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Authorization for field planting of *Nicotiana attenuata* null segregant plants

NO CBI

Dr. Michael J. Firko
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737

September 3, 2018

Dear Dr. Firko,

I am writing to receive authorization from the USDA Biotechnology Regulatory Services for field plantations of *Nicotiana attenuata* null segregant plants derived from a parental transgenic plant and not containing or inheriting any transgenes.

Since 2002 we have planted transgenic *N. attenuata* lines at the Lytle preserve field site in Utah (recent permit number 18-054-101r, GPS coordinates 37.146302, -114.019753). Starting in 2019 we also will plant transgenic *N. attenuata* lines at the WCCER-SEGA exclosure in Arizona (permit number 18-072-103r, GPS coordinates 34.921630, -112.844870). Our intention is to include *N. attenuata* null segregant plants in future releases at both sites.

The CRISPR/Cas9 system was used to generate these non-transgenic segregants containing small insertion or deletion (indel) mutations at a specific target site. We first transformed wild-type *N. attenuata* (Utah accession) with T-DNA from the vector pHAtC, containing the codon-optimized gene for the *Streptococcus pyogenes* Cas9 protein and the DNA sequences for two specific single guide RNAs (sgRNA), via an *Agrobacterium*-mediated transformation method [Krügel *et al*. (2002), Chemoecology 12 (4), 177-183]. After segregation, we used hygromycin (25 mg/L) resistance tests and PCR analyses to identify lines without T-DNA insertions and to confirm the absence of all transgenes. The PCR primers used for this are described in **Table 1**. The mutation patterns of the transgene-free lines were determined by targeted deep Illumina sequencing.

Target genes for genome editing
The *N. attenuata* target genes for the induction of indel mutations were *NEC3a* (NIATv7_g24152), *NEC1b* (NIATv7_g08730) and *NEC1d* (NIATv7_g28565). The DNA sequences for the specific sgRNAs are given in **Table 2**.

DNA amplification and Sanger sequencing
We isolated genomic DNA from *N. attenuata* null segregant candidate plants using a genomic DNA extraction kit (HiGene™ Genomic DNA Prep kit, BIOFACT GD141-100). PCR amplifications were performed for 30 cycles with the specific primer sets shown in **Table 1**. The PCR products were separated by agarose gel electrophoresis and directly sequenced at Bioneer (Daejeon, South Korea) using the Sanger dideoxy method.

Identified and confirmed *N. attenuata* null segregant lines we want to use for field planting
- NEC3a-#6 [two single-base (A) insertions in the *NEC3a* gene 78 bases apart]
- NEC1b1d-4.10 [single-base (T) insertion in the *NEC1b* gene and single-base (T) insertion in the *NEC1d* gene]
- Cross of lines NEC3a-#6 and NEC1b1d-4.10

Plasmid and organism information
After cloning the specific DNA fragments for the two sgRNAs (gRNA scaffold), the T-DNA of the binary plasmid pHAtC (https://www.addgene.org/78098/, Fig. 1) was used to transform *N. attenuata*.

The pHAtC T-DNA contains expression cassettes for the following elements:
- *hptII* hygromycin resistance gene (plant selectable marker)
- codon-optimized *Streptococcus pyogene Cas9* gene

- two sgRNA sequences

The lack of the *hptII* and the *Cas9* genes in the null segregant plants was confirmed by PCR analysis.

The organism used for genome editing was *Nicotiana attenuata* (Utah accession), 38th inbred generation. The intended phenotype of the null segregants is the lack of nectarin (superoxide dismutase) proteins in floral nectar.

The bacterial vector used for plant transformation was *Agrobacterium tumefaciens* LBA4404.

Developer name
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Sincerely,

Prof. Ian T. Baldwin

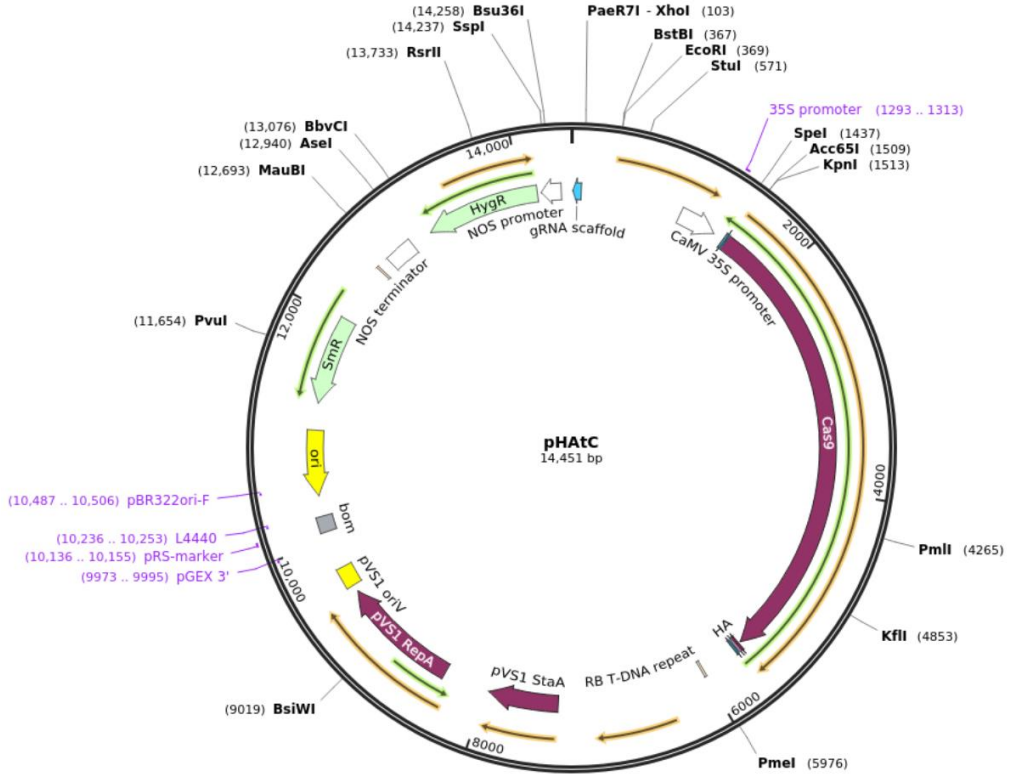

Fig. 1. Map of the binary plant transformation plasmid pHAtC.

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Figure 2. Transgene PCR analysis of NEC3a. The presence of the PCR product band indicates the existence of the *Cas9* gene sequence in T2 transgenic plants. Wild type *N. attenuata* genomic DNA was used as template for the negative control (NC) and the pHAtC vector was used as template for the positive control (PC). Blue arrows indicate the null segregants (#2, #6, #11). Progeny of the NEC3a #6 plant will be used for further experiments.

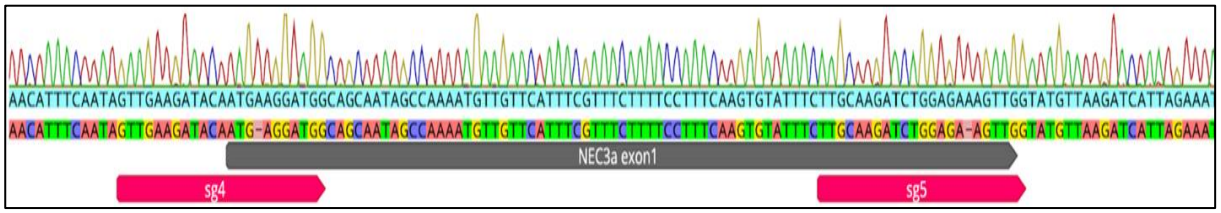

Figure 3. Mutation pattern of the NEC3a-#6 transgenic plant. NEC3a-#6 null segregant has a single-base (A) insertion in the site where the CAS9 + guide RNA4 (sg4) cleaves and a single-base (A) insertion in the site where the CAS9 + sg5 complex cleaves. The guide RNA binding site in NEC3a-#6 was analyzed by Sanger dideoxy sequencing (chromatogram and ATGC characters in cyanine blue box) and compared with the wild-type sequence (A, T, G, C characters in red, green, yellow, and purple boxes, respectively).

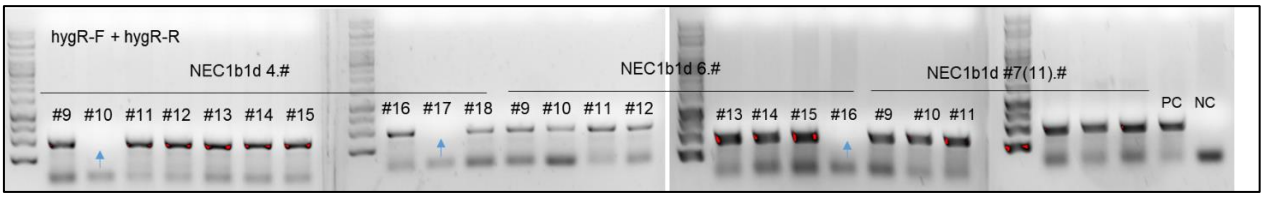

Figure 4. Transgene PCR analysis of NEC1b1d. The presence of the PCR product band indicates the existence of the hygromycin-resistance gene sequence in T2 transgenic plants. Wild type *N. attenuata* genomic DNA was used as template for the negative control (NC) and the pHAtC vector was used as template for the positive control (PC). Blue arrows indicate the null segregants (NEC1b1d-4.10, -4.17, and -6.16 transgenic plants). Progeny of the NEC1b1d-4.10 plant will be used for further experiments.

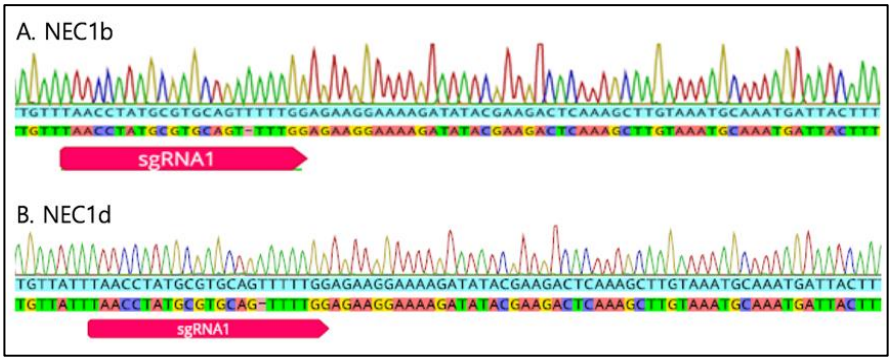

Figure 5. Mutation pattern of the NEC1b1d-4.10 transgenic plant. The NEC1b1d-4.10 null segregant has a single-base (T) insertion both in the *NEC1b* (A) and *NEC1d* (B) genes where the CAS9 + guide RNA1 (sgRNA1) complex cleaves. Guide RNA binding sites in *NEC1b* and *NEC1d* were analyzed by Sanger dideoxy sequencing (chromatogram and ATGC characters in cyanine blue box) and compared with wild type sequence (A, T, G, C characters in red, green, yellow, and purple boxes, respectively).

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Table 1. Information on primers used in transgenic plant analysis.

Primer name	Sequence	Amplicon size (bp)	Purpose
Cas9-F	CCTACCACGACCTGCTGAAG	617	Detection of Cas9 gene
Cas9-R	CTCCTTCAGGATCTGGCTGC	617	Detection of Cas9 gene
hygR-F	TTGCTGATCCCCATGTGT	508	Detection of Cas9 gene
hygR-R	TCTACACAGCCATCGGTC	508	Detection of Cas9 gene
NEC3a_sg4,5_deep1_F	ACTTTGGAAAGGGTTGGGCA	799	Sanger sequencing of *NEC3a* gene to analyze mutation pattern
NEC3a_sg4,5_deep1_R	AGGGTGGCATTCGATGGTTT	799	Sanger sequencing of *NEC3a* gene to analyze mutation pattern
NEC1b_seqF	AGTCGTCATATAAAGATGTTG	258	Sanger sequencing of *NEC1b* gene to analyze mutation pattern
NEC1d_seqF	ATCTAGTCAAACTACGTCATAT	268	Sanger sequencing of *NEC1d* gene to analyze mutation pattern
NEC1b_sgRNA1,2_deep1_R1	AGTGGTACGAGAGCTAATGGC	258 or 268	Used with NEC1b_seqF and NEC1d_seqF

Table 2. Information on sgRNAs used in genome editing.

Name	DNA sequence for sgRNA (**bold**: PAM sequence)	Location
NEC3a sg4	GTTGAAGATACAATGAGGA**TGG**	1st exon of *Nicotiana attenuata NEC3a* (NIATv7_g24152)
NEC3a sg5	TTGCAAGATCTGGAGAAGT**TGG**	1st exon of *Nicotiana attenuata NEC3a* (NIATv7_g24152)
NEC1b1d sg1	TAACCTATGCGTGCAGTTT**TGG**	2nd exon of *Nicotiana attenuata NEC1b* (NIATv7_g08730) and *NEC1d* (NIATv7_g28565)
NEC1b1d sg2	CCAATGGTTTGAATTGATT**AGG**	2nd exon of *Nicotiana attenuata NEC1b* (NIATv7_g08730) and *NEC1d* (NIATv7_g28565)

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