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Bernadette Juarez
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United States Department of Agriculture Animal and Plant Health Inspection Service
Biotechnology Regulatory Services
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Dear Ms. Juarez,

The enclosed document (**Request 23-226-01rsr**), submitted in accordance with the Guidance for Requesting a Regulatory Status Review under 7 CFR part 340, has been revised per the request of USDA-BRS following your technical completeness check. To this end, we removed specific internally generated data that has not yet been published.

As background, PlantArcBio is seeking confirmation of a novel protoporphyrinogen oxidase (PPO) herbicide tolerant soy plant transformed with an PPO expressing gene (*PABHrD0047*). This fungal-derived PPO enzyme has a similar mode of action as endogenous PPO genes of plants, and confers resistance to PPO-type inhibitor herbicides, thereby providing soybean (*Glycine max*) with a desirable trait for Herbicide Tolerance (HT). While this submission is focused on soybean, we believe that this gene could be used in a variety of crops to improve weed control, reduce herbicide use, and ultimately increase crop yields.

CBI Justification: The attached documents also contain Confidential Business Information. PlantArcBio claims specific information in this permit application as confidential business information. The Freedom of Information Act (FOIA), 5 U.S.C. Section 552, expressly exempts the release of protected information that would likely cause substantial financial competitive harm. Information that is designated as confidential business information in this application include proprietary information on how PlantArcBio develops and evaluates products including specific genetic modifications, construct details, the origin of genetic material utilized, as well as proprietary transformation methods. Disclosure of any or all the designated confidential business information could aid other entities in the development of products that replicate and/or compete with PlantArcbio's inventions and products and would likely result in substantial competitive harm to PlantArcBio that would materially damage their ability to realize a return on their investment.

Please let us know if you have any further questions at this time or would like any additional information as your team proceeds with their review of our submission.

Regards,

Dror Shalitin, PhD
Founder and CEO
PlantArcBio Ltd.

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Contains Confidential Business Information

1. Introduction:

PlantArcBio is intending to develop a transgenic soybean plant that is resistant to Protoporphyrinogen oxidase (PPO) herbicides group 14 as designated by the Global HRAC (Herbicide Resistance Action Committee). PPO is an enzyme found in the chloroplasts and mitochondria that oxidizes protoporphyrinogen IX to produce protoporphyrin IX which is a precursor molecule for both chlorophyll and heme. PPO inhibitors block the production of chlorophyll and heme and cause the formation of highly reactive molecules that attack and destroy lipids and protein within membranes, which ultimately leads to disruption of cell membranes and plant death. The new soybean variety described in this submission will be genetically modified to produce a novel PPO protein (*PABHrD0047)* with decreased binding affinity for PPO inhibitor herbicides. This will effectively 'protect' soybeans from the negative effects of PPO herbicidal applications while effectively controlling surrounding susceptible weeds. This is summarized in Figure 1.

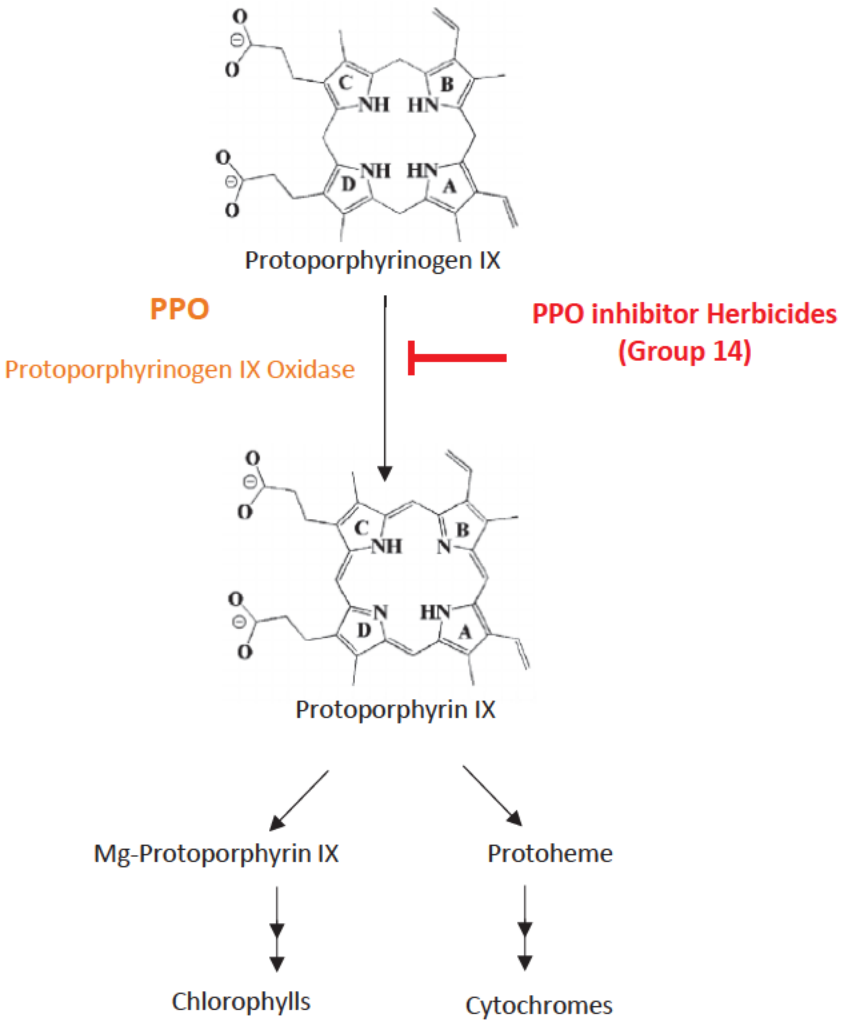

Figure 1: Protoporphyrinogen oxidase catalyzes six electrons oxidation of protoporphyrinogen to protoporphyrin. Protoporphyrinogen oxidase (PPO)-inhibiting herbicides are used to control weeds in a variety of crops. The inhibition of this enzyme results in an accumulation of protoporphyrinogen IX in the cytoplasm, where it reacts with molecular oxygen and produces singlet oxygen ($^1O_2$), causing cell death.

2. Description of comparator plant:

Conventional soybeans will be genetically modified to express resistance to PPO herbicides. Therefore, the appropriate comparator plant is soybean with details as follows:

- Order: Fabales
- Family: Fabaceae
- Genus: Glycine

- Species: Glycine max (L.) Merr.

3. Genotype of Modified Plant:

PlantArcBio intends to develop Soybean (*Glycine max*) plants produced by transformation of Soybean tissues using horizontal gene transfer (Agrobacterium-mediated) resulting in a single insert of a complete linear fragment. The PPO gene *pabHrD0047* is derived from the fungus [CBI

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] Since only the coding sequence of the cloned *pabHrD0047* PPO gene CBI
(producing the novel PPO protein) and no other DNA derived from the original host is included in the transformation construct employed, neither the potential source organism nor its safety profile should be relevant to the safety of the resulting PABHrD0047 protein[1].

The detailed genotype for the novel PPO-inhibitor resistant soy plant is as follows. The 'blue' bases represent the chloroplast transit proteins.

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The binary vector is ~7.3kb. It is composed of a transformed portion delineated by right border (RB) and left border (LB) sequences of T-DNA as well as a backbone vector sequences for bacterial replication functions (Figure 2, 3). The T-DNA contains an expression cassette of the chloroplast transit peptide CTP2 [CBI
fused to PABHr0047 resulting in the chloroplast targeted protein CBI
and leading to a plant which is highly resistant to group 14 herbicides. As the expression cassette can serve as selectable marker for transgenic plants by applying the herbicide, the resulting plant will not contain any additional sequences. An annotation of the different genetic elements is provided in Table 1.

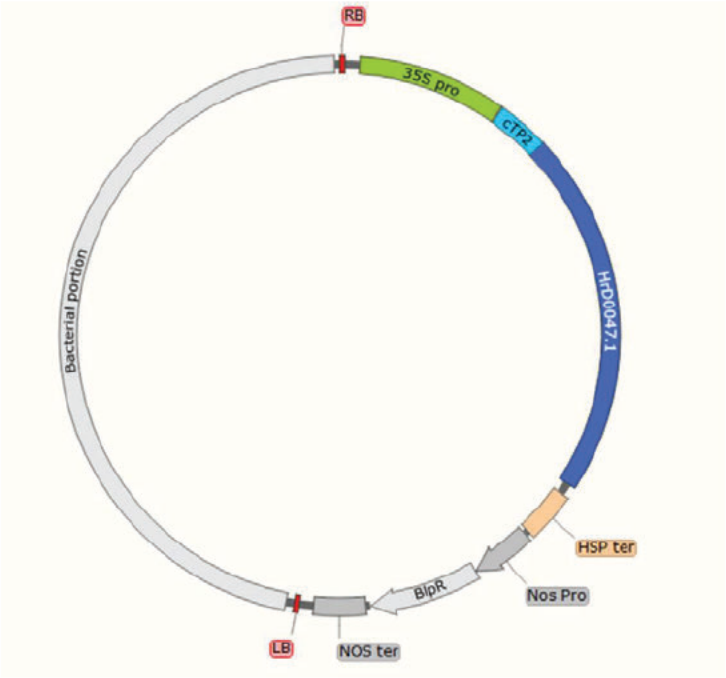

Figure 2: Binary vector for T-DNA insertion by Agrobacterium mediated transformations.

Construct Description:

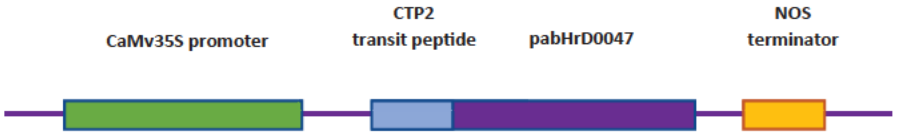

Figure 3: pabHrD0047.1 expression construct for soybean transformation. pabHrD0047 expression cassette is represented by the following string: "CaMV35S promoter, CTP2-pabHrD0047 expressed gene, NOS-terminator."

Table 1: Annotation of the inserted genetic material

Nucleotide position (bp)	Inserted component	Function	Construct Component Donor	Sequence ID	
1-25	Right border	Used for transfer of the T-DNA	Synthetic construct octopine right border sequence		
26-98	Intervening sequence	Used in DNA cloning	"Synthetic"* sequence		
99-971	CaMV35S	CaMV35S promoter with a duplicated enhancer region to regulate expression	*Cauliflower mosaic virus*	NC_001497.2	
972-977	Noncoding linker				
978-1091	CTP2	Chloroplast transit peptide [derived from PPOX gene]	*Arabidopsis thaliana*	[ ]	CBI
1092-1094	Noncoding linker				
1095-2887	pabHrD0047	Protoporphyrinogen oxidase (PPO)	[ ]	[ ]	CBI(2)
2888-2944	Noncoding linker	used in DNA cloning	"Synthetic"* sequence		
2945-3192	Nos-terminator	Transcription terminator from the 3' UTR of the nopaline synthase with a polyadenylation signal	*Agrobacterium tumefaciens*	FN436278.1	
4355-4425	Noncoding linker				
4426-4453	Left border	Used for transfer of the T-DNA	Synthetic construct octopine left border sequence		

* The term "synthetic" used in this table is defined in the USDA/APHIS-BRS Guidance Document BRS-GD-2020-0003. In the context of this table, the word "synthetic" indicates that the sequence does not have an assign function and was not intentionally derived from a known source, although some homology may exist to known DNA sequences.

A description of each element of the inserted transgene is as follows:

CaMV35S promoter:

The CaMV35S promoter with a duplicated enhancer region sequence is derived from the *Cauliflower mosaic* virus. The CaMV35S promoter is considered a constitutive promoter facilitating transgene expression[2].

AtCTP2:

The transit peptide [ ] that is fused to *pabHrD0047* was derived from *Arabidopsis thaliana* [ ]. This facilitates transition of the PABHrD0047.1 protein to the chloroplast as the native PPO, and thus, is expected to preserve PABHrD0047's native PPO mechanism of action. CBI CBI

pabHrD0047:

The pabHrD0047 gene encodes for a] protein[3], consisting of [ ] amino acids. CBI(2)

NOS terminator:

NOS terminator is an efficient transcription terminator from the 3' untranslated region of the nopaline synthase from *Agrobacterium tumefaciens* which is a polyadenylation signal. The NOS terminator sequence was used to produce event FG72 soybean, which has been previously reviewed and granted nonregulated status by the USDA (petition number 09-328-01p).

Phenotypic evaluations will be employed to demonstrate the integrity, stability, and consistent inheritance of the transgene in the expected Mendelian pattern over multiple generations. Molecular characterization by applying Next Generation Sequencing will determine the integrity of the desirable T-DNA, the loci in the genomic DNA to ensure that gene fragments are not integrated into an existing gene, the single insert, the absence of the bacterial backbone of the plasmid. These analyses will confirm that the intended genetic modification has been made without the unintended presence and expression of new fragments, exogenous DNA, or fusion genes.

PlantArcBio also completed confirmatory analyses to show that the genetic sequence appropriately encoded for the novel PPO enzyme. These assessments provided conclusive results which are summarized in the following figures. The amino acid sequence encoded by the inserted genetic material PABHrD0047, and the Blast search conducted is as follows:

PABHrD0047 amino acid sequence:

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4. Intended Phenotype/ Trait:

As previously described, the intended soybean plant will confer resistance to Group 14 (Weed Science Society of America (WSSA)) mode of action products: PPO inhibiting herbicides including active ingredients such as: Flumioxazin, Carfentrazone, Oxyfluorfen and Oxadiazon. Herbicide tolerance will be achieved by the insertion and expression of the gene that encodes for a form of PPO (protoporphyrinogen oxidase) with decreased binding affinity for PPO inhibitor herbicides. Through the creation of an insensitive form of PPO to group 14 herbicides, the introduced genetic sequence will ultimately render the plant resistant to PPO herbicides without changing the normal function of the PPO enzyme.

5. Mechanism of Action (MOA) resulting in desired Herbicide-tolerant phenotype

The MOA or the biochemical process through which the inserted genetic material will confer this resistance is achieved via the modified plant's ability to produce an insensitive form of PPO. Since PPO serves an essential function by catalyzing the oxidation of protoporphyrinogen IX to protoporphyrin IX, inhibiting this enzyme will

have a lethal effect on plants. Therefore, the novel insensitive form of PPO will not be affected by herbicides that target this enzyme, thus rendering it insensitive to the herbicide's function. This MOA has been previously described in a number of publications [5,6,7].

6. Summary

A PPO gene was identified that confers resistance to group 14 PPO inhibitor herbicides when expressed in plants. The introduction of this gene into soybean should not increase plant pest risks as many organisms in the environment express this gene. Moreover, the expression of an exogenous PPO enzyme should not have an impact on human health or the environment due to the fact that the donor organism is widely used in the production of proteins for food grade and medical applications, and the resulting proteins are generally regarded as safe (GRAS). Therefore, this novel PPO inhibitor-resistant soybean should facilitate the safe and effective management of hard-to-control or resistant weeds in the field.

7. References

1. [CBI
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2. Amack, S,C, Antunes, M,S (2020) CaMV35S promoter – A plant biology and biotechnology workhorse in the era of synthetic biology. Current Plant Biology, v. 24, 100179 (1-9).
3. Calculated using the protein molecular weight calculator available at: https://www.bioinformatics.org/sms/protmw.html
4. Arnould, S, Camadro, J, M (1998) The domain structure of protoporphyrinogen oxidase, the molecular target of diphenyl ether-type herbicides. Proc Natl Acad Sci USA 95(18):10553-8.
5. Li, X, Nicholl, D (2005) Development of PPO inhibitor-resistant cultures and crops. Pest Manag Sci 61:277-285.
6. Lermontova, I, Grimm, B (2000) Overexpression of plastidic protoporphyrinogen IX oxidase leads to resistance to the diphenyl-ether herbicide acifluorfen. Plant Physiol 122(1):75-84.
7. Larue, CT, Ream, JE, Zhou, X, Moshiri, F, Howe, A, Goley, M, Sparks, OC, Voss, ST, Hall, E, Ellis, C, Weihe, J, Qi, Q, Ribeiro, D, Wei, X, Guo, S, et al. (2020) Microbial HemG-type protoporphyrinogen IX oxidase enzymes for biotechnology applications in plant herbicide tolerance traits. Pest Manag Sci 76:1031–1038.