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By apmball for BRS Document Control Officer at 5:49 pm, Jun 28, 2019

February 6th, 2019

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

Sent via Email to : AIRinquiries@aphis.usda.gov

Ref: APHIS Inquiry Letter Virus Resistant Tomato Lines NP-TV101-1, NP-TV101-2, NP-TV101-3, NP-TV201-1, NP-TV201-2 and NP-TV201-3,

Dear Dr Firko

Nexgen Plants is an Australian based plant trait company delivering novel solutions for a range of pathogens, production traits and consumer traits. Nexgen Plants wishes to evaluate in the field six virus resistant tomato lines (*Solanum Lycopersicum* NP-TV101-1, NP-TV101-2, NP-TV101-3, NP-TV201-1, NP-TV201-2, NP-TV201-3) that were developed at the Universtiy of Queensland using an intragenic method that produces events that mimic natural recombination processes. Nexgen Plants respectfully seeks confirmation from the Biotechnology Regulatory Services (BRS) that the virus resistant tomato lines do not meet the definition of a regulated article under 7 CFR Part 340.

Nexgen Plants provides the information summarized in this letter to assist BRS in making its determination. Key information in support of our request includes:

1. The methodology used to create the virus resistant tomatoes did not introduce any foreign DNA sequences into the plant. The entirety of introduced DNA originates from the tomato genome and no foreign DNA, marker or selection gene sequences were introduced.
2. Expression of the introduced DNA constructs forms a double stranded “hairpin” structure that is processed into numerous short interfering RNAs (siRNAs) along the length of the

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double stranded hairpin. The siRNAs silence complementary gene transcript sequences, thereby reducing the amount of the targeted gene product[1,2].

3. RNAi approaches have been widely used to directly silence plant viruses to develop resistance[3].
4. The transfer of the introduced DNA constructs used direct gene delivery via particle bombardment of gel purified linearized DNA.
5. *S. Lycopersicum* is not a plant pest and does not pose a weed potential[4].
6. The method used to generate the virus resistant tomatoes results in plants with no introduced plant pest sequences, no expressed protein. The production of siRNAs do not generate a plant pest or pose an increase to weed potential.

Based on this information, Nexgen Plants considers the methodology to be a form a speed breeding and therefore the virus resistant tomato lines do not meet the definition of a regulated article based on 7 CFR Part 340.

The Intended Phenotype

Plant viruses remain a major threat to horticultural crops, in particular if the control of the vectors is not efficient or novel resistant virus strains occur. For example, Tomato Spotted Wilt Virus (TSWV)[5,6] is a persistent challenge faced by tomato growers.

TSWV is the type member of the genus *Tospovirus*, which contains the only plant-infecting members of the family *Bunyaviridae*. Control of TSWV in crop plants has proven difficult because of the wide range of plant hosts (>1000 species) and effective spread of TSWV by thrip vectors[7]. Intense efforts have been made around the world to obtain durable resistant cultivars against

[1] Voinnet O, Pinto YM, Baulcombe DC. (1999). Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proc Natl Acad Sci U S A. 96:14147–14152. doi: 10.1073/pnas.96.24.14147.
[2] Hamilton A, Baulcombe D (1999). A species of small antisense RNA in post-transcriptional gene silencing in plants. Science. 286 (5441): 950–2
[3] Weiwei Chen, Xian Zhang, Yaya Fan, Bin Li, Eugene Ryabov, Nongnong Shi, Mei Zhao, Zhiming Yu, Cheng Qin, Qianqian Zheng, Pengcheng Zhang, Huizhong Wang, Stephen Jackson, Qi Cheng, Yule Liu, Philippe Gallusci, Yiguo Hong. (2018). A Genetic Network for Systemic RNA Silencing in Plants. Plant Physiology 176 (4) 2700-2719; DOI: 10.1104/pp.17.01828
[4] OECD (2017), Safety Assessment of Transgenic Organisms in the Environment, Volume 7: OECD Consensus Documents, Harmonisation of Regulatory Oversight in Biotechnology, OECD Publishing, Paris. http://dx.doi.org/10.1787/9789264279728-en
[5] Saidi M, Warade SD (2008) Tomato breeding for resistance to Tomato spotted wilt virus (TSWV): an overview of conventional and molecular approaches. Czech J Genet Plant Breed 44:83–92
[6] Sherwood, J.L., German, T.L., Moyer, J.W. and D.E. Ullman (2003). Tomato spotted wilt. The Plant Health Instructor. DOI:10.1094/PHI-I-2003-0613-02, accessed January 2019 from https://www.apsnet.org/edcenter/disandpath/viral/pdlessons/Pages/TomatoSpottedWilt.aspx
[7] Hanssen, I. M., Lapidot, M. and Thomma, B. P. H. J. (2010). Emerging viral diseases of tomato crops. Mol Plant Microbe Interact 23, 539–548

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TSWV. Only the dominant gene *Sw-5*[8,9] has been found to confer resistance to a wide spectrum of TSWV isolates and this has been deployed in commercial cultivars worldwide. *Sw-5* is also effective against two other tospoviruses, Tomato chlorotic spot virus (TCSV) and Groundnut ringspot virus (GRSV)[10]. However, *Sw-5* resistance-breaking (SRB) isolates of TSWV have been detected in a number of countries[11] threatening commercial production.

The virus resistance approach of Nexgen Plants is based on using short interfering RNAs that bring a more durable resistance to such diseases. Our approach can also be applied to other viruses and other crops and contributes to an integrated plant health management framework. For example, the Nexgen Plants approach offers efficient resistance in tomato against the Cucumber Mosaic Virus (CMV) and TSWV by applying our methodology as described herein.

The introduced tomato DNA sequences are associated with increased virus resistance via the production of short interfering RNAs (Figure 1).

[8] Rosello S., Soler S., Diez M.J., Rambla J.L., Richarte C., Nuez F. (1999): New sources for high resistance of tomato to the tomato spotted wilt virus from *Lycopersicon peruvianum*. Plant Breeding, 118: 425–429.
[9] Rosello S., Ricarte B., Diez M.J., Nuez F. (2001): Resistance to tomato spotted wilt virus introgressed from *Lycopersicon peruvianum* in line UPV 1 may be allelic to Sw-5 and can be used to enhance the resistance of hybrid cultivars. Euphytica, 119: 357–367.
[10] Soler, S., Cebolla-Cornejo, J. and Nuez, F. (2003). Control of diseases induced by tospoviruses in tomato: an update of the genetic approach. Phytopathol Mediterr 42, 207–219
[11] Aramburu, J. and Martí, M. (2003). The occurrence in north-east Spain of a variant of Tomato spotted wilt virus (TSWV) that breaks resistance in tomato (Lycopersicon esculentum) containing the Sw-5 gene. Plant Pathol 52, 407.

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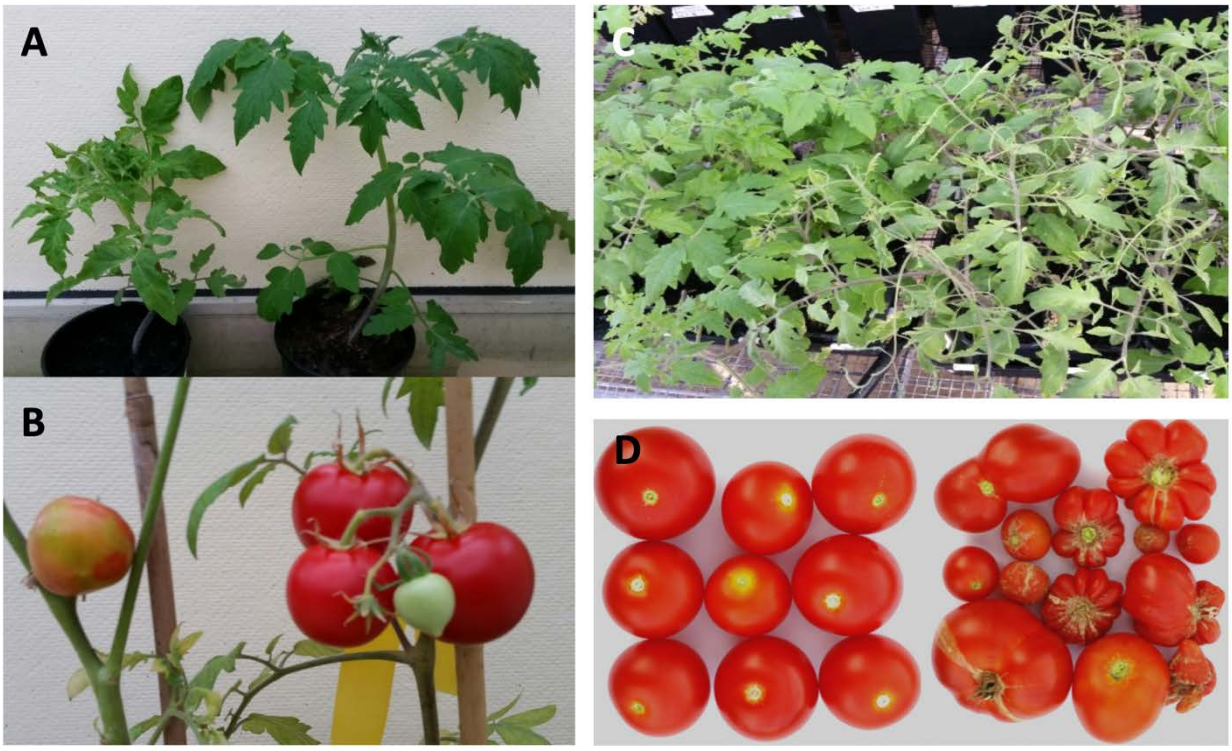

Figure 1. Virus resistant tomato using Nexgen Plants innovation.
A and B. *Left*, Tomato Spotted Wilt Virus (TSWV) susceptible and *Right*, TSWV resistant tomato plants; **C and D.** *Left*, Cauliflower Mosaic Virus (CMV) resistant and *Right*, CMV susceptible tomato plants.

The Intented Activity

Nexgen Plants intends to export virus resistant tomato seeds from Australia to the United States in accordance with USDA permits for the importation of tomato and tomato related articles under the authority of 7 CFR 319.55. The purpose is to conduct field evaluation trials and introgression of resistance into US tomato cultivars.

Developer name and contact information, including email address

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Development of Virus resistant tomato cultivars

The methodology described herein utilizes characterized tomato sequences to confer unique virus resistance. The entirety of introduced DNA sequences originates from the tomato genome. No foreign DNA, foreign marker or selection gene sequences are introduced.

RNA interference (RNAi) refers to a gene silencing technique. It involves designing a specific nucleotide sequence of the targeted gene sequence (typically >100 nucleotides) next to an inverted repeat of the same sequence. These forward and reverse sequences are linked by a loop sequence. When expressed, the construct forms a double stranded "hairpin" structure that can be processed into numerous short interfering RNAs (siRNAs) along the length of the double stranded hairpin. The siRNAs silence complementary specific gene transcript sequences, thereby reducing the amount of the targeted gene product. RNAi approaches have been widely used to directly silence plant viruses to develop resistance.

Construct Design and Insertion of the Intragenic DNA

Only native sequences from Tomato were used to design and develop a fully intragenic DNA expression cassette. Nexgen Plants selected the native promoter sequence from the tomato [ ] gene[12] and the native termination sequence from the tomato [ ] gene. The cassette contains the following genetic components : CBI-deleted, CBI-deleted

1. A native tomato [ ] promoter DNA sequence [ ]. CBI deleted, CBI deleted CBI deleted
2. A native tomato [ ] terminator DNA sequence [ ] with an additional native sequence at the end of the terminator [ ] containing enzyme cut sites and, CBI deleted, CBI deleted CBI-deleted, CBI-deleted CBI deleted
3. A specific tomato-derived siRNA DNA sequence. Nexgen Plants identified specifically siRNA DNA sequences (forward and reverse sequences) that confer increased resistance to viruses CMV and TSWV respectively. For Lines NP-TV101-1, NP-TV101-2, NP-TV101-3 conferring CMV resistance, a derived 399 bp siRNA DNA sequence was obtained by the assembly of ≥20nt native DNA fragments from the tomato genome sequence with an overall homology of 86% targeting three regions of the CMV RNA genome (see Table 1 for the full sequence information). For Lines NP-TV201-1, NP-TV201-2 and NP-TV201-3 conferring TSWV resistance, a derived 776 bp siRNA DNA sequence was obtained by the assembly of ≥20nt native DNA fragments from the tomato genome sequence with an

[12] [ ] CBI-deleted

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overall homology of 75% targeting four locations of the TSWV RNA genome (see Table 2 for the full sequence information). The forward and reverse tomato-derived siRNA DNA sequences were linked by a fully intragenic loop sequence of 74 bp that was obtained by the assembly of three native DNA fragments from the tomato genome [CBI-deleted
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]. The maps of the cloning vectors are provided CBI-deleted
in Figures 2 and 3.

The entirety of the introduced DNA originates from the tomato genome. The constructs contain no foreign DNA, foreign marker or selection gene sequences. Each of the tomato sequences used are publically available in the scientific literature and/or in the NCBI GenBank online resource.

The fully intragenic DNA expression cassettes were synthetised, cloned it into a pUC57 plasmid and the sequences were verified to confirm that the cloned expression cassettes contained no errors.

The tomato DNA constructs were linearized by restriction digestion using PmeI (a restriction endonuclease that recognizes the sequence GTTT^AAAC) and PmlI (a restriction endonuclease that recognizes the sequence CAC^GTG) that left only tomato sequences and separated from the plasmid backbone by agarose gel electrophoresis. The tomato constructs were subsequently sliced from the agarose gel, column purified, and used for tomato transformation via gold particle bombardment transformation.

The resulting tomato events mimic theoretically possible naturally occurring recombination events, with the production of siRNAs derived from native tomato DNA sequences and targeted against virus genomes.

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Table 1. Composite of tomato-derived native DNA sequences used to build the fully intragenic siRNA DNA sequence to target three regions of the CMV RNA genome.

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Table 2. Composite of tomato-derived native DNA sequences used to build the fully intragenic siRNA DNA sequence to target four regions of the TSWV RNA genome.

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Figure 2. Cloning Vector containing the fully intragenic CMV resistance expression cassette used to make Lines NP-TV101-1, NP-TV101-2, NP-TV101-3

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Figure 3. Cloning Vector containing the fully intragenic TSWV resistance expression cassette used to make Lines NP-TV201-1, NP-TV201-2 and NP-TV201-3

Phenotype of Virus Resistant Tomato Cultivars

Several experiments confirmed the virus resistance phenotype against the targeted virus and the trait inheritability. For example, virus resistance of T1 generation plants expressing the CMV RNAi intragenic cassette (Lines NP-TV101-1, NP-TV101-2, NP-TV101-3) was observed (see Figure 3).

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ELISA measurements were used to confirm a low virus titre in Lines NP-TV101-1, NP-TV101-2, NP-TV101-3 conferring CMV resistance and Lines NP-TV201-1, NP-TV201-2 and NP-TV201-3 conferring TSWV resistance (Figures 4 and 5 respectively)

CMV and TSWV plants expressing intragenic RNAi cassettes were healthy and did produce quality fruits without showing any negative phenotypic effect (data not shown).

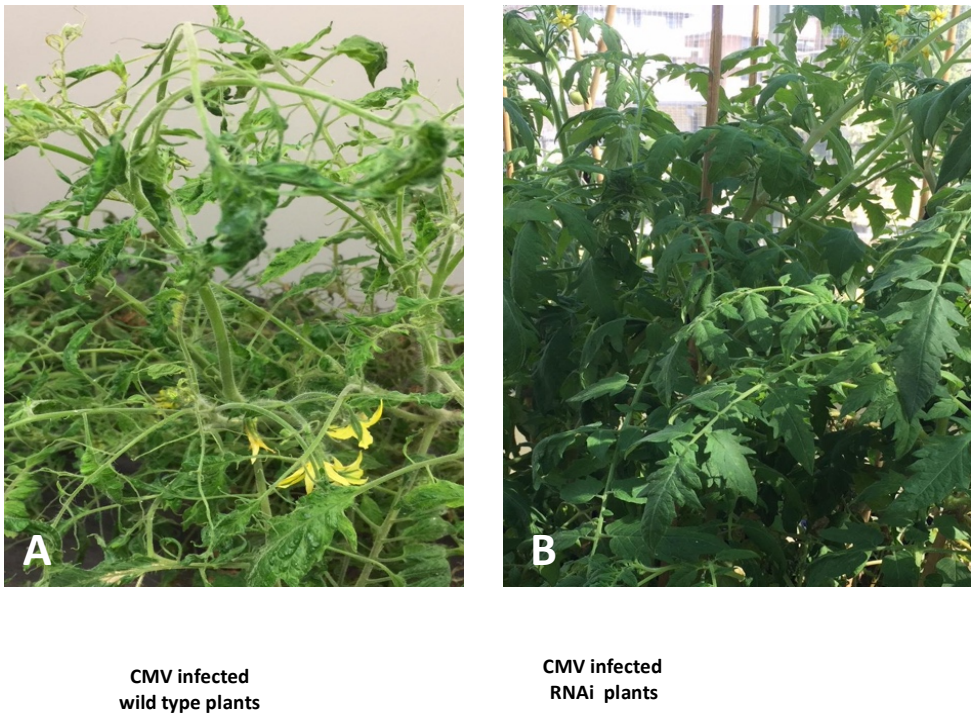

Figure 3. Tomato wild type and CMV RNAi plants (Line NP-TV101-1).
A Cauliflower Mosaic Virus (CMV) susceptible and **B.** CMV resistant tomato plants.

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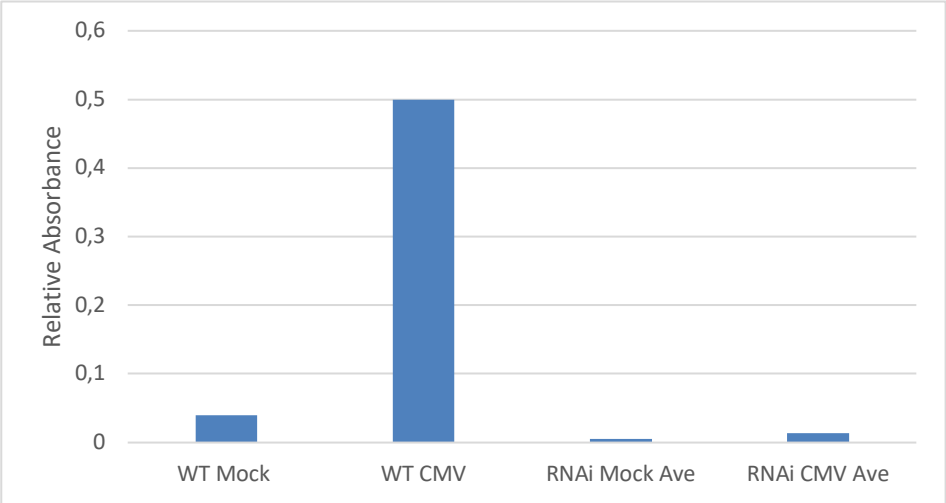

Figure 4. ELISA measurement of Wild type Mock, TSWV inoculated and T1 CMV RNAi plants (Lines NP-TV101-1, NP-TV101-2, NP-TV101-3)

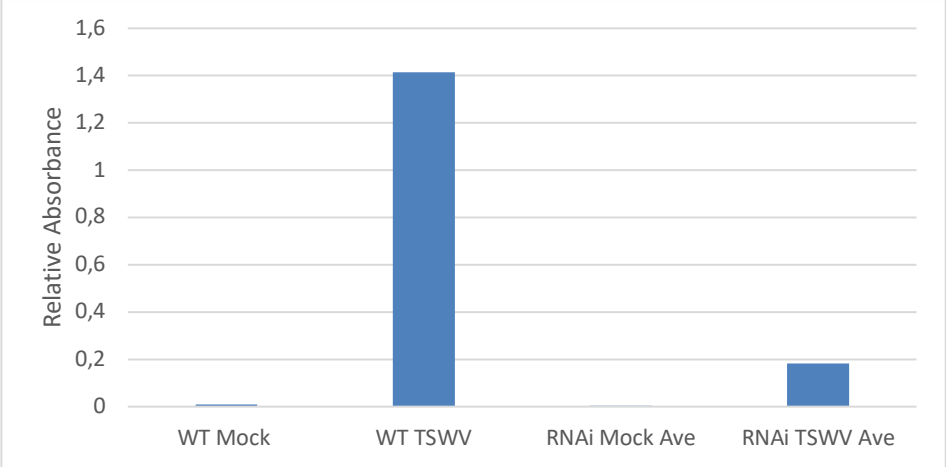

Figure 5. ELISA measurement of Wild type Mock, TSWV inoculated and T1 TSWV RNAi plants (Lines NP-TV201-1, NP-TV201-2 and NP-TV201-3)

Tomato is not a Regulated Article

Tomato (*Solanum Lycopersicum*) is not a federal noxious weed pursuant to 7 CFR 360[13].

[13] OECD (2017), Safety Assessment of Transgenic Organisms in the Environment, Volume 7: OECD Consensus Documents, Harmonisation of Regulatory Oversight in Biotechnology, OECD Publishing, Paris. http://dx.doi.org/10.1787/9789264279728-en

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No Plant Pest Risk or Increased Weed Potential

There is unlikely to be any effects to non-target organisms including beneficial, threatened or endangered species because the virus resistance trait is conferred by small interfering RNAs that are produced by the plant and not released to the environment. The introduced DNA does not lead to the production of any protein that may be toxic or allergenic.

Conclusions

Nexgen Plants has developed a methodology to generate virus resistant tomato plants using only native DNA sequences derived from the tomato genome. The method used to generate the virus resistant tomato results in plants with no introduced plant pest sequences, no expressed protein and the production of the intended small interfering RNAs will neither generate a plant pest nor pose a weed potential.

Nexgen Plants requests confirmation from BRS that the six virus resistant tomato lines described do not meet the definition of a regulated article under 7 CFR Part 340.

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