Biotechnology Regulatory Services
Animal and Plant Health Inspection Service
United States Department of Agriculture
4700 River Road
Riverdale, MD 20737

RECEIVED
By hjohnson for BRS Document Control 10:21a.m., Oct 28, 2021

Attn: Bernadette Juarez
USDA-APHIS Deputy Administrator
Biotechnology Regulatory Services
ConfirmationRequests@USDA.gov

Subject: Request **21-264-01cr:** 7 CFR § 340.1(e) Request for Confirmation that PlantArcBio's HPPD Herbicide Tolerant Soy is Exempt from Regulation Under §340.1(c)(2)

Dear Bernadette,

PlantArcBio is seeking confirmation of an exemption for novel HPPD herbicide tolerant soy plants transformed with an HPPD expressing gene (*pabHrA001*) under USDA-APHIS article 7 CFR §340.1(c)(2). This specific exemption pertains to plants modified to contain a plant-trait-MOA (PMOA) combination that is the same as that in another plant of the same species previously reviewed and determined by APHIS not to be regulated under USDA's previous petition process. PlantArcBio suggests that PABHrA001 protein expressing soy modified plants contain a PMOA combination that is the same as FG72, SYHTOH2 soybean (petition # 09-328-01p and 12-215-01p respectively) related to HPPD inhibitor resistant upland soybean (*Glycine max*) which was previously reviewed and determined not to be regulated. We are pleased to provide the following information in support of our request in accordance with the guidelines provided by USDA-APHIS Biotechnology Regulatory Services.

Requester's Information and Contact:

Company: Plantarc Bio Ltd.
Contact Person: Dror Shalitin, PhD, Chief Technical Officer
Address: 23b Hateena St., Raanana, 4357724, Israel
Phone: 972-50-3161062
Email: dror@plantarcbio.com

Description of the plants:

Soybean details are as follows:

- Order: Fabales
- Family: Fabaceae
- Genus: Glycine
- Species: Glycine max (L.) Merr.

Rationale for regulatory exemption of PABHrA001 Soybean

We claim the exemption for our modified Soybean under the fourth exemption defined in USDA APHIS regulations at 7 CFR §340.1(c)2. This exemption covers plants modified to contain a plant-trait-MOA (PMOA) combination that is the same as that in another plant of the same species previously reviewed and determined by APHIS not to be regulated. USDA-APHIS article 7 CFR Part 340.1(c)(2) specifically states that "a plant-trait-MOA combination that is the same as that in a plant of the same species APHIS determined to be nonregulated in response to a petition submitted prior to October 1, 2021, pursuant to 340.6 of the previous regulations found at 7 CFR part 340". PlantArcBio believes that its proposed Soybean product qualifies for this exemption as USDA has previously reviewed FG72, SYHTOH2 soybean (petition # 17-138-01p and 12-215-01p respectively) related to HPPD inhibitor resistant soybeans (*Glycine Max*). In this case, the plant was genetically engineered to produce an insensitive form of 4-Hydroxyphenylpyruvate dioxygenase (HPPD) with decreased binding affinity for HPPD inhibitor herbicides which ultimately enhances resistance of plants sprayed with this class of herbicides.

Section 1:

Description of plant trait and phenotype:

PlantArcBio is intending to develop a transgenic Soybean that is resistant to HPPD herbicides. 4-Hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors (HPPD inhibitors) are a class of herbicides that prevent growth in plants by blocking 4-Hydroxyphenylpyruvate dioxygenase enzyme. HPPD catalyzes the conversion of 4-hydroxyphenyl pyruvate (HPP) to homogentisate (HGA) which is an intermediate in the biosynthesis of plastoquinone (PQ) and tocopherols in photosynthetic organisms (figure 1). PQ is essential for the phytoene desaturase reaction of carotenoid biosynthesis as well as for photosynthetic electron transfer from photosystem II. Carotenoids are essential for

the correct assembly of new photosynthetic units and thus HPPD herbicides characteristically cause shoot tip tissues to emerge as white and bleached [(2)]. HPPD inhibitor herbicides are competitive inhibitors with high affinity to HPPD enzyme binding site. By binding, they form a complex with the enzyme and prevent the binding of HPP substrate and its conversion to HGA. HPPD inhibitors possess a higher dissociation constant (K_D) decreasing the inhibitor-binding affinity [(3)]. The new Soybean varieties will be genetically modified to produce a form of novel HPPD protein with decreased binding affinity for HPPD inhibitor herbicides. This will effectively 'protect' these specific plants from the negative effects of HPPD herbicidal sprays while controlling surrounding susceptible weeds and plants.

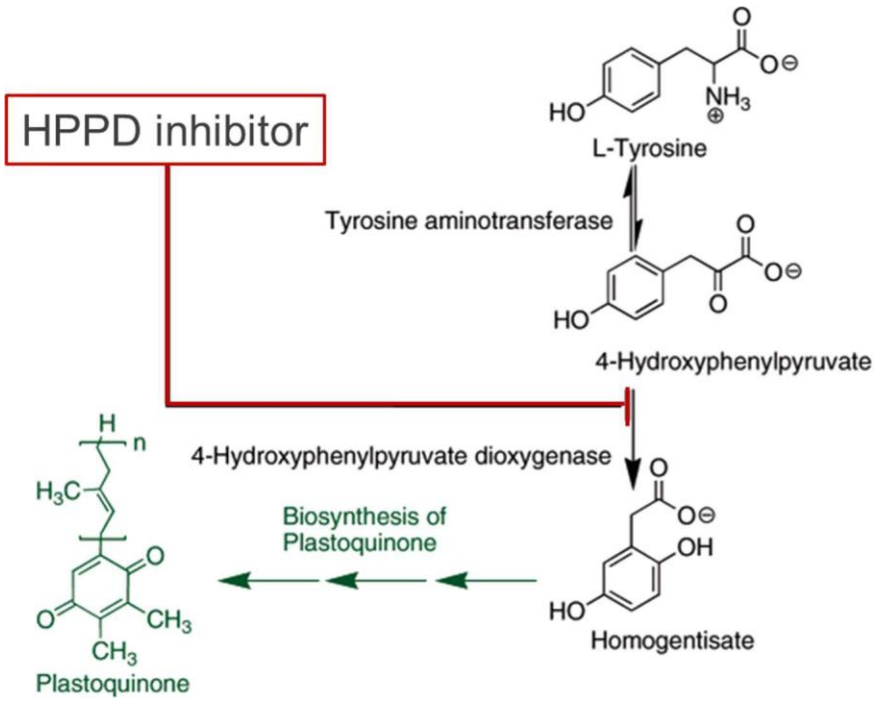


Figure 1. Plants Tyrosine metabolic pathway. The metabolic pathway of tyrosine catabolism produces energetically exploitable glucogenic and ketogenic products in variety of organisms of different kingdoms. In plants it has particular importance where an anabolic branch from homogentisate leads to the production of the essential quinonoid redox cofactors, plastoquinone and tocopherol. HPPD inhibitors binds to the HPPD enzyme in and prevents the catalysis of HPP to HGA by HPPD [(1)].

An insensitive form of HPPD with decreased binding affinity for HPPD inhibitor herbicides was previously reviewed by APHIS in petition numbers 09-328-01p, 12-215-01p (FG72 Soybean and SyHT0H2 soybean respectively). Regarding the development of FG72 soybean, the wild-type (wt) hppd gene (*hppdPf*) was isolated and cloned from *Pseudomonas fluorescens* with a TPotp Y transit peptide at the N-terminal end of the protein. A single amino acid substitution introduced in *hppdPf* gene resulted in the

modified *hppdPfW336* gene. The modified protein possesses greater than 99.5% identity to the native HPPD protein from *P. fluorescens* and is tolerant to isoxaflutole (IFT). The SYHT0H2 soybean contains the transgene avhppd-03 encoding for an HPPD enzyme that is more than 99.7% identical in its amino acid sequence to the native HPPD in common oat (*Avena sativa*) and has lower binding affinity for HPPD-inhibiting herbicides, such as mesotrione and isoxaflutole.

Similarly, PlantArc Bio's HPPD resistant Soybean will be transformed with a native unmodified hppd (*pabHrA001*) gene isolated and cloned from *Trichoderma harzianum spp.* Trichoderma are a ubiquitous species in the environment and present in most soils [(4)]. As further presented here, the newly produced protein's (PABHrA001) enzymatic activity was characterized and found to possess the exact same biochemical mechanism of action as the plant's HPPD enzymes. Moreover, PABHrA001 HPPD's herbicide tolerance was established in genetically modified plants (*Arabidopsis thaliana, Camelina sativa* and *Nicotiana tabacum* (Tobacco) grown in the presence of several widely use HPPD inhibitors (Isoxaflutole, Tembotrione, Mesotrione, Topramezone and Pyrazoxyfen).

Genetic modification and components:

PlantArc Bio intends to develop Soybean (*Glycine max*) plants produced by transformation of Soybean tissues using either direct gene transfer (biolistic) or horizontal gene transfer (Agrobacterium-mediated) with a single insert of a complete linear fragment. PlantArc Bio's HPPD herbicide tolerant Soybean will contain the following transgene features represented by the following string: "AtUbi10 or CaMV35S-CTP2::pabHrA001::Nos-ter", similar to constructs previously used (e.g., petition 09-328-01p) (Figure 2).

Potential risk of integration of unintended vector backbone sequences, including confirmation of insertion location, will be dealt with, and discussed in **Section 2**

CaMV35S promoter:

The CaMV35S promoter with a duplicated enhancer region sequence is derived from the Cauliflower mosaic virus. The CaMV35S promoter is considered a strong constitutive promoter facilitating high levels of transgene expression [(5)]. The CaMV35S promoter sequence was also used to produce HB4 soybean line (Verdeca LLC), which have been previously reviewed and granted nonregulated status by the USDA (petition number 17-223-01p).

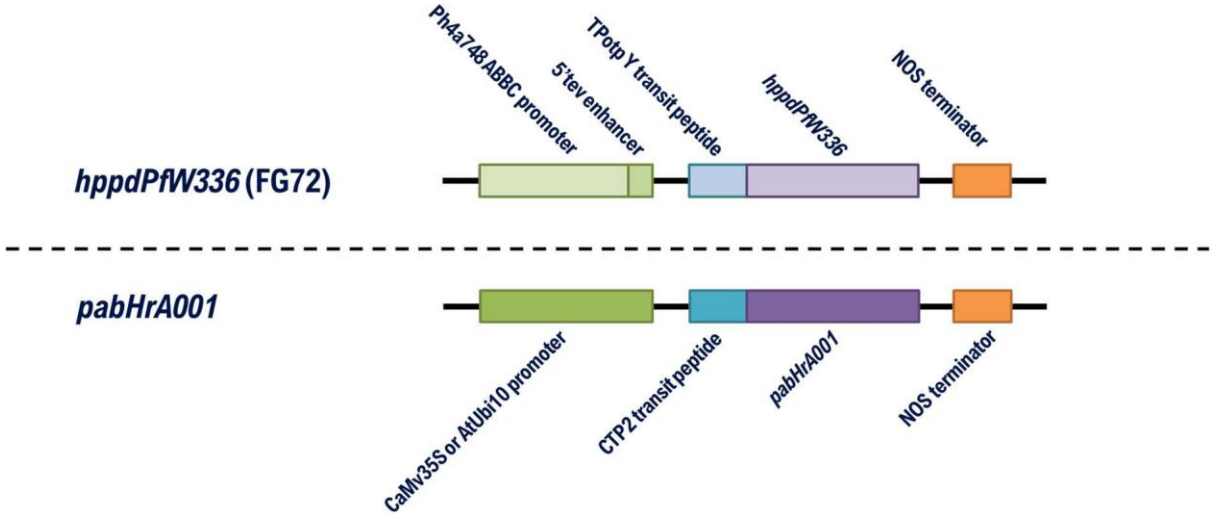


Figure 2. Expression constructs used soybean for plant transformation. *pabHrA001* vs. *hpdPfW336* gene expression cassettes. The two cassettes share similar motifs. *pabHrA001* gene expression cassette is represented by the following string: "AtUbi10 or CaMV35S-CTP2::*pabHrA001*::NOS-ter" and the *hppdPfW336* gene expression cassette is represented by the following string: "Ph4a748 ABBC-5'tev -TPotpY::*hppdPfW336*::3'nos".

AtUbi10 promoter:

Polyubiquitin promoter (AtUbi10) promoter from *Arabidopsis thaliana* is known to drive constitutive expression of the genes it controls [(14)]. The Ubi10 promoter sequence was also used to produce Dow DAS-81419-2 soybean line [(15)], which have been previously reviewed and granted nonregulated status by the USDA (petition number 12-272-01p).

EcTP transit peptide:

The chloroplast transit peptide (CTP2) of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme was derived from Arabidopsis thaliana. This facilitates the transition of the HPPD enzyme to the chloroplast as the native HPPD. The CTP2 coding region used to produce event MON 87705 soybean was also previously reviewed and granted nonregulated status by the USDA (petition number 09-201-01p).

(**Or) AtcTP:**

The transit peptide of the HPPD was derived from *Arabidopsis thaliana.* This facilitates the transition of the HPPD enzyme to the chloroplast as the native HPPD, and, thus, expected to preserve PABHrA001's native HPPD MOA [(3)].

***pabHrA001*:**

The HPPD gene *pabHrA001* is derived from *Trichoderma harzianum spp.* (Accession number – DSM 33749, given by the International Depository Authority). The *pabHrA001* gene encodes for a 46.4 kDa protein, PABHrA001, consisting of 415 amino acids.

NOS terminator:

Efficient transcription terminator from the 3' untranslated region of the nopaline synthase from *Agrobacterium tumefaciens* which is a polyadenylation signal [(6)]. The NOS terminator sequence was used to produce event FG72 soybean, which have been previously reviewed and granted nonregulated status by the USDA (petition number 09-328-01p).

Trait and associated MOA:

The trait of herbicide resistance in the intended plant will be resistant to the group 27 of HPPD inhibitor herbicides including active ingredients such as: Tembotrione, Mesotrione, Isoxaflutole, Topramezone and Pyrazoxyfen. Tolerance will be achieved by the insertion of a gene that encodes for a form of HPPD (p-hydroxyphenylpyruvate dioxygenase) with decreased binding affinity for HPPD inhibitor herbicides. This mechanism of action is the same as the antecedent HPPD-resistant soybean plants (Bayer CropScience FG72 Soybean- petition 09-328-01p; Syngenta and Bayer SYHT0H2 Soybean- petition 12-215-01p). Through the creation of an insensitive form of HPPD, the introduced genetic sequence will ultimately result in the same biochemical process as detailed in previously deregulated products and, thus, render the plant resistant to HPPD herbicides.

Section 2:

Scientific methodology used, or intended to use, to verify the plant and modifications:

Molecular characterization of plant/ insert

The HPPD gene *pabHrA001* is derived from *Trichoderma harzianum spp*. PABHrA001 protein sequence which is aligned with three common Trichoderma HPPD proteins (*T. harzianum, T. reesei* and *T. gamsii*), found to be identical to *Trichoderma harzianum* HPPD and highly conserved through the Trichoderma genus (a complete description of the

method is found at "Efficacy of methodology" under "Cloning and sequencing of *pabHrA001*") (figure 3).

Figure 3. Sequence alignment of PABHrA001 protein with common Trichoderma HPPD sequences. PABHrA001 protein sequence was aligned with three common Trichoderma HPPD proteins (*T. harzianum, T. reesei and T. gamsii*) and found to be identical to *T. harzianum* HPPD and highly conserved through the Trichoderma genus. Alignment is colored by percentage identity.

Risk assessment of Soybean plants transformed with *pabHrA001: Confirming Insertion of Intended Sequence and absence of vector backbone*

As mentioned, pabHrA001 Soybean plants will either be transformed using either biolistic or Agrobacterium mediated methods. To ensure that no unintended exogenous DNA from the vector will be introduced in the transformed plant, the following methods will be employed: phenotypic evaluations that demonstrate the integrity, stability, and consistent inheritance of the inserted genetic material; plant compositional assessments that may demonstrate any unintended compositional changes in the transformed Soybean events; and genetic/ segregation assays to demonstrate that a single insertion (in a single loci) was achieved that is consistent with an intact copy of the desirable T-DNA. PCR assays and genome walking using vector specific sets of primers (figure 4) will also be performed to ensure the presence of the desired gene and the absence of unintended DNA fragments derived from the vector (plasmid) backbone.

Sequence analysis of flanking genomic DNA regions will also be used to determine the loci of the transformed DNA to ensure that gene fragments are not integrated into an existing gene, and that the inserted gene is stably inherited in the expected Mendelian pattern over multiple generations. Therefore, 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.

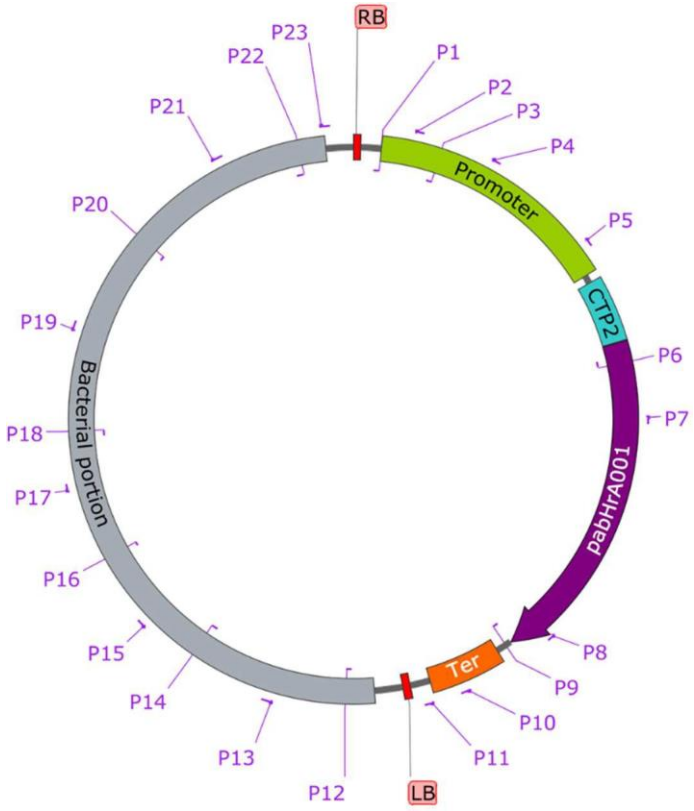


Figure 4. Binary vector for T-DNA insertion by Agrobacterium mediated transformations. The binary vector is approximately 6kb. It contains a transformed elements portion delineated by a right border (RB) and left border (LB) sequences of T-DNA (T-DNA expression construct is presented in figure 2, as well as backbone vector sequences outside of the two T-DNA border sequences. P1-23 representing primers that shall be used to determine the integrity of the T-DNA portion, the loci in the crop genomic DNA and to ensure the absence of DNA fragments derived from the vector backbone of plasmid.

Novel enzyme/ protein characterization

Sequence alignment analysis of PABHrA001 protein with *Arabidopsis thaliana* HPPD (At_HPPD), soybean HPPD (Gm_HPPD), cotton HPPD (Gh_HPPD) and the non-regulated HPPD of the granted petition HPPDPfW336 (FG72 Soybean) and avHPPD-03 (SYHT0H2 Soybean) concluded that PABHrA001 possesses a typical HPPD sequence conservation pattern (figure 5). The sequence similarities between the Soybean HPPD (Gm_HPPD), cotton HPPD (Gh_HPPD) and Arabidopsis HPPD (At_HPPD) support the assumption that the enzymes possess similar structural features and MOA mechanism.

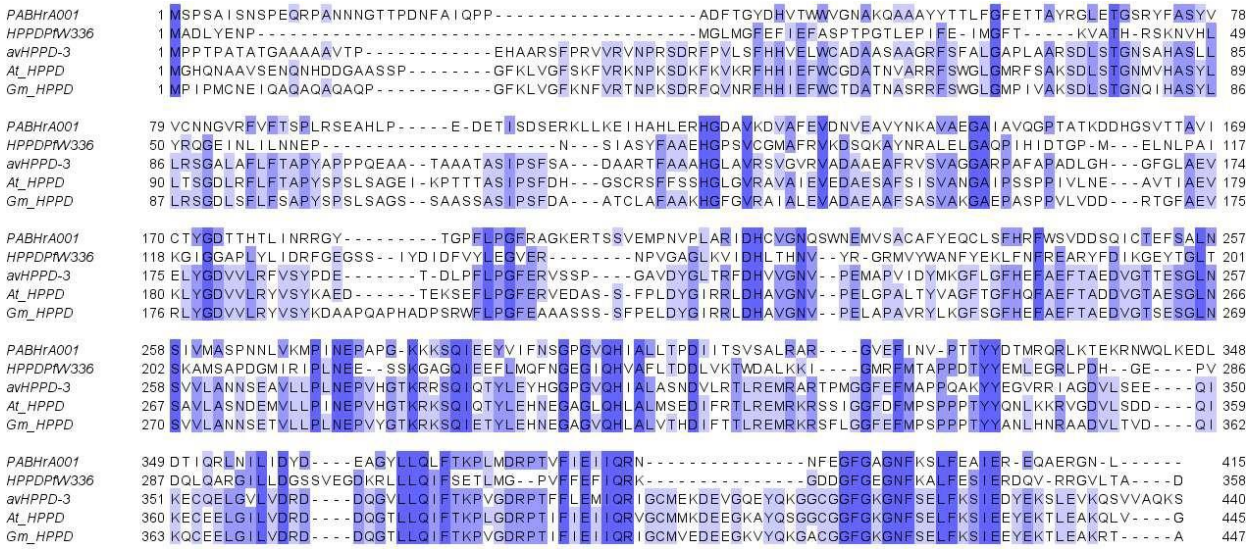


Figure 5. Sequence alignment of PABHrA001 protein with Arabidopsis HPPD (At_HPPD), Soybean HPPD (Gm_HPPD), cotton (Gh_HPPD) and the non-regulated HPPDs of the granted petitions - HPPDPfW336 (FG72 Soybean) and avHPPD-03 (SYHT0H2 Soybean). The predicted conserved residues of the catalytic pocket of PABHrA001 interacting with the HPPD inhibitors are indicated by orange triangles (His215, His298, Glu370, Phe383 and Phe398).

Moreover, structural analysis of the PABHrA001 model and its comparison with plant HPPD structures with the presence of NTBC (a common HPPD inhibitor) revealed that PABHrA001 possesses a structure typical of HPPD proteins (figure 6) with a distinct structure of the catalytic region (figure 7). Despite possessing a typical HPPD sequence conservation pattern and a distinct catalytic region consistent with other HPPD enzymes, PABHrA001 (as with most of the fungal HPPDs) has an overall low sequence similarity to plant HPPDs and HPPDGfW336. Those dissimilarities most likely result in low binding affinity of the plant-HPPD-targeted inhibitors to PABHrA001 and imparts PABHrA001 with HPPD-herbicide tolerance (a complete description of the method is found at "Efficacy of methodology" under "Structural analysis to determine PABHrA001 MOA and catalytic mechanism").

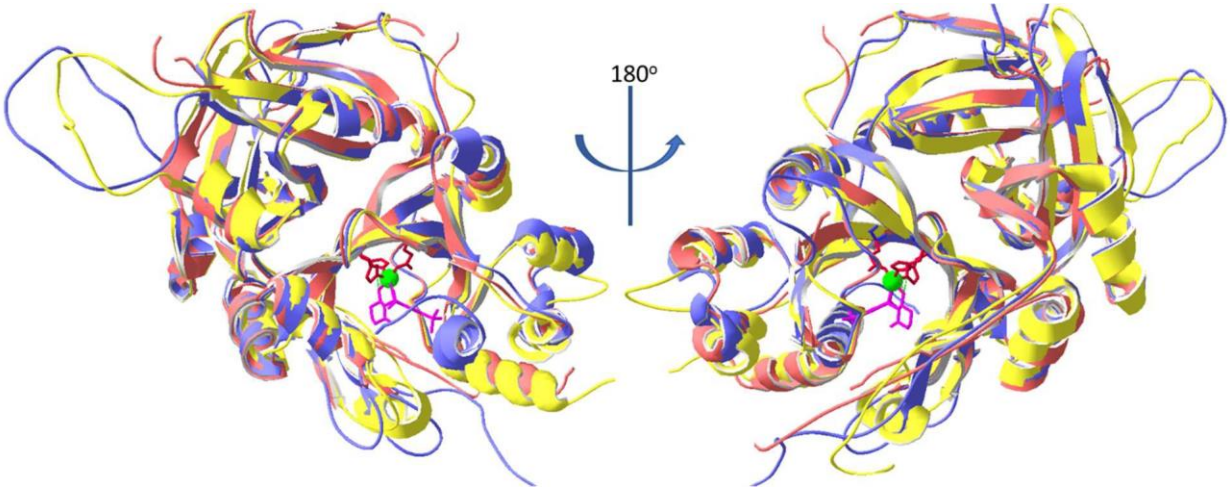
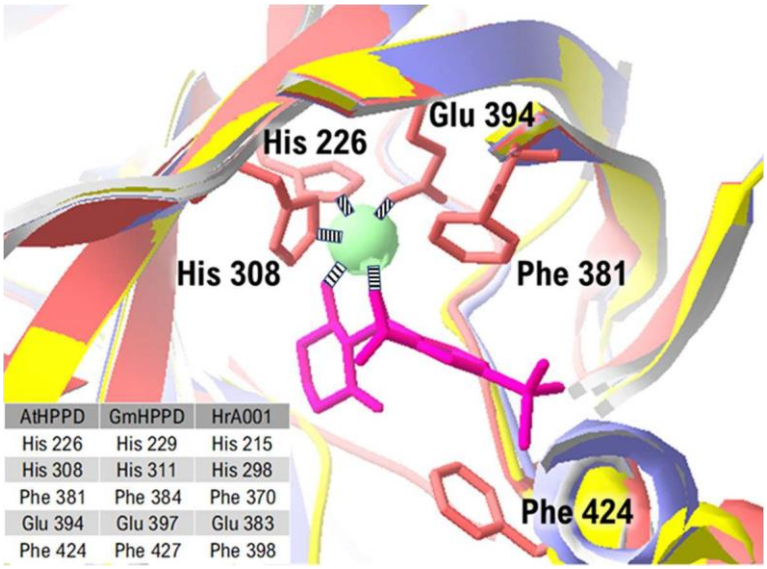


Figure 6. Structural models of HPPD proteins. Superposition of PABHrA001 (yellow) and soybean HPPD (purple) structure models over Arabidopsis HPPD (red) with NTBC [10] (pink). The inhibitor, NTBC is displayed in sticks, and the metal ion is shown in green sphere.

Figure 7. Binding modes in the catalytic pocket of Arabidopsis HPPD (AtHPPD) to the NTBC inhibitor. The coordination interactions of the conserved His226, His308 and Glu394 residues are indicated with black dash. The conserved Phe381 and Phe424 performs hydrophobic interactions between the cyclohexane moiety of NTBC and AtHPPD. The table at the left bottom indicates the conserved residues of AtHPPD a side to the corresponding residues at Soy HPPD (GmHPPD) and PABHrA001.



AtHPPD	GmHPPD	HrA001
His 226	His 229	His 215
His 308	His 311	His 298
Phe 381	Phe 384	Phe 370
Glu 394	Glu 397	Glu 383
Phe 424	Phe 427	Phe 398

HPPD inhibitor binding efficiency assay of PABHrA001

HPPD enzymes catalyze the conversion of p-hydroxyphenylpyruvate (HPP) into homogentisic acid (homogentisate) (2,5-dihydroxyohenylacetate) (HGA). When a HPPD gene is expressed in bacterial host cells, the bacteria secret a melanin-like pigment, a product of the natural oxidization process of the HGA (figure 8). Here, we used an activity assay to detect HPPD activity in *E. coli BL21 (DE3)* cells transformed with HPPD genes

cloned to an expression vector. The transformed bacteria were cultivated on agar media in petri dishes and subjected to increasing concentrations of HPPD inhibitors herbicides active ingredients. The HPPD activity was detected by the formation of a brown halo around the bacterial colony (a complete description of the method is found at "Efficacy of methodology" under "Bacterial HPPD-activity assay to determine PABHrA001 MOA").

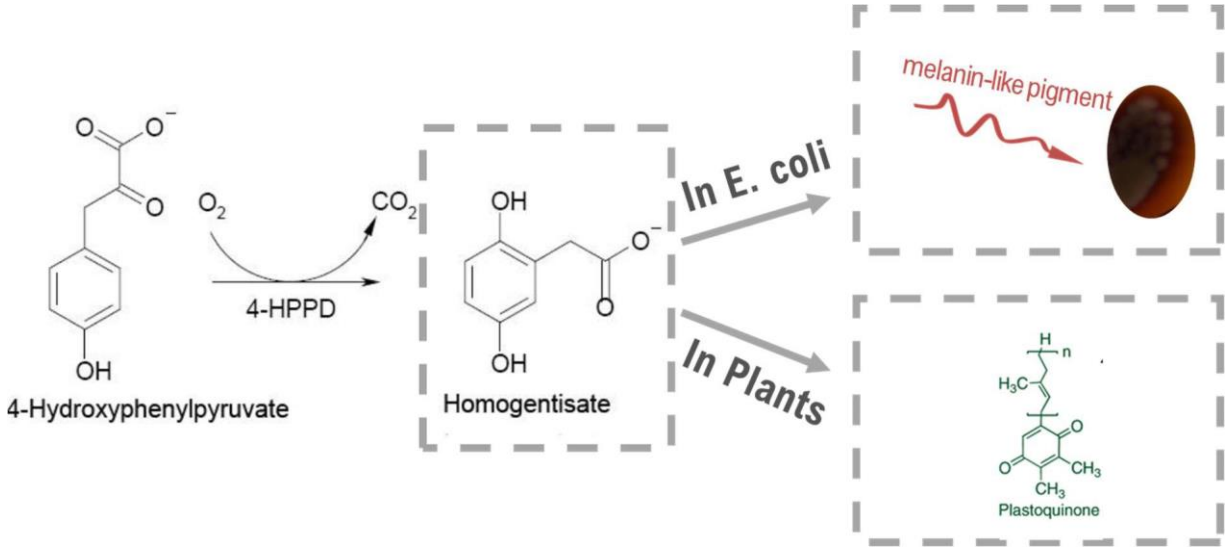


Figure 8. The mechanism of HPPD activity bacterial assay. HPPD enzymes catalyzes the conversion of p-hydroxyphenylpyruvate (HPP) into homogentisic acid, homogentisate, (2,5-dihydroxyohenylacetate) (HGA). When a HPPD gene is expressed in bacterial host cells the bacteria secret a melanin-like pigment (bottom right), a product of the natural oxidization process of the HGA.

PABHrA001 HPPD activity was detected on plates supplemented with 250 µg/ml of Tembotrione, 250 µg/ml of Mesotrione, 25 µg/ml of Isoxaflutole, 25 µg/ml of Topramezone and 1250 µg/ml of Pyrazoxyfen. On the other hand, *E. coli BL21 (DE3)* transformed with the *Arabidopsis thaliana* HPPD (AtHPPD) showed poor HPPD activity (figure 9). These results verified that PABHrA001 is translated to an HPPD enzyme with the same catalytic function as plant HPPDs and with lower affinity to the group 27 HPPD inhibitors tested.

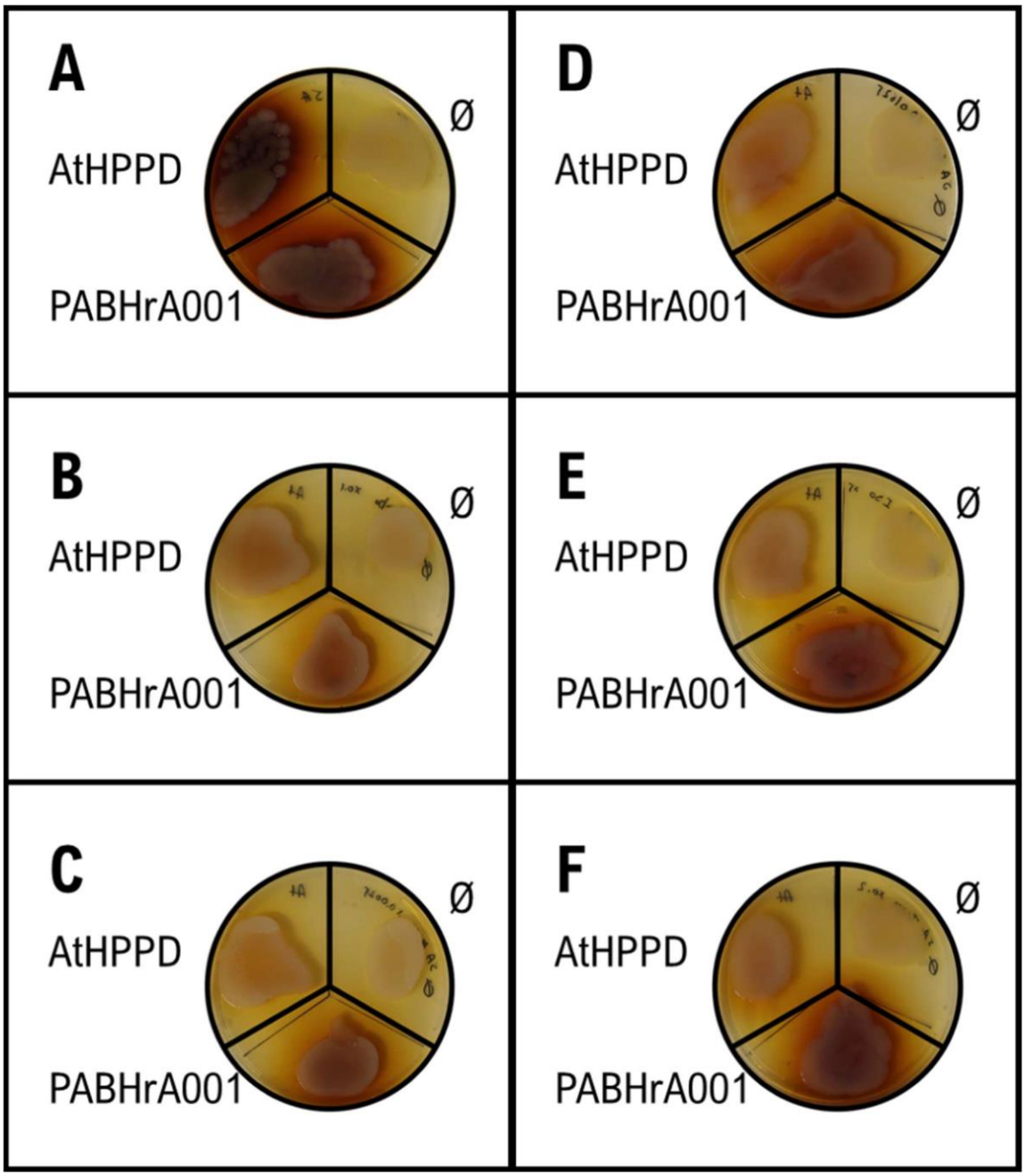


Figure 9. HPPD activity bacterial assay. Images obtained from a HPPD-activity assay of *E. coli BL21 (DE3) expressing the* **transformed genes of** *pabHrA001*, **Arabidopsis HPPD (AtHPPD) and empty vector (Ø), grown in the presence of HPPD inhibitors. A.** No inhibitor; **B.** Tembotrione 250 µg/lit; **C.** Mesotrione 250 µg/lit; **D.** Pyrazoxyfen 1250 µg/lit; **E.** Isoxaflutole 25 µg/lit; **F.** Topramezone 15 µg/lit. Brown Halo indicating the HPPD activity of the transformed gene due to the HPPD-mediated conversion of p-hydroxyphenylpyruvate (HPP) into homogentisate (HGA), following by oxidization into a melanin-like pigment.

HPPD resistance of transformed plant

The HPPD tolerance of PABHrA001 transformed plants were tested on Arabidopsis (*Arabidopsis thaliana*), camelina (*Camelina sativa*) and tobacco (*Nicotiana tabacum*) plants. The transformed plants were treated with HPPD inhibitors (a complete description of the method is found at "Efficacy of methodology" under "In-planta screen of plants transformed with pabHrA001").

T2 generation of Arabidopsis plants transformed with *pabHrA001* gene grew without inhibition in the presence of the Tembotrione (x1= 2500 µg/ml and x0.2= 500 µg/ml) Mesotrione (x1= 50 µg/ml), Topramezone (x1= 150 µg/ml and x0.2= 30 µg/ml) and Isoxaflutole (x1= 50 µg/ml) herbicides. Meanwhile, acute growth inhibition and leaf bleaching were observed in the control (wt plants) treated with the same HPPD herbicides (figure 10A).

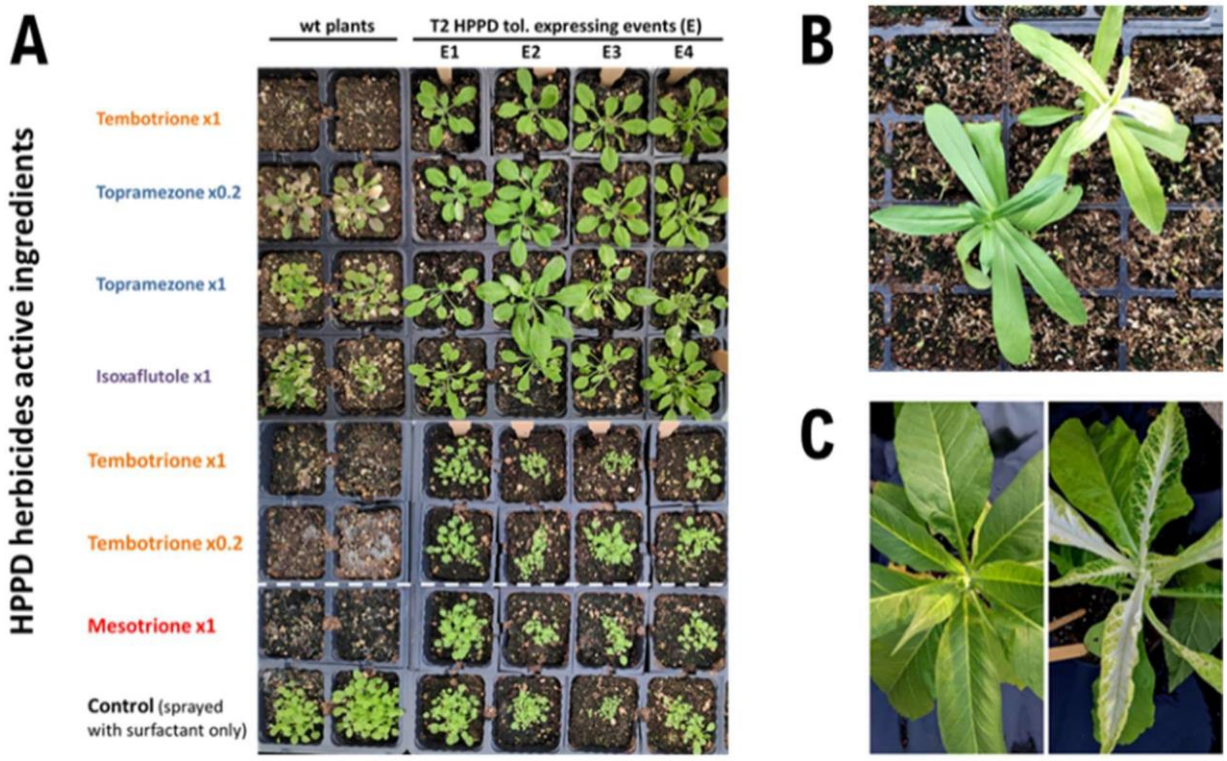


Figure 10. ***pabHrA001*** **transformed plants tolerant to HPPD inhibitors. A.** *A. thaliana* plants treated with different HPPD inhibitors. x1 indicate the concentration of active ingredient as recommended by the manufacturer; **B.** Bottom left - tolerant *pabHrA001* modified Camelina plant treated with 0.075% Laudis (Bayer CropSience LLC, Tembotrione active ingredient) and at the top right a wildtype plant with bleaching affect caused by the herbicide; **C.** At the left - tolerant *pabHrA001* transformed tobacco plant treated with 0.075% Laudis (Bayer CropSience LLC, Tembotrione active ingredient) and at the right a wild-type plant with bleaching affect caused by the herbicide.

In addition, T1 generation transformed camelina (figure 10B) and tobacco (figure 10C) plants transformed with the *pabHrA001* gene were treated with Laudis (Tembotrione) 0.075%. Similarly, the transformed plants were largely unaffected following treatment with the herbicide, while the control wild-type plants exhibited significant inhibition and leaf bleaching.

Based on these assessments with surrogate plants, we anticipate similar phenotypic responses in transformed Soybean plants.

Section 3

Efficacy of methodology:

Cloning and sequencing of pabHrA001

The HPPD gene *pabHrA001* is derived from *Trichoderma harzianum spp.* fungus (Accession number – DSM 33749, given by the International Depository Authority). The fungus was shown to belong to the genus Trichoderma spp., by sequencing several genes including 18S Internal Transcribed spacer1 (ITS1), translation elongation factor 1-alpha (tef1), endochitinase 42 (ech42) and RNA polymerase II (rpb2), followed by whole genome sequencing of the fungus. A 4-Hydroxyphenylpyruvate dioxygenase (HPPD) gene of the isolated fungus was detected by homology search (BLASTx) and cloned. PABHrA001 protein sequence was aligned with common Trichoderma HPPD proteins (*T. harzianum, T. reesei, and T. gamsii*) using CLUSTALW alignment tool [(7)] and found as identical to *Trichoderma harzianum* HPPD and highly conserved through the Trichoderma genus (figure 3).

Structural analysis to determine PABHrA001 MOA and catalytic mechanism

Structural prediction of PABHrA001 and Soybean HPPD (GmHPPD; GenBank accession AIN39549) was performed using MODELLER [(8)] via the hhpred server [(9)] using PDB entries 1T47, 1SP8 and 1FTZ as best fit templates (48.1%, 31.7% and 33.8% seq ID respectively) for PABHrA001 and PDB entries 1SQD and 1SP8 (72.4% and 64.9% seq ID respectively).

The predicted structures were fitted on the HPPD structure of *A. thaliana* with NTBC inhibitor (Nitisinone) (PDB: 5YWG[(10)]) with ExPASy Swiss-PdbViewer [(11)] iterative magic fit (RMS= 1.01Å for HrA001, RMS = 0.61Å for GmHPPD and RMS = 0.96Å for HrA001 over GmHPPD) (Figure 6 and 7). NTBC (2-(2-nitro-4-(triflouromethyl) benzoyl)-1,3-cyclohexanedione) is a member of the benzoylcyclohexane-1,3-dione (triketone)

inhibitors family of herbicides that include also Tembotrione and Mesotrione. Thus, its binding to the HPPD active site is like other HPPD herbicides from this group.

As seen in figure 6, PABHrA001 possesses a typical structure of HPPD proteins with the distinct structure of the catalytic region. Figure 7 Illustrates the catalytic binding pocket of PABHrA001 and GmHPPD by superposing the structural models over AtHPPD structure with NTBC inhibitors. The residues of AtHPPD interacting with the inhibitor are conserved in GmHPPD and PABHrA001 as seen by the sequence alignment (Figure 5).

Multiple sequence alignment of PABHrA001 with HPPD sequences of Arabidopsis thaliana (At_HPPD; RefSeq accession NM_100536.4), Glycine max (Soybean; Gm_HPPD; GenBank accession AIN39549.1) was performed via T-coffee server with expresso mode [(12)]. The alignment was verified over the structures fit of PABHrA001 and GmHPPD models over the *A. thaliana* HPPD structure with NTBC inhibitor (Nitisinone) (PDB: 5YWG).

To compare PABHrA001 protein sequence with the non-regulated HPPDs of the granted petition HPPDPfW336 (FG72 Soybean) and avHPPD-03 (SYHT0H2 Soybean), the protein sequence was aligned with PABHrA001, AtHPPD (Arabidopsis) and GmHPPD (Soybean) using the pre-aligned sequences of PABHrA001, AtHPPD and GmHPPD sequences as a template (figure 5). The alignment shows the resemblance in these enzymes structure, supporting the previous data that PABHrA001 and other HPPDs used previously in the non-regulated petition of Soybean plants possess a similar MOA with the same catalytic activity. PABHrA001, however, retains sequence dissimilarities that differentiates it from other plant endogenous HPPDs, resulting in its low binding affinity of the HPPD inhibitors causing tolerance to group 27 HPPD targeted herbicides.

Bacterial HPPD-activity assay to determine PABHrA001 MOA

HPPD enzymes catalyzed the conversion of p-hydroxyphenylpyruvate (HPP) into homogentisate (2,5-dihydroxyohenylacetate) (HGA; enzyme commission number: EC 1.13.11.27; gene ontology molecular function: GO:0003868. https://www.rhea-db.org). The HPPD activity of PABHrA001 and its resistance to HPPD inhibitors was determined using a bacterial assay.

The coding sequence of *pabHrA001* gene was cloned to a pET16a expression vector. *E. coli BL21 (DE3)* were transformed with the expression vector and monitored for HPPD activity using a colorimetric method as essentially explained in E. Rocaboy-Faquet *et al*. (2014) [(13)] with two minor modifications, namely: 1) The transformed bacteria were inoculated on an agar based medium instead of a liquid medium; 2) The screen was performed at 25°C for 4 days.

The screen media was prepared with active ingredients of group 27 HPPD inhibitors at the indicated concentrations, and the transformed bacteria were inoculated on the screening agar plates. HPPD activity was detected by a brown halo resulting from the HPPD-mediated conversion of p-hydroxyphenylpyruvate (HPP) into homogentisate, which later is oxidized to a melanin-like pigment (figure 8). HPPD inhibitors were compared with *E. coli BL21 (DE3)* bacteria transformed with either empty pET16a vector or a pET16a vector transformed with *Arabidopsis thaliana* HPPD cDNA (AtHPPD; accession no. AF047834).

As seen in Figure 9, transformation of *E. coli BL21 (DE3)* with the *pabHrA001* resulted in detection of HPPD activity (brown halo) on plates with as much as to 250 µg/ml of Tembotrione, 250 µg/ml of Mesotrione, 25 µg/ml of Isoxaflutole, 25 µg/ml of Topramezone and 1250 µg/ml of Pyrazoxyfen. On the other hand, *E. coli BL21 (DE3)* transformed with the Arabidopsis thaliana HPPD (AtHPPD) showed poor HPPD activity. No activity was detected in the empty vector control plates (Ø). These results verified that *pabHrA001* is translated to an HPPD enzyme with the same catalytic function as plants HPPDs and with lower affinity to the group 27 HPPD inhibitors tested.

Sequences of PABHrA001 for use for Soybean

pabHrA001 DNA sequence

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ATGTCCCCTTCAGCCATCTCTAATAGCCCCGAGCAGCGCCCTGCTAACAACAATGGTACTACCCCCGATAACTTCGCAAT
TCAGCCCCCCGCTGATTTCACAGGATACGATCATGTTACTTGGTGGGTAGGAAACGCTAAACAGGCCGCCGCTTACTACA
CTACCTTGTTTGGCTTTGAAACTACTGCATATCGTGGTCTCGAGACTGGCTCAAGGTACTTTGCTAGCTATGTGGTATGC
AACAATGGGGTACGCTTTGTGTTCACAAGTCCACTCAGAAGCGAGGCACATCTCCCAGAAGATGAAACCATTAGTGACAG
CGAACGCAAATTGCTCAAAGAAATCCACGCACATCTTGAACGCCACGGGGATGCTGTCAAGGATGTCGCCTTCGAAGTAG
ATAATGTGGAAGCAGTGTATAATAAAGCCGTAGCAGAGGGAGCCATCGCCGTTCAGGGTCCCACAGCAACAAAAGATGAT
CATGGGAGCGTTACTACCGCCGTTATATGTACCTACGGAGATACTACCCACACTCTGATCAACCGTCGTGGGTACACAGG
GCCCTTCCTGCCCGGTTTTAGAGCCGGCAAGGAGCGTACAAGCAGCGTAGAAATGCCTAACGTGCCCCTCGCAAGGATAG
ACCACTGTGTTGGAAACCAGTCTTGGAATGAAATGGTGTCCGCCTGTGCATTCTATGAGCAATGCCTCAGCTTCCACAGG
TTCTGGTCTGTTGATGATTCCCAGATATGCACAGAGTTTAGCGCACTGAACAGTATCGTAATGGCCTCACCAAACAATCT
GGTCAAGATGCCAATCAATGAACCCGCACCCGGAAAAAAGAAAAGCCAAATCGAAGAGTATGTCATATTCAATTCTGGAC
CCGGCGTTCAACACATTGCCCTCCTGACACCCGATATAATCACCTCTGTAAGCGCACTGAGGGCCCGCGGTGTAGAATTT
ATAAATGTACCAACCACTTATTATGACACAATGAGACAGCGCCTCAAGACAGAAAAAAGGAATTGGCAGCTTAAAGAGGA
TCTGGACACCATACAAAGGCTTAATATTCTGATTGATTACGATGAAGCTGGCTATCTCCTCCAACTCTTCACAAAGCCTC
TGATGGATCGTCCCACAGTTTTTATCGAAATCATACAACGCAACAACTTTGAAGGATTTGGGGCCGGAAATTTTAAATCT
CTGTTTGAAGCTATCGAGAGAGAACAAGCAGAGAGAGGTAATCTCTGA
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PABHrA001 amino acid sequence

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MSPSAISNSPEQRPANNNGTTPDNFAIQPPADFTGYDHVTWWVGNAKQAAAYYTTLFGFETTAYRGLETGSRYFASYVVCNNGVRF
VFTSPLRSEAHLPEDETISDSERKLLKEIHAHLERHGDAVKDVAFEVDNVEAVYNKAVAEGAIAVQGPTATKDDHGSVTTAVICTY
GDTTHTLINRRGYTGPFLPGFRAGKERTSSVEMPNVPLARIDHCVGNQSWNEMVSACAFYEQCLSFHRFWSVDDSQICTEFSALNS
IVMASPNNLVKMPINEPAPGKKKSQIEEYVIFNSGPGVQHIALLTPDIITSVSALRARGVEFINVPTTYYDTMRQRLKTEKRNWQL
KEDLDTIQRLNILIDYDEAGYLLQLFTKPLMDRPTVFIEIIQRNNFEGFGAGNFKSLFEAIEREQAERGNL
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In-planta screen of plants transformed with *pabHrA001*

The *pabHrA001* coding region was cloned into pPA35H binary vector under the control of a constitutive promoter and terminator (WO 2018/178975) (fig. 2) and was used to transform Arabidopsis plants using agrobacterium tumefaciens according to the dipping flower method, as described in WO 2018/178975. Transformed plants were treated with HPPD inhibitors 1 week post germination. HPPD resistant plants were detected 10-14 days post germination.

As seen from figure 10A, T2 generation Arabidopsis plants harboring *pabHrA001* gene enabled growth in the presence of the Tembotrione (x1= 2500 µg/ml and x0.2= 500 µg/ml), Mesotrione (x1= 50 µg/ml), Topramezone (x1= 150 µg/ml and x0.2= 30 µg/ml) and Isoxaflutole (x1= 50 µg/ml) herbicides, while plant growth or an acute growth inhibition and leaf bleaching were observed in the control (wt plants). This shows that plants that possess the *pabHrA001* gene produces an enzymatic protein that enables their growth in the presence of the herbicides Tembotrione, Mesotrione, Topramezone and Isoxaflutole, which are highly effective in weed control of plants, in particular broad leaf plants.

Camelina sativa plants were transformed as Arabidopsis, with the same vector, using agrobacterium according to the dipping flower method. Tobacco plants were transformed with same vector as Arabidopsis in tissue culture as described in WO 2018/178975. T1 generation transformed camelina and tobacco plants harboring the *pabHrA001* gene and wt control plants were treated with Laudis (Tembotrione) 0.075% 2 weeks post germination. Images taken 2 weeks post treatment. As seen from figure 10B, *pabHrA001* gene enabled growth to the transformed plants in the presence the herbicide while acute growth inhibition and leaf bleaching were observed in the control (wt) plants. This shows that transformation of plants with the *pabHrA001* gene results in greater tolerance to group 27 herbicide Tembotrione.

Summary

We have demonstrated the following elements which support our exemption request:

- The HPPD gene *pabHrA001* is derived from the Trichoderma sp. Fungus, and its protein sequence PABHrA001 is identical to *Trichoderma harzianum* HPPD and highly conserved through the Trichoderma genus.
- PABHrA001 possesses a typical HPPD sequence conservation pattern and a typical structure of HPPD proteins with a distinct catalytic region consistent with other plant HPPDs and the non-regulated HPPD of the previously granted petition for HPPDPfW336 (FG72 Soybean) and avHPPD-03 (SYHT0H2 Soybean).
- PABHrA001 is a HPPD enzyme with the same catalytic function as HPPD's found in wild-type plants as well as the non-regulated HPPDs, including HPPDPfW336 (FG72 Soybean) and avHPPD-03 (SYHT0H2 Soybean).
- PlantArc Bio's *pabHrA001*-transformed plants produce an enzymatic protein *(*PABHrA001) which has a lower affinity to group 27 herbicides / HPPD inhibitors. This reduced sensitivity results in tolerance to HPPD pesticides, and, thus, retains the same PMOA as the previously deregulated FG72 and SYHT0H2 Soybean (petition # 09-328-01p and 12-215-01p respectively).

Please let me know if you have any further questions or need additional information as you review our exemption request. Thank you.

Sincerely,

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