February 6, 2023

VIA ELECTRONIC SUBMISSION

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
U.S. Department of Agriculture
4700 River Road, Unit 98
Riverdale, MD 20737
E-mail: RSRrequests@usda.gov

Subject: Request for Regulatory Status Review of THC null C.sativa

Dear Ms. Juarez,

GT Research Inc., ("the Requestor"), respectfully requests a Regulatory Status Review as required by USDA's Biotechnology Regulatory Services (BRS) under 7 C.F.R. part 340 of *Cannabis sativa* developed using genetic engineering for altered cannabinoids profile. Information supporting our request is provided herein.

We would be pleased to answer any questions you may have regarding this information. Thank you in advance for your attention to this matter.

Sincerely,

C. Michael Francis, PhD
Chief Science Officer
GT Research Inc.

cc: Jinbo Wang, USDA (jinbo.wang@usda.gov) (via e-mail)

Requestor

- First Name – Michael
- Last Name - Francis
- Position – Chief Science Officer
- Organization Name (if applicable) – Growing Together Research Inc.

Contact information

- Telephone – 251-599-0345
- Email address – michael@gtresearch.io

Confidential Business Information (CBI) Statement

This request contains Confidential Business Information

Description of Comparator Plant

Scientific name - *Cannabis sativa*. Common Name - Marijuana

Genotype of the Modified Plant

Position (nt)	Sequence	Function	Genbank ID	Donor
1-26	TGGCAGGAT ATATTGTGGT GTAAACA	pCAMBIA 2300 binary vector Left Border T-DNA repeat	*AF234315.1: 6173-6198*	*Agrobacterium tumefaciens C58*
27-298	AATTGACGCT TAGACAACTT AATAACACAT TGCGGACGTT TTTAATGTAC TGAATTAACG CCGAATTAAT TCGGGGGAT CTGGATTTTA GTACTGGATT TTGGTTTTAG GAATTAGAAA	pCAMBIA 2300 binary vector AF234315.1 spacer	*AF234315.1:6198-6469*	*Agrobacterium tumefaciens C58*

	TTTTATTGAT AGAAGTATTT TACAAATACA AATACATACT AAGGGTTTCT TATATGCTCA ACACATGAGC GAAACCCTAT AGGAACCCTA ATTCCCTTAT CTGGGAACTA CTCACACATT ATTATGGAGA AACTCGAGCT TGTCGATCGA CT			
299-473	GATCTGGATT TTAGTACTGG ATTTTGGTTT TAGGAATTAG AAATTTTATT GATAGAAGT ATTTTACAAA TACAAATACA TACTAAGGGT TTCTTATATG CTCAACACAT GAGCGAAAC CCTATAGGAA CCCTAATTCC TTATCTGGGA ACTACTCACA CATTATTATG GAGAAA	CaMV poly(A) signal, cauliflower mosaic virus polyadenylation signal, terminates transcription	MK896900.1:8618-8792	Plant binary expression vector pNC-Cam1304-SubN artificial sequences, hybrid with *Caulimovirus Cauliflower Mosaic Virus*
474-479	GAGCTC	Xhoi restriction enzyme site		Artificial sequence
480-1032	ATGAGCCCA GAACGACGC CCGGCCGAC ATCCGCCGTG CCACCGAGG	Bialaphos resistance protein gene, translated	MG719235.1: 284-835	Streptomyces hygroscopicus

	CGGACATGCC GGCGGTCTG CACCATCGTC AACCACTACA TCGAGACAA GCACGGTCA ACTTCCGTAC CGAGCCGCA GGAACCGCA GGAGTGGAC GGACGACCTC GTCCGTCTGC GGGAGCGCT ATCCCTGGCT CGTCGCCGA GGTGGACGG CGAGGTCGC CGGCATCGCC TACGCGGGC CCCTGGAAG GCACGCAAC GCCTACGACT GGACGGCCG AGTCGACCGT GTACGTCTCC CCCCGCCACC AGCGGACGG GACTGGGCT CCACGCTCTA CACCCACCTG CTGAAGTCCC TGGAGGCAC AGGGCTTCAA GAGCGTGGT CGCTGTCATC GGGCTGCCC AACGACCCG AGCGTGCGC ATGCACGAG GCGCTCGGA TATGCCCCCC GCGGCATGC TGCGGGCGG CCGGCTTCAA GCACGGGAA	protein at AYD60114.1		

	CTGGCATGAC GTGGGTTTCT GGCAGCTGG ACTTCAGCCT GCCGGTACC GCCCCGTCCG GTCCTGCCCG TCACCGAGAT TTGA			
1033-1709	TGAGACTTTT CAACAAAGG GTAATATCGG GAAACCTCCT CGGATTCCAT TGCCCAGCTA TCTGTCACTT CATCAAAGAC AGTAGAAAA GGAAGGTGG CACCTACAAA TGCCATCATT GCGATAAAG GAAAGGCTAT CGTTCAAGAT GCCTCTGCCG ACAGTGGTCC CAAAGATGG ACCCCCACCC ACGAGGAGC ATCGTGGAAA AAGAAGACG TTCCAACCAC GTCTTCAAAG CAAGTGGATT GATGTGATAA CATGGTGGA GCACGACACT CTCGTCTACT CCAAGAATAT CAAAGATACA GTCTCAGAAG ACCAAAGGG CTATTGAGAC TTTTCAACAA AGGGTAATAT	BAR promoter	MG561370.1:8669-9346	*Streptomyces hygroscopicus*

	CGGGAAACC TCCTCGGATT CCATTGCCCA GCTATCTGTC ACTTCATCAA AAGGACAGT AGAAAAGGA AGGTGGCAC CTACAAATGC CATCATTGCG ATAAAGGAA AGGCTATCGT TCAAGATGCC TCTGCCGACA GTGGTCCCAA AGATGGACC CCCACCCACG AGGAGCATC GTGGAAAAA GAAGACGTTC CAACCACGTC TTCAAAGCAA GTGGATTGAT GTGATATCTC CACTGACGTA AGGGATGAC GCACAATCCC ACTATCCTTC GCAAGACCTT CCTCTATATA AGGAAGTTCA TTTCATTTGG AGAGGACAC GCTGA			
1710-1905	TGTGGATAGC ACGTACATTG GGAACCCAA AGCCGTACAT TGGGAACCG GAACCCGTAC ATTGGGAACC CAAAGCCGTA CATTGGGAAC CGGTCACACA TGTAAGTGAC	Binary vector pCAMBIA-BAR promoter	KP795973.1:3415-3609	*Escherichia coli*

	TGATATAAAA GAGAAAAAA GGCGATTTTT CCGCCTAAAA CTCTTTAAAA CTTATTAAAA CTCTTAAAAC CCGCCTGGCC TGTGCATA				
[ ]	[ ]	[ ]		[ ]	4x CBI-deleted
[ ]	[ ]	[ ]		[ ]	4x CBI-deleted
[ ]	[ ]	[ ]		[ ]	4x CBI-deleted
[ ]	[ ]	[ ]		[ ]	4x CBI-deleted
[ ]	[ ]	[ ]		[ ]	4x CBI-deleted

		[ ]			CBI-deleted
[ ]	[ ]	[ ]		[ ]	4x CBI-deleted
[ ]	[ ]	[ ]		[ ]	4x CBI-deleted
[ ]	[ ]	[ ]		[ ]	4x CBI-deleted
[ ]	[ ]	[ ]		[ ]	4x CBI-deleted
[ ]	[ ]	[ ]		[ ]	4x CBI-deleted
[ ]	[ ]	[ ]		[ ]	4x CBI-deleted
2223-2473	ATATGAAGAT GAAGATGAA ATATTTGGTG TGTCAAATAA ATAGCTTGTG TGCTTAAGTT TGTGTTTTTTT CTTGGCTTGT TGTGTTATGA ATTTGTGGCT TTTTCTAATAT TAAATGAATG TAAGATCTCA TTATAATGAA TAAACAAATG TTTCTATAAT CCATTGTGAA TGTTTTGTTG GATCTCTTCT GCAGCATATA ACTACTGTAT GTGCTATGGT	Heat shock protein 18.2 terminator to increase expression.	MT896417.1:4091-4340	*Arabidopsis Thaliana*	

	ATGGACTATG GAATATGATT AAAGATAAG			
2474-2500	GTAAACCTAA GAGAAAAGA GCGTTTA	pCAMBIA 2300 binary vector AF234315.1, Right Border T-DNA repeat	AF234315.1:8656-8681	*Agrobacterium tumefaciens C58*

Description of New Traits

Intended Trait 1

~0% tetrahydrocannabinol (THC)

Intended Trait 2

Bialaphos resistance

Intended Phenotype 1

Cannabis plant with 0% THC and 0% cannabichromene (CBC), otherwise having the traits of the recipient plant

Intended Phenotype 2

Cannabis plant with resistance to the herbicide Bialaphos

Description of the Mechanism of Action for Trait 1 (MOA 1)

RNA interference with two double stranded RNA molecules targeting the *C. Sativa* tetrahydrocannabinolic acid synthase (*THCAS*) and cannabichromenic acid synthase (*CBCAS*) genes, respectively. Selectable marker using a Bialaphos resistance gene.

Background

Under the 2018 Farm Bill, industrial hemp is defined as Cannabis sativa having a THC content of less than 0.3%. Many cultivars of Cannabis have great potential for utility as industrial hemp but are not reliably below 0.3% THC. This creates an economic and regulatory risk to farmers who want to grow hemp, but cannot risk a “hot” crop with THC levels above the permissible threshold,

which must be destroyed. Plants having levels of THC that are higher than 0.3% are classified, under the Controlled Substances Act, as marijuana.

Tetrahydrocannabinolic acid (THCA) is the acid form of THC and is produced in the plant from precursor molecule cannabigerolic acid (CBGA), mediated by the enzyme THCA synthase. THC accumulates primarily in trichomes of mature flowers. Plants that do not express THCA synthase cannot produce THCA in "normal" amounts and typically show little to no THCA in mature flowers.

The cultivar that is the subject of this application is a transgenic modification of a parent marijuana plant that has been transformed with the construct described above to interfere with THCA synthase mRNA accumulation and, thereby, THCA synthase expression. This genetically modified form shows little or no THCA in mature flowers.

Mechanism of Action 1 (MOA 1)

Reduction or elimination of THC accumulation in mature flowers via RNA interference with expression of both THCA and cannabichromenic acid (CBCA) synthase enzymes (RNAi is complementary to a 23-bp sequence common to both synthase enzymes) and subsequent selection via bialaphos resistance. The promoter was taken directly from the Cannabis U6 gene. It will be constitutively expressed in all tissues.

The expected reduction of THC accumulation is not expected to result in any other material changes to metabolism, physiology or development.

Other information on the MOA 1

This MOA has been used widely in numerous studies for several decades.
The resulting plants are, in terms of their impact on insects and other plants, not generally distinguishable from other plants in widespread cultivation. However, such a MOA only recently been studied in *C. sativa.*[1,2]. To date, *C. sativa* organisms which have been subject to this mechanism of action have not been characterized as dissimilar to their wild type counterparts[3–5]. Given that the proposed MOA only targets the *THCAS* and *CBCAS* coding sequences, and not adjacent loci, we hypothesize no effect on pathogen resistance in the resulting plants.

Description of Mechanism of Action for Trait 2 (MOA 2)

The Bialaphos resistance (Bar) gene codes for phosphinothricin acetyltransferase which converts phosphinothricin, an irreversible inhibitor of glutamine synthetase into a non-herbicidal acetylated form. This confers plant resistance to the herbicide Bialaphos, which contains phosphinothricin[6].

Mechanism of Action 2 (MOA 2)

The resistance to the herbicide Bialaphos that the Bar gene confers to the plant serves as a marker that the intended T-DNA, also containing the Bar gene, has been incorporated into the plant. The expected resistance to Bialaphos is not expected to result in any other material changes to metabolism, physiology or development of the plant.

References

1. Zhang, X. *et al.* Establishment of an Agrobacterium-mediated genetic transformation and CRISPR/Cas9-mediated targeted mutagenesis in Hemp (Cannabis Sativa L.). *Plant Biotechnol J* (2021) doi:10.1111/pbi.13611.
2. Deguchi, M. *et al.* Establishment and optimization of a hemp (Cannabis sativa L.) agroinfiltration system for gene expression and silencing studies. *Sci Rep* **10**, 3504 (2020).
3. McKernan, K. J. *et al.* Sequence and annotation of 42 cannabis genomes reveals extensive copy number variation in cannabinoid synthesis and pathogen resistance genes. *bioRxiv* 2020.01.03.894428 (2020) doi:10.1101/2020.01.03.894428.
4. Kojoma, M., Seki, H., Yoshida, S. & Muranaka, T. DNA polymorphisms in the tetrahydrocannabinolic acid (THCA) synthase gene in “drug-type” and “fiber-type” Cannabis sativa L. *Forensic Science International* **159**, 132–140 (2006).
5. Laverty, K. U. *et al.* Supplemental figures and table for: A physical and genetic map of Cannabis sativa identifies extensive rearrangement at the THC/CBD acid synthase locus. 10.
6. D'Halluin, K. *et al.* The bar Gene as a Selectable and Screenable Marker in Plant Engineering. *Selected Methods in Enzymology*, 157-168 (1995).