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21-025-01cr **No-CBI**

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Description of the modification, exemption category.

The specific genetic modifications described in this proposal involve single nucleotide changes resulting from the cellular repair of a targeted DNA break in the absence of an externally provided repair template. Hence this CR application seeks exemptions based on the following category:
340.1(b)(1): NaASAT1-like [NaASAT1 CRISPR-6. #6]; NaASAT2-like [NaASAT2 CRISPR-6. #6]; NaASAT3-like [NaASAT3-1. #1]; NaTPS1-like [NaTPS-1. #8].

Specifically, we seek exemption for 4 lines of *Nicotiana attenuata*, with the following nucleotide changes:
NaASAT1-like [NaASAT1 CRISPR-6. #6 – three-base homozygous deletion (A/C/A)].
NaASAT2-like [NaASAT2 CRISPR-6. #6- a single-base homozygous insertion (A)].
NaASAT3-like [NaASAT3-1. #1 –a single-base homozygous insertion (T)].
NaTPS1-like [NaTPS-1. #8 –a single-base T to C replacement].

The factual grounds demonstrating that the proposed modification could be achieved through conventional plant breeding.

It has been reported that the *de novo* spontaneous mutation rate was 7×10^{-9} base replacements per site per generation in all the nuclear genomes of five *Arabidopsis thaliana* accumulation lines sustained by single seed descent (SSD) over 30 generations (Wani et al., 2014; Weng et al., 2019). This is expected to be true for the genomes of most other plant species: for example, about 20 billion mutations occur each year in a one-hectare wheat field (Krasileva et al., 2017). Breeding techniques based on efficient mutation techniques are commonly used by plant breeders. These spontaneous mutations as well as those elicited by physical and chemical mutagens are known to result from damage to DNA (Menda et al., 2004; Watanabe et al., 2007; Wani et al., 2014). Plants are particularly vulnerable to DNA-damaging environmental factors, which result in double-strand breaks (DSBs) in chromosomes. DSBs can also be caused by environmental exposure to irradiation, other chemical agents, or ultraviolet light (UV)(Aguilera and Gómez-González, 2008). Hence, plants have evolved a complex network of mechanisms of DNA damage detection and repair dedicated to ensure the stability of their genomes. If not correctly repaired, DSBs can cause a deletion or insertion (also known as an indel)(Puchta, 2005). Modern genome engineering methods as well as conventional breeding methods can generate indels at precisely targeted genomic locations. Genome-editing techniques allow researchers to achieve the same alterations as is achieved by conventional breeding, albeit much faster and in a highly targeted manner (Huang et al., 2016). As CRISPR (clustered regularly interspaced short palindromic repeats) lead to DSB in DNA in absence of an external repair template, it can target single base-pair substitution or deletion. A plant containing a modification involving DNA breaks induced through genome editing should be eligible for regulatory exemption.

The published scientific literature that supports the proposal demonstrating that the stated modification(s) can routinely be achieved in conventional breeding:

Scientific Literature

1. Wani, M.R., et al., Mutation breeding: a novel technique for genetic improvement of pulse crops particularly Chickpea (*Cicer arietinum* L.), in Improvement of crops in the era of climatic changes. 2014, Springer. p. 217-248.
2. Krasileva, K.V., et al., Uncovering hidden variation in polyploid wheat. Proceedings of the National Academy of Sciences, 2017. 114(6): p. E913-E921.
3. Menda, N., et al., *In silico* screening of a saturated mutation library of tomato. The Plant Journal, 2004. 38(5): p. 861-872.
4. Watanabe, S., et al., Ethylmethanesulfonate (Bai et al.) mutagenesis of *Solanum lycopersicum* cv. Micro-Tom for large-scale mutant screens. Plant Biotechnology, 2007. 24(1): p. 33-38.
5. Aguilera, A. and B. Gómez-González, Genome instability: a mechanistic view of its causes and consequences. Nature Reviews Genetics, 2008. 9(3): p. 204-217.
6. Puchta, H., The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. Journal of experimental botany, 2005. 56(409): p. 1-14.
7. Huang, S., et al., A proposed regulatory framework for genome-edited crops. Nature genetics, 2016. 48(2): p. 109-111.

Unpublished studies

Since 2019 we have been conducting research on *Nicotiana attenuata*'s acyl sugars and triterpenes using the CRISPR-Cas9 system. A major aspect of this project involved segregating descendants of transformed plants in order to attain null segregants that do not contain a T-DNA insertion, but contain the targeted mutations induced by the plants own repair mechanisms. In 2021 we propose to plant non-transgenic *N. attenuata* lines to perform field experiments at the Lytle preserve field site in Utah. The intended trials will include **observations of herbivore interactions with tobacco plants with different acyl sugar and triterpene properties, as well as tracking of growth and fitness of the plants**.

A CRISPR/Cas9 system was used to generate these non-transgenic segregants containing small insertion or deletion (indel) mutations at a specific target site. Our CRISPR vector was developed by Professor Sang-Gyu Kim (Center for Genome Engineering, Institute for Basic Science, Yuseong-gu, Daejeon, 34047, South Korea) and was published in (Kim et al., 2016). Here are the details of the development of the pHAtC CRISPR vectors used to construct the 4 lines of plants.

To design a new CRISPR-Cas vector for plant transformation, we first considered that a short guide sequence (19 or 20 bp) of an sgRNA can be directly cloned into a binary vector (pHAtC) through a single ligation step without any PCR. To do this, we chose the type IIs restriction enzyme AarI, which cuts 4 and 8 bp outside of its binding sequence 5`-CACCTGC(N)4/8-3` and generates a non-palindromic overhang of any sequence. We added two AarI recognition sites between the U6 promoter and sgRNA scaffold to place a guide sequence precisely after the 3' end of the U6 promoter and before the 5` end of the sgRNA scaffold (Figure 1). The AarI enzyme allows for the assembly of two DNA overhangs without an extra sequence in the junction. This U6 promoter-AarI-XhoI-AarI-sgRNA scaffold cassette was inserted into pH2GW7 harboring the hygromycin-resistance gene, developed for *Agrobacterium*-mediated plant transformation (Figure 1) (Karimi et al., 2002). We modified the original pB2GW7 to remove the AarI recognition site in the BastaTM-resistance gene. The engineered SpCas9 coding sequence (Cho et al., 2013) was replaced with a standard GatewayTM cassette (attR1-ccdBattR2) located downstream of the CaMV 35S promoter in both pH2GW7 vectors (Figure 1). The Arabidopsis U6 or the rice U3 promoter is generally used to express sgRNA in dicot and monocot plants, respectively (Cho et al., 2013). However, in some cases U6 promoters derived from the target plants function better than heterologous U6 promoters (Belhaj et al., 2013). Therefore, we added the EcoRI site at 50bp upstream of the U6 promoter and the XhoI site between two AarI sites at 30bp downstream of the U6 promoter to easily replace the U6 promoter (Figure 1).

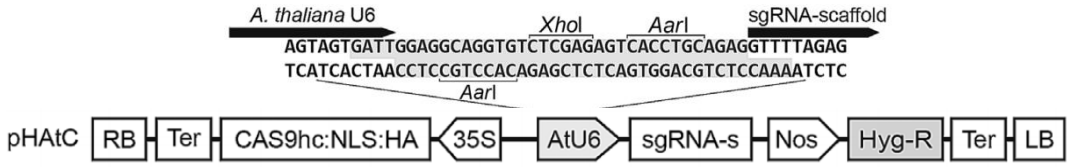

Figure 1. Schematic maps of complete plant RGEN binary vector. The nomenclature of our vector system is as follows: *Hyg-R*, hygromycin resistance gene; AtU6, Arabidopsis U6-26 promoter driving the single-guide RNA (sgRNA) cassette; CAS9hc, human codon-optimized Cas9 expressing cassette. The gray shaded sequence represents nucleotides that are removed after AarI cutting in RGEN binary vector. RB, right border sequences; Ter, terminator sequence; 35S, CaMV 35S promoter; Nos, Nos promoter; LB, left border sequences; For the complete vector, see **Supplemental Figure 1**, below.

To assess possible off-target effects, we uploaded the *Nicotiana attenuata* genome to the Cas-Designer tool (http://www.rgenome.net/cas-designer/), and it provided the targets found in the genome for gRNAs that we used to create the lines. The analysis revealed that there were only single targets in the genome.

We first transformed wild-type *N. attenuata* with T-DNA from the vector pHAtC plasmid, 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). After segregation, we used hygromycin (25 mg/L) resistance tests, PCR and NanoString (nCounter: see He et al. 2019, see Figure S10 panels B—vector-- and C—nCounter data) analyses to identify lines that lacked T-DNA insertions and to confirm the absence of transgenes. The PCR primers used for this are described in **Table 1**.

The nCounter probe set contains three probes for the vector backbone outside of the T-DNA (pVS1_3, nptII_1 and nptIII_3), of which only one (pVS1_3) would work in the *pHCASATs* line due to spectinomycin resistance. All other probes were designed to characterize the T-DNA. All of the T-DNA probes hptII_3 (no mismatch), T35S_1 (1 mismatch), PNOS_1 (1 mismatch) and P35S_1 (2 mismatches) detect the *pHCASATs* and *pHCTPS1* plasmids quantitatively and with high sensitivity. We have also included the AOC (allene oxide cyclase—from jasmonate biosynthesis) probe as a positive control, which will provide a strong signal for a single copy plant gene and support

the technical validation/normalization objectives of the analysis to quantify the number of T-DNA copies inserted into the genome (Kallenbach et al., 2012). The nCounter targets (vector map given in Figure S10 of He et al. 2019) are:

>S-RNase-2_1
TCACCAATGCAATTGAGGTTAGGATACGCTTGAGTAACTTCTCTGATGGCTTCTTCAATCTTGTTAACG
GTGTAAGTTTTTCCAGGAATAACTCCTTGAT

>AOC_2
CAAAAAGGAACGTACTTTGTTGCTAAAGGGGACAAGATCTCCAAGGGAATTGACAGTCTTTTGGCTCA
AGCGAAGATAGGCAGGGCTACCACGGTCACGT

>sulfite_2
TCTTTCCATAATACTGTTCAGTGACAGATCGAAACTTCTCGATTCCCCATGAGCTGAGTAAATATTTCA
ATCTGCTGTATCTGCGATCATCTCTTCTGCC

>TNOS+LB_1
GGTACCAGCTCGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTT
GCCGGTCTTGCGATGATTATCATATAATTTC

>hptII_3
ATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTT
GGGCGCAGGGTCGATGCGACGCAATCGTCCG

>PNOS_1
GACGCGGGACAAGCCGTTTTACGTTTGGAACTGACAGAACCGCAACGTTGAAGGAGCCACTCAGCCG
CGGGTTTCTGGAGTTTAATGAGCTAAGCACATA

>P35S_1
ATATCAAAGATACAGTCTCAGAAGACCAAAGGGCAATTGAGACTTTTCAACAAAGGGTAATATCCGG
AAACCTCCTCGGATTCCATTGCCCAGCTATCTG

>T35S_1
TGAGTAGTTCCCAGATAAGGGAATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAA
ACCCTTAGTATGTATTTGTATTTGTAAAATAC

>pVS1_3
GAACGCAGTGGCAGCGCCGGAGAGTTCAAGAAGTTCTGTTTCACCGTGCGCAAGCTGATCGGGTCAAA
TGACCTGCCGGAGTACGATTTGAAGGAGGAGG

>sat-1_1
ATGATGACTCTGATGAAGACTCTGCTTGCTATGGCGCATTCATCGACCAAGAGCTTGTCGGGAAGATT
GAACTCAACTCAACATGGAACGATCTAGCCTC

>nptII_1
TTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCT
ACCTGCCCATTCGACCACCAAGCGAAACATCG

>nptIII_3
ACAAGTGGTATGACATTGCCTTCTGCGTCCGGTCGATCAGGGAGGATATCGGGGAAGAACAGTATGTC
GAGCTATTTTTTGACTTACTGGGGATCAAGCC

Sanger sequencing of the targeted gene determined the mutation patterns of the transgene-free lines. The complete bioinformatics analysis of ASATs and TPS1 CRISPR lines can be found in the Appendix and **Figures S5-S8**.

Target genes for genome editing: *NaASATs* and *NaTPS1*

In this research, the targeted genes were identified using Quantitative trait loci (QTL) analysis of the recently established Multi-parent Advanced Generation Inter-Cross (MAGIC) population, which captures the genetic diversity of *N. attenuata*. A number of significant QTLs for acyl sugars were identified and acyltransferase-like candidate genes from *N. attenuata* were selected according to the QTL results. The *N. attenuata* target genes for the induction of indel mutations were NaASATs that catalyze the sequential addition of specific acyl chains to the sucrose molecule using acyl CoA donors. In a separate study, the analysis of transcriptome datasets from *N.*

attenuata led to the identification of a *NaTPS1*, a triterpene cyclase that catalyzes the first committed step of the triterpene biosynthetic pathway.

The *N. attenuata* target genes are *NaASAT1* (NIATv7_g04553), *NaASAT2* (NIATv7_g17261), *NaASAT3* (NIATv7_g08565) and *NaTPS1* (NIATv7_g21921). The DNA sequences for the specific sgRNAs are given in **Table 2**. The genomic gene sequences are given in the **Appendix.** The specific lines that we would like to use for the field releases are given in the Figure caption of **Figure 3** below, and named above**.**

DNA amplification and Sanger sequencing
We isolated genomic DNA from *N. attenuata* null segregant candidate plants from leaves of two week-old seedlings using Edwards buffer (Edwards et al., 1991). Next, 2 µL of gDNA was used as a template in a standard 10 µL volume PCR reaction with ALLin™ Red Taq Mastermix. A 600–700 bp genomic region spanning the predicted Cas9 cut site was amplified and the amplicon sequenced by standard capillary sequencing. 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 sequenced by standard capillary sequencing using the Sanger dideoxy method.

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

The lack of the hptII and the *Cas9* genes in the null segregant plants was confirmed by PCR and the NanoString (nCounter) analysis shown in **Figure 2** (He et al., 2019), which is briefly described below:

For the nCounter analysis, a 12 code probe set was designed from 12 target regions, which comprised 3 calibrator genes that occur as a single copy in the genome of *N. attenuata* [AOC_2: allene oxide cyclase (GenBank LOC109240002); S-RNase-2_1: ribonuclease S-7-like (GenBank LOC109235079); sulfite_2: sulfite reductase 1 (GenBank LOC109217753)], and 9 functional regions present on the pNAT(Krügel et al., 2002), pRESC(Zavala et al., 2004), pSOL (Krügel et al., 2002), and pPOP6 (Schäfer et al., 2013) binary vectors used in our group for the transformation of *N. attenuata*. These target sequences are indicative of complete T-DNA insertions (TNOS+LB_1: terminator of the nopaline synthase gene; hptII_3: hygromycin phosphotransferase gene; PNOS_1: promoter of the nopaline synthase gene; P35S_1: cauliflower mosaic virus 35S promoter: T35S_1: cauliflower mosaic virus 35S terminator; sat-1_1: streptothricin 6 acetyltransferase; nptII_1: neomycin phosphotransferase II gene) or T-DNA overreads (pVS1_3: pVS1 vector backbone; nptIII_3: neomycin phosphotransferase III gene) of the specific transformation vectors. The oligonucleotides were designed by NanoString (Seattle, WA, USA) and synthesized by IDT (Integrated DNA technologies).

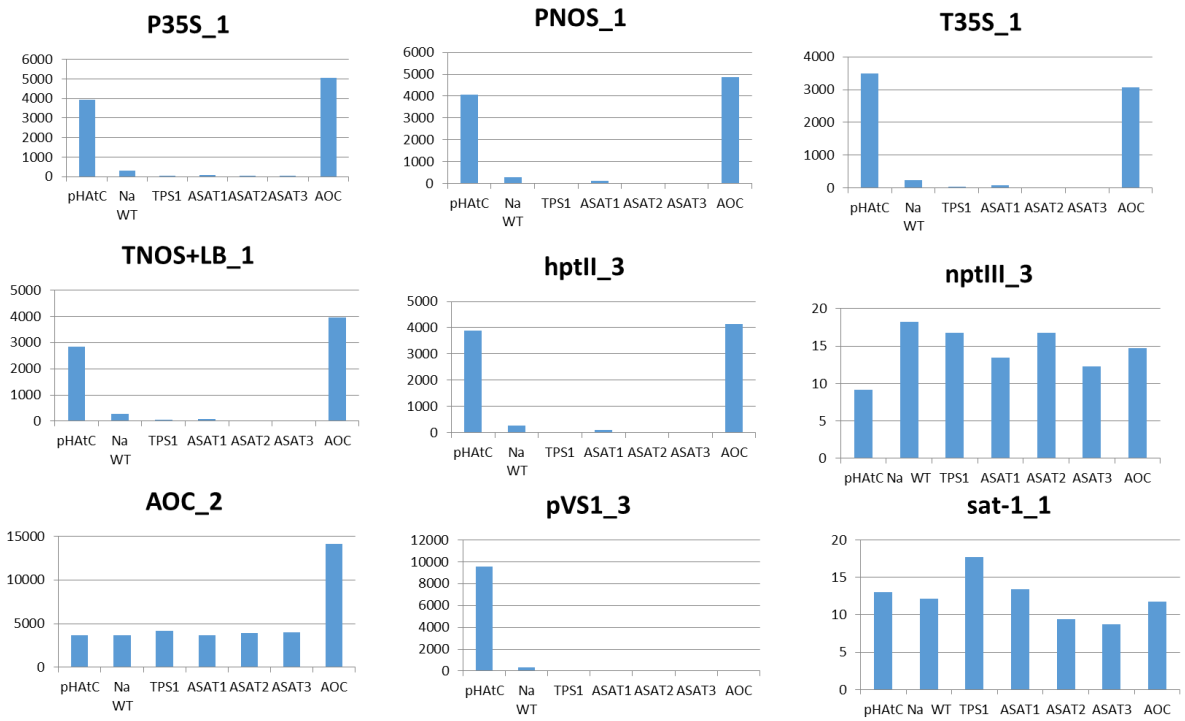

Figure 2. Confirmation of single and complete T-DNA insertions in ectopic expression lines and complete segregation of the T-DNA from the 4 lines of this request by Nanostring nCounter® technology. pHATC (positive control) is a *NaASAT1* CRISPR line harboring a single, complete T-DNA insertions with pHAtC vector screened from CAS9 gene genotyping by PCR. **Na WT,** *N. attenuata* wild type plant. ***NaTPS-1*** #8, *NaASAT1*-#6, ***NaASAT2***-#6, and ***NaASAT3*** #1 are the *NaASATs* and *NaTPS1* CRISPR lines of this request. **IrAOC** (Positive control), *IrAOC* (A-07-457-1) line harboring single, complete T-DNA insertion with pRESC5AOC vector, which harbors an inverted repeat of the same AOC fragment sequence as the probe, and hence is expected to return a 3x signal as compared to WT plants.

One published *IrAOC* (A-07-457-1) line harboring single, complete T-DNA insertion with pRESC5AOC vector and one *NaASAT1* CRISPR line harboring single, complete T-DNA insertions with the pHAtC vector, as verified by several NanoString probes (Fig. 2), were selected as positive controls; and *N. attenuata* wild type plant was selected as negative control. Two positive controls and one negative control along with *NaASAT1*-#6, *NaTPS-1* #8 and *NaASAT3* #1 CRISPR lines plants are used for the nCounter analysis. Hybridization of 5 probes designed from transgene promotor (PNOS, P35S) and terminator (TNOS, T35S), as well as the selective marker gene (hptII) sequences indicated a single and complete insertion of the transformation cassette only in IrAOC and one NaASAT1 CRISPR line harboring single, complete T-DNA insertions with pHAtC vector, no signals from *NaASAT1*-#6, *NaASAT2*-#6, *NaTPS-1* #8 and *NaASAT3* #1 CRISPR lines plants were found. Moreover, 2 probes designed from the transformation vector backbone outside the right and left transfer borders (nptII, pVS1) showed that only IrAOC and one *NaASAT1* CRISPR line harboring single, complete T-DNA insertions with pHAtC vector harbor T-DNA overreads, signals from *NaASAT1*-#6, *NaTPS-1* #8 and *NaASAT3* #1 CRISPR lines plants were not detected. These results are consistent with the PCR results presented in **Figures S4 and S6**, and confirm that all 4 lines are complete null segregants that do not harbor transgenes or any part thereof.

The organism used for genome editing was *Nicotiana attenuata* (Utah accession), 31st inbred generation.

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

The anticipated herbivore and fungal susceptibility phenotype(s) of the modified plants.

For the ***NaASAT1*-#6 CRISPR line**, we anticipated a lack of acyl sugar production and this phenotype was confirmed. We also observed a decreased number of trichomes on leaves and stems, the primary site of acyl sugar biosynthesis.

For the *NaASAT1*-#6, *NaASAT2* #6, *NaASAT3* #1 CRISPR lines, our group initially reported that trichome-derived O-acyl sugars are a first meal for caterpillars that tags them for predation (Weinhold and Baldwin, 2011).

Subsequently, we also reported that O-acyl sugars protect *Nicotiana attenuata* from both native fungal pathogens and a specialist herbivore (Luu et al., 2017). Our unpublished glasshouse tests of *NaASAT1*-#6, *NaTPS-1* #8 and *NaASAT3* #1 reveal that *NaASAT1*-#6 and *NaASAT3* #1 are highly susceptible to herbivory by *Manduca sexta* larvae, which have substantially enhanced growth rates when feeding on these lines. Therefore, we expect that, when planted out at our field station in Utah, these acyl sugar CRISPR lines will be more susceptible to the native fungal pathogens and specialist herbivores.

The *NaTPS-1* #8 CRISPR line is edited in a triterpenes cyclase that plays a central role in triterpene biosynthesis. Since triterpenes has been shown to be involved in the recruitment of microbes (Huang et al., 2019), we hypothesize that *NaTPS-1* #8 will have an altered microbiome compared to wild type plants. Moreover, as simple triterpenes are components of surface waxes and specialized membranes and may potentially act as signaling molecules, we hypothesize that triterpenes might protect tobacco from the native fungal pathogens and specialist herbivore. Therefore, we predict that the *NaTPS-1* #8 CRISPR line will also be more susceptible to the native fungal pathogens and specialist herbivores, when planted into native habitats.

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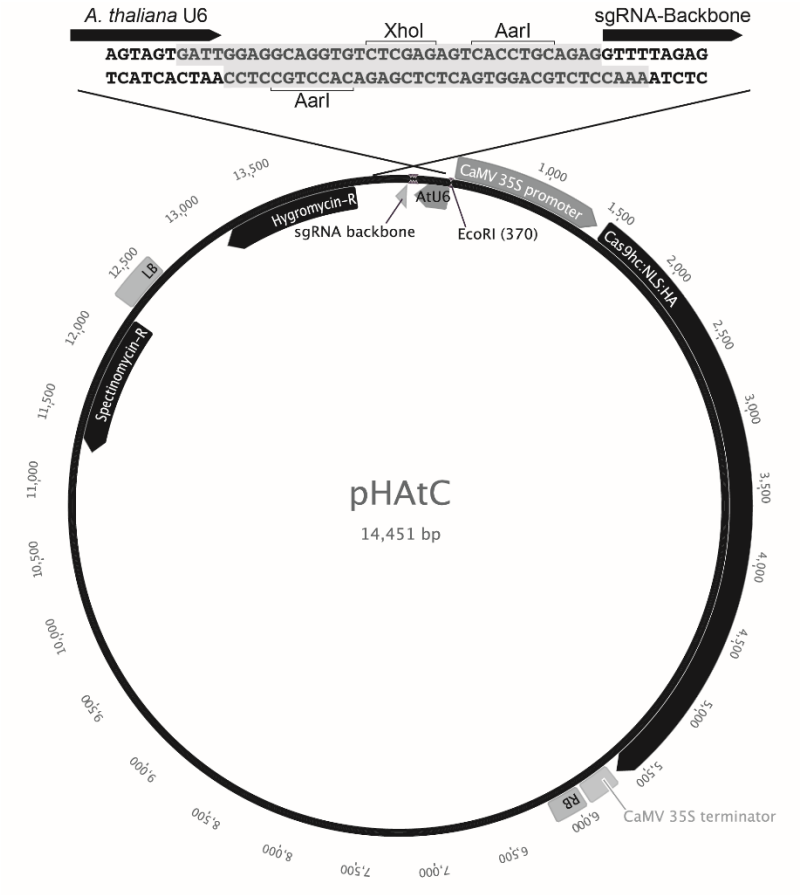
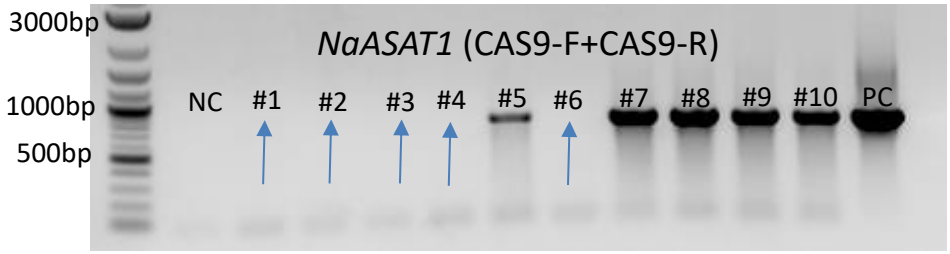
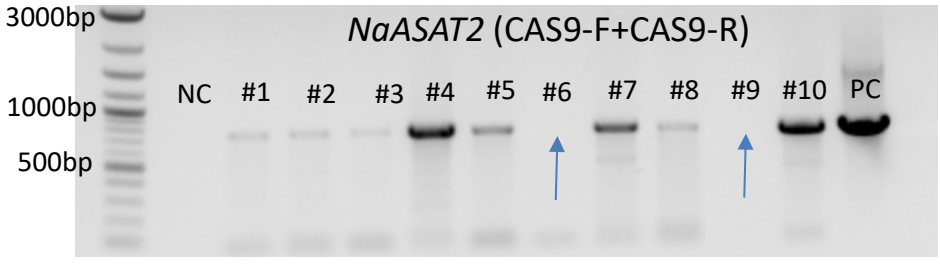
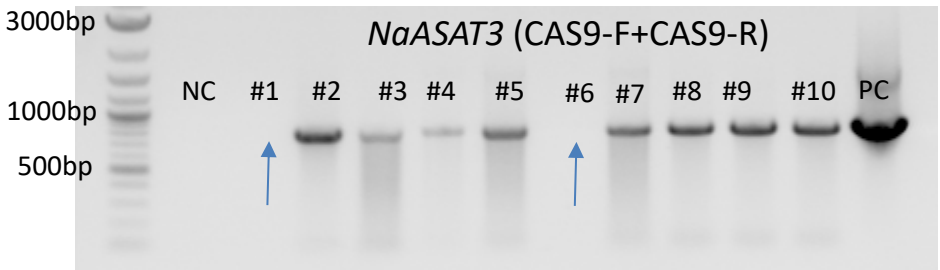
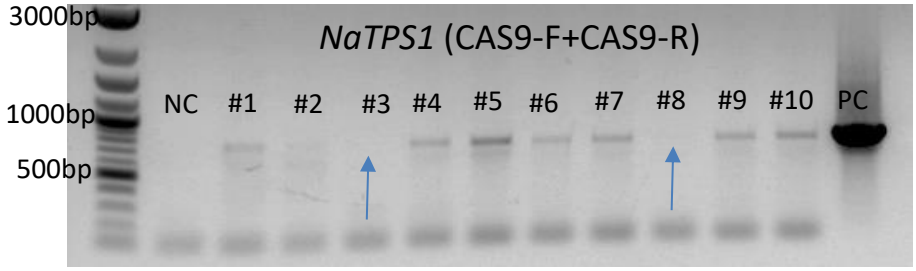


Supplementary Figure. 1. Map of the binary plant transformation plasmid pHAtC.



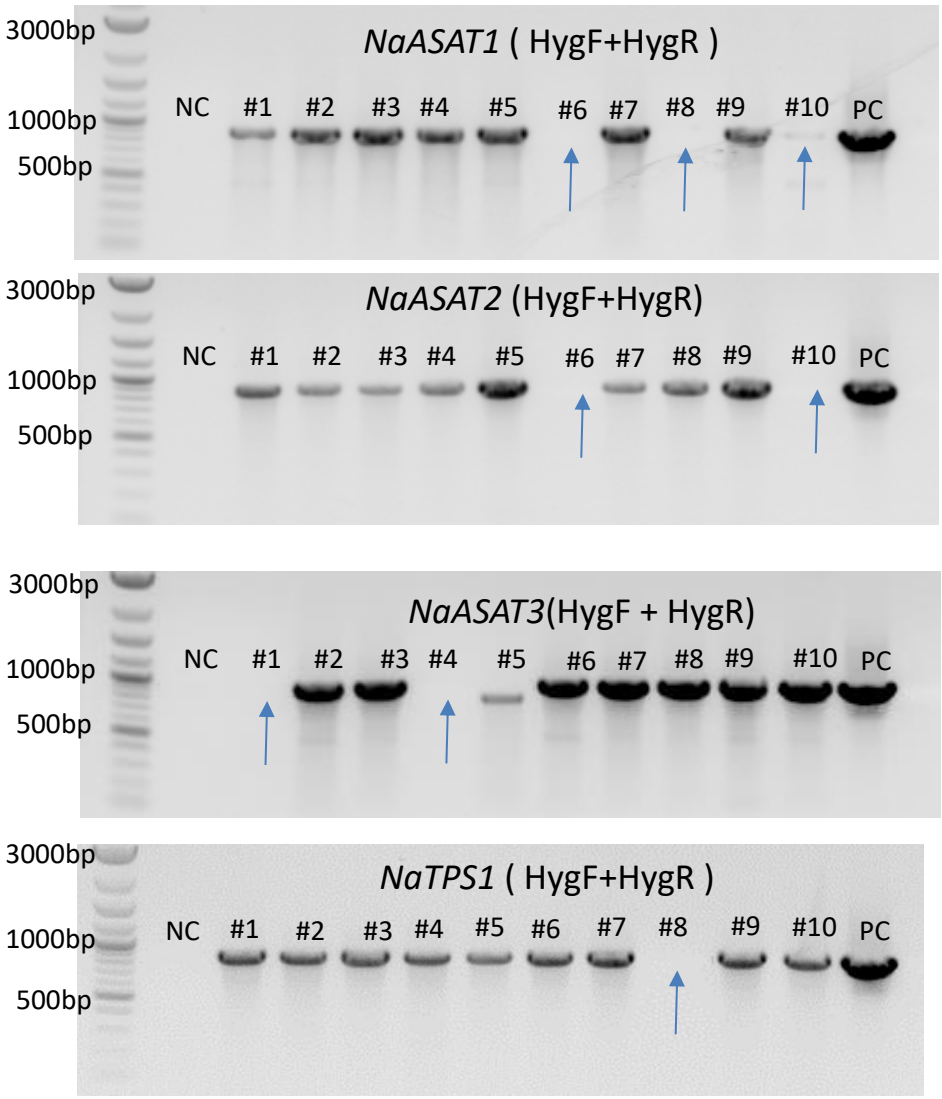






Supplementary Figure. 2.. Transgene PCR analysis of *NaASAT1, NaASAT2, NaASAT3 and NaTPS1* CRISPR lines. The presence of the PCR product band indicates the existence of the Cas9 gene sequence in T2 transgenic plants. Wild type *N. attenuata* genomic DNA was used as template for the negative control (NC) and the pHAtC vector was used as template for the positive control (PC). For the *NaASAT1* lines, blue arrows indicate the null segregants (#2, #3, #4, #6). Progeny of the ***NaASAT1 #6* plant** will be used for field experiments. For the *NaASAT2* lines, Blue arrows indicate the null segregants (and #9). Progeny of the ***NaASAT2 #6* plant** will be used for field experiments. For the *NaASAT3* lines, Blue arrows indicate the null segregants (and #6). Progeny of the ***NaASAT3* #1 plant** will be used for field experiments. For the NaTPS1 lines, blue arrows indicate the null segregants (*NaTPS1*-1.3, -and -1.8 lines). Progeny of the ***NaTPS1-1*. #8 plant** will be used for field experiments.

Supplementary Figure. 3. **Mutation pattern of the *NaASAT1*-#6, *NaASAT2* #6, *NaASAT3* #1 and *NaTPS1* #8 CRISPR lines**. *NaASAT1*-#6 null segregant harbors a three-base homozygous deletion in the site where the CAS9 + guide RNA2 (gRNA2) cleaves. *NaASAT2*-#6 null segregant harbors a single base homozygous insertion (A) in the site where the CAS9 + guide RNA1 (gRNA1) cleaves. *NaASAT3*-#1 null segregant harbors a single base homozygous insertion (T) in the site where the CAS9 + guide RNA1 (gRNA1) cleaves. *NaTPS1*-#8 null segregant harbors a single base homozygous T to C replacement in the site where the CAS9 + guide RNA2 (gRNA2) cleaves. The guide RNA binding site in *NaASAT1*-#6, *NaASAT2* #6, *NaASAT3* #1 and *NaTPS1* #8 were analyzed by Sanger dideoxy sequencing and compared with the wild-type sequence (A, T, G, C characters in red, green, yellow, and purple, respectively). The PAM sequences and gRNA sequence are labeled with boxes and lines, respectively, in the figure.



Supplementary Figure. 4. Transgene PCR analysis of *NaASAT1*-#6, *NaASAT2* #6, *NaASAT3* #1 and *NaTPS1* #8 CRISPR lines. 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). For the *NaASAT1* lines, blue arrows indicate the null segregants (*NaASAT1*-6.6, -6.8, and -6.10 lines). **Progeny of the *NaASAT1-6*. #6 plant** will be used for field experiments. For the *NaASAT2* lines, blue arrows indicate the null segregants (NaASAT2-6.6, -and -6.10 lines). **Progeny of the *NaASAT2-6. #6* plant** will be used for further experiments. For the *NaASAT3* lines, blue arrows indicate the null segregants (NaASAT3-1.1, -and -1.4 lines). **Progeny of the *NaASAT3-1*. #1 plant** will be used for field experiments. For the *NaTPS1* lines, blue arrows indicate the null segregants (NaTPS1-1.8 line). **Progeny of the *NaTPS1-1*. #8 plant** will be used for field experiments.

Supplementary Table 1. **Primers used in transgenic plant analysis**.

Primer name	Sequence	Amplicon size (bp)	Purpose
Cas9 longF	TCACCGACGAGTACAAGGTG	923	Detection of Cas9 gene
Cas9 longR	AGGTCGTCGTCGTAGGTGTC	923	Detection of Cas9 gene
NaASAT1_F	AAGTGGCCTGCAGTTAGAGAC	909	Sanger sequencing of *NaASAT1* gene to analyze mutation pattern
NaASAT1_R	GCTGCTGATGCAACGTAAGG	909	Sanger sequencing of *NaASAT1* gene to

			analyze mutation pattern
NaASAT2_F	ATGGCCTCAATTGCTCAATCGC	1374	Sanger sequencing of *NaASAT2* gene to analyze mutation pattern
NaASAT2_R	TAACAACTTGGAACTAATGAAG	1374	Sanger sequencing of *NaASAT2* gene to analyze mutation pattern
NaASAT3_F	TACTCAGGCAGCTCTATCAACAA	627	Sanger sequencing of *NaASAT3* gene to analyze mutation pattern
NaASAT3_R	AATCACGAGCAATTGCAGCCC	627	Sanger sequencing of *NaASAT3* gene to analyze mutation pattern
NaTPS1_F	TTGTGGACGGTTAATTGTAGTTGT	909	Sanger sequencing of *NaTPS1* gene to analyze mutation pattern
NaTPS1_R	TTCCACACATCCGATGGTGAT		Sanger sequencing of *NaTPS1* gene to analyze mutation pattern
HygR-F	CGTCTGTCGAGAAGTTCTG	874	Detection of hygromycin gene
HygR-R	CCGGATCGGACGATTGCG	874	Detection of hygromycin gene

Supplementary **Table 2. sgRNAs used in genome editing.**

Name	DNA sequence for sgRNA (**bold**: PAM sequence)	Location
NaASAT1 sg1	**CCC**AAAACAAGACACTGCAAGC	1st exon of *Nicotiana attenuata NaASAT1-like* gene (NIATv7_g04553)
NaASAT1 sg2	**CCC**ACAAGGTTGCTGATGGTTA	1st exon of *Nicotiana attenuata NaASAT1-like* gene (NIATv7_g04553)
NaASAT2 sg1	**CCT**ACTAAAAGATGGCACAAAC	1st exon of *Nicotiana attenuata NaASAT2-like* gene (NIATv7_g17261)
NaASAT2 sg2	TGTGTATATCACACAAGAC**TGG**	1st exon of *Nicotiana attenuata NaASAT2-like* gene (NIATv7_g17261)
NaASAT3 sg1	**CCA**AGTGATATGTGTAAGGTTA	1st exon of *Nicotiana attenuata NaASAT3-like* gene (NIATv7_g08565)
NaASAT3 sg2	CATGTGTATCACACAAAAT**TGG**	1st exon of *Nicotiana attenuata NaASAT3-like* gene (NIATv7_g08565)
NaTPS1 sg1	ATATTTGTATGAGGATCCT**CGG**	3rd exon of *Nicotiana attenuata NaTPS1-like* gene (NIATv7_g21921)
NaTPS1 sg2	GCCACTGTCTTATCTCTAT**GGG**	5th exon of *Nicotiana attenuata NaTPS1-like* gene (NIATv7_g21921)

Appendix

Genomic sequence received from NCBI assembly. Organism: *Nicotiana attenuata* (eudicots). Infraspecific name: Strain: UT Submitter: Max Planck Institute for Chemical Ecology Date: 2016/11/15. Assembly level: Chromosome. Genome representation: fullRefSeq. Category: representative genome GenBank assembly accession: GCA_001879085.1 (latest). RefSeq assembly accession: GCF_001879085.1 (latest). IDs: 889921 [UID] 3726648 [GenBank] 3745728 [RefSeq]. All of these three *NaASATs* genes have no introns for the DNA sequences, *NaTPS1* has 18 exons. PAM sequences are in green, gRNAs are in yellow, putative ATG and stop codons (assigned through ORF analysis) are indicated in red.

***NaASAT1* (NCBI ACCESSION: XM_019408441)**

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***NaASAT2* (NCBI ACCESSION: XM_019390325)**

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***NaASAT3* (NCBI ACCESSION: XM_019389028)**

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***NaTPS1* (NCBI ACCESSION: XM_019391273.1)**

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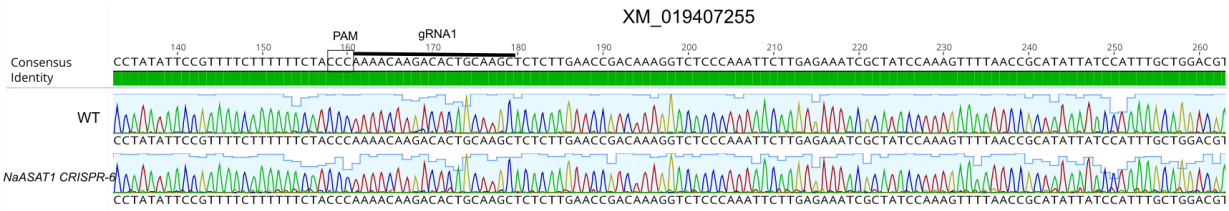
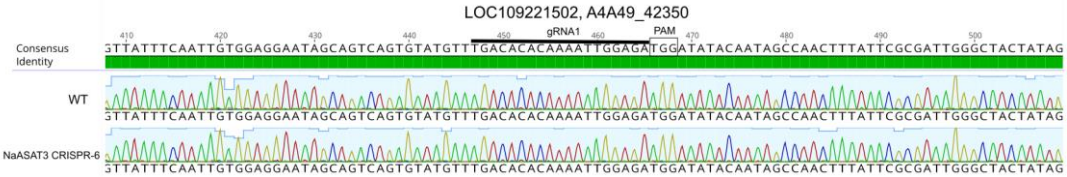
GTATGCCATTTGGTCCTTATAAAAATTTCCAGAAAAAATGTAGTTTATTTTTTCTTGTGTTAATGTAAGT
GATGTAACAATGTCAAAAATCATAGCCTAA

A. We designed primers and conducted a PCR to check the *NaASAT1* homologous gene (LOC109240593, A4A49_18877, MJEQ01000296) in the *NaASAT1-6* CRISPR mutant, and we found that there was no modification of this gene. The sequence alignment results of WT and *NaASAT1-6* CRISPR line are presented in Supplementary Figure 5.



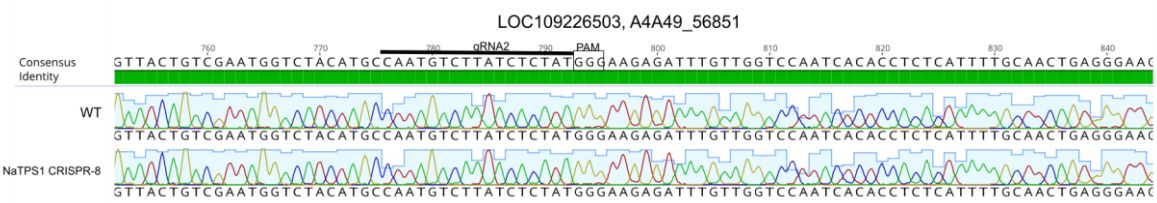
Supplementary Figure 5. Off-target detection of *NaASAT1* homologous gene (LOC109240593, A4A49_18877, MJEQ01000296) in the *NaASAT1-6* CRISPR mutant by Sanger sequencing.

B. We designed primers and conducted a PCR to evaluate the *NaASAT3* homologous gene (LOC109221502, A4A49_42350) in the *NaASAT3-6* CRISPR mutant, and we found no modification of this gene. The sequence results *NaASAT3-6* CRISPR line for the *NaASAT3* homologous gene (LOC109221502, A4A49_42350) are in Supplementary Figure 6.

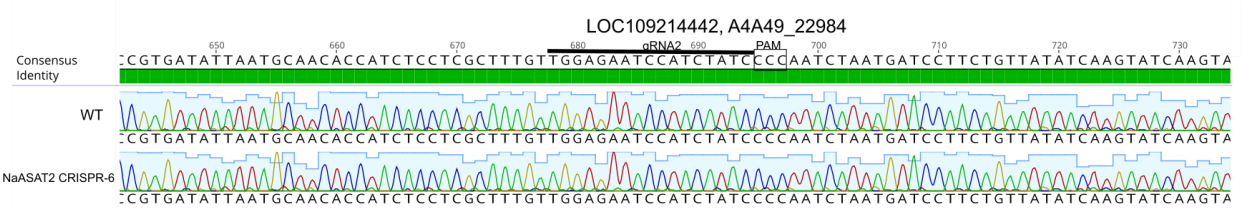


Supplementary Figure 6. Off-target detection of *NaASAT3* homologous gene (LOC109221502, A4A49_42350) in the *NaASAT3-6* CRISPR mutant by Sanger sequencing.

C. We designed primers and conducted a PCR to evaluate the *NaTPS1* homologous gene (LOC109226503, A4A49_56851) in the *NaTPS1-8* CRISPR mutant, and found no modifications of this gene. The sequencing results of *NaTPS1-8* CRISPR mutant for the *NaTPS1* homologous gene (LOC109226503, A4A49_56851) are in Supplementary Figure 7.



D. Supplementary Figure 7. Off-target detection of *NaTPS1* homologous gene (LOC109226503, A4A49_56851) in the *NaTPS1-8* CRISPR mutant by Sanger sequencing We designed primers and conducted a PCR to evaluate the *NaASAT2* homologous gene (LOC109214442, A4A49_22984) in the *NaASAT2-6* CRISPR mutant, and found no modifications of this gene. The sequence results of the *NaASAT2-6* CRISPR mutant for the *NaASAT2* homologous gene (LOC109214442, A4A49_22984) are in Supplementary Figure 8.



Supplementary Figure 8. Off-target detection of the *NaASAT2* homologous gene (LOC109214442, A4A49_22984) in the *NaASAT2-6* CRISPR mutant by Sanger sequencing.

Supplementary Table 3. Primers used for the off-target sequencing in the mutants.

Primer name	Sequence	Amplicon size (bp)	NCBI- Locus tag
18877-F	ACGCGTCGACCGATGGCTATTTCAAGGCTTGT	1266	A4A49_18877
18877-R	CCGCTCGAGCGAGTCCTGAGCTTGGAG	1266	A4A49_18877
22984_F	ACGCGTCGACCGATGGCTGCATTGTCAGT	1311	A4A49_22984
22984_R	CCGCTCGAGCATGATTTGGAATTGGTATAGCAAATTC	1311	A4A49_22984
42350_F	ACGCGTCGACCGATGTATGCATCAAACCTTGTATCAT	1300	A4A49_42350
42350_R	CCGCTCGAGCGTTGCTTGGAGAAGTAAACTC	1300	A4A49_42350
56851_F	AAAAAGCAGGCTTAATGTGGAAGTTGAAGATTG	2300	A4A49_56851
56851_R	A GAA AGC TGG GTATTAGTTGTTTTGTAATGGTG	2300	A4A49_56851

Information unfavorable to the proposal
None