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Engineering resistance against *Tobacco streak virus* (TSV) in sunflower and tobacco using RNA interference

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Abstract

The coat protein (CP) gene of *Tobacco streak virus* (TSV) from sunflower (*Helianthus annuus* L.) was amplified, cloned and sequenced. A 421 bp fragment of the TSV coat protein gene was amplified and a gene construct encoding the hairpin RNA (hpRNA) of the TSV-CP sequence was made in the plasmid pHANNIBAL. The construct contains sense and antisense CP sequences flanking a 742 bp spacer sequence (*Pdk* intron) under the control of the constitutive CaMV35S promoter. A 3.6 kb *Not* I fragment containing the hpRNA cassette (TSV-CP) was isolated from pHANNIBAL and sub-cloned into the binary vector pART27. This chimeric gene construct was then mobilized into *Agrobacterium tumefaciens* strain LBA4404 *via* triparental mating using pRK2013 as a helper. Sunflower (cv. Co 4) and tobacco (cv. Petit Havana) plants were transformed with *A. tumefaciens* strain LBA4404 harbouring the hpRNA cassette and *in vitro* selection was performed with kanamycin. The integration of the transgene into the genome of the transgenic lines was confirmed by PCR analysis. Infectivity assays with TSV by mechanical sap inoculation demonstrated that both the sunflower and tobacco transgenic lines exhibited resistance to TSV infection and accumulated lower levels of TSV compared with non-transformed controls.

Additional key words: *Agrobacterium tumefaciens, Helianthus annuus*, kanamycin, *Nicotiana tabacum,* PCR.

Introduction

Tobacco streak virus (TSV) is one of the most widespread and economically important plant viruses. TSV belongs to subgroup I of the *Ilarvirus* genus (Ramiah *et al*. 2001, Bhat *et al*. 2002) and is characterized by icosahedral particles measuring 27 - 35 nm in diameter. Its genome consists of three single stranded positive sense RNA species of 2.9, 2.7 and 2.2 kb designated as RNA-1, RNA-2 and RNA-3, respectively. It has been reported that the coat protein (CP), encoded by RNA-3, is required for infectivity (Van Vloten-Doting 1975). TSV has a wide host range and more than 140 genera of plants were identified worldwide that can be infected with TSV (Kaiser *et al*. 1982, Hull 2002). The economically important host crops for TSV include peanut (Reddy *et al*. 2002), sunflower (Dijkstra 1983, Prasada Rao *et al*. 2000), safflower (Chander Rao *et al*. 2003), soybean (Fagbenle and Ford 1970), potato (Salazar *et al*. 1982), tomato (Cupertino *et al*. 1984), pepper (Gracia and Feldman 1974), cucumber and gherkin (Krishnareddy *et al*. 2003) and strawberry (Spiegel and Cohen 1985). Several weeds including *Parthenium hysterophorus* (Sharman *et al*. 2009) are also known to be infected with TSV which could serve as a source of inoculums in the field. TSV can be transmitted mechanically, but the usual transmission route in the field is through thrips. Several species of thrips, including *Microcephalothrips abdominalis* (Greber *et al*. 1991), *Thrips tabaci* (Sdoodee and Teakle 1987), *Megalurothrips usitatus, Frankliniella schultzei* and *Scirtothrips dorsalis* (Prasada Rao *et al*. 2003) have been implicated in transmission of TSV. Development of crop plants resistant to TSV is crucial for sustenance of resource-poor farmers depending on their livelihood on agricultural lands where options are few. Advances in plant transformation have opened up the possibility of controlling plant virus diseases through genetic

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Abbreviations: CP - coat protein, PDR - pathogen derived resistance, TSV - *Tobacco streak virus.*
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engineering. Several strategies have been used to engineer resistance in plants to viruses (Lomonossoff 1995, Baulcombe 1996). The so-called pathogen derived resistance (PDR) strategy has been successfully used to protect transgenic crop plants against several genera of viruses (Goldbach *et al*. 2003). PDR is a phenomenon whereby genetically modified plants containing genes or sequences of a parasite are protected against cognate or related pathogens (Sanford and Johnston 1985). PDR strategies can be classified into groups: those that involve production of transgenic virus proteins (*e.g.* coat protein or movement protein-mediated resistance) and those that function on the RNA level only (*e.g*. dsRNA mediated resistance) (Kalantidis *et al*. 2002). By using the latter approach several virus resistant papaya and zucchini squash cultivars have been developed for commercial applications (Tricoli *et al*. 1995, Ferreira *et al*. 2002). Analysis of the transgenic plants revealed that PDR in many cases is due to transgene-induced post-transcriptional gene silencing (PTGS) or RNA silencing (Baulcombe 2002). Genetically modified crop plants producing dsRNA with homology to viral sequences are known to exhibit resistance to the virus (Kalantidis *et al*. 2002). Engineering virus-resistant transgenic crops through RNA silencing takes advantage of the natural defense mechanism in plants against viruses. Resistance engineered through RNA silencing, especially through dsRNA production, is very strong, in most cases reaching immunity. In the present study sunflower and tobacco plants were transformed with a plant expression vector containing inverted repeats of the partial CP gene of TSV. The plants were challenged by TSV by mechanical sap inoculation in the greenhouse, and their level of resistance was evaluated.

Materials and methods

Cloning and characterization of the TSV coat protein gene: Total RNA was extracted from TSV-infected sunflower (*Helianthus annuus* L.) leaves with the *RNeasy* plant mini kit according to the manufacturer's protocol (*Qiagen*, Germantown, Maryland, USA), eluted with 0.05 cm^3 of RNase-free water, and stored at -20 °C.

Reverse transcription- polymerase chain reaction (RT-PCR) was carried out with a *OneStep* RT-PCR kit (*Qiagen*) in 0.05 cm^3 reaction volume containing 100 ng of RNA and 2 units of enzyme mix and 0.6 μM of each primer following the protocol suggested by the manufacturer. Amplification was performed in a *Palm-Cycler* model *CG1-960* (*Corbett Research*, Mortlake, NSW, Australia) programmed for one cycle of 37 °C for 30 min for cDNA synthesis followed by initial denaturation at 95 °C for 15 min and 40 cycles of 94 °C for 45 s, 48 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 1 h. The primers, CP-F (5'-ATGAATACTTTGATCCAAGG-3') and CP-R (5'-TCAGTCTTGATTCACCAG-3') (Bhat *et al*. 2002) were used for amplification of the entire CP gene of TSV. Amplification products were separated by electrophoresis on 1.2 % agarose gels using Tris-acetate-EDTA (TAE) buffer for 1 h at 80 V constant current. The gels were stained with ethidium bromide solution and visualized with a gel documentation system (*AlphaImager 2000*; *Alpha Innotech Corporation*, San Leandro, CA, USA).

Cloning of PCR products and sequence analysis: The RT-PCR products were excised from the gel and purified with a *QIAquick* gel extraction kit (*Qiagen*). The PCR fragment was ligated into the *pGEM-T Easy* vector (*Promega*, Madison, WI, USA) at 4 °C overnight. Ligated DNA was used for transformation of competent *Escherichia coli* DH5∝ cells. Plasmid DNA was isolated from the potential recombinant clones by using *Wizard Plus* plasmid DNA purification kit (*Promega*) according to the manufacturer's recommendations. The clones were sequenced in both the forward and reverse directions, by using universal M13 and T7 primers. Sequencing was done at *1st Base Pvt Ltd*. (Singapore). Database search was performed with the *BLAST 2.0* program from the National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD World Wide Web server. The CP-gene sequence was deposited in the *GenBank* database with the accession number GQ167767.

Construction of vector: Primer pairs, CP(S)-F and CP(S)-R, and CP(AS)-F and CP(AS)-R containing appropriate restriction sites were synthesized to amplify a 421 bp fragment containing the partial TSV coat protein gene, reflecting nucleotide positions 176 to 596 in order to clone into pHANNIBAL (Wesley *et al*. 2001) in the sense and antisense orientations, respectively (Table 1).

Table 1. Primers used in plasmid construction

Primer	Sequence (5' to 3')	Introduced enzyme site
CP(S)-F	ATCTCGAGATGCCGCTAGAG	*XhoI*
CP(S)-R	GCGAATTCACGATAGCTCTATC	*EcoRI*
CP(AS)-F	GCTCTAGAATGCCGCTAGAG	*XbaI*
CP(AS)-R	ATATCGATACGATAGCTCTATC	*ClaI*

PCR reactions were performed using the plasmid pGEM-T(TSV-CP) as template under the following conditions; 1 cycle of 95 °C for 3 min; 40 cycles at 94 °C for 45 s, 58 °C for 1 min and 72 °C for 1 min; and a final extension cycle at 72 °C for 10 min. The PCR products were separated by electrophoresis on 1.2 % agarose gels, recovered from the gel and purified. These PCR products were cloned into the plasmid pHANNIBAL in opposite orientation on either side of a *pdk* intron sequence to create an inverted repeat construct driven by the CaMV

35S promoter (Fig.1). Correct plasmid construction was identified by restriction endonuclease digestion of plasmid minipreps (Sambrook and Russell 2001). A 3.6 kb *Not I* fragment containing the hpRNA cassette was isolated from the plasmid pHANNIBAL(TSV-CP) and sub-cloned into the binary vector pART27 (Gleave 1992). This chimeric gene construct was then inserted into *Agrobacterium tumefaciens* strain LBA4404 (Jefferson *et al*. 1987) *via* triparental mating using pRK2013 as a helper as described by An (1987). Insertion of the chimeric gene construct was confirmed by colony PCR. *A. tumefaciens* harbouring the plasmid with the hpRNA cassette was used for transformation of sunflower and tobacco.

Transformation of sunflower: Seeds of sunflower (cv. Co 4) were immersed in sterile distilled water and placed under vacuum for 2 min. The seeds were then surface sterilized for 2 min with 70 % (v/v) ethanol, rinsed in a 4 % (v/v) sodium hypochlorite (2.25 % active Cl) solution containing few drops of *Tween-20* and dried for 30 min. Then, the seeds were washed five times (1 min each) with sterile distilled water and soaked in sterile distilled water for 16 h at 25 °C in the dark. The disinfected seeds were peeled, radicle and the cotyledons were removed by cutting them off at their base and the meristem-containing explants were cut through the middle to make 4 pieces (Lewi *et al*. 2006), each piece containing ¼ of the apical meristem and the lateral cotyledonal meristems. The explants were placed into Petri dishes with 20 cm^3 of *A. tumefaciens* grown in YEP medium (10 g dm^{-3} yeast extract, 10 g dm^{-3} peptone, 5 g dm^{-3} NaCl; pH 7.0) supplemented with 100 mg dm^{-3} acetosyringone. Co-culture was performed for 3 d in darkness at 25 °C in Murashige and Skoog (1962; MS) medium supplemented with 0.5 mg dm^{-3} benzylamino-purine (BAP), 0.1 mg dm^{-3} gibberellic acid (GA_3). After co-cultivation for 3 d, the explants were washed twice in 500 mg dm^{-3} cefotaxime, blotted on sterile *Whatman 3 MM* filter paper and transferred to solid MS supplemented with 0.5 mg dm^{-3} BAP, 0.1 mg dm^{-3} GA_3, 50 mg dm^{-3} kanamycin and 500 mg dm^{-3} cefotaxime. After 15 d the explants were transferred to the regeneration medium (MS medium supplemented with 0.1 mg dm^{-3} GA_3, 50 mg dm^{-3} kanamycin and 500 mg dm^{-3} cefotaxime). The plants with well developed roots were transferred to small pots containing sterile pot mixture (*Vermicompost*: sand: soil mixed at 1:1:1 ratio) and were grown in the greenhouse. The primary transformants were tested for the integration of the TSV-CP gene fragment into their genome by PCR analysis and for their resistance to TSV infection as described below.

Transformation of tobacco: Tobacco (*Nicotiana tabacum* L. cv. Petit Havana) leaf discs were transformed with *A. tumefaciens* LBA4404 harbouring the plasmid with the hpRNA cassette according to the method described by Horsch *et al.* (1985). Approximately 200 explants were used during transformation experiments. Putative transformants were screened on MS medium containing kanamycin (100 µg cm^{-3}). Regenerated (transgenic) plants (T_0) were grown in the greenhouse for transgene confirmation and seed production. Twenty four T_0 independent transgenic lines resistant to kanamycin were obtained. The integration of the TSV-CP gene fragment into the genome of these transgenic lines was verified by PCR analysis of the genomic DNA from regenerated tobacco plants.

Molecular detection of transgenic plants: PCR analysis was performed using approximately 100 ng of plant genomic DNA and with the oligonucleotide primers 5'-ATCTCGAGATGCCGCTAGAG-3' and 5'-GCG AATTCACGATAGCTCTATC-3' under the following conditions; 1 cycle of 95 °C for 3 min; 40 cycles at 94 °C for 45 s, 58 °C for 1 min and 72 °C for 1 min; and a final extension cycle at 72 °C for 1 h. PCR products were fractionated by electrophoresis in 1.2 % agarose gels, stained with ethidium bromide dye and visualized under UV radiation.

Testing of transgenic lines for resistance to TSV: Inoculation of the transgenic sunflower and tobacco plants with TSV was performed using a standard mechanical rubbing method as described by Gutha and Reddy (2008). To prepare the inoculum, TSV-infected cowpea leaves (1 g) were ground in 5 cm^3 of 50 mM potassium phosphate buffer (pH 7.2) using a pre-chilled pestle and mortar, and 0.01 % (m/v) celite was mixed with the buffer immediately before inoculation. The transgenic sunflower (T_0) and tobacco (T_1) plants were inoculated with TSV by gently rubbing the upper leaf surface with 0.1 cm^3 of the viral suspension inoculums using a pestle. Excess inoculum was washed off with water using a squeeze bottle. Following inoculation, plants were maintained in an insect-proof glasshouse at temperatures ranging from 25 to 30 °C and observed for symptom expression. Leaf samples were collected and tested for TSV by direct antigen coating-enzyme linked immunosorbent assay (DAS-ELISA) as described by Ramiah *et al*. (2001). Leaf samples were ground in carbonate buffer, pH 9.6, (1:10, m/v) and added to wells of *Nunc* ELISA plates (*Fisher Scientific*, Loughborough, UK). Polyclonal antiserum (a gift of Dr. Stephan Winter, DSMZ, Germany) was used at 1:5 000 dilution in phosphate buffered saline containing 0.2 % ovalbumin and 2 % polyvinylpyrrolidone (PVP), at 37 °C for 45 min. Alkaline phosphatase (ALP)-labelled goat anti-rabbit immunoglobulin G (IgG) (*Sigma*, St. Louis, USA) was used at 1:5 000 dilution for detecting the immobilized antigen-antibody complex, and *p*-nitrophe-nyl phosphate as the substrate for the ALP at 1.0 mg cm^{-3} in 0.2 M Tris buffer (*Sigma Fast p*-nitrophenyl phosphate tablet sets). Absorbance readings at 405 nm (A_{405} nm) were taken after incubation at room temperature for 30 min, in an ELISA reader (*EL800*, *Bio-Tek Instruments*, Winooski, VT, USA). All tests were made in duplicate wells and replicated three times in different plates.

Results and discussion

Total RNA was extracted from the infected plants and the coat protein gene of TSV was amplified by RT-PCR using CP gene specific primers. The amplified DNA fragment of 717 bp was cloned into pGEM-T Easy vector and sequenced. Sequence analysis revealed that the cloned DNA fragment shared 100 % nucleotide sequence identity with those of TSV isolates from sunflower (acc. No. AF400664) and cotton (acc. No. AF515824) (data not shown). The CP-gene sequence was deposited in *GenBank* database with the accession number GQ167767. A 421 bp fragment of the coat protein gene was amplified from the TSV coat protein gene and a gene construct encoding the hairpin RNA (hpRNA) of the TSV-coat protein gene sequence was made in pHANNIBAL. The construct contains sense and antisense CP gene sequences flanking a 742 bp spacer sequence (PDK intron) under the control of the constitutive CaMV35S promoter. A fragment containing the hpRNA cassette was sub-cloned into the binary vector pART27 (Fig. 1) which was then mobilized into *Agrobacterium tumefaciens* strain LBA4404. *A. tumefaciens* harbouring the plasmid with the hpRNA cassette was used for transformation of sunflower and tobacco.

Eight putative transgenic sunflower lines (T_0) resistant

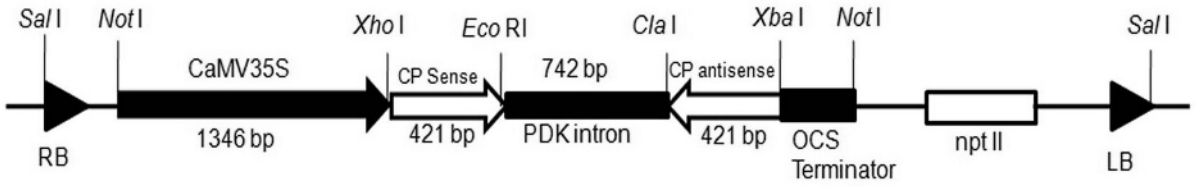

Fig. 1. Transformation cassette used to transform sunflower and tobacco plants. The construct contains sense and antisense TSV-CP sequences flanking a 742 bp spacer sequence (PDK intron) under the control of the constitutive CaMV35S promoter and OCS terminator.

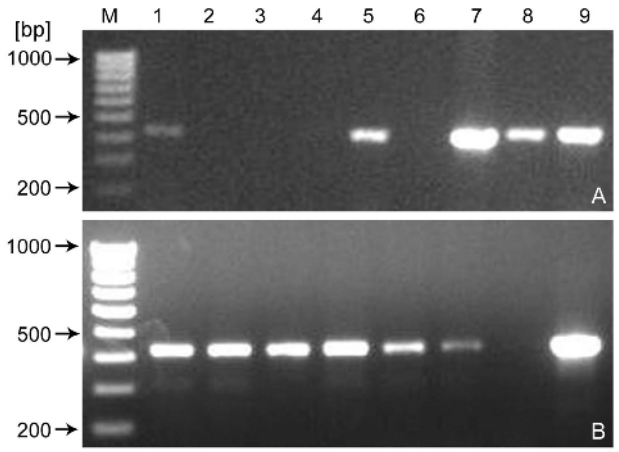

Fig. 2. PCR analysis of total genomic DNA from putative transgenic sunflower (*A*) and tobacco (*B*) plants Total DNA from primary transformants (T_0 generation) were analyzed by PCR for the integration of the TSV-CP gene in sunflower and tobacco using CP-F (5'-GCTCTAGAATGCCGCTAGAG-3') and CP-R (5'-ATATCGATACGATAGCTCTATC-3') primers. *A*: M - 100-bp ladder, 1 to 8 - total DNA from kanamycin-resistant sunflower plants, 9 - pHANNIBAL(TSV-CP) DNA (positive control). *B*: M - 100-bp DNA ladder, 1 to 6 - total DNA from putative transgenic tobacco plants, 7 - total DNA from WT (negative control), 8 - pHANNIBAL(TSV-CP) DNA (positive control).

to kanamycin were obtained. No significant differences in morphology and growth were observed. Molecular analysis of these plants by PCR indicated that the CP gene sequence was present in 4 out of 8 plants tested

Table 2. Bioassay of T_0 transgenic sunflower plants for resistance to TSV measured by ELISA (A_{405}). In the second column (–) or (+) indicate plants lacking or containing the TSV CP gene, respectively, as determined by PCR analysis. (+) or (-) in the last column indicate plants displaying local necrotic lesions within 7 d after TSV inoculation or no symptoms, respectively.

Plant	CP gene	TSV (A_{405})	Symptoms
SF-IR-CP-1	+	0.09	-
SF-IR-CP-2	-	0.57	+
SF-IR-CP-3	-	0.61	+
SF-IR-CP-4	-	0.42	+
SF-IR-CP-5	+	0.04	-
SF-IR-CP-6	-	0.69	+
SF-IR-CP-7	+	0.03	-
SF-IR-CP-8	+	0.02	-
Wild-type	-	0.65	+

(Fig. 2*A*). As expected, the TSV-CP primer set amplified a fragment of 421 bp in the transformants. The bioassay results indicated that necrotic lesions were observed on the leaves of the wild-type (WT) plants 5 - 7 d after inoculation whereas the transgenic plants (T_0 generation) did not develop symptoms. ELISA results indicated that the WT plants inoculated with TSV recorded the higher virus content as compared to the transgenic lines (Table 2).

In the case of tobacco, 24 T_0 independent transgenic lines resistant to kanamycin were obtained. No significant differences in morphology and growth were observed. To confirm the presence of the target gene in the putative

transgenic plants, the T_0 plants were subjected to PCR analysis with the primers specific for the TSV-CP gene. Agarose gel electrophoresis of PCR products from the transgenic plant samples showed the expected 417 bp band in all the putative transformants tested (Fig. 2*B*). No PCR product was obtained from non-transformed control plants. The self-pollinated seeds from PCR-positive plants in the T_0 populations were harvested and sown in soil. WT and the T_1 progeny derived from the selfed primary transformant (Tob-IR-CP-4) was challenged with TSV inoculum and monitored for viral symptoms and accumulation of virus by ELISA, using TSV antibody. The results indicated that all the transgenic lines inoculated with TSV did not develop symptoms, whereas the mosaic and necrotic lesions and vein necrosis were observed after 25 - 30 d on young uninoculated leaves of the wild type plants. The transgenic lines inoculated with TSV recorded the lowest virus concentration as compared to the non-transgenic control plants (Table 3).

Several studies demonstrated that plant viral and viroid diseases can be efficiently controlled using RNA interference (Kalantidis *et al*. 2002, Bonfim *et al*. 2007, Tyagi *et al*. 2008, Schwind *et al*. 2009, Fahim *et al*. 2010, Jiang *et al*. 2011, Patil *et al*. 2011, Shimizu *et al*. 2011). Kalantidis *et al*. (2002) developed transgenic tobacco lines that express dsRNA homologous to the 3' portion of the *Cucumber mosaic virus* (CMV) RNA3 genome and demonstrated that the presence of homologous CMV siRNAs in the plants prior to infection can be used as a molecular marker to predict the resistance. Plant lines that failed to produce siRNA at a detectable level were susceptible to CMV infection, whereas plants producing siRNAs were resistant to CMV. Bonfim *et al*. (2007) used the RNAi concept to silence the *AC1* viral gene that encodes a complex, multifunctional protein (Rep) essential for viral genome replication, in common bean to generate transgenic lines with strong resistance to *Bean golden mosaic virus* (BGMV). Fahim *et al*. (2010) demonstrated that transgenic wheat plants expressing an RNAi construct targeting the nuclear inclusion protein a (*NIa*) gene of *Wheat streak mosaic virus* (WSMV) showed complete resistance to WSMV. Patil *et al*. (2011) recently demonstrated that tobacco plants transformed with RNAi constructs consisting of full-length or partial sequences of the coat protein gene of a Ugandan isolate of *Cassava brown streak uganda virus* (CBSUV) showed resistance to diverse isolates belonging to two different virus species, *Cassava brown streak virus* and CBSUV.

The resistance to TSV in crop plants was previously reached by transformation with the viral CP gene (Van Dun *et al*. 1988, Bag *et al*. 2007). Van Dun *et al*. (1988) demonstrated that transgenic tobacco plants expressing the TSV CP gene were highly resistant to infection with TSV nucleoproteins but were susceptible to infection with *Alfalfa mosaic virus* (AlMV) nucleoproteins. The present study demonstrates the successful application of RNAi technology for the management of TSV in sunflower and tobacco. All the transformants were completely resistant to TSV as these lines neither developed symptoms nor contained virus particles up to 30 d after inoculation. The present findings suggest that introduction of inverted repeats of the CP gene of TSV may be an effective and reliable strategy for developing economically important crops with resistance to TSV.

Table 3. Bioassay of transgenic tobacco lines (T_1) and non-transformed controls for their resistance against TSV measured by ELISA (A_{405}). In the second column (–) or (+) indicate plants lacking or containing the TSV CP gene, respectively, as determined by PCR analysis. (+) or (-) in the last column indicate plants displaying local necrotic lesions within 7 d after TSV inoculation or no symptoms, respectively.

Plant	CP gene	TSV (A_{405})	Symptoms
Tob-IR-CP-4-1	+	0.086	-
Tob-IR-CP-4-2	+	0.093	-
Tob-IR-CP-4-3	+	0.084	-
Tob-IR-CP-4-4	+	0.066	-
Tob-IR-CP-4-5	+	0.034	-
Tob-IR-CP-4-6	+	0.078	-
Tob-IR-CP-4-7	+	0.021	-
Tob-IR-CP-4-8	+	0.054	-
Tob-IR-CP-4-9	+	0.019	-
Tob-IR-CP-4-10	+	0.072	-
Tob-IR-CP-4-11	+	0.038	-
Tob-IR-CP-4-12	+	0.053	-
Control #1	-	0.714	+
Control #2	-	0.668	+

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