.*, 2022) by suppression of ZmGA20ox3 and ZmGA20ox5 through RNA interference (RNAi) process in plant.

B.3(b) Whether the substance has previously been safely consumed in Food

B.3(b)(i) The History of Safe Use of RNAi-mediated Gene Suppression in Plants

RNAi-mediated gene suppression is a naturally occurring, ubiquitous process in eukaryotes, including plants and animals consumed as food and feed. Endogenous RNAi-mediated gene modulation is responsible for certain characteristics, such as virus-induced gene silencing, of conventional crops (Della Vedova *et al.*, 2005; Kamthan *et al.*, 2015; Kusaba *et al.*, 2003; Tuteja *et al.*, 2004; Xu *et al.*, 2019) and has also been utilized in many biotechnology-derived crops approved for cultivation and food and feed use (Arpaia *et al.*, 2020; Ivashuta *et al.*, 2009; Kleter, 2020; Parrott *et al.*, 2010; Petrick *et al.*, 2013). Therefore, there is a history of safe consumption of the RNA molecules mediating gene suppression in plants, including those with sequence homology to genes in humans and other animals (Fletcher *et al.*, 2020; Frizzi and Huang, 2010; Ivashuta *et al.*, 2009; Jensen *et al.*, 2013; Petrick *et al.*, 2016).

There is no evidence to suggest that dietary consumption of nucleic acids is associated with toxicity (Petrick *et al.*, 2013; U.S. FDA, 1992). U.S. Environmental Protection Agency (EPA) has an established tolerance exemption for nucleic acids that encode for plant-incorporated

protectant (PIP) products (U.S. EPA, 2001). U.S. Food and Drug Administration (FDA) recognizes that all food allergens are proteins (U.S. FDA, 1992) and there is also no evidence of allergenicity of dietary RNA in the peer-reviewed scientific literature. This lack of toxicity or allergenicity for ingested RNA also extends to RNA molecules associated with RNAi-mediated gene regulation. Therefore, an extensive history of safe consumption of dietary RNAs, including double-stranded RNA (dsRNA), small interfering RNA (siRNA), and microRNA (miRNA), has been established as reviewed in Petrick *et al.* (2013). One of the reasons for this history of safe consumption of dietary RNAs is that extensive sequence-independent physiological and biochemical barriers are known to exist in humans and other animals that limit the potential for uptake or activity of ingested nucleic acids (Juliano *et al.*, 2009; O'Neill *et al.*, 2011; Petrick *et al.*, 2013; Rodrigues and Petrick, 2020).

Additionally, regulatory agencies have concluded that ingestion of RNA molecules does not present a hazard to humans or other mammals. The following are published considerations from different regulatory agencies regarding the history of safe consumption of nucleic acids including RNAs:

- Introduced nucleic acids, in and of themselves, do not raise safety concerns (U.S. FDA, 1992).
- DNA and RNA are common to all forms of plant and animal life, and the Agency knows of no instance where these nucleic acids have been associated with toxic effects related to their consumption as a component of food (U.S. EPA, 2010).
- Based on the current knowledge, gained in pharmaceutical research and development, RNAi molecules show limited bioavailability (EFSA, 2014).
- A history of safe human consumption of RNAi mediators exists, including those with homology to human genes. The evidence published to date also does not indicate that dietary uptake of these RNAs from plant food is a widespread phenomenon in vertebrates (including humans) or, if it occurs, that sufficient quantities are taken up to exert a biologically relevant effect (FSANZ, 2015).
- There is no reliable evidence that exogenous dsRNAs are taken up from the gut into mammalian circulation to exert their functions in the ingesting organism (U.S. EPA, 2016).

Small RNAs (sRNAs) are mainly classified into siRNAs and miRNAs according to their origin. siRNAs originate from dsRNA precursors that are synthesized by RNA-dependent RNA polymerases, whereas miRNAs are produced by the specific cleavage of dsRNA that originates from hairpin-containing primary transcripts (Li *et al.*, 2018; Liu *et al.*, 2014; Sanan-Mishra *et al.*, 2021). Processed miRNAs are a naturally occurring class of endogenous single-stranded non-coding RNAs of approximately 20-24 nucleotides that play an important role in post-transcriptional gene regulation in plants and animals (Liu *et al.*, 2017; Lou *et al.*, 2018; Sanan-Mishra *et al.*, 2021). In crop plants, endogenous miRNA-mediated gene modulation is responsible for several plant processes including development and defense responses to pathogens, insects and environmental stresses (Basso *et al.*, 2019; Hackenberg *et al.*, 2015; Yi *et al.*, 2015) and has also been utilized in genetic engineering for improving the agronomic properties of crops as food and feed use (Gupta, 2015; Zhang and Wang, 2015; Zhou and Luo, 2013). Therefore, plant-derived RNA molecules, including miRNAs, present in the diet of humans and other animals have a history of safe consumption (Ivashuta *et al.*, 2009; Jensen *et al.*, 2013; Ramesh, 2013; Rodrigues and Petrick, 2020; Sherman *et al.*, 2015).

B.3(b)(ii) B.3(b)(ii) Safety of the GA20ox_SUP miRNA produced in MON 94804 Maize

GA20ox_SUP miRNA produced in MON 94804 maize is processed to a miRNA that causes gene suppression of the targeted ZmGA20ox genes within the maize plant (Paciorek *et al.*, 2022). An extensive literature search indicates no evidence for the presence of GA biosynthetic pathway or GA20ox genes in humans or animals (Keswani *et al.*, 2022; Salazar-Cerezo *et al.*, 2018). From the ubiquitous nature of gene suppression utilizing miRNAs in a wide variety of extensively consumed plant species, the long history of safe consumption of RNA molecules including miRNAs from a range of sources, and the apparent lack of toxicity or allergenicity of dietary RNAs including miRNA, it can be concluded that the GA20ox_SUP miRNA produced in MON 94804 maize poses negligible risks to humans or animals. Therefore, the GA20ox_SUP miRNA from MON 94804 maize or its progeny is considered safe for humans and animals.

The principal reasons contributing to this history of safe consumption of dietary miRNAs are attributed to the differences in processing pathways between plant and animal miRNAs, and lack of evidence of cross-kingdom effect.

1. Processing pathway is different between plant and animal miRNAs

Although biogenesis of miRNAs in animals and plants initiates in the nucleus, differences exist in the subsequent maturation of miRNA. The Drosha gene that generates precursor miRNA (pre-miRNA) from primary miRNA (pri-miRNA) in animals is absent from plant genomes. A Dicer-like 1 (DCL1) protein catalyzes the processing of the pri-miRNA in plants (Li *et al.*, 2018; Millar and Waterhouse, 2005; Rogers and Chen, 2013). In animals, miRNAs are excised from stem-loop structures in the cytoplasm, while in plants, this reaction occurs in the nucleus (Millar and Waterhouse, 2005; Rogers and Chen, 2013). Plant miRNAs possess higher complementarity to their target genes than animal miRNAs (Doench and Sharp, 2004; Parizotto *et al.*, 2004). The location of the miRNA binding sites within the target genes is also different between the two kingdoms. While plant miRNAs bind in one site on the target gene, animal miRNA binding occurs in multiple sites in the gene and is always within the 3′ untranslated region (3′-UTR) (Lewis *et al.*, 2003; Millar and Waterhouse, 2005; Rogers and Chen, 2013). Considering all these differences, the ingested plant-derived dietary miRNAs pose negligible risks to humans or animals.

2. Cross-kingdom effect of exogenous miRNAs is not evident

Humans regularly consume plants that contain miRNAs. Several physiological and biochemical barriers are known to prevent the uptake of plant miRNAs by mammalian cells. Nucleases in the saliva break down the food-derived miRNAs after ingestion (Park *et al.*, 2006; Rodrigues and Petrick, 2020). As ingested food reaches the stomach, degradation of dietary miRNAs occurs due to the low pH and hydrolysis by digestive enzymes acids (Huang *et al.*, 2018; Rodrigues and Petrick, 2020). A feeding study in non-human primates with a miRNA rich food source concluded that there is little evidence for the presence of these plant miRNA in non-human primate blood prior to or following dietary intake of a plant miRNA-rich substance (Witwer *et al.*, 2013). A study investigating the bioavailability of maize miRNAs after gavage feeding to mice concluded that maize miRNAs are extensively degraded early in the digestion process and are not taken up into circulation or tissues in the mouse model (Huang *et al.*, 2018). The same study noted that no evidence of increased levels of maize miRNAs in whole blood or tissues after supplementation of maize miRNAs in the diet was observed in a

mouse model and concluded that cross-kingdom effect of ingested exogenous miRNAs was insignificant and not biologically relevant. A recent paper found no evidence that broccoli sprouts miRNA sequences affect human target genes and suggested that dietary RNAs are not functional in mammals (Xiao *et al.*, 2020).

As presented above, the known extensive sequence-independent physiological and biochemical barriers to systemic and cellular uptake of exogenous RNA molecules, the differences in processing pathways between animals and plants miRNAs, and the lack of cross-kingdom effect of miRNAs contribute to the safety of miRNAs. The established history of safe consumption of miRNA in a wide variety of food and feed lead us to conclude that ingested GA20ox_SUP miRNA produced in MON 94804 maize does not cause any biologically meaningful effects and poses negligible risks to humans and animals.

B.3(c) Potential dietary exposure to the GA20ox_SUP miRNA from MON 94804

MON 94804 maize does not have a toxic mode of action, nor any pest control properties. Lack of oral toxicity and history of safe consumption of miRNA in higher organisms (B.3(b)) indicate that there would be negligible risks to human and animal health associated with consumption of GA20ox_SUP miRNA in food and feed products derived from MON 94804 maize.

B.3(d) where RNA interference has been used

B.3(d)(i) the role of any endogenous target gene and any changes to the food as a result of silencing that gene

MON 94804 contains a suppression cassette expressing an inverted repeat sequence designed to target endogenous maize gibberellic acid 20 oxidase (GA20ox) genes, *ZmGA20ox3* and *ZmGA20ox5* which are involved in GA biosynthesis. Suppresion of those genes leads to reduction of GA level in stalk resulting reduced plant height.

B.3(d)(i)(i) Plant Hormone Gibberellins and Mechanism of Action of MON 94804 Maize

Being sessile organisms, plants need to continually adapt and modulate their rate of growth and development in accordance with the changing environmental conditions (Lymperopoulos *et al.*, 2018). Plant hormones, a series of plant endogenous small molecules, play versatile roles in regulating plant growth and development in response to these conditions (Jiang and Asami, 2018; Santner *et al.*, 2009). A recent study reported that auxin, gibberellin, cytokinin, abscisic acid, ethylene, brassinosteroids, jasmonic acid, salicylic acid, and strigolactones have been confirmed to function as plant hormones (Jiang and Asami, 2018).

Gibberellic acids (GAs), also known as gibberellins, are plant hormones that are essential for modulating many developmental processes in plants, including stem elongation, seed germination, leaf growth and floral transition (Achard and Genschik, 2009; Nelissen *et al.*, 2012). Mutations in the genes involved with the action or production of GA have been utilized in breeding programs for introduction of dwarfing/short stature traits into staple crops. This genetic variation resulting in shorter statured crops was one of the cornerstones of the so-called

'Green Revolution' and led to significant increases in global wheat and rice yields (Hedden, 2003).

The biosynthesis of GA has been studied in many plant species, and is catalyzed by multiple enzymes (Yamaguchi, 2008). GA biosynthesis is divided into three stages leading to bioactive GAs as shown in Figure 19. Illustration of GA biosynthesis pathway in plants (adapted and recreated from Binenbaum *et al.* (2018)). Bioactive GAs are biologically functional plant hormones which control diverse aspects of plant growth and development (Yamaguchi, 2008). The major bioactive GAs identified in many plant species are GA_1 and GA_4 (Binenbaum *et al.*, 2018). In maize, GA_1 is the major bioactive form of GA controlling internode elongation (Phinney, 1985), and a reduction of the GA_1 level in maize has been shown to reduce total plant height (Chen *et al.*, 2020; Chen *et al.*, 2019).

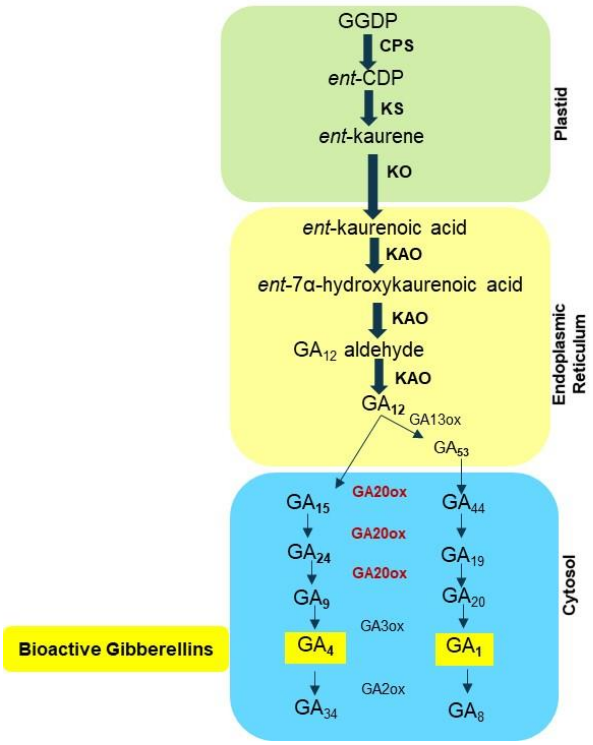


Figure 19. Illustration of GA biosynthesis pathway in plants (adapted and recreated from Binenbaum *et al.* (2018))

GGDP: geranylgeranyl diphosphate; *ent-CDP: ent-* copalyl diphosphate; CPS: *ent*-copalyl diphosphate synthase; KS: *ent*-kaurene synthase ; KO: *ent*-kaurene oxidase ; KAO: *ent*-kaurenoic acid oxidase ; GA20ox (colored red in the figure): Gibberellin 20-oxidase enzyme that is encoded by the *ZmGA20ox* gene family of which *ZmGA20ox3* and *ZmGA20ox5* genes are targeted for suppression in MON 94804 maize.

B.3(d)(i)(ii) Description of the Mechanism of Action (MOA) for MON 94804 Maize

The biosynthesis of GA has been characterized in many plant species and is catalyzed by multiple enzymes (Yamaguchi, 2008). Among these enzymes, GA20ox is a key enzyme in synthesizing bioactive GAs in the later steps of the GA biosynthesis pathway (Oikawa *et al.*,

2004; Qin *et al.*, 2013)(Figure 19. Illustration of GA biosynthesis pathway in plants (adapted and recreated from Binenbaum *et al.* (2018))).

In maize, five *ZmGA20ox* genes were initially identified (Song *et al.*, 2011). Four additional putative *ZmGA20ox* genes have also been identified in the Maize Genetics and Genomics database (Woodhouse *et al.*, 2021). According to the publicly available atlas of global transcription profiles for maize genes (Sekhon *et al.*, 2011; Winter *et al.*, 2007), *ZmGA20ox3* and *ZmGA20ox5* genes showed relatively higher expression levels in vegetative tissues and lower expression levels in reproductive tissues among the nine *ZmGA20ox* genes (Li *et al.*, 2010). In addition, phylogenetic analysis of the *ZmGA20ox* gene family revealed that *ZmGA20ox3* and *ZmGA20ox5* shared high sequence homology with the rice semi-dwarf gene, *OsGA20ox2* known as the "Green Revolution gene", that was one of the most important genes deployed in modern rice breeding (Monna *et al.*, 2002; Sasaki *et al.*, 2002; Spielmeyer *et al.*, 2002). Phylogenetic analysis shows that all three of these genes, *ZmGA20ox3*, *ZmGA20ox5*, and *OsGA20ox2*, are grouped together in the same clade (Song *et al.*, 2011). With these considerations, *ZmGA20ox3* and *ZmGA20ox5* genes were selected as the target genes for suppression of gene expression in developing MON 94804 maize.

To precisely and effectively suppress gene expression of the above-mentioned target maize endogenous genes, a microRNA (miRNA)-mediated suppression cassette that works through an RNA interference (RNAi) process was developed. The RNAi mechanism is a natural process in eukaryotic organisms for the down regulation of endogenous gene expression (Fire *et al.*, 1998; Jones-Rhoades *et al.*, 2006). A miRNA can trigger the RNAi mechanism in a manner similar to other small non-coding RNAs such as small interfering RNA (siRNA)(Carthew and Sontheimer, 2009).

MON 94804 contains a suppression cassette expressing an inverted repeat sequence designed to target endogenous maize gibberellic acid 20 oxidase (GA20ox) genes, *ZmGA20ox3* and *ZmGA20ox5*. The expressed inverted repeat transcript is recognized by the endogenous RNA interference (RNAi), resulting in down-regulation of the targeted GA20ox gene expression. Specifically, miRNA-encoding genes, such as *GA20ox_SUP* in MON 94804, are transcribed by Polymerase II, and folded into stem loop structures called primary miRNA (pri-miRNA). In plants, Dicer Like 1 (DCL1) processes the RNA initially into a duplex precursor miRNA (pre-miRNA), from which the mature miRNA (~21 nt) is incorporated into the functional Argonaute (AGO). AGO facilitates target sequence recognition and messenger RNA (mRNA) cleavage which leads to specific suppression of the target mRNA (Borges and Martienssen, 2015; Siomi and Siomi, 2009).

The GA20ox_SUP miRNA was expressed under transcriptional control of the rice tungro bacilliform virus (RTBV) promoter which showed the highest expression level within internodes in maize and low activity in reproductive tissues, which makes it a desired tool for targeted suppression of GA in vegetative tissues (Paciorek *et al.*, 2022).

The MOA responsible in generating the short stature phenotype in MON 94804[4] was studied and described in detail in Paciorek et al. (2022). It is important to demonstrate the target gene specificity of the *GA20ox_SUP* suppression cassette to evaluate the potential for unintended off-target gene suppression. To demonstrate the specificity of the *GA20ox_SUP* miRNA,

[4] The events, which are described as event-1 and event-2 in Paciorek *et al.* (2022) are MON 94804 and an experimental event containing a miRNA-mediated suppression cassette named *GA20ox_SUP* suppression cassette.

expression levels of *ZmGA20ox1* mRNA, the closest homolog gene to the two target genes in maize, were quantified (Paciorek *et al.*, 2022). Unlike the targeted *ZmGA20ox3* and *ZmGA20ox5* genes, no consistent suppression of expression levels of *ZmGA20ox1* was observed in tissues evaluated, including internode, leaf and reproductive tissues. The results demonstrate suppression of gene expression by the GA20ox_SUP miRNA[5] produced from the *GA20ox_SUP* suppression cassette is specific to the targeted *ZmGA20ox3* and *ZmGA20ox5* genes (Paciorek *et al.*, 2022).

The effect of suppression of *ZmGA20ox3* and *ZmGA20ox5* gene expression on endogenous bioactive GA levels was also assessed. Levels of bioactive GAs, GA_1 and GA_4, were reduced significantly in the vegetative tissues of stalk internode and leaf with no significant reduction of GA levels being detected in reproductive tissues (Paciorek *et al.*, 2022). Therefore, suppression of both targeted genes resulted in the reduction of bioactive GA levels in the stalk internode, leading to a reduction of stalk internode length, which consequently reduced overall plant height without affecting the reproductive potential when compared to the control maize (Paciorek *et al.*, 2022).

B.3(d)(ii) The Expression levels of GA20ox_SUP RNA in MON 94804 Maize in Multiple Tissues

Northern blots were performed to analyze the transcripts produced from the *GA20ox_SUP* suppression cassette in MON 94804 maize in multiple tissue types collected from a 2021 US greenhouse[6]. Because the transcripts from the *GA20ox_SUP* suppression cassette can exist as full length transcripts, or as processed intermediate transcripts, or as fully processed small RNA transcripts, two types of northern blot analyses, HMW and LMW, were performed to determine the presence or absence of the transcripts from *GA20ox_SUP* suppression cassette in over season leaf (OSL1), over season root (OSR1), stalk, forage, and grain tissues collected from MON 94804 maize.

The HMW northern blot was probed with sequences spanning the *GA20ox_SUP* suppression cassette in MON 94804. A map of the plasmid vector PV-ZMAP527892 annotated with the DIG-labeled probes used for northern analysis, is presented in Figure 15. Using this approach, medium or large (~0.2 kb to 6.0 kb) RNA molecules that are derived from the *GA20ox_SUP* sequences in the suppression cassette will produce a hybridization signal.

The LMW northern blot was probed with a synthesized 5´end DIG labeled 21 nucleotide DNA oligo with sequence derived from the *GA20ox_SUP* suppression cassette. This oligo was used to target the mature miRNA transcript and will detect any small RNA molecules (~15 nucleotides to 1.0 kb) from *GA20ox_SUP* suppression cassette in MON 94804.

Samples with a defined quantity of RNA were used to establish the limit of detection in the northern blot analyses. In vitro-transcribed RNA, GA20ox_SUP RNA (719 nt) from the *GA20ox_SUP* suppression cassette (1.0% agarose/~7% formaldehyde gel) or a 21 nucleotide RNA oligo with sequences derived from the *GA20ox_SUP* suppression cassette in plasmid

[5] GA20ox_SUP miRNA includes the original transcript, intermediate processing products and the GA20ox_SUP mature miRNA (21 nt).

[6] Grain samples were produced in an earlier field production. The same grain material was used for northern analyses and planting of the 2021 greenhouse production for collection of other tissue types

PV-ZMAP527892 (15% TBE-Urea gels) were spiked into conventional maize RNA in serial dilutions and loaded on the gel.

Northern blot analyses showed that transcripts from the *GA20ox_SUP* suppression cassette are detected in over season leaf (OSL) 1, over season root (OSR) 1, stalk, and forage tissues in both populations of high and low molecular weight RNA molecules and in grain tissue in the population of LMW RNA molecules, that are consistent with gene transcription and miRNA processing.

The details of the materials and methods are described in Appendix 6

B.3(d)(ii)(i) Northern Blot Analysis of GA20ox_SUP HMW & LMW Transcripts in MON 94804 OSL1 Tissue

Northern Blot Analysis of GA20ox_SUP HMW Transcripts in MON 94804 OSL1 Tissue

Approximately 10 µg of total RNA extracted from conventional or MON 94804 OSL1 tissue were resolved on a 1.0% agarose/~7% formaldehyde gel. Prior to transfer of the RNA onto the nylon membrane, fluorescent image of the ethidium bromide stained molecular weight marker and samples was taken to assess RNA loading and quality. The ethidium bromide signal is at similar intensity across all lanes indicating that the total RNA from the conventional control and MON 94804 OSL1 tissues were of comparable loading and quality (Figure 20B).

After the transfer of the RNA onto a nylon membrane, the blot was hybridized with DIG-labeled GA20ox_SUP probes (Figure 20A). As the positive hybridization controls in HMW northern blot, probe templates with 2 pg each spiked into conventional RNA showed the hybridization signals as expected (Figure 20A, lane 1 & 2). Detection of the positive controls indicates that the probes hybridized to the target sequences. The RiboRuler High Range RNA ladder serves as a broad reference for the approximate positions of the northern signals from RNA lanes and it is not applicable to the DNA probe template lanes. Total RNA isolated from the OSL1 tissue of the conventional control showed no detectable GA20ox_SUP hybridization bands as expected (Figure 20A, lane 3). Total RNA isolated from OSL1 tissue of MON 94804 produced a hybridization band at ~0.9 kb (Figure 20A, lane 4), which is the expected size of pri-miRNA transcript in MON 94804 from HMW Northern analysis. No pre-miRNA band was detected likely due to the rapid processing of this RNA. 5 pg, 10 pg, 25 pg, and 50 pg of the in vitro-transcribed GA20ox_SUP RNA spiked into conventional RNA produced the expected hybridization bands at ~0.7 kb (Figure 20A, lane 5-8, respectively) indicating that the limit of detection of this northern blot is 5 pg per 10 µg total RNA.

Northern Blot Analysis of GA20ox_SUP LMW Transcripts in MON 94804 OSL1 Tissue

Approximately 10 µg of total RNA extracted from conventional or MON 94804 OSL1 tissue were resolved on a 15% TBE-Urea gel. Prior to transfer of the RNA onto the nylon membrane, a fluorescent image of the ethidium bromide stained molecular weight marker and samples was taken to assess RNA loading and quality. The ethidium bromide signal is at similar intensity across all lanes indicating that the total RNA from the conventional control and MON 94804 OSL1 tissues were of comparable loading and quality (Figure 21B).

After the transfer of the RNA onto a nylon membrane, the blot was hybridized with DIG-labeled oligo probe which was designed to target the mature miRNA sequence in MON 94804 (Figure 21A). Total RNA isolated from the OSL1 tissue of the conventional control showed no detectable hybridization bands as expected (Figure 21A, lane 1). Total RNA isolated from OSL1 tissue of MON 94804 produced a hybridization band at ~21 nt (Figure 21A, lane 2), which is the expected size of transcript as mature miRNA in MON 94804 from LMW Northern analysis. 5 pg, 10 pg, 25 pg, and 50 pg of the synthesized 21 nucleotide RNA oligo spiked into conventional RNA produced the expected hybridization bands at ~21 nt (Figure 21A, lane 3-6, respectively) indicating that the limit of detection of this northern blot is 5 pg per 10 µg total RNA.

B.3(d)(ii)(ii) Northern Blot Analysis of GA20ox_SUP HMW & LMW Transcripts in MON 94804 OSR1 Tissue

Northern Blot Analysis of GA20ox_SUP HMW Transcripts in MON 94804 OSR1 Tissue

Approximately 10 µg of total RNA extracted from conventional or MON 94804 OSR1 tissue were resolved on a 1.0% agarose/~7% formaldehyde gel. Prior to transfer of the RNA onto the nylon membrane, fluorescent image of the ethidium bromide stained molecular weight marker and samples was taken to assess RNA loading and quality. The ethidium bromide signal is at similar intensity across all lanes indicating that the total RNA from the conventional control and MON 94804 OSR1 tissues were of comparable loading and quality (Figure 22B).

After the transfer of the RNA onto a nylon membrane, the blot was hybridized with DIG-labeled GA20ox_SUP probes (Figure 22 A). As the positive hybridization controls in HMW northern blot, probe templates with 2 pg each spiked into conventional RNA showed the hybridization signals as expected (Figure 22A, lane 1 & 2). Detection of the positive controls indicates that the probes hybridized to the target sequences. The RiboRuler High Range RNA ladder serves as a broad reference for the approximate positions of the northern signals from RNA lanes and it is not applicable to the DNA probe template lanes. Total RNA isolated from the OSR1 tissue of the conventional control showed no detectable GA20ox_SUP hybridization bands as expected (Figure 22A, lane 3). Total RNA isolated from OSR1 tissue of MON 94804 produced a hybridization band at ~0.9 kb (Figure 22A, lane 4), which is the expected size of pri-miRNA transcript in MON 94804 from HMW northern analysis. No pre-miRNA band was detected likely due to the rapid processing of this RNA. 5 pg, 10 pg, 25 pg, and 50 pg of the in vitro-transcribed GA20ox_SUP RNA spiked into conventional RNA produced the expected hybridization bands at ~0.7 kb (Figure 22A, lane 5-8, respectively) indicating that the limit of detection of this northern blot is 5 pg per 10 µg total RNA.

Northern Blot Analysis of GA20ox_SUP miRNA LMW Transcripts in MON 94804 OSR1 Tissue

Approximately 10 µg of total RNA extracted from conventional or MON 94804 OSR1 tissue were resolved on a 15% TBE-Urea gel. Prior to transfer of the RNA onto the nylon membrane, a fluorescent image of the SYBR® Gold stained molecular weight marker and samples was taken to assess RNA loading and quality. The SYBR® Gold signal is at similar intensity across all lanes indicating that the total RNA from the conventional control and MON 94804 OSR1 tissues were of comparable loading and quality (Figure 23B).

After the transfer of the RNA onto a nylon membrane, the blot was hybridized with DIG-labeled oligo probe which was designed to target the mature miRNA sequence in MON 94804

(Figure 23A). Total RNA isolated from the OSR1 tissue of the conventional control showed no detectable hybridization bands as expected (Figure 23A, lane 1). Total RNA isolated from OSR1 tissue of MON 94804 produced a hybridization band at ~21 nt (Figure 23A, lane 2), which is the expected size of transcript as mature miRNA in MON 94804 from LMW northern analysis. 2.5 pg, 5 pg, 12.5 pg, and 25 pg of the synthesized 21 nucleotide RNA oligo spiked into conventional RNA produced the expected hybridization bands at ~21 nt (Figure 23A, lane 3-6, respectively) indicating that the limit of detection of this northern blot is 2.5 pg per 10 µg total RNA.

B.3(d)(ii)(iii) Northern Blot Analysis of GA20ox_SUP HMW & LMW Transcripts in MON 94804 Stalk Tissue

Northern Blot Analysis of GA20ox_SUP HMW Transcripts in MON 94804 Stalk Tissue

Approximately 10 µg of total RNA extracted from conventional or MON 94804 stalk tissue were resolved on a 1.0% agarose/~7% formaldehyde gel. Prior to transfer of the RNA onto the nylon membrane, fluorescent image of the ethidium bromide stained molecular weight marker and samples was taken to assess RNA loading and quality. The ethidium bromide signal is at similar intensity across all lanes indicating that the total RNA from the conventional control and MON 94804 stalk tissues were of comparable loading and quality (Figure 24B).

After the transfer of the RNA onto a nylon membrane, the blot was hybridized with DIG-labeled GA20ox_SUP probes (Figure 24A). As the positive hybridization controls in HMW northern blot, probe templates with 2 pg each spiked into conventional RNA showed the hybridization signals as expected (Figure 24A, lane 1 & 2). Detection of the positive controls indicates that the probes hybridized to the target sequences. The RiboRuler High Range RNA ladder serves as a broad reference for the approximate positions of the northern signals from RNA lanes and it is not applicable to the DNA probe template lanes. Total RNA isolated from the stalk tissue of the conventional control showed no detectable GA20ox_SUP hybridization bands as expected (Figure 24A, lane 3). Total RNA isolated from stalk tissue of MON 94804 produced a hybridization band at ~0.9 kb (Figure 24A, lane 4), which is the expected size of pri-miRNA transcript in MON 94804 from HMW northern analysis. No pre-miRNA band was detected likely due to the rapid processing of this RNA. 10 pg, 25 pg, 50 pg, and 75 pg of the in vitro-transcribed GA20ox_SUP RNA spiked into conventional RNA produced the expected hybridization bands at ~0.7 kb (Figure 24A, lane 5-8, respectively) indicating that the limit of detection of this northern blot is 10 pg per 10 µg total RNA.

Northern Blot Analysis of GA20ox_SUP LMW Transcripts in MON 94804 Stalk Tissue

Approximately 10 µg of total RNA extracted from conventional or MON 94804 stalk tissue were resolved on a 15% TBE-Urea gel. Prior to transfer of the RNA onto the nylon membrane, a fluorescent image of the SYBR® Gold stained molecular weight marker and samples was taken to assess RNA loading and quality. The SYBR® Gold signal is at similar intensity across all lanes indicating that the total RNA from the conventional control and MON 94804 stalk tissues were of comparable loading and quality (Figure 25B).

After the transfer of the RNA onto a nylon membrane, the blot was hybridized with DIG-labeled oligo probe which was designed to target the mature miRNA sequence in MON 94804 (Figure 25A). Total RNA isolated from the stalk tissue of the conventional control showed no

detectable hybridization bands as expected (Figure 25A, lane 1). Total RNA isolated from stalk tissue of MON 94804 produced a hybridization band at ~21 nt (Figure 25A, lane 2), which is the expected size of transcript as mature miRNA in MON 94804 from LMW northern analysis. 5 pg, 10 pg, 25 pg, and 50 pg of the synthesized 21 nucleotide RNA oligo spiked into conventional RNA produced the expected hybridization bands at ~21 nt (Figure 25A, lane 3-6, respectively) indicating that the limit of detection of this northern blot is 5 pg per 10 µg total RNA.

B.3(d)(ii)(iv) Northern Blot Analysis of GA20ox_SUP HMW & LMW Transcripts in MON 94804 Forage Tissue

Northern Blot Analysis of GA20ox_SUP HMW Transcripts in MON 94804 Forage Tissue

Approximately 10 µg of total RNA extracted from conventional or MON 94804 forage tissue were resolved on a 1.0% agarose/~7% formaldehyde gel. Prior to transfer of the RNA onto the nylon membrane, fluorescent image of the ethidium bromide stained molecular weight marker and samples was taken to assess RNA loading and quality. The ethidium bromide signal is at similar intensity across all lanes indicating that the total RNA from the conventional control and MON 94804 forage tissues were of comparable loading and quality (Figure 26B).

After the transfer of the RNA onto a nylon membrane, the blot was hybridized with DIG-labeled GA20ox_SUP probes (Figure 26A). As the positive hybridization controls in HMW northern blot, probe templates with 2 pg each spiked into conventional RNA showed the hybridization signals as expected (Figure 26A, lane 1 & 2). Detection of the positive controls indicates that the probes hybridized to the target sequences. The RiboRuler High Range RNA ladder serves as a broad reference for the approximate positions of the northern signals from RNA lanes and it is not applicable to the DNA probe template lanes. Total RNA isolated from the forage tissue of the conventional control showed no detectable GA20ox_SUP hybridization bands as expected (Figure 26A, lane 3). Total RNA isolated from forage tissue of MON 94804 produced a hybridization band at ~0.9 kb (Figure 26A, lane 4), which is the expected size of pri-miRNA transcript in MON 94804 from HMW northern analysis. No pre-miRNA band was detected likely due to the rapid processing of this RNA. 5 pg, 10 pg, 25 pg, and 50 pg of the in vitro-transcribed GA20ox_SUP RNA spiked into conventional RNA produced the expected hybridization bands at ~0.7 kb (Figure 26A, lane 5-8, respectively) indicating that the limit of detection of this northern blot is 5 pg per 10 µg total RNA.

Northern Blot Analysis of GA20ox_SUP LMW Transcripts in MON 94804 Forage Tissue

Approximately 10 µg of total RNA extracted from conventional or MON 94804 forage tissue were resolved on a 15% TBE-Urea gel. Prior to transfer of the RNA onto the nylon membrane, a fluorescent image of the SYBR® Gold stained molecular weight marker and samples was taken to assess RNA loading and quality. The SYBR® Gold signal is at similar intensity across all lanes indicating that the total RNA from the conventional control and MON 94804 forage tissues were of comparable loading and quality (Figure 27B).

After the transfer of the RNA onto a nylon membrane, the blot was hybridized with DIG-labeled oligo probe which was designed to target the mature miRNA sequence in MON 94804 (Figure 27A). Total RNA isolated from the forage tissue of the conventional control showed no detectable hybridization bands as expected (Figure 27A, lane 1). Total RNA isolated from

forage tissue of MON 94804 produced a hybridization band at ~21 nt (Figure 27A, lane 2), which is the expected size of transcript as mature miRNA in MON 94804 from LMW northern analysis. 5 pg, 10 pg, 25 pg, and 50 pg of the synthesized 21 nucleotide RNA oligo spiked into conventional RNA produced the expected hybridization bands at ~21 nt (Figure 27A, lane 3-6, respectively) indicating that the limit of detection of this northern blot is 5 pg per 10 µg total RNA.

B.3(d)(ii)(v) Northern Blot Analysis of GA20ox_SUP HMW & LMW Transcripts in MON 94804 Grain Tissue

Northern Blot Analysis of GA20ox_SUP HMW Transcripts in MON 94804 Grain Tissue

Approximately 10 µg of total RNA extracted from conventional or MON 94804 grain tissue were resolved on a 1.0% agarose/~7% formaldehyde gel. Prior to transfer of the RNA onto the nylon membrane, fluorescent image of the ethidium bromide stained molecular weight marker and samples was taken to assess RNA loading and quality. The ethidium bromide signal is at similar intensity across all lanes indicating that the total RNA from the conventional control and MON 94804 grain tissues were of comparable loading and quality (Figure 28B).

After the transfer of the RNA onto a nylon membrane, the blot was hybridized with DIG-labeled GA20ox_SUP probes (Figure 28A). As the positive hybridization controls in HMW northern blot, probe templates with 2 pg each spiked into conventional RNA showed the hybridization signals as expected (Figure 28A, lane 1 & 2). Detection of the positive controls indicates that the probes hybridized to the target sequences. The RiboRuler High Range RNA ladder serves as a broad reference for the approximate positions of the northern signals from RNA lanes and it is not applicable to the DNA probe template lanes. Total RNA isolated from the grain tissue of the conventional control showed no detectable GA20ox_SUP hybridization bands as expected (Figure 28A, lane 3). Total RNA isolated from grain tissue of MON 94804 showed no detectable GA20ox_SUP hybridization bands (Figure 28A, lane 4), at the level of detection of this northern blot, indicated below. 5 pg, 10 pg, 25 pg, and 50 pg of the in vitro-transcribed GA20ox_SUP RNA spiked into conventional RNA produced the expected hybridization bands at ~0.7 kb (Figure 28A, lane 5-8, respectively) indicating that the limit of detection of this northern blot is 5 pg per 10 µg total RNA.

Northern Blot Analysis of GA20ox_SUP LMW Transcripts in MON 94804 Grain Tissue

Approximately 10 µg of total RNA extracted from conventional or MON 94804 grain tissue were resolved on a 15% TBE-Urea gel. Prior to transfer of the RNA onto the nylon membrane, a fluorescent image of the SYBR® Gold stained molecular weight marker and samples was taken to assess RNA loading and quality. The SYBR® Gold signal is at similar intensity across all lanes indicating that the total RNA from the conventional control and MON 94804 grain tissues were of comparable loading and quality (Figure 29B).

After the transfer of the RNA onto a nylon membrane, the blot was hybridized with DIG-labeled oligo probe which was designed to target the mature miRNA sequence in MON 94804 (Figure 29A). Total RNA isolated from the grain tissue of the conventional control showed no detectable hybridization bands as expected (Figure 29A, lane 1). Total RNA isolated from grain tissue of MON 94804 produced a hybridization band at ~21 nt (Figure 29A, lane 2), which is the expected size of transcript as mature miRNA in MON 94804 from LMW northern analysis.

2.5 pg, 5 pg, 12.5 pg, and 25 pg of the synthesized 21 nucleotide RNA oligo spiked into conventional RNA produced the expected hybridization bands at ~21 nt (Figure 29A, lane 3-6, respectively) indicating that the limit of detection of this northern blot is 2.5 pg per 10 µg total RNA.

B.3(d)(ii)(vi) Expression of GA20ox_SUP Transcripts in MON 94804 Maize in Multiple Tissues Conclusions

Multiple tissues from MON 94804, except grain/seed, grown from a greenhouse production were collected and analyzed for the GA20ox_SUP transcripts using HMW & LMW northern blot analyses. Grain/seed samples were produced in an earlier field production. The same grain/seed material was used for northern analyses and planting of the 2021 greenhouse production for other tissue types. Northern blot analyses showed two populations of transcripts from the *GA20ox_SUP* suppression cassette, consistent with the expected pri-miRNA and mature miRNA, were present in MON 94804 OSL1, OSR1, stalk, and forage tissues. Only the low molecular weight RNA molecules, consistent with the mature miRNA, were detected in grain tissue.

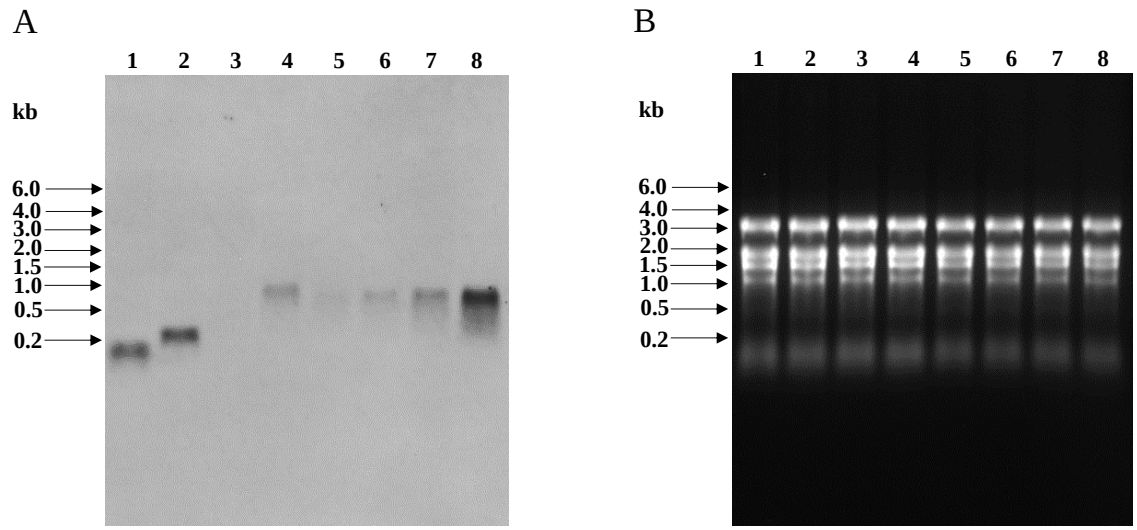


Figure 20. Northern Blot Analysis of HMW Transcript from *GA20ox_SUP* Suppression Cassette in MON 94804 Maize OSL1 Tissue

Approximately 10 µg total RNA isolated from conventional control and MON 94804 OSL1 tissue was resolved on a 1.0% agarose/~7% formaldehyde gel. Panel A is the HMW northern blot hybridized with the DIG-labeled GA20ox_SUP probes. Panel B is the fluorescent image of the ethidium bromide stained formaldehyde/ agarose gel prior to transfer onto a nylon membrane. Lane designations are as follows:

Lane
1 Conventional control + GA20ox_SUP probe template-1 (2 pg)
2 Conventional control + GA20ox_SUP probe template-2 (2 pg)
3 Conventional control
4 MON 94804 (OSL1)
5 Conventional control + 5 pg in vitro-transcribed GA20ox_SUP RNA
6 Conventional control + 10 pg in vitro-transcribed GA20ox_SUP RNA
7 Conventional control + 25 pg in vitro-transcribed GA20ox_SUP RNA
8 Conventional control + 50 pg in vitro-transcribed GA20ox_SUP RNA

Arrows denote the size of the RNA, in kilo bases, obtained from the RiboRuler High Range RNA Ladder (Thermo Fisher) on ethidium bromide stained gel.

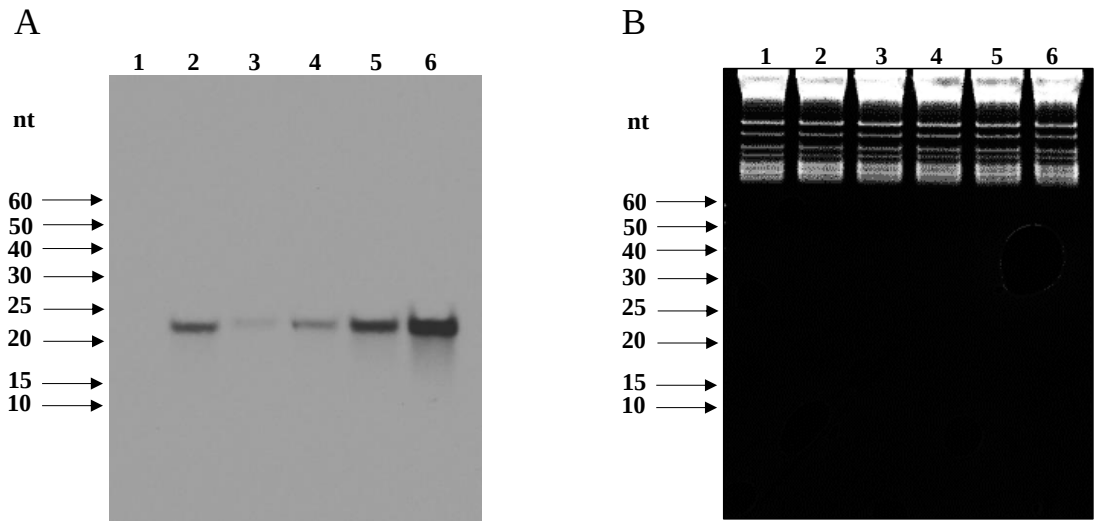


Figure 21. Northern Blot Analysis of LMW Transcript from *GA20ox_SUP* Suppression Cassette in MON 94804 Maize OSL1 Tissue

Approximately 10 µg of total RNA isolated from conventional control and MON 94804 OSL1 tissue was resolved on a precast 15% TBE Urea gel. Panel A is the LMW northern blot hybridized with the DIG-labeled oligo probe. Panel B is the fluorescent image of the ethidium bromide stained TBE Urea gel prior to transfer onto a nylon membrane. Lane designations are as follows:

Lane	
1	Conventional control
2	MON 94804 (OSL1)
3	Conventional control + 5 pg RNA oligo
4	Conventional control + 10 pg RNA oligo
5	Conventional control + 25 pg RNA oligo
6	Conventional control + 50 pg RNA oligo

Arrows denote the size of the RNA, in nucleotides, obtained from the 10/60 Oligo Ladder (Integrated DNA Technologies, Inc.) on the ethidium bromide stained gel.

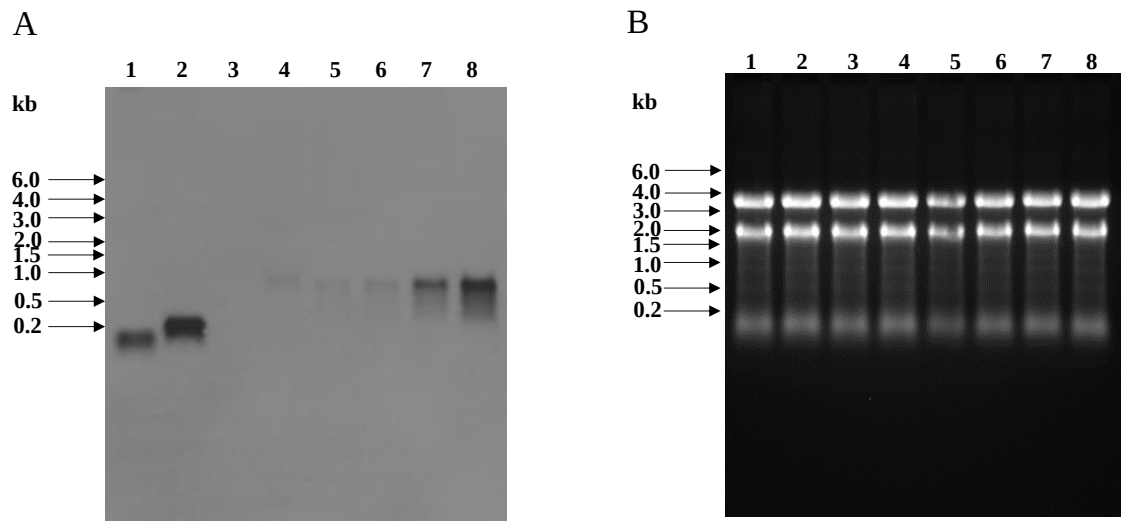


Figure 22. Northern Blot Analysis of HMW Transcript from *GA20ox_SUP* Suppression Cassette in MON 94804 Maize OSR1 Tissue

Approximately 10 µg total RNA isolated from conventional control and MON 94804 OSR1 tissue was resolved on a 1.0% agarose/~7% formaldehyde gel. Panel A is the HMW northern blot hybridized with the DIG-labeled GA20ox_SUP probes. Panel B is the fluorescent image of the ethidium bromide stained formaldehyde/ agarose gel prior to transfer onto a nylon membrane. Lane designations are as follows:

Lane

1 Conventional control + GA20ox_SUP probe template-1 (2 pg)
2 Conventional control + GA20ox_SUP probe template-2 (2 pg)
3 Conventional control
4 MON 94804 (OSR1)
5 Conventional control + 5 pg in vitro-transcribed GA20ox_SUP RNA
6 Conventional control + 10 pg in vitro-transcribed GA20ox_SUP RNA
7 Conventional control + 25 pg in vitro-transcribed GA20ox_SUP RNA
8 Conventional control + 50 pg in vitro-transcribed GA20ox_SUP RNA

Arrows denote the size of the RNA, in kilo bases, obtained from the RiboRuler High Range RNA Ladder (Thermo Fisher) on ethidium bromide stained gel.

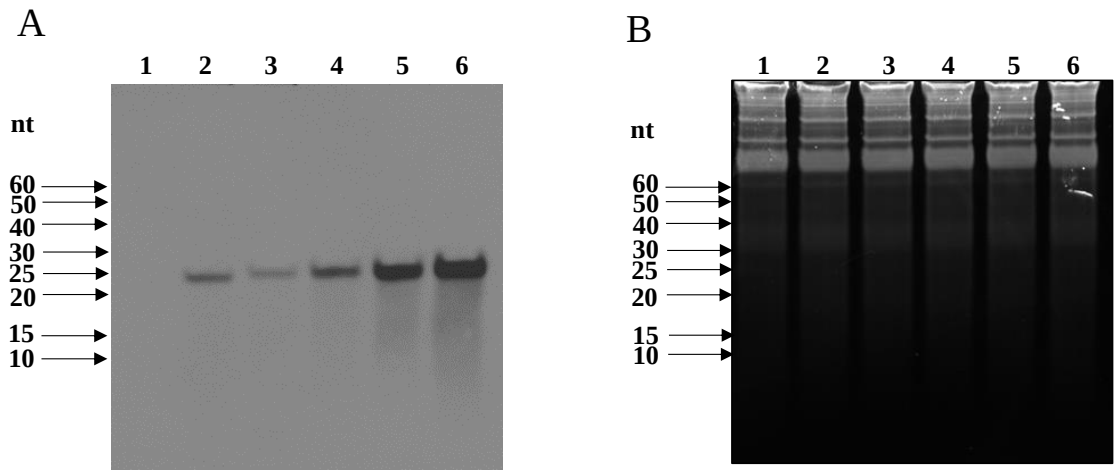


Figure 23. Northern Blot Analysis of LMW Transcript from *GA20ox_SUP* Suppression Cassette in MON 94804 Maize OSR1 Tissue

Approximately 10 µg of total RNA isolated from conventional control and MON 94804 OSR1 tissue was resolved on a precast 15% TBE Urea gel. Panel A is the LMW northern blot hybridized with the DIG-labeled oligo probe. Panel B is the fluorescent image of the SYBR® Gold stained TBE Urea gel prior to transfer onto a nylon membrane. Lane designations are as follows:

Lane
1 Conventional control
2 MON 94804 (OSR1)
3 Conventional control + 2.5 pg RNA oligo
4 Conventional control + 5 pg RNA oligo
5 Conventional control + 12.5 pg RNA oligo
6 Conventional control + 25 pg RNA oligo

Arrows denote the size of the RNA, in nucleotides, obtained from the 10/60 Oligo Ladder (Integrated DNA Technologies, Inc.) on the SYBR® Gold stained gel.

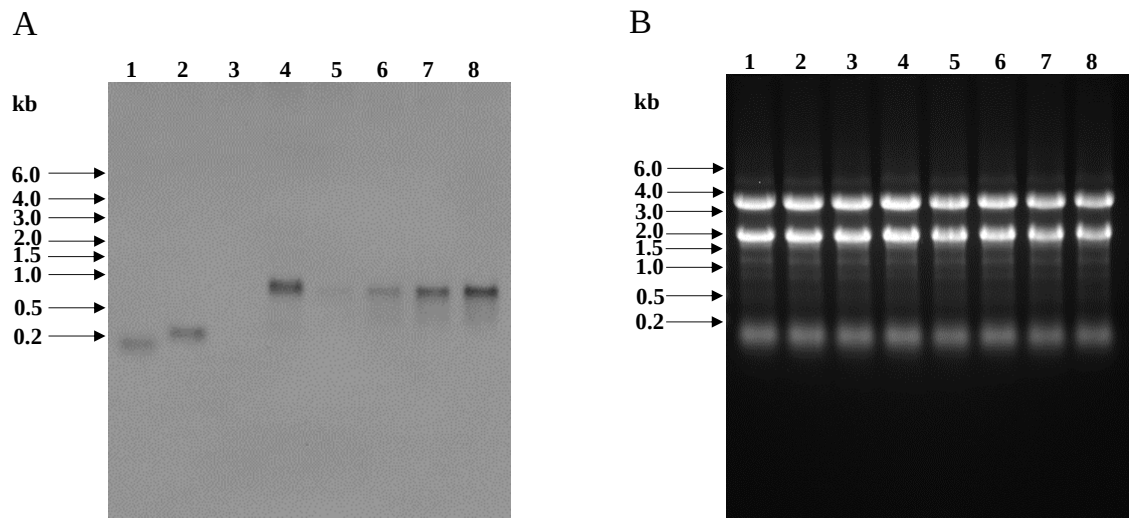


Figure 24. Northern Blot Analysis of HMW Transcript from *GA20ox_SUP* Suppression Cassette in MON 94804 Maize Stalk Tissue

Approximately 10 µg total RNA isolated from conventional control and MON 94804 stalk tissue was resolved on a 1.0% agarose/~7% formaldehyde gel. Panel A is the HMW northern blot hybridized with the DIG-labeled GA20ox_SUP probes. Panel B is the fluorescent image of the ethidium bromide stained formaldehyde/ agarose gel prior to transfer onto a nylon membrane. Lane designations are as follows:

Lane
1 Conventional control + GA20ox_SUP probe template-1 (2 pg)
2 Conventional control + GA20ox_SUP probe template-2 (2 pg)
3 Conventional control
4 MON 94804 (Stalk)
5 Conventional control + 10 pg in vitro-transcribed GA20ox_SUP RNA
6 Conventional control + 25 pg in vitro-transcribed GA20ox_SUP RNA
7 Conventional control + 50 pg in vitro-transcribed GA20ox_SUP RNA
8 Conventional control + 75 pg in vitro-transcribed GA20ox_SUP RNA

Arrows denote the size of the RNA, in kilo bases, obtained from the RiboRuler High Range RNA Ladder (Thermo Fisher) on ethidium bromide stained gel.

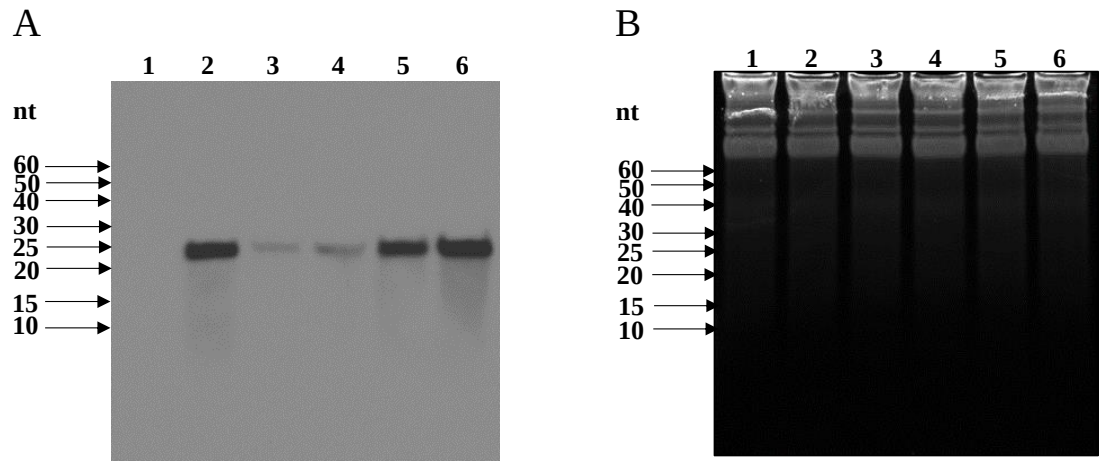


Figure 25. Northern Blot Analysis of LMW Transcript from *GA20ox_SUP* Suppression Cassette in MON 94804 Maize Stalk Tissue

Approximately 10 µg of total RNA isolated from conventional control and MON 94804 stalk tissue was resolved on a precast 15% TBE Urea gel. Panel A is the LMW northern blot hybridized with the DIG-labeled oligo probe. Panel B is the fluorescent image of the ethidium SYBR® Gold TBE Urea gel prior to transfer onto a nylon membrane. Lane designations are as follows:

Lane	
1	Conventional control
2	MON 94804 (Stalk)
3	Conventional control + 5 pg RNA oligo
4	Conventional control + 10 pg RNA oligo
5	Conventional control + 25 pg RNA oligo
6	Conventional control + 50 pg RNA oligo

Arrows denote the size of the RNA, in nucleotides, obtained from the 10/60 Oligo Ladder (Integrated DNA Technologies, Inc.) on the SYBR® Gold stained gel.

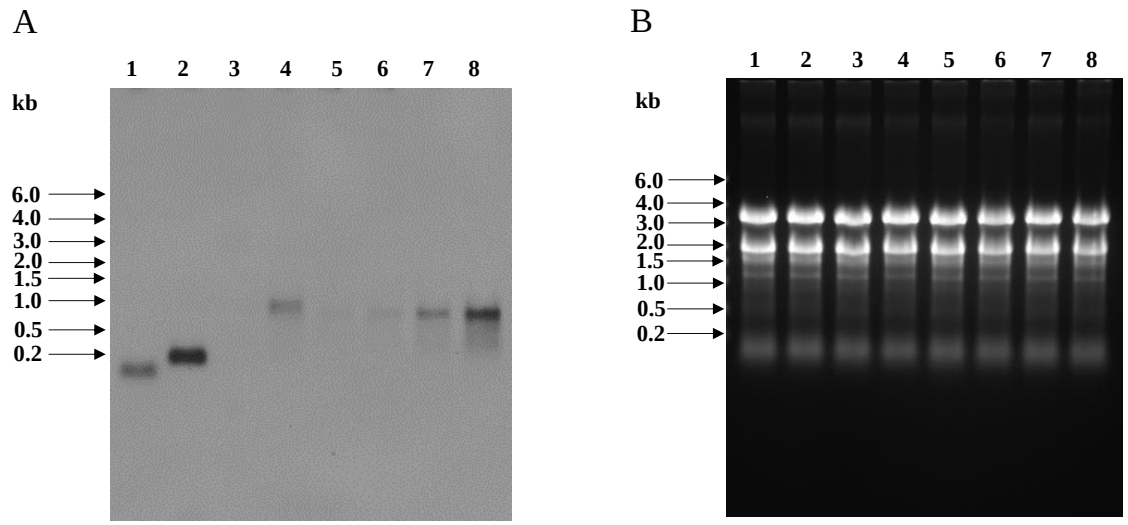


Figure 26. Northern Blot Analysis of HMW Transcript from *GA20ox_SUP* Suppression Cassette in MON 94804 Maize Forage Tissue

Approximately 10 µg total RNA isolated from conventional control and MON 94804 forage tissue was resolved on a 1.0% agarose/~7% formaldehyde gel. Panel A is the HMW northern blot hybridized with the DIG-labeled GA20ox_SUP probes. Panel B is the fluorescent image of the ethidium bromide stained formaldehyde/ agarose gel prior to transfer onto a nylon membrane. Lane designations are as follows:

Lane

1 Conventional control + GA20ox_SUP probe template-1 (2 pg)
2 Conventional control + GA20ox_SUP probe template-2 (2 pg)
3 Conventional control
4 MON 94804 (Forage)
5 Conventional control + 5 pg in vitro-transcribed GA20ox_SUP RNA
6 Conventional control + 10 pg in vitro-transcribed GA20ox_SUP RNA
7 Conventional control + 25 pg in vitro-transcribed GA20ox_SUP RNA
8 Conventional control + 50 pg in vitro-transcribed GA20ox_SUP RNA

Arrows denote the size of the RNA, in kilo bases, obtained from the RiboRuler High Range RNA Ladder (Thermo Fisher) on ethidium bromide stained gel.

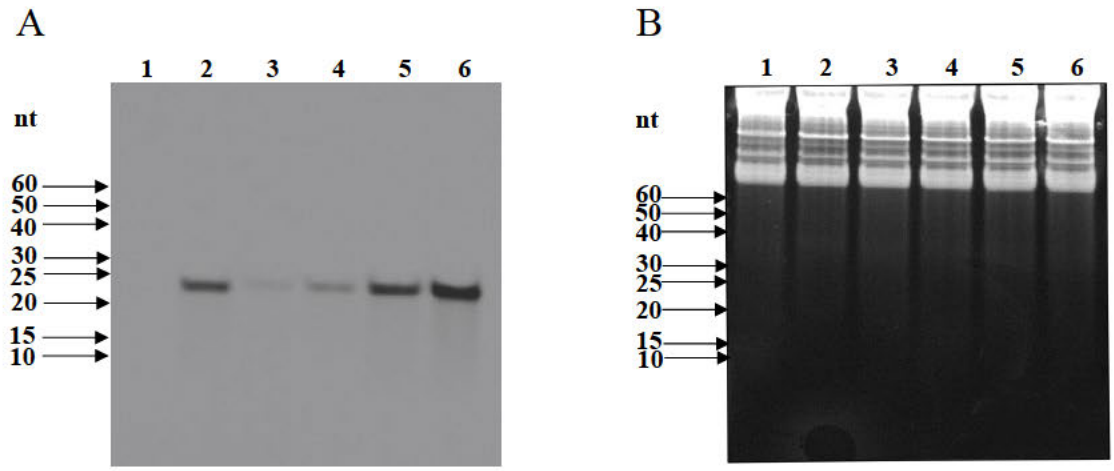


Figure 27. Northern Blot Analysis of LMW Transcript from *GA20ox_SUP* Suppression Cassette in MON 94804 Maize Forage Tissue

Approximately 10 µg of total RNA isolated from conventional control and MON 94804 forage tissue was resolved on a precast 15% TBE Urea gel. Panel A is the LMW northern blot hybridized with the DIG-labeled oligo probe. Panel B is the fluorescent image of the SYBR® Gold stained TBE Urea gel prior to transfer onto a nylon membrane. Lane designations are as follows:

Lane	
1	Conventional control
2	MON 94804 (Forage)
3	Conventional control + 5 pg RNA oligo
4	Conventional control + 10 pg RNA oligo
5	Conventional control + 25 pg RNA oligo
6	Conventional control + 50 pg RNA oligo

Arrows denote the size of the RNA, in nucleotides, obtained from the 10/60 Oligo Ladder (Integrated DNA Technologies, Inc.) on the SYBR® Gold stained gel.

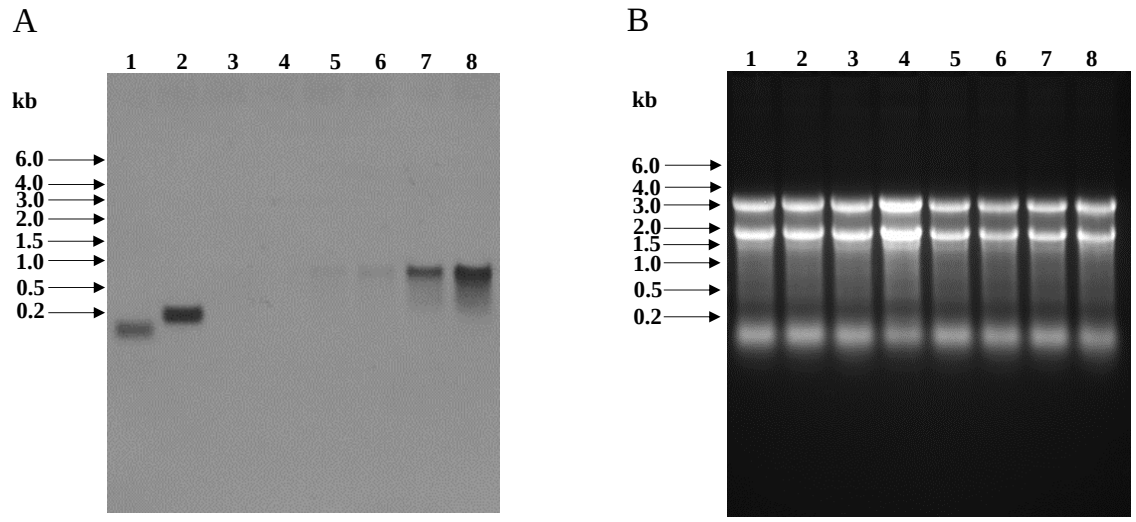


Figure 28. Northern Blot Analysis of HMW Transcript from *GA20ox_SUP* Suppression Cassette in MON 94804 Maize Grain Tissue

Approximately 10 µg total RNA isolated from conventional control and MON 94804 grain tissue was resolved on a 1.0% agarose/~7% formaldehyde gel. Panel A is the HMW northern blot hybridized with the DIG-labeled GA20ox_SUP probes. Panel B is the fluorescent image of the ethidium bromide stained formaldehyde/ agarose gel prior to transfer onto a nylon membrane. Lane designations are as follows:

Lane
1 Conventional control + GA20ox_SUP probe template-1 (2 pg)
2 Conventional control + GA20ox_SUP probe template-2 (2 pg)
3 Conventional control
4 MON 94804 (Grain)
5 Conventional control + 5 pg in vitro-transcribed GA20ox_SUP RNA
6 Conventional control + 10 pg in vitro-transcribed GA20ox_SUP RNA
7 Conventional control + 25 pg in vitro-transcribed GA20ox_SUP RNA
8 Conventional control + 50 pg in vitro-transcribed GA20ox_SUP RNA

Arrows denote the size of the RNA, in kilo bases, obtained from the RiboRuler High Range RNA Ladder (Thermo Fisher) on ethidium bromide stained gel.

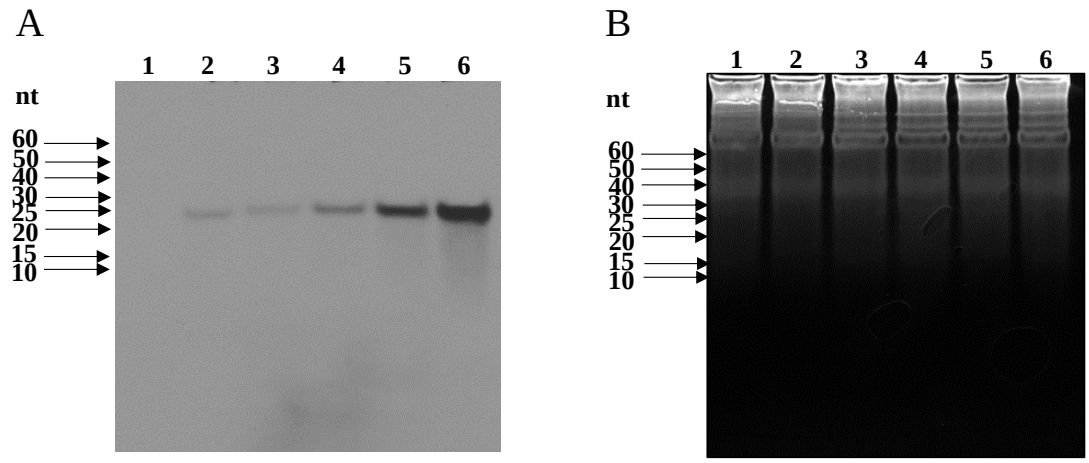


Figure 29. Northern Blot Analysis of LMW Transcript from *GA20ox_SUP* Suppression Cassette in MON 94804 Maize Grain Tissue

Approximately 10 µg of total RNA isolated from conventional control and MON 94804 grain tissue was resolved on a precast 15% TBE Urea gel. Panel A is the LMW northern blot hybridized with the DIG-labeled oligo probe. Panel B is the fluorescent image of the SYBR® Gold stained TBE Urea gel prior to transfer onto a nylon membrane. Lane designations are as follows:

Lane
1 Conventional control
2 MON 94804 (Grain)
3 Conventional control + 2.5 pg RNA oligo
4 Conventional control + 5 pg RNA oligo
5 Conventional control + 12.5 pg RNA oligo
6 Conventional control + 25 pg RNA oligo

Arrows denote the size of the RNA, in nucleotides, obtained from the 10/60 Oligo Ladder (Integrated DNA Technologies, Inc.) on the SYBR® Gold stained gel.

B.3(d)(iii) The specificity of the RNA interference

Assessment of the Endogenous *ZmGA20ox3* and *ZmGA20ox5* mRNA Levels in Maize Tissues of MON 94804 Maize

The *GA20ox_SUP* suppression cassette was designed to target *ZmGA20ox3* and *ZmGA20ox5*, which have relatively higher expression levels in vegetative tissues such as stalk and leaf and relatively lower expression levels in reproductive tissues such as grain (http://bar.utoronto.ca/efp_maize/cgi-bin/efpWeb.cgi). In addition, to further refine the target suppression to tissue types that are more directly associated with plant height, the rice tungro bacilliform virus (RTBV) promoter, which is preferentially expressed in vascular bundles (Yin and Beachy, 1995), was used to control the transcription of the *GA20ox_SUP* suppression cassette. Those tissues, especially stalks with a relatively high number of vascular bundles, are expected to exhibit expression suppression of *ZmGA20ox3* and *ZmGA20ox5* in MON 94804 maize. To assess the effectiveness of the *GA20ox_SUP* suppression cassette that expresses GA20ox_SUP miRNA, relative mRNA levels of *ZmGA20ox3* and *ZmGA20ox5* were determined in various maize tissues collected from MON 94804 and the corresponding conventional control maize.

The details of the materials and methods are described in Appendix 7.

Relative mRNA levels of *ZmGA20ox3*

Relative mRNA levels of *ZmGA20ox3* were determined in five tissue types of MON 94804 and conventional control maize using TaqMan qPCR assay and the results are summarized Table 7. Among the five tissue types analyzed, the relative mRNA levels of *ZmGA20ox3* in MON 94804 are significantly reduced in OSL1, stalk, and grain compared to that of the corresponding conventional control maize tissues. This observation is consistent with the MON 94804 mode of action that GA20ox_SUP miRNA leads to suppression of *ZmGA20ox3* expression in those tissues through the endogenous RNAi process. No statistically significant change of *ZmGA20ox3* mRNA levels was observed in OSR1, and forage of MON 94804 compared to the conventional control maize.

Relative mRNA levels of *ZmGA20ox5*

Relative mRNA levels of *ZmGA20ox5* were determined in five tissue types of MON 94804 and conventional control maize using TaqMan qPCR assay and the results are summarized in Table 8. Among the five tissue types analyzed, the relative mRNA levels of *ZmGA20ox5* in MON 94804 are significantly reduced in OSL1, stalk, and grain tissues compared to those of the corresponding conventional control maize tissues. This observation is consistent with the MON 94804 mode of action that GA20ox_SUP miRNA leads to suppression of *ZmGA20ox5* expression in those tissues through the endogenous RNAi process. No statistically significant change of *ZmGA20ox5* mRNA levels was observed in OSR1, and forage tissues of MON 94804 compared to the conventional control maize.

Conclusion of Assessment of the Endogenous *ZmGA20ox3* and *ZmGA20ox5* mRNA Levels in Maize Tissues of MON 94804 Maize

Statistical analyses of the relative mRNA levels of GA20ox_SUP miRNA target genes, *ZmGA20ox3* and *ZmGA20ox5*, in MON 94804 and conventional control maize were conducted. Compared to that of the corresponding conventional control tissues, expression reduction of both GA20ox_SUP miRNA target genes, *ZmGA20ox3* and *ZmGA20ox5*, was

detected in several tissues including vegetative tissues such as OSL1 and stalk. These results thus demonstrate that the *GA20ox_SUP* suppression cassette that expresses the GA20ox_SUP miRNA suppresses the expression of target genes *ZmGA20ox3* and *ZmGA20ox5* in MON 94804 maize.

Table 7. Summary of Mean, Standard Error (SE), and p-value of *ZmGA20ox3* Relative mRNA Levels in Tissues of Conventional Control Maize and MON 94804 Maize

Tissue Type[1]	Product	Mean (SE)[2]	p-value
OSL1	Conventional Control	1615.1 (149.5)	<0.0001*
	MON 94804	546.6 (39.9)	
OSR1	Conventional Control	178.2 (39.7)	0.3695
	MON 94804	139.7 (12.0)	
Stalk	Conventional Control	4488.5 (348.0)	<0.0001*
	MON 94804	797.0 (37.1)	
Forage	Conventional Control	1736.1 (212.5)	0.0787
	MON 94804	1285.4 (106.3)	
Grain	Conventional Control	47.7 (10.3)	0.0144*
	MON 94804	18.6 (1.4)	

[1]Tissues were collected at the following growth stages:
OSL1: V3-V4; OSR1: V3-V4; Stalk: V10-V12; Forage: R5; Grain: R6.
[2]The sample means and SEs were calculated for each tissue type (n=8). Since these are values relative to *EF1-α* mRNA level, no unit was given.
*Denotes a statistical difference between the conventional control maize and MON 94804 at $p < 0.05$ using t-tests.

Table 8. Summary of Mean, Standard Error (SE), and p-value of *ZmGA20ox5* Relative mRNA Levels in Tissues of Conventional Control Maize and MON 94804 Maize

Tissue Type[1]	Product	Mean (SE)[2]	p-value
OSL1	Conventional Control	34659.6 (3078.1)	<0.0001*
	MON 94804	11830.2 (802.7)	
OSR1	Conventional Control	814.1 (110.5)	0.9558
	MON 94804	822.0 (86.0)	
Stalk	Conventional Control	4072.4 (122.4)	<0.0001*
	MON 94804	2728.8 (180.1)	
Forage	Conventional Control	35097.3 (3879.3)	0.0645
	MON 94804	26644.2 (1642.0)	
Grain	Conventional Control	2071.7 (119.0)	<0.0001*
	MON 94804	992.2 (26.1)	

[1]Tissues were collected at the following growth stages:
OSL1: V3-V4; OSR1: V3-V4; Stalk: V10-V12; Forage: R5; Grain: R6.
[2]The sample means and SEs were calculated for each tissue type (n=8, except in conventional control pollen where n=7 due to one sample had no amplification). Since these are values relative to *EF1-α* mRNA level, no unit was given.
*Denotes a statistical difference between the conventional control maize and MON 94804 at $p < 0.05$ using t-tests.

B.4 Novel Herbicide Metabolites in GM Herbicide-Tolerant Plants

Not applicable.

B.5 Compositional Analyses of the Food Produced Using Gene Technology

Food and feed safety assessments of biotechnology-derived crops follow the comparative safety assessment process (Codex Alimentarius, 2009) in which the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop are compared to the appropriate conventional control that has a history of safe use. For maize, assessments are performed based on the general principles outlined in the OECD consensus document for maize composition (OECD, 2002a).

A review of compositional assessments, that encompassed a total of seven biotechnology-derived crop traits, four maize and three soybean, nine countries, and eleven growing seasons, concluded that incorporation of biotechnology-derived agronomic traits has had little impact on crop composition (Harrigan *et al.*, 2010). Most compositional variation is attributable to growing region, agronomic practices, and genetic background (Harrigan *et al.*, 2010). Numerous scientific publications have further documented the extensive variability in the concentrations of crop nutrients, anti-nutrients, and secondary metabolites that reflect the influence of environmental and genetic factors as well as extensive conventional breeding efforts to improve nutrition, agronomics, and yield (Harrigan *et al.*, 2010; Harrigan *et al.*, 2009; Ridley *et al.*, 2011; Zhou *et al.*, 2011). Compositional equivalence between biotechnology-derived and conventional crops supports an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD, 2002b). OECD consensus documents on compositional considerations for new crop varieties emphasize quantitative measurements of essential nutrients and known anti-nutrients or toxicants. These quantitative measurements effectively discern compositional changes that imply potential nutritional or safety (*e.g.*, anti-nutritional) concerns. Levels of the components in grain and/or other raw agricultural commodities of the biotechnology-derived crop product are compared to: 1) corresponding levels in a conventional control, a genetically similar conventional line, grown concurrently under similar field conditions, and 2) natural ranges from data published in the scientific literature or in publicly available databases (*e.g.* Agriculture and Food Systems Institute Crop Composition Database (AFSI CCDB)) (AFSI, 2020). This second comparison places any potential differences between the assessed biotechnology-derived crop and its comparator in the context of the well-documented variation within and among maize lines in the concentrations of crop nutrients, anti-nutrients and secondary metabolites.

This section provides a summary of the analyses conducted to evaluate key nutrients, anti-nutrients and secondary metabolites in grain and forage of MON 94804 maize compared to that of a conventional control maize HCL301+HCL617 grown and harvested under similar conditions. The production of materials for compositional analyses used a sufficient variety of field trial sites, robust experimental design (randomized complete block design with four block replicates), and sensitive analytical methods that allow accurate assessments of compositional characteristics over a range of environmental conditions typical for maize production. The information provided in this section addresses relevant factors in Codex Plant Guidelines, Section 4, paragraphs 44 and 45 for compositional analyses (Codex Alimentarius, 2009).

See Appendix 8 for details on composition methods.

B.5(a) The levels of relevant key nutrients, toxicants and anti-nutrients in the food produced using gene technology compared with the levels in an appropriate comparator

B.5(a)(i) Compositional equivalence of MON 94804 maize grain and forage to conventional maize

Grain and forage samples were harvested from MON 94804 and the conventional control grown in the United States during the 2020 season. The field production was conducted at five sites. The field sites were planted in a randomized complete block design with four replicates at each site. MON 94804 and the conventional control were grown under normal agronomic field conditions for their respective growing regions.

The compositional analysis provided a comprehensive comparative assessment of the levels of key nutrients, anti-nutrients and secondary metabolites in grain and forage of MON 94804 and the conventional control.

The evaluation of MON 94804 followed considerations relevant to the compositional quality of maize as defined by the OECD consensus document (OECD, 2002a). Harvested grain samples were assessed for moisture and levels of nutrients including proximates (protein, total fat and ash), amino acids (18 components), fatty acids (22 components), carbohydrates by calculation, fiber (ADF, NDF and TDF), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc) and vitamins (vitamin A, vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6, vitamin B_9 and vitamin E). Grain samples were assessed for levels of other components including anti-nutrients (phytic acid and raffinose) and secondary metabolites (ferulic acid, furfural and p-coumaric acid). Harvested forage samples were assessed for moisture and levels of nutrients including proximates (protein, total fat and ash), carbohydrates by calculation, fiber (ADF and NDF) and minerals (calcium and phosphorus). In all, 78 different components were analyzed. Of these, 15 components (caprylic acid, capric acid, lauric acid, myristic acid, myristoleic acid, pentadecanoic acid, pentadecenoic acid, heptadecanoic acid, heptadecenoic acid, gamma linolenic acid, eicosadienoic acid, eicosatrienoic acid, arachidonic acid, sodium and furfural in grain) had more than 50% of the observations below the assay limit of quantitation (LOQ) and were excluded from statistical analysis. Moisture values for grain and forage were measured for conversion of components from fresh to dry weight but were not statistically analyzed. Therefore, 61 components were statistically analyzed.

The statistical comparisons of MON 94804 and the conventional control were based on compositional data combined across all field sites. Statistically significant differences were identified at the 5% level ($\alpha = 0.05$). A statistically significant difference between MON 94804 and the conventional control does not necessarily imply biological relevance from a food and feed safety perspective. Therefore, any statistically significant differences observed between MON 94804 and the conventional control were evaluated further to determine whether the detected difference indicated a biologically relevant compositional change or supported a conclusion of compositional equivalence, as follows:

Step 1 – Determination of the Magnitude of Difference between Test and Conventional Control Means

The difference in mean values between MON 94804 and the conventional control was determined for use in subsequent steps. For protein and amino acids only, the relative magnitude of the difference (percent change relative to the control) between MON 94804 and

the conventional control was determined to allow an assessment of any observed difference in amino acids in relation to the difference in protein.

Step 2 – Assessment of the Difference in the Context of Natural Variation within the Conventional Control across Multiple Sites

The difference between MON 94804 and the conventional control was evaluated in the context of variation within the conventional control germplasm grown across multiple sites (i.e., variation due to environmental influence) by determining the range of replicate values for the conventional control (range value = maximum value minus the minimum value). A mean difference less than the variability seen due to natural environmental variation within the single, closely related germplasm is typically not a food or feed safety concern (Venkatesh *et al.*, 2014).

Step 3 – Assessment of the Difference in the Context of Natural Variation Due to Multiple Sources

The relative impact of MON 94804 on composition was also evaluated in the context of sources of natural variation such as environmental and germplasm influences. This assessment determined whether the component mean value of MON 94804 was within the natural variability defined by the literature values and/or the AFSI Crop Composition Database (AFSI CCDB) values. This natural variability is important in assessing the biological relevance to food and feed safety of statistically significant differences in composition between MON 94804 and the conventional control.

These evaluations of natural variation are important as crop composition is known to be greatly influenced by environment and variety (Harrigan *et al.*, 2010). Although used in the comparative assessment process, detection of statistically significant differences between MON 94804 and the conventional control mean values does not imply a meaningful contribution by MON 94804 to compositional variability. Only if the impact of MON 94804 on levels of components is large relative to natural variation inherent to conventional maize would the difference in composition be potentially meaningful from a food and feed safety and nutritional perspective. Differences between MON 94804 the conventional control that are within the observed natural variation for maize are not meaningful, therefore the results support a conclusion of compositional equivalence.

There were no statistically significant differences ($p<0.05$) for 52 of the 61 components analyzed. There were nine components (stearic acid, TDF, calcium and ferulic acid in grain and protein, carbohydrates by calculation, ADF, NDF and ash in forage) that showed a statistically significant difference ($p<0.05$) between MON 94804 and the conventional control. For these components, the mean difference between MON 94804 and the conventional control was less than the conventional control range values. The MON 94804 mean component values were also within the range of values observed in the literature and/or the AFSI CCDB values. These results support the overall conclusion that MON 94804 was not a major contributor to variation in component levels in maize grain and forage and confirmed the compositional equivalence of MON 94804 to the conventional control in levels of these components. A detailed description of the assessment of statistically significant differences observed between MON 94804 and the conventional control is provided in the following sections.

B.5(a)(ii) Results of compositional analysis of MON 94804 maize grain and forage compared to conventional maize

Nutrient Levels in MON 94804 Grain

Maize grain is composed of nutrients including protein and constituent amino acids, fat and constituent fatty acids, carbohydrates, ash, minerals, and vitamins. The grain contains approximately 10% protein and 4% total fat (OECD, 2002a). In addition to protein and total fat, major biomass components assessed in grain include carbohydrates and fiber (ADF, NDF, and TDF). The ash content, which is included in this analysis, is a measure of the total amount of minerals present in the grain; minerals include calcium, copper, iron, magnesium, manganese, phosphorus, potassium and zinc. Maize grain contains both water-soluble vitamins (folic acid, niacin, B1, B2, and B6) and fat-soluble vitamins [vitamins A (β-carotene) and E]. Nutrient levels in maize can vary widely depending on growing conditions and genetic background (Egesel *et al.*, 2003; Harrigan *et al.*, 2009; Ridley *et al.*, 2011; Zhou *et al.*, 2011).

Protein and Amino Acids

Of the 19 components (protein and 18 amino acids) analyzed in this category, no statistically significant differences ($p<0.05$) between MON 94804 and the conventional control were observed (Table 9).

The data demonstrated that MON 94804 was not a major contributor to variation in protein or amino acid levels in maize and confirmed the compositional equivalence of MON 94804 to the conventional control in levels of these components.

Total Fat and Fatty Acids

Of the 10 components (total fat and nine fatty acids) analyzed in this category, no statistically significant difference ($p<0.05$) between MON 94804 and the conventional control were observed for nine components (total fat, palmitic acid, palmitoleic acid, oleic acid, linoleic acid, linolenic acid, arachidic acid, eicosenoic acid and behenic acid) (Table 10). Statistically significant difference ($p<0.05$) between MON 94804 and the conventional control was observed for stearic acid (Table 2). This difference was evaluated using considerations of natural variation in composition as outlined above.

Step-1. Mean differences between MON 94804 and the conventional control components were determined. For stearic acid, the magnitude of difference was 0.056% Total FA (Table 10).

Step-2. As shown in Table 2, the magnitude of difference for stearic acid between MON 94804 and the conventional control were less than the corresponding conventional control range values. This indicates that MON 94804 does not impact the level of stearic acid more than the natural variation within the conventional control grown at multiple locations.

Step-3. The mean level of stearic acid was within the natural variability of this component as published in the scientific literature on maize composition and/or the AFSI CCDB (Table 16).

The data demonstrated that MON 94804 was not a major contributor to variation in total fat or fatty acid levels in maize and confirmed the compositional equivalence of MON 94804 to the conventional control in levels of these components.

Carbohydrates by Calculation and Fiber

Of the four components analyzed in this category, no statistically significant difference ($p<0.05$) between MON 94804 and the conventional control were observed for three components (carbohydrates by calculation, ADF and NDF) (Table 11). Statistically significant differences ($p<0.05$) between MON 94804 and the conventional control were observed for TDF (Table 11). The difference was evaluated using considerations of natural variation in composition as outlined above.

Step-1. Mean differences between MON 94804 and the conventional control components were determined. For TDF, the difference was -0.83% dw (Table 11).

Step-2. As shown in Table 3, the magnitude of difference for TDF between MON 94804 and the conventional control was less than the corresponding conventional control range value. This indicates that MON 94804 does not impact the level of TDF more than the natural variation within the conventional control grown at multiple locations.

Step-3. The mean level of TDF was within the natural variability of this component as published in the scientific literature on maize composition and/or the AFSI CCDB (Table 16).

The data demonstrated that MON 94804 was not a major contributor to variation in carbohydrates by calculation or fiber levels in maize and confirmed the compositional equivalence of MON 94804 to the conventional control in levels of these components.

Ash and Minerals

Of the nine components analyzed in this category, no statistically significant difference ($p<0.05$) between MON 94804 and the conventional control were observed for eight components (ash, copper, iron, magnesium, manganese, phosphorus, potassium and zinc) (Table 12). Statistically significant difference ($p<0.05$) between MON 94804 and the conventional control was observed for calcium (Table 12). This difference was evaluated using considerations of natural variation in composition as outlined above.

Step-1. Mean differences between MON 94804 and the conventional control components were determined. For calcium, the difference was 0.00052% dw (Table 12).

Step-2. As shown in Table 4, the magnitude of differences for calcium between MON 94804 and the conventional control were less than the corresponding conventional control range value. This indicates that MON 94804 does not impact the level of calcium more than the natural variation within the conventional control grown at multiple locations.

Step-3. The mean level of calcium was within the natural variability of this component as published in the scientific literature on maize composition and/or the AFSI CCDB (Table 16).

The data demonstrated that MON 94804 was not a major contributor to variation in ash or mineral levels in maize and confirmed the compositional equivalence of MON 94804 to the conventional control in levels of these components.

Vitamins

Of the seven components analyzed in this category (vitamin A, vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6, vitamin B_9 and vitamin E), no statistically significant differences ($p<0.05$) between MON 94804 and the conventional control were observed (Table 13).

The data demonstrated that MON 94804 was not a major contributor to variation in vitamin levels in maize and confirmed the compositional equivalence of MON 94804 to the conventional control in levels of these components.

Anti-Nutrient and Secondary Metabolite Levels in MON 94804 Grain

Anti-nutrients present in maize grain include phytic acid and raffinose. Phytic acid, the major form of phosphorus in maize grain, is considered an anti-nutrient due to its mineral-chelating properties (Landoni *et al.*, 2013). Raffinose, a low molecular weight non-digestible carbohydrate, is considered an anti-nutrient due to its gas-producing characteristics that cause flatulence (Liener, 2000).

Secondary metabolites in maize grain include furfural, ferulic acid, and p-coumaric acid (OECD, 2002a). Ferulic acid and p-coumaric acid are secondary metabolites derived from the amino acids phenylalanine and tyrosine (Buchanan *et al.*, 2000), and are precursors of phenylpropanoid compounds and fiber. Furfural is an organic compound that is typically prepared from agricultural byproducts such as maize cobs (Li *et al.*, 2015), and is either present at low levels in maize grain (OECD, 2002a) or below the limit of quantification (Harrigan *et al.*, 2009; Ridley *et al.*, 2011). The levels of anti-nutrients and secondary metabolites can vary widely depending on growing conditions and genetic background (Harrigan *et al.*, 2009; Ridley *et al.*, 2011).

Of the four components analyzed in this category, no statistically significant difference ($p<0.05$) between MON 94804 and the conventional control were observed for three components (phytic acid, raffinose and p-coumaric acid) (Table 14). Statistically significant difference ($p<0.05$) between MON 94804 and the conventional control was observed for ferulic acid (Table 14). The difference was evaluated using considerations of natural variation in composition as outlined above.

Step-1. Mean differences between MON 94804 and the conventional control components were determined. For ferulic acid, the difference was -55.08 µg/g dw (Table 14).

Step-2. As shown in Table 6, the magnitude of difference for ferulic acid between MON 94804 and the conventional control was less than the corresponding conventional control range value. This indicates that MON 94804 does not impact level of ferulic acid more than the natural variation within the conventional control grown at multiple locations.

Step-3. The mean level of ferulic acid was within the natural variability of this component as published in the scientific literature on maize composition and/or the AFSI CCDB (Table 16).

The data demonstrated that MON 94804 was not a major contributor to variation in anti-nutrient or secondary metabolite levels in maize and confirmed the compositional equivalence of MON 94804 to the conventional control in levels of these components.

Nutrient Levels in MON 94804 Forage

Of the eight components analyzed in this category, no statistically significant difference ($p<0.05$) between MON 94804 and the conventional control were observed for three components (total fat, calcium and phosphorus) (Table 15). Statistically significant differences ($p<0.05$) between MON 94804 and the conventional control were observed for protein, carbohydrates by calculation, ADF, NDF and ash (Table 15). These differences were evaluated using considerations of natural variation in composition as outlined above.

Step-1. Mean differences between MON 94804 and the conventional control components were determined. For protein, carbohydrates by calculation, ADF, NDF and ash, the differences were 0.56, 1.53, 3.01, 4.27 and 0.61 % dw respectively (Table 15).

Step-2. As shown in Table 7, the magnitude of differences for protein, carbohydrates by calculation, ADF, NDF and ash between MON 94804 and the conventional control were less than the corresponding conventional control range values. This indicates that MON 94804 does not impact levels of these components more than the natural variation within the conventional control grown at multiple locations.

Step-3. The mean levels of protein, carbohydrates by calculation, ADF, NDF and ash were within the natural variability of these components as published in the scientific literature on maize composition and/or the AFSI CCDB (Table 16).

The data demonstrated that MON 94804 was not a major contributor to variation in vitamin levels in maize and confirmed the compositional equivalence of MON 94804 to the conventional control in levels of these components.

B.5(a)(iii) Compositional assessment of MON 94804 maize conclusion

Compositional analysis was conducted on grain and forage of MON 94804 and the conventional control grown at five sites in the United States during the 2020 field season. Of the 61 components statistically assessed, 52 showed no statistically significant differences ($p<0.05$) between MON 94804 and the conventional control. A total of nine components (stearic acid, TDF, calcium and ferulic acid for grain and protein, carbohydrates by calculation, ADF, NDF and ash in forage) showed a statistically significant difference ($p<0.05$) between MON 94804 and the conventional control. For these components, the mean difference in component values between MON 94804 and the conventional control was less than the range of the conventional control values. The MON 94804 mean component values were within the range of values observed in the literature and/or the AFSI-CCDB.

These results support the overall conclusion that MON 94804 maize was not a major contributor to variation in component levels in grain or forage and confirmed the compositional equivalence of MON 94804 to the conventional control in levels of these components. The statistically significant differences observed were not compositionally meaningful from a food and feed safety perspective.

Table 9. Summary of Maize Grain Protein and Amino Acids for MON 94804 Maize and the Conventional Control

				Difference (Test minus Control)		
Component (% dw)[1]	MON 94804 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Mean (S.E.)	p-Value	% Relative[4]
Protein	9.83 (0.38) 8.06 – 11.34	9.70 (0.38) 8.01 – 10.97	2.96	0.13 (0.22)	0.580	1.35
Alanine	0.76 (0.034) 0.58 – 0.96	0.75 (0.034) 0.60 – 0.88	0.28	0.0082 (0.022)	0.731	1.09
Arginine	0.49 (0.017) 0.39 – 0.59	0.49 (0.017) 0.37 – 0.54	0.18	-0.0031 (0.011)	0.787	-0.64
Aspartic Acid	0.64 (0.020) 0.51 – 0.73	0.64 (0.020) 0.51 – 0.75	0.24	0.00017 (0.013)	0.989	0.03
Cystine	0.22 (0.012) 0.18 – 0.29	0.22 (0.012) 0.17 – 0.25	0.084	0.00029 (0.0070)	0.968	0.13
Glutamic Acid	1.87 (0.088) 1.43 – 2.37	1.83 (0.088) 1.44 – 2.13	0.69	0.035 (0.058)	0.578	1.90
Glycine	0.37 (0.011) 0.30 – 0.42	0.37 (0.011) 0.30 – 0.41	0.11	-0.0049 (0.0060)	0.463	-1.31
Histidine	0.28 (0.010) 0.23 – 0.32	0.27 (0.010) 0.22 – 0.31	0.097	0.0058 (0.0047)	0.227	2.15

Table 9. Summary of Maize Grain Protein and Amino Acids for MON 94804 Maize and the Conventional Control (Continued)

				Difference (Test minus Control)		
Component (% dw)[1]	MON 94804 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Mean (S.E.)	p-Value	% Relative[4]
Isoleucine	0.36 (0.015) 0.28 - 0.45	0.35 (0.015) 0.28 - 0.42	0.14	0.0095 (0.0092)	0.305	2.72
Leucine	1.28 (0.061) 0.96 - 1.65	1.24 (0.061) 0.99 - 1.52	0.54	0.036 (0.041)	0.434	2.89
Lysine	0.29 (0.0065) 0.24 - 0.33	0.30 (0.0065) 0.23 - 0.33	0.099	-0.011 (0.0060)	0.131	-3.77
Methionine	0.21 (0.013) 0.16 - 0.28	0.21 (0.013) 0.15 - 0.27	0.11	0.00039 (0.0067)	0.956	0.18
Phenylalanine	0.51 (0.021) 0.39 - 0.62	0.50 (0.021) 0.40 - 0.60	0.20	0.0086 (0.013)	0.513	1.73
Proline	0.89 (0.038) 0.71 - 1.09	0.88 (0.038) 0.70 - 0.99	0.30	0.014 (0.028)	0.629	1.63

Table 9. Summary of Maize Grain Protein and Amino Acids for MON 94804 Maize and the Conventional Control (Continued)

				Difference (Test minus Control)		
Component (% dw)[1]	MON 94804 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Mean (S.E.)	p-Value	% Relative[4]
Serine	0.48 (0.019) 0.38 - 0.59	0.48 (0.019) 0.38 - 0.55	0.16	0.0056 (0.013)	0.695	1.16
Threonine	0.35 (0.012) 0.28 - 0.42	0.35 (0.012) 0.27 - 0.38	0.11	0.0011 (0.0084)	0.903	0.31
Tryptophan	0.076 (0.0028) 0.067 - 0.085	0.075 (0.0028) 0.060 - 0.084	0.024	0.00045 (0.0012)	0.726	0.60
Tyrosine	0.44 (0.018) 0.34 - 0.56	0.43 (0.018) 0.33 - 0.49	0.17	0.0074 (0.012)	0.577	1.73
Valine	0.47 (0.017) 0.38 - 0.56	0.47 (0.017) 0.37 - 0.53	0.16	0.0037 (0.0093)	0.693	0.79

[1]dw=dry weight
[2] Mean (S.E.) = least-square mean (standard error)
[3]Maximum value minus minimum value for the control maize hybrid
[4]The relative magnitude of the difference in mean values between MON 94804 and the control, expressed as a percent of the control.

Table 10. Summary of Maize Grain Total Fat and Fatty Acids for MON 94804 Maize and the Conventional Control

				Difference (Test minus Control)	
Component	MON 94804 Mean (S.E.)[1] Range	Control Mean (S.E.) Range	Control Range Value[2]	Mean (S.E.)	p-Value
Total Fat (% dw)[3]	3.70 (0.058) 3.21 – 3.96	3.80 (0.058) 3.37 – 4.14	0.77	-0.095 (0.064)	0.212
16:0 Palmitic (% total FA)[4]	12.80 (0.12) 12.43 – 13.37	12.65 (0.12) 12.13 – 13.10	0.97	0.15 (0.085)	0.158
16:1 Palmitoleic (% total FA)	0.085 (0.0061) 0.045 – 0.10	0.098 (0.0061) 0.049 – 0.11	0.058	-0.013 (0.0061)	0.096
18:0 Stearic (% total FA)	1.40 (0.017) 1.35 – 1.47	1.46 (0.017) 1.38 – 1.55	0.17	-0.056 (0.012)	0.010
18:1 Oleic (% total FA)	27.51 (0.11) 26.74 – 28.07	27.53 (0.11) 26.47 – 28.21	1.74	-0.014 (0.16)	0.930
18:2 Linoleic (% total FA)	56.36 (0.13) 55.92 – 57.28	56.42 (0.13) 55.61 – 57.41	1.80	-0.058 (0.11)	0.615
18:3 Linolenic (% total FA)	1.10 (0.0099) 1.03 – 1.17	1.09 (0.0099) 1.05 – 1.13	0.078	0.00072 (0.0068)	0.920

Table 10. Summary of Maize Grain Total Fat and Fatty Acids for MON 94804 Maize and the Conventional Control (Continued)

				Difference (Test minus Control)	
Component	MON 94804 Mean (S.E.)[1] Range	Control Mean (S.E.) Range	Control Range Value[2]	Mean (S.E.)	p-Value
20:0 Arachidic (% total FA)	0.33 (0.0030) 0.32 - 0.35	0.34 (0.0030) 0.32 - 0.36	0.039	-0.0042 (0.0029)	0.224
20:1 Eicosenoic (% total FA)	0.28 (0.0018) 0.27 - 0.29	0.28 (0.0018) 0.27 - 0.29	0.022	0.0036 (0.0023)	0.190
22:0 Behenic (% total FA)	0.13 (0.0025) 0.11 - 0.14	0.13 (0.0025) 0.10 - 0.15	0.045	-0.0057 (0.0030)	0.108

[1] Mean (S.E.) = least-square mean (standard error)
[2]Maximum value minus minimum value for the control maize hybrid
[3]dw=dry weight
[4]FA=Fatty Acid
The following components with more than 50% of observations below the assay LOQ were excluded from statistical analysis: caprylic acid, capric acid, lauric acid, myristic acid, myristoleic acid, pentadecanoic acid, pentadecenoic acid, heptadecanoic acid, heptadecenoic acid, gamma linolenic acid, eicosadienoic acid, eicosatrienoic acid and arachidonic acid.

Table 11. Summary of Maize Grain Carbohydrates by Calculation and Fiber for MON 94804 Maize and the Conventional Control

Component (% dw)[1]	MON 94804 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
Carbohydrates by Calculation	85.13 (0.46) 83.54 – 87.63	85.20 (0.46) 83.63 – 87.05	3.41	-0.069 (0.27)	0.807
ADF	3.23 (0.11) 2.51 – 4.26	3.40 (0.11) 2.56 – 4.14	1.57	-0.17 (0.13)	0.210
NDF	9.61 (0.32) 8.14 – 12.60	9.87 (0.32) 8.09 – 12.27	4.19	-0.26 (0.30)	0.445
TDF	11.46 (0.28) 9.31 – 14.50	12.29 (0.28) 9.51 – 14.93	5.42	-0.83 (0.32)	0.017

[1]dw=dry weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Maximum value minus minimum value for the control maize hybrid

Table 12. Summary of Maize Grain Ash and Minerals for MON 94804 Maize and the Conventional Control

				Difference (Test minus Control)	
Component	MON 94804 Mean (S.E.)[1] Range	Control Mean (S.E.) Range	Control Range Value[2]	Mean (S.E.)	p-Value
Ash (% dw)[3]	1.33 (0.042) 0.91 – 1.45	1.28 (0.042) 1.03 – 1.50	0.47	0.047 (0.032)	0.163
Calcium (% dw)	0.0039 (0.00013) 0.0034 – 0.0044	0.0034 (0.00013) 0.0029 – 0.0040	0.0011	0.00052 (0.000050)	<0.001
Copper (mg/kg dw)	1.95 (0.093) 1.47 – 2.76	1.88 (0.093) 1.45 – 2.31	0.86	0.068 (0.12)	0.601
Iron (mg/kg dw)	16.84 (0.71) 14.43 – 20.57	16.87 (0.71) 13.98 – 20.41	6.43	-0.035 (0.28)	0.901
Magnesium (% dw)	0.11 (0.0041) 0.090 – 0.13	0.11 (0.0041) 0.091 – 0.12	0.031	0.0040 (0.0025)	0.175
Manganese (mg/kg dw)	5.67 (0.51) 3.72 – 7.35	5.33 (0.51) 3.97 – 6.91	2.94	0.34 (0.23)	0.216
Phosphorus (% dw)	0.33 (0.011) 0.27 - 0.36	0.33 (0.011) 0.27 - 0.38	0.11	0.00051 (0.0065)	0.941

Table 12. Summary of Maize Grain Ash and Minerals for MON 94804 Maize and the Conventional Control (Continued)

				Difference (Test minus Control)	
Component	MON 94804 Mean (S.E.)[1] Range	Control Mean (S.E.) Range	Control Range Value[2]	Mean (S.E.)	p-Value
Potassium (% dw)	0.34 (0.0069) 0.32 - 0.38	0.34 (0.0069) 0.31 - 0.38	0.068	-0.0022 (0.0070)	0.769
Zinc (mg/kg dw)	19.64 (1.14) 15.23 - 25.03	19.71 (1.14) 16.55 - 23.46	6.91	-0.071 (0.50)	0.893

[1]Mean (S.E.) = least-square mean (standard error)
[2]Maximum value minus minimum value for the control maize hybrid
[3]dw=dry weight
The following components with more than 50% of observations below the assay LOQ were excluded from statistical analysis: sodium.

Table 13. Summary of Maize Grain Vitamins for MON 94804 Maize and the Conventional Control

				Difference (Test minus Control)	
Component (mg/kg dw)[1]	MON 94804 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Mean (S.E.)	p-Value
Vitamin A	0.94 (0.041) 0.69 – 1.12	0.98 (0.041) 0.74 – 1.19	0.46	-0.039 (0.033)	0.300
Vitamin B1	3.63 (0.14) 3.05 – 4.03	3.71 (0.14) 3.28 – 4.53	1.25	-0.08 (0.055)	0.221
Vitamin B2	1.66 (0.055) 1.20 – 2.03	1.60 (0.055) 1.24 – 2.01	0.77	0.059 (0.071)	0.409
Vitamin B3	22.14 (1.44) 16.10 – 35.31	20.82 (1.44) 16.70 – 27.49	10.79	1.32 (1.79)	0.501
Vitamin B6	6.52 (0.26) 5.06 – 10.48	6.08 (0.26) 4.51 – 9.23	4.71	0.44 (0.34)	0.262
Vitamin B9	0.37 (0.017) 0.28 – 0.48	0.37 (0.017) 0.25 – 0.61	0.36	-0.003 (0.022)	0.892
Vitamin E	11.66 (0.45) 8.27 – 16.65	11.92 (0.45) 9.93 – 16.54	6.60	-0.26 (0.53)	0.622

[1]dw=dry weight; Common names of vitamins: A=β-Carotene; B_1=Thiamine HCl; B_2=Riboflavin; B_3=Niacin; B_6=Pyridoxine HCl; B_9=Folic Acid; E=α-Tocopherol
[2]Mean (S.E.) = least-square mean (standard error)
[3]Maximum value minus minimum value for the control maize hybrid

Table 14. Summary of Maize Grain Anti-Nutrients and Secondary Metabolites for MON 94804 Maize and the Conventional Control

				Difference (Test minus Control)	
Component	MON 94804 Mean (S.E.)[1] Range	Control Mean (S.E.) Range	Control Range Value[2]	Mean (S.E.)	p-Value
Phytic Acid (% dw)[3]	0.87 (0.027) 0.69 – 1.10	0.92 (0.027) 0.72 – 1.05	0.32	-0.049 (0.025)	0.118
Raffinose (% dw)	0.21 (0.013) 0.17 – 0.25	0.22 (0.013) 0.17 – 0.28	0.10	-0.0098 (0.0080)	0.284
Ferulic Acid (µg/g dw)	1842.57 (32.59) 1662.87 – 2013.50	1897.65 (32.59) 1751.99 – 2155.76	403.77	-55.08 (23.00)	0.022
p-Coumaric Acid (µg/g dw)	161.84 (5.21) 141.39 – 186.73	154.05 (5.21) 127.66 – 181.61	53.95	7.80 (3.91)	0.117

[1]Mean (S.E.) = least-square mean (standard error)
[2]Maximum value minus minimum value for the control maize hybrid
[3]dw=dry weight
The following components with more than 50% of observations below the assay LOQ were excluded from statistical analysis: furfural.

Table 15. Summary of Maize Forage Proximates, Carbohydrates by Calculation, Fiber and Minerals for MON 94804 Maize and the Conventional Control

				Difference (Test minus Control)	
Component (% dw)[1]	MON 94804 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Mean (S.E.)	p-Value
Protein (% dw)	7.66 (0.23) 5.74 – 9.08	7.10 (0.23) 5.98 – 8.72	2.75	0.56 (0.22)	0.022
Total Fat (% dw)	3.03 (0.13) 2.27 – 3.84	2.71 (0.13) 1.79 – 3.89	2.11	0.32 (0.16)	0.121
Carbohydrates by Calculation (% dw)	84.89 (0.34) 81.99 – 88.10	86.42 (0.34) 84.14 – 88.51	4.37	-1.53 (0.34)	<0.001
ADF (% dw)	19.24 (0.54) 16.52 – 23.40	22.25 (0.54) 16.26 – 27.59	11.33	-3.01 (0.64)	<0.001
NDF (% dw)	32.08 (1.07) 23.92 – 37.99	36.35 (1.07) 29.30 – 43.29	13.99	-4.27 (1.09)	<0.001
Ash (% dw)	4.40 (0.13) 2.97 – 6.10	3.79 (0.13) 3.03 – 4.97	1.94	0.61 (0.18)	0.002
Calcium (% dw)	0.19 (0.013) 0.12 – 0.24	0.20 (0.013) 0.14 – 0.28	0.15	-0.0067 (0.0083)	0.429
Phosphorus (% dw)	0.22 (0.0080) 0.14 – 0.29	0.21 (0.0080) 0.14 – 0.27	0.13	0.010 (0.010)	0.341

[1]dw=dry weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Maximum value minus minimum value for the control maize hybrid

B.5(b) Information on the range of natural variation for each constituent measured to allow for assessment of biological significance should any statistically significant difference be identified

Table 16. Literature and AFSI Database Ranges for Components in Maize Grain and Forage

Tissue Components[1]	Literature Range[2]	AFSI Range[3]
Grain Nutrients		
Proximates		
protein (% dw)	8.27-13.33[a]; 9.17-12.19[b]	5.72-17.26
total fat (% dw)	2.95-4.40[a]; 3.18-4.23[b]	1.363-7.830
ash (% dw)	1.17-2.01[a]; 1.27-1.63[b]	0.616-6.282
Amino Acids		
alanine (% dw)	0.60-1.04[a]; 0.68-0.96[b]	0.40-1.48
arginine (% dw)	0.34-0.52[a]; 0.34-0.50[b]	0.12-0.71
aspartic acid (% dw)	0.52-0.78[a]; 0.59-0.76[b]	0.30-1.21
cystine (% dw)	0.19-0.26[a]; 0.20-0.26[b]	0.12-0.51
glutamic acid (% dw)	1.54-2.67[a]; 1.71-2.44[b]	0.83-3.54
glycine (% dw)	0.33-0.43[a]; 0.33-0.42[b]	0.184-0.685
histidine (% dw)	0.25-0.37[a]; 0.27-0.34[b]	0.14-0.46
isoleucine (% dw)	0.30-0.48[a]; 0.32-0.44[b]	0.18-0.69
leucine (% dw)	1.02-1.87[a]; 1.13-1.65[b]	0.60-2.49
lysine (% dw)	0.26-0.33[a]; 0.28-0.31[b]	0.129-0.668
methionine (% dw)	0.17-0.26[a]; 0.16-0.30[b]	0.11-0.47
phenylalanine (% dw)	0.43-0.72[a]; 0.45-0.63[b]	0.24-0.93
proline (% dw)	0.74-1.21[a]; 0.78-1.11[b]	0.46-1.75
serine (% dw)	0.39-0.67[a]; 0.43-0.6[b]	0.15-0.77
threonine (% dw)	0.29-0.45[a]; 0.31-0.39[b]	0.17-0.67
tryptophan (% dw)	0.047-0.085[a]; 0.042-0.07[b]	0.027-0.215
tyrosine (% dw)	0.13-0.43[a]; 0.12-0.41[b]	0.10-0.73
valine (% dw)	0.42-0.62[a]; 0.45-0.58[b]	0.27-0.86
Fatty Acids		
palmitic acid (% total FA)	8.80-13.33[a]; 9.84-12.33[b]	6.81-26.55
palmitoleic acid (% total FA)	0.059-0.23[a]	0.067-0.453
stearic acid (% total FA)	1.36-2.14[a]; 1.3-2.1[b]	1.02-3.83
oleic acid (% total FA)	19.50-33.71[a]; 19.59-29.13[b]	16.38-42.81
linoleic acid (% total FA)	49.31-64.70[a]; 56.51-65.65[b]	34.27-67.68
linolenic acid (% total FA)	0.89-1.56[a]; 1.03-1.38[b]	0.55-2.33
arachidic acid (% total FA)	0.30-0.49[a]; 0.30-0.41[b]	0.267-0.993
eicosenoic acid (% total FA)	0.17-0.29[a]; 0.17-0.27[b]	0.098-1.952
behenic acid (% total FA)	0.069-0.28[a]; 0.059-0.18[b]	0.093-0.417
Carbohydrates By Calculation		
carbohydrates by calculation (% dw)	81.31-87.06[a]; 82.10-85.98[b]	77.4-89.7
Fiber		
ADF (% dw)	1.82-4.48[a]; 1.83-3.39[b]	1.41-11.34
NDF (% dw)	6.51-12.28[a]; 6.08-10.36[b]	4.28-24.30
TDF (% dw)	10.65-16.26[a]; 10.57-14.56[b]	5.78-35.31
Minerals		
calcium (% dw)	0.0036-0.0068[a]; 0.0035-0.007[b]	0.001-0.101
copper (mg/kg dw)	0.85-3.54[c]	0.55-21.20
iron (mg/kg dw)	14.17-23.40[a]; 15.90-24.66[b]	9.51-191.00
magnesium (% dw)	0.091-0.14[a]; 0.1-0.14[b]	0.06-0.19
manganese (mg/kg dw)	4.83-8.34[a]; 4.78-9.35[b]	1.69-14.30
phosphorus (% dw)	0.24-0.37[a]; 0.27-0.38[b]	0.13-0.55
potassium (% dw)	0.29-0.39[a]; 0.36-0.43[b]	0.18-0.60

Tissue Components[1]	Literature Range[2]	AFSI Range[3]
zinc (mg/kg dw)	16.78-28.17[a]; 18.25-30.44[b]	6.5-42.6
Vitamins		
vitamin A (mg/kg dw)	0.14-11.27[d]	0.32-5.81
vitamin B_1 (mg/kg dw)	2.33-4.17[a]; 2.71-4.33[b]	1.26-40.00
vitamin B_2 (mg/kg dw)	0.94-2.42[a]; 1.64-2.81[b]	0.50-7.35
vitamin B_3 (mg/kg dw)	15.07-32.38[a]; 13.64-42.06[b]	7.42-46.94
vitamin B_6 (mg/kg dw)	4.93-7.53[a]; 4.97-8.27[b]	1.18-12.14
vitamin B_9 (mg/kg dw)	0.19-0.35[a]; 0.23-0.42[b]	0.09-3.50
vitamin E (mg/kg dw)	5.96-18.44[a]; 2.84-15.53[b]	0.84-68.67
Grain Other		
Anti-Nutrients		
phytic acid (% dw)	0.69-1.09[a]; 0.60-0.94[b]	0.111-1.940
raffinose (% dw)	0.079-0.22[a]; 0.061-0.15[b]	0.020-0.466
Secondary Metabolites		
ferulic acid (µg/g dw)	1205.75-2873.05[a]; 1011.40-2539.86[b]	291.93-4,397.30
p-coumaric acid (µg/g dw)	94.77-327.39[a]; 66.48-259.68[b]	53.4-820.0
Forage Nutrients		
Proximates		
protein (% dw)	5.80-10.24[a]; 5.56-9.14[b]	2.37-16.32
total fat (% dw)	1.28-3.62[a]; 0.20-1.76[b]	0.296-6.755
ash (% dw)	2.67-8.01[a]; 4.59-6.9[b]	0.66-13.20
Carbohydrates By Calculation		
carbohydrates by calculation (% dw)	81.88-89.26[a]; 84.11-87.54[b]	73.3-92.9
Fiber		
acid detergent fiber (% dw)	19.11-30.49[a]; 20.73-33.39[b]	5.13-47.39
neutral detergent fiber (% dw)	27.73-49.62[a]; 31.81-50.61[b]	18.30-67.80
Minerals		
calcium (% dw)	0.12-0.33[a]; 0.21-0.41[b]	0.04-0.58
phosphorus (% dw)	0.090-0.26[a]; 0.13-0.21[b]	0.07-0.44

[1]dw=dry weight; FA=Fatty Acid; mg/kg/ dw

[2]Literature range references: [a](Harrigan *et al.*, 2009) (see U.S. Field data);[b](Harrigan *et al.*, 2009) (see Chile field data);[c](Ridley *et al.*, 2011);[d](Egesel *et al.*, 2003)

[3]AFSI range is from AFSI CCDB, 2020 (Accessed January 02, 2021 and January 04, 2021).

B.5(c) The levels of any other constituents that may potentially be influenced by the genetic modification.

Considering mode of action of *GA20ox_SUP miRNA* described in B.3, and composition analysis in B.5(b), it is not anticipated that any other constituents would be influenced by the genetic modification.

C. INFORMATION RELATED TO THE NUTRITIONAL IMPACT OF THE FOOD PRODUCED USING GENE TECHNOLOGY

There are no nutritional impacts on the food derived from MON 94804. This product is developed to confer altered plant height. It is not a nutritionally altered product.

D. OTHER INFORMATION

The data and information presented in this submission demonstrate that the food derived from MON 94804 are as safe and nutritious as those derived from commercially-available, conventional maize for which there is an established history of safe consumption. No additional studies are considered necessary to demonstrate the safety of MON 94804.

PART 3 STATUTORY DECLARATION – AUSTRALIA

STATUTORY DECLARATION pursuant to Statutory Declarations Act 1959

I, [redacted] at Bayer CropScience Pty Ltd, Level 4, 109 Burwood Road, Hawthorn, Vic. 3122, make the following declaration under the Statutory Declarations Act 1959:

1. the information provided in this application fully sets out the matters required,

2. the information provided in this application is true to the best of my knowledge and belief,

3. no information has been withheld that might prejudice this application, to the best of my knowledge and belief.

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the Statutory Declarations Act 1959, and I believe that the statements in this declaration are true in every particular.

[redacted]

Signature of person making the declaration

Declared at Hawthorn on 8 September 2023

Before me

[redacted] 8/9/2023

[redacted] an Australian Legal Practitioner within the meaning of the Legal Profession Uniform Law (Victoria)

////////////////

08 September 2023

Bayer CropScience Pty Ltd
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PART 4 REFERENCES

UNPUBLISHED REPORTS BEING SUBMITTED

Appendix 1. [redacted] 2022. Amended from TRR0001453: Molecular Characterization of Short Stature Maize MON 94804. M-811545-03-1. (CCI)

Appendix 2. [redacted] 2022. Amended from TRR0001535: Bioinformatic Evaluation of the T-DNA in MON 94804 Utilizing the AD_2022, TOX_2022, and PRT_2022 Databases. M-814383-02-1.

Appendix 3. [redacted] 2022. Bioinformatic Evaluation of Putative Flank-Junction Peptides in MON94804 Utilizing the AD_2022, TOX_2022, and PRT_2022 Databases. M-813988-01-2.

Appendix 4. [redacted] 2022. Amended Report for M-814604-02-1/TRR0001576: Segregation Analysis of the T-DNA Insert in Short Stature Maize MON 94804 Across Three Generations. M-814604-03-1.

Appendix 5. [redacted] 2022. Demonstration of the Presence or Absence of GA20ox SUP Transcripts in Maize Leaf Samples Across Multiple Generations of MON 94804. M-822071-01-1.

Appendix 6. [redacted] 2022. Analysis of Transcripts from *GA20ox_SUP* Suppression Cassette in OSL1, OSR1, Stalk, Forage, and Grain Maize Tissues Collected from MON 94804. M-814626-01-1.

Appendix 7. [redacted] 2022. Assessment of the Endogenous *ZmGA20ox3* and *ZmGA20ox5* mRNA Levels in Maize OSL1, OSR1, Stalk, Forage, and Grain Tissues of MON 94804. M-816677-01-1.

Appendix 8. [redacted] 2021. Compositional Analyses of Maize Grain and Forage from MON 94804 Grown in United States During the 2020 Season. M-819925-01-1.

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