TITLE

Segregation of the *cp4 epsps* Coding Sequence in MON 88302 in the F_2, F_3 and F_4 Populations

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SPONSOR

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RPN-10-085

CERTIFICATION

This is an accurate and authentic representation of the results from a study (RPN-10-085) entitled "Segregation of the *cp4 epsps* Coding Sequence in MON 88302 in the F_2, F_3 and F_4 Populations". The Monsanto Regulatory Quality Assurance Unit audited this summary.

Signature of Approval:

Author: [redacted] Date: _____

SUMMARY

Monsanto Company has developed a second generation herbicide-tolerant canola product, MON 88302 that is tolerant to glyphosate application(s) from emergence to first flowering at a rate up to 1800 g a.e. (acid equivalents) per hectare. With a wide window of application and higher spray rates, MON 88302 will provide farmers with expanded weed control options.

MON 88302 was developed through *Agrobacterium*-mediated transformation of conventional canola using plasmid vector PV-BNHT2672. PV-BNHT2672 contains the codon optimized coding sequence of *cp4 epsps* from *Agrobacterium* sp. strain CP4 (Barry et al., 1997; Padgette et al., 1996).

The purpose of this project was to demonstrate that the *cp4 epsps* coding sequence in MON 88302 resided at a single locus within the canola genome and is inherited according to Mendelian principles of inheritance. Segregation data to illustrate Mendelian inheritance of the trait was generated from the MON 88302 breeding path described in Figure 1. The transformed R_0 plant was self-pollinated to generate R_1 seed. From the R_1 segregating population, an individual plant (Plant #: P000000000125792706233 82) homozygous for the *cp4 epsps* coding sequence was identified via TaqMan PCR copy number assay and Southern blot copy number analysis. The *cp4 epsps* homozygous R_1 plant was self-pollinated to give rise to R_2 plants that were self-pollinated to produce R_3 seed. At each generation, the homozygous plants were tested for the expected segregation pattern of 1:0 (positive: negative) for the *cp4 epsps* gene using a glyphosate spray test and/or the TaqMan PCR assay.

An individual *cp4 epsps* positive R_3 plant, which was confirmed by Endpoint TaqMan PCR assay, was crossed to a Monsanto proprietary canola inbred, which does not contain the MON 88302 insert, via traditional breeding techniques to produce hemizygous F_1 seed. The resulting F_1 plant, was shown to contain a single copy of the *cp4 epsps* gene by real-time TaqMan PCR, and was then self-pollinated to produce F_2 seed. A *cp4 epsps* hemizygous F_2 plant from the F_2 population was shown to contain a single copy of the *cp4 epsps* gene by real-time TaqMan PCR and was then self-pollinated to produce the F_3 population. A *cp4 epsps* hemizygous F_3 plant from the F_3 population was shown to contain a single copy of the *cp4 epsps* gene by real-time TaqMan PCR and was self-pollinated to produce the F_4 population. The copy number of the *cp4 epsps* gene in plants of the F_2, F_3, and F_4 population was assessed using a real-time TaqMan PCR assay.

Real-time TaqMan PCR captures fluorescent readings throughout the entire PCR process, while endpoint Taqman PCR only captures sample fluorescence reading following the completion of the PCR reaction. Both real-time TaqMan PCR and endpoint Taqman PCR were designed to detect specific DNA sequences. In this study, a real-time TaqMan PCR assay was used to determine the copy number of *cp4 epsps* coding sequence in individual R_1, F_1, F_2, F_3, and F_4 plants, thus identifying the homozygous positive, hemizygous, and homozygous negative *cp4 epsps* plants. An endpoint Taqman PCR assay was used to confirm the presence or absence of *cp4 epsps* in the R_3 plants.

A Chi-square (χ^2) analysis was performed on each of the F_2, F_3, and F_4 populations using the statistical program R Version 2 10 1 (2009-12-14) to compare the observed segregation ratio of *cp4 epsps* coding sequence to the expected ratio according to Mendelian principles of inheritance. 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% (α=0.05).

The results of the χ^2 analysis of the MON 88302 segregating progeny are presented in Table 1. The χ^2 value in the F_2, F_3, and F_4 populations indicated no statistically significant difference between the observed and expected 1:2:1 segregation ratio (homozygous positive: hemizygous: homozygous negative) of *cp4 epsps* coding sequence. These results support the conclusion that the *cp4 epsps* coding sequence in MON 88302 resides at a single locus within the canola genome and is inherited according to Mendelian principles of inheritance. These results are also consistent with the molecular characterization data indicating that MON 88302 contains a single, intact copy of the *cp4 epsps* expression cassette inserted at a single locus in the canola genome [redacted].

References

Barry, G.F., G.M. Kishore, S.R. Padgette, and W.C. Stallings. 1997. Glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthases. United States Patent 5.633.435.

Padgette, S.R., D.B. Re, G.F. Barry, D.E. Eichholtz, X. Delannay, R.L. Fuchs, G.M. Kishore, and R.T. Fraley. 1996. New weed control opportunities: Development of soybeans with a Roundup Ready™ gene., CRC Press, New York.

[redacted] 2010. Molecular Analysis of Glyphosate-Tolerant Roundup Ready® 2 (RR2) Canola MON 88302. Monsanto St. Louis Technical Report, MSL0022523, St. Louis, MO.

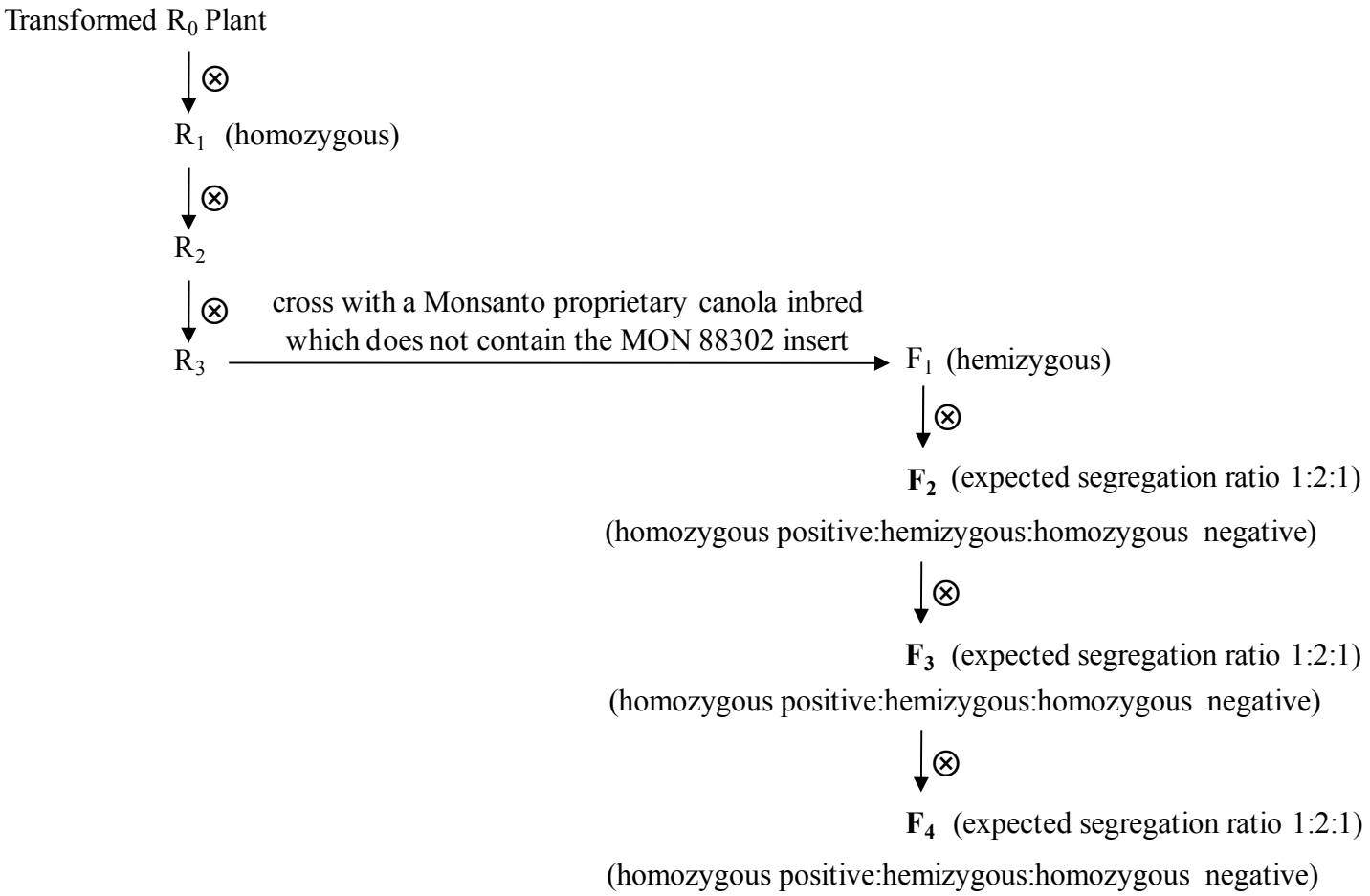

Figure 1. Breeding Path for Generating Segregation Data on MON 88302.

An individual hemizygous plant from each of the F_1, F_2, and F_3 populations was self-pollinated to produce the population of the next generation. Chi-square analysis was conducted on segregation data from the F_2, F_3, and F_4 populations.

Table 1. Segregation Results for MON 88302

					1:2:1 Segregation				
Generation	**Total Plants**	**Observed # Plants Homozygous Positive**	**Observed # Plants Hemizygous**	**Observed # Plants Homozygous Negative**	**Expected # Plants Homozygous Positive**	**Expected # Plants Hemizygous**	**Expected # Plants Homozygous Negative**	**χ^2**	**Probability**
F_2	220	51	122	47	55.00	110.00	55.00	2.76	0.2511
F_3	166	39	94	33	41.50	83.00	41.50	3.35	0.1874
F_4	198	53	97	48	49.50	99.00	49.50	0.33	0.8465