Infinite Enzymes, Inc.; Jonesboro, AR

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Ms. Bernadette Juarez
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Regulatory Status Review Submission for corn (*Zea mays,* L.) expressing manganese peroxidase from *Phanerochaete chrysosporium*, a white rot fungus.

Submitted by Infinite Enzymes, Inc.; 504 University Loop East, 130B; Jonesboro, AR 72401

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Introduction:

Infinite Enzymes (IE) is submitting this document to USDA-APHIS Biotechnology Regulatory Services for a Regulatory Status Review as described in 7 CFR part 340.4. IE has been growing this corn for many years under USDA-APHIS permits and intends to scale up production to increase sales of its manganese peroxidase enzyme products for a variety of potential environmental detoxification uses. The manganese peroxidase of note here has never been produced in large enough quantities or at low enough cost for the intended applications.

Corn as a plant and crop

Zea mays, L., is a food, feed, fiber, and fuel crop plant. It is grown on approximately 85-95 million acres in the U.S. every year. Roughly forty percent of the crop goes to ethanol production as a gasoline additive. Another 30-40% goes to animal feed for cattle, hogs, and poultry. A small percentage is exported, and small amounts are used in human food products.

Corn is an open pollinated crop with separate male and female flowers. It is produced as a hybrid, and thus the male and female cross to produce the hybrid must be controlled. This is fortunate for the IE team in that when doing back crosses to generate inbred elite lines for the hybrid, selection for higher expression of the transgene can be accomplished over the several generations required for breeding.

Genes inserted into corn

The manganese peroxidase gene (MnP) inserted into this corn is for the major (first) isozyme from *Phanerochaete chrysosporium*, a white rot fungus. Transformation was achieved using the disarmed *A. tumefaciens* strain, EHA101 with the super binary plasmid from Ishida et al. (1996). The construct used is described in the following table:

Table 1: Genetic Elements and Their Functions in Infinite Enzymes MnP-producing corn.

Genetic Element	Location in Plasmid	Function (Reference)
T-DNA regions		
Right Border Region	10,403-10,427	DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Intervening sequence	10,428-10,612	Sequence used in DNA cloning
Globulin-1 promoter	1-1442	DNA region from *Zea mays* globulin-1 gene (1.4 kb) (Belanger and Kriz 1991) AH001354.2
Intervening Sequence	1443	Sequence used in DNA cloning
BAASS	1444-1516	Alpha amylase signal sequence from barley, *Hordeum vulgare* (Rogers, 1985). ABBO1247.1
manganese peroxidase gene	1516-2589	Manganese peroxidase gene from *Phanerochaete chrysosporium*—a white rot fungus. (Tien and Kirk, 1985) J04980.1
Intervening Sequence	2590-2596	Sequence used in DNA cloning
Pin II terminator	2597-2852	Protease inhibitor II gene terminator from potato, *Solanum tuberosum* (An et al., 1989) X04118.1
Intervening Sequence	2853-2961	Sequence used in DNA cloning
35S promoter	2962-3503	Cauliflower mosaic virus promoter for the 35S rna. (Franck et al., 1980) NC_001497.2
Intervening Sequence	3504-3523	Sequence used in DNA cloning
Maize optimized PAT	3524-4075	Phosphinothricin acetyl transferase from *Streptomyces viridochromogenes* (Strauch et al., 1988) WP_003988626.1
Intervening sequence	4076-4093	Sequence used in DNA cloning
35S terminator	4094-4288	Cauliflower mosaic virus terminator for the 35S rna. (Franck et al., 1980) NC_001497.2
Intervening Sequence	4289-4355	Sequence used in DNA cloning
Left Border Region	4356-4380	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Vector Backbone		
Vector	4381-10,402	Sequence used in DNA cloning; spectinomycin resistance; origin of replication;

The plasmid map used for the transformation is found on the following page and the intended sequence insertion (between the right and left borders) is found in Appendix I.

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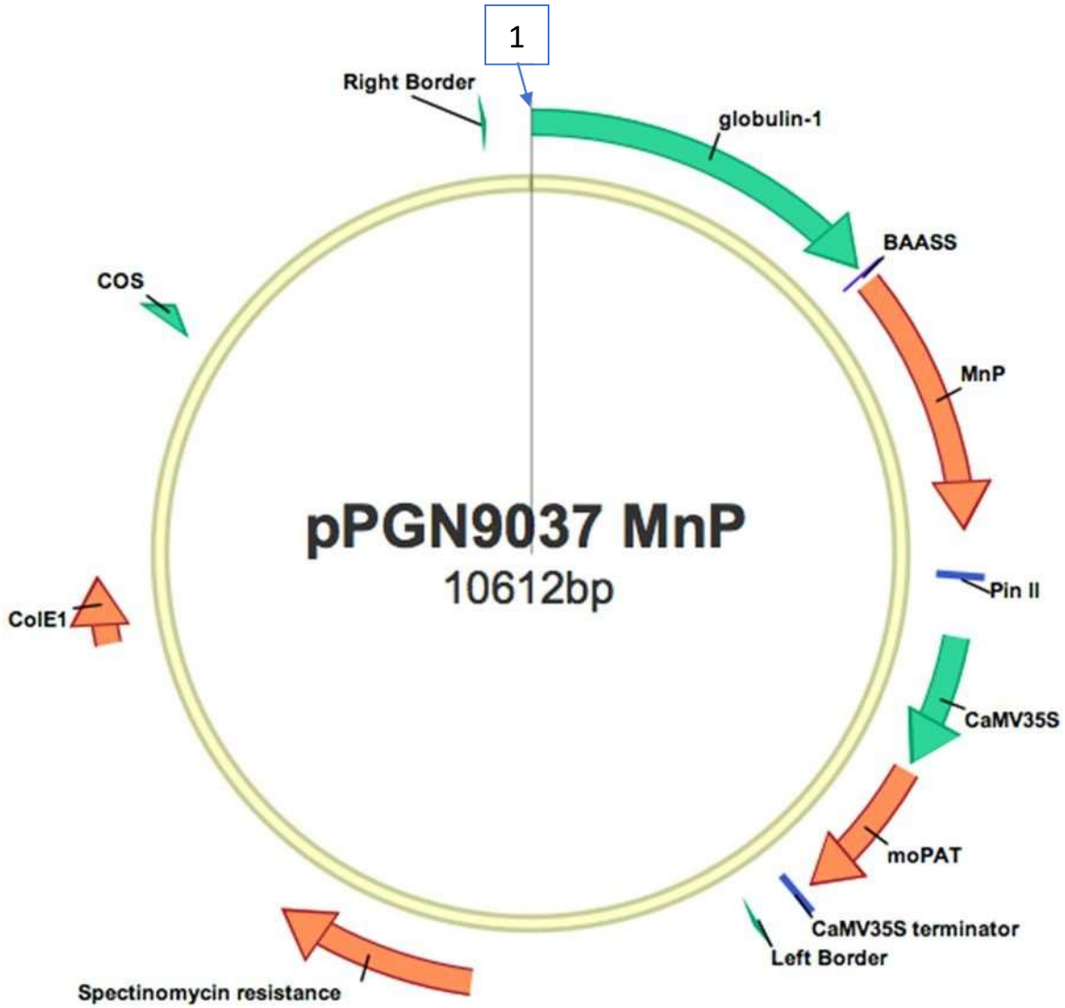

Figure 1: Plasmid map of pPGN9037 MnP

Agronomic Characteristics:

IE has grown the MnP event in the field for a number of years. IE spent several years in the backcross program putting the gene into two inbred lines to produce a hybrid. The lines are Stine male and female elite lines MBS411 and 16308-025. Two self-pollinated generations made them essentially homozygous. In our observations of the production fields over the last 2 years, we have not observed any issues with germination percent, growth, or insect susceptibility. The enzyme appears to have no detrimental effects on the grain or overall plant health (Infinite Enzymes, unpublished; Clough, et al, 2006). We surmise that this is because no peroxide, or manganese, the MnP substrates, are present in significant quantities in the grain. This contrasts with noted adverse effects on plant health when the MnP gene was put under control of a constitutive polyubiquitin-1 promoter (Clough, et al, 2006). In those instances, adverse impacts were noted on leaves, stems, and seeds. All growing of the MnP lines to date has been at 3 locations in Arkansas.

The yield of protein from the ground grain is approximately 0.4% of dry weight. This estimate is from enzyme assays and is based on the active ingredient. IE has not observed inactive protein.

Mechanism of Action

As intended, the trait expressed in these plants is identified as embryo-preferred production of manganese peroxidase. This is accomplished by use of the *Zea mays* globulin-1 promoter in combination with the barley alpha amylase signal sequence which further targets production to the cell walls of embryo tissues. This construct has been shown to be highly effective for this purpose (Hood, et. al, 2003). IE has not noted production of MnP in other tissues that have been tested (i.e., leaf, root, stem, endosperm) (Infinite Enzymes, unpublished). Expression of MnP in other tissues or times of plant development, other than embryo growth and development, is not expected. Secondarily, constitutive production of PAT using the cauliflower mosaic virus 35S promoter results in plant tolerance to glufosinate ammonium herbicides.

IE would not expect this event to have impacts on non-target organisms because it is not a plant-incorporated protectant, nor is the trait expressed to a measurable extent in the roots or other vegetative parts of the plant.

After many years of field production under APHIS permit, IE has not identified any phenotypic characteristics of these corns lines from which one could identify any plausible pathway to an increased plant pest risk compared to other corn varieties.

Characteristics of manganese peroxidase

The basic reaction of manganese peroxidase is shown below. Adding hydrogen peroxide begins the reaction by accepting the 2 electrons from the Fe center of the enzyme. The Fe core accepts 2 electrons from chelated Mn++ creating 2 Mn+++ ions that are thus activated to remove electrons from other molecules—the work of the enzyme completed. MnP has been studied extensively for use in the pulp and paper industry where it could be used for degrading lignin and bleaching pulp and has other potential applications in the food industry, textile manufacturing, and for bioremediation (Twala, et al, 2020). MnP may also have applications for industrial

wastewater treatment (Xu, et al, 2017).

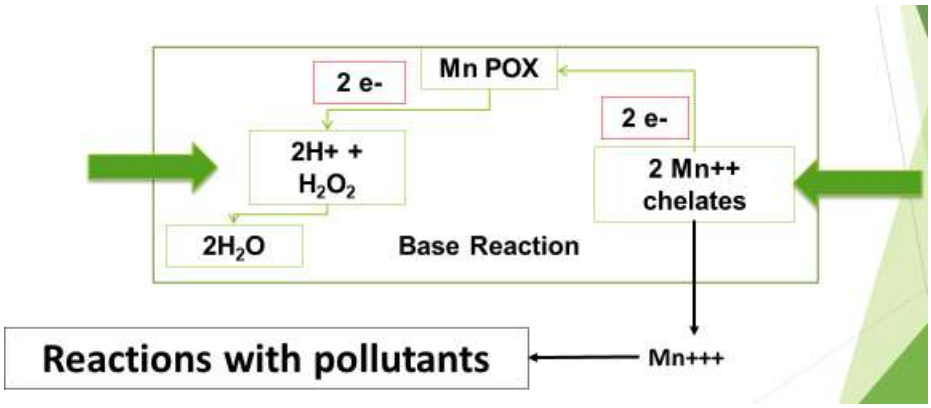

Figure 2: The basic manganese peroxidase (MnP) reaction scheme: MnP catalysis occurs in a series of oxidation-reduction reactions. In step one, hydrogen peroxide (or an organic peroxide) enters the active site of the enzyme. The oxygen binds to the iron core, then two electrons are transferred from Fe3+ to peroxide, breaking the bond to form water and a radical. The Fe3+ core than binds to one Mn2+, which donates an electron to the iron core, generating Mn3+, followed by a second electron extraction from a second Mn2+ to form the second Mn3+, regenerating the ground state of the enzyme. The activated unstable Mn3+ can then extract electrons from secondary compounds (modified from Wikipedia: Manganese peroxidase - Wikipedia).

Maize contains numerous peroxidases of its own. A white paper describing those was submitted to APHIS-BRS as part of early IE permit applications.

Conclusion

Infinite Enzymes has been growing its MnP-producing corn lines under USDA-APHIS permit since 2014 and has not noted any phenotypic changes in these lines that would indicate any plausible path to increased plant pest risk compared to other corn varieties. As such, Infinite Enzymes is requesting that USDA-APHIS-BRS determine that the MnP-producing corn described in this document is not subject to its regulations at 7 CFR part 340.

Respectfully submitted,

Elizabeth Hood, Ph.D.

Infinite Enzymes, Inc.

Jonesboro, Arkansas

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Appendix I: Sequence of intended insert in Infinite Enzymes MnP corn

Right border sequence
Sequences used in DNA cloning (throughout the construct)
Maize globulin-1 promoter
BAASS—barley alpha amylase signal sequence
Manganese peroxidase gene from *Phanerochaete chrysosporium*
Pin II terminator from potato
Cauliflower mosaic virus 35S promoter sequences
Maize optimized phosphinothricin N-acetyl transferase [*Streptomyces viridochromogenes*]
Cauliflower Mosaic Virus 35S terminator sequence
Left border sequence

Right border

gtttacccgccaatatatcctgtcaaacactgatagtttaaactgaaggcgggaaacgacaatctgatcatgagcggagaattaagggagtcacgttatg
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