Westhoff Vertriebsgesellschaft mbH Petition (19-099-01p) for the Determination of Non-regulated Status for Petunias Containing the A1 Gene of Maize (A1-DFR petunias)

Plant Pest Risk Assessment

December 2020

Agency Contact
Cindy Eck
Biotechnology Regulatory Services
4700 River Road
USDA, APHIS
Riverdale, MD 20737

TABLE OF CONTENTS

A. Introduction

Westhoff Vertriebsgesellschaft mbH has petitioned the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) for a determination that genetically engineered (GE) petunia (*Petunia hybrida* Vilm.), which includes multiple events that will be collectively referred to as A1-DFR petunias, are unlikely to pose a greater plant pest risk than the unmodified organism from which they were derived and, therefore, should no longer be a regulated article under the APHIS' 7 Code of Federal Regulations (CFR) part 340. This petition was assigned the number 19-099-01p (hereafter referred to as Westhoff 2019). APHIS administers 7 CFR part 340 under the authority of the plant pest provisions of the Plant Protection Act (PPA) of 2000 (7 U.S.C. 7702 *et seq*.)[1]. This plant pest risk assessment was conducted to determine if A1-DFR petunias are unlikely to pose a greater plant pest risk than the unmodified organism with which they are compared or from which they were derived, which are referred to as "non-GE petunias" hereafter.

APHIS regulations in 7 CFR part 340 regulate the introduction (importation, interstate movement, or release into the environment) of certain GE organisms and products. A GE organism is no longer subject to the plant pest provisions of the PPA or the regulatory requirements of part 340 when APHIS determines that it is unlikely to pose a greater plant pest risk than the unmodified organism with which they are compared or from which they were derived. A GE organism is considered a regulated article under part 340 if the donor organism, recipient organism, or vector, or vector agent used in engineering the organism belongs to any genera or taxa designated in 7 CFR 340.2 and meets the definition of a plant pest, or is an unclassified organism and/or an organism whose classification is unknown, or any product which contains such an organism, or any other organism or product altered or produced through genetic engineering which the Administrator determines is a plant pest or has reason to believe is a plant pest[2]. A1-DFR petunias were produced by genetic transformation using a vector that contains, in addition to the dihydroflavonol 4-reductase (*A1*) gene from maize (*Zea mays*), the 35S promoter and terminator from cauliflower mosaic virus (CaMV), the nopaline synthase (*nos*) gene promoter and the octopine synthase (*ocs*) gene terminator from *Agrobacterium tumefaciens*. Because the donor organisms CaMV and *A. tumefaciens* are plant pests listed in 7 CFR 340.2, the GE A1-DFR petunias are considered regulated articles under APHIS regulations at 7 CFR part 340.

Westhoff Vertriebsgesellschaft mbH has provided information to support the conclusion that A1-DFR petunias are unlikely to pose a greater plant pest risk than non-GE petunias, in support of their request for a determination of nonregulated status.

---

[1] Plant Protection Act in 7 U.S.C. 7702 § 403(14) defines plant pest as: "Plant Pest - The term "plant pest" means any living stage of any of the following that can directly or indirectly injure, cause damage to, or cause disease in any plant or plant product: (A) A protozoan. (B) A nonhuman animal. (C) A parasitic plant. (D) A bacterium. (E) A fungus. (F) A virus or viroid. (G) An infectious agent or other pathogen. (H) Any article similar to or allied with any of the articles specified in the preceding subparagraphs."

[2] Limited exclusions or exemptions apply for certain engineered microorganisms and for interstate movement of some organisms, as in 7 CFR 340.1 and 340.2.(b).

Potential impacts in this Plant Pest Risk Assessment are those that pertain to plant pest risk associated with A1-DFR petunias and their progeny and their use in the absence of confinement relative to the unmodified recipient and/or other appropriate reference varieties. APHIS utilizes data and information submitted by the applicant, in addition to current literature, to determine if A1-DFR petunias are unlikely to pose a greater plant pest risk than the unmodified organism from which they were derived. APHIS regulations in 7 CFR 340.6(c) specify the information needed for consideration in a petition for a determination of nonregulated status. APHIS will assess applicant submitted A1-DFR petunia information related to plant pest risk characteristics; expression of the gene product, new enzymes, or changes to plant metabolism; disease and pest susceptibilities and indirect plant pest effects on other agricultural products; effects of the regulated article on non-target organisms; weediness of the regulated article; impact on the weediness of any other plant with which they can interbreed; changes to agricultural or cultivation practices that may impact diseases and pests of plants; and transfer of genetic information to organisms with which they cannot interbreed.

APHIS may also consider information relevant to reviews conducted by other agencies that are part of the 'Coordinated Framework for the Regulation of Biotechnology'(51 FR 23302 1986; 57 FR 22984 1992). Under the Coordinated Framework, the oversight of biotechnology-derived plants rests with APHIS, the U.S. Food and Drug Administration (FDA), and the Office of Pesticide Programs of the U.S. Environmental Protection Agency (EPA). However, because A1-DFR petunias are not intended to be used for food and feed and do not contain Plant Incorporated Protectants, additional regulatory reviews by the FDA and EPA are not required.

B. Cultivation, Development and the Regulatory History of A1-DFR Petunias

Petunia cultivation and usage

The garden petunia (*Petunia* x *hybrida* Vilm.) (syn. *P. atkinsiana* D. Don, or *Petunia hybrida*) was originally developed by Atkins in Great Britain in 1834 from *P. integrifolia* and *P. axillaris*, two of 14 Petunia species endemic to South America (Wijsman 1982; Stehmann et al. 2009; Reck-Kortmann et al. 2014; USDA-NRCS 2018; USDA ARS NGRS 2018; ITIS 2019).

Ornamental hybrid petunias were sold in the U.S. in and before the 1890s (Craib 2016), and at least 1591 petunia varieties are available in the U.S. (All-America Selections 2018; Costa Farms 2018; National Gardening Association 2018; Swallowtail Garden Seeds 2018; Vite Greenhouses 2018). The USDA's National Agricultural Statistics Service documents large scale production of horticultural varieties of petunia in every state but Nevada (USDA-NASS 2015), but it can be assumed that petunias are also grown in Nevada.

Petunias are sold as seeds or plants in pots and baskets for display or bed planting (USDA-NASS 2015). For commercial production, petunia is cultivated by a limited number of experienced growers inside horticultural greenhouse settings before the mature plants being sold to consumers (Westhoff 2019). F_1-hybrids accounts for 99% of the varieties on the market. To maintain the key ornamental traits of F_1-hybrids, propagation is done through cuttings, primarily by highly specialized nurseries as petunia is sensitive to many plant viruses that can be spread through the vegetative propagation process. For this reason, most consumers (except hobbyists) usually do not harvest their own seeds nor propagate plants via cuttings (Westhoff 2019).

Petunia x hybrida is often grown as an annual but is also a tender perennial in hardiness zones 9 to 11 of the USDA Plant Hardiness Zone Map (Old Farmer's Almanac n.d.). The plant can be severely injured at 5°C if un-acclimated, prefers full sun and is not adapted to full shade or wet sites (Pennycooke et al. 2003; Ohio State University 2018). Petunia hybrids are drought tolerant, which is not surprising as its parent species, *P. axillaris* and *P. integrifolia* are known to inhabit rocky sites or are able to grow in sand, respectively (Stehmann et al. 2009).

A1-DFR petunia development

The anthocyanin biosynthesis pathway

Flower color is one of the most important traits in the floriculture industry. It determines the consumer's preference and affects commercial values. A broad range of flower colors exists in nature. However, some colors are available only in certain ornamental plant species. The three major floral pigments are flavonoids, carotenoids, and betalains. Among these three floral pigments, the flavonoids contribute most to the range and type of colored pigments in plants. Flavonoids consist of several classes of compounds such as anthocyanins, aurones, chalcones, flavones, and flavonols. Anthocyanins confer orange, red, magenta, violet, and blue colors. Aurones and chalcones are yellow pigments while flavones and flavonols are colorless or very pale yellow. Anthocyanins and flavonols are the pigments responsible for flower color in petunia (Fig.1).

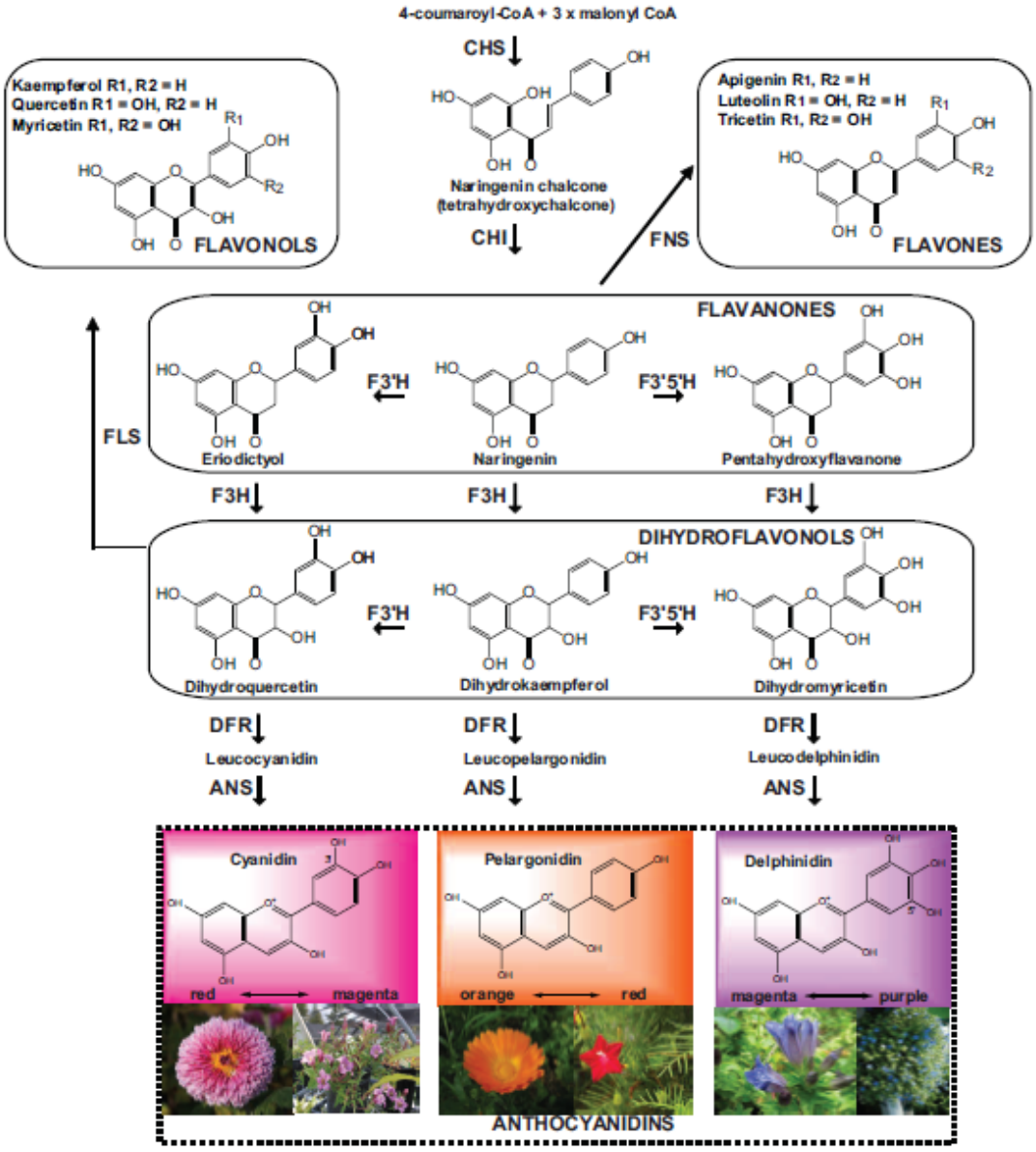

Fig.1 A simplified scheme of the biosynthesis of anthocyanidins- cyanidin, pelargonidin, and delphinidin. The enzymes catalyzing each step are indicated in bold. Abbreviations include: CHS- chalcone synthase; CHI- chalcone isomerase; FNS- flavone synthase; F3H-flavanone 3-hydroxylase; F3'H- flavonoid-3'-hydroxylase; F3'5'H- flavonoid 3',5'-hydroxylase; DFR- dihydroflavonol 4-reductase; ANS-anthocyanidin synthase (from Falcone Ferreyra et al. 2012)

Petunia x hybrida has been used as one of the classical plant species for studying anthocyanin pigmentation since the 1980s, so the biochemistry and genetics of anthocyanin biosynthesis had been well studied (Meyer et al. 1987). Dihydroflavonol 4-reductase (DFR) is specifically involved in anthocyanin biosynthesis and catalyzes the

reduction of the dihydroflavonols dihydroquercetin, dihydromyricetin and dihydrokaempferol to the flavan-3,4-cis diols leucocyanidin, leucodelphinidin and leucopelargonidin, which are the immediate precursors for the respective anthocyanidins cyanidin (red to magenta), delphinidin derivatives (magenta to purple) and pelargonidin (orange to red). Petunia DFR, in contrast to maize A1-DFR, cannot use dihydrokaempferol as a substrate for conversion to leucopelargonidin. Therefore petunias synthesize cyanidin- and delphinidin- derivatives, but not pelargonidin-derivatives.

To easily identify the color of pelargonidin derivatives when the maize A1-DFR gene was expressed in petunia, a mutant line RL01 was chosen as the parental line for genetic transformation because its genetic background does not allow synthesis of the normal amount of cyanidin and delphinidin, the two pigments normally expressed in petunia.

Transformation System

A1-DFR petunias were developed by direct gene transfer of synchronized M-phase protoplasts in osmoticum (Meyer et al. 1985). The protoplasts isolated from RL01 parental petunia lines were transformed with p35A1 plasmid DNA that contains both the gene of interest maize A1-DFR, and the selectable marker gene *nptII* (Meyer et al. 1987;). The transformed protoplasts were cultured using the agarose bead technique with kanamycin as the selection agent (Meyer et al. 1985). The transformed microcalli were then cultured on a regeneration medium containing kanamycin (50 mg/L) to select cells that express the *nptII* gene. Two transformed plants showed a brick red coloration on the flower rather than the pale pink from the remnants of cyanidin and delphinidin (Meyer et al. 1987). One of them (RP235-15) showed strong mRNA expression of the maize *A1* gene and an anthocyanin peak at 512 nm, a shift from 528 nm of RL01 parental line. These analyses and the many publications since then confirmed that Meyer et al. (1985) indeed modified petunia with the maize *A1* gene.

A1-DFR petunia developing, marketing, and regulatory history

Originally generated for scientific research purposes more than 30 years ago, the petunia with genetically engineered flower color was of interest to the horticultural industry, as color is a major contributor to the total value of ornamental flowers. In 1992, APHIS issued a permit (92-260-01r) to allow a field trial of genetically engineered petunias in Florida for two independent transformation events (USDA-APHIS 1992). In 1995, Oud et al. published the Florida field trial results, which indicated that the orange F4 and F5 breeding lines generated from events 235/1-15 and 235/1-17 had improved orange flower color in combination with good general performance. In May 2017, USDA became aware that GE petunia that could be tracked back to the A1-DFR transformation had been imported, moved between states in the United States, and released into the environment without the required USDA authorization. In April of 2019, USDA received the Westhoff petition 19-099-01p to deregulate a total of 23 A1-DFR events, which were listed in Linn et al. (1990) paper as well as Meyer et al. (1987), Pröls and Meyer (1992), and Oud et al. (1995). The Westhoff petition, on the other hand, listed 15 GE varieties, including 11 in which the maize A1-DFR construct region was sequenced, and confirms

that, the vector construct that was used to obtain the 23 transformants is maintained in the unintentional GE petunia varieties (Bashandy and Teeri 2017; Haselmair-Gosch et al. 2018; Westhoff 2019). However, the relationship between each event and the variety is not established (Westhoff 2019).

It is possible that petunias with A1-DFR gene were crossed to various conventional varieties and sold on the market for more than a decade (Servick 2017; Westhoff 2019) without breeders, growers, or sellers realizing their GE origin. The varieties bearing the new and brilliant color from A1-DFR trait were best sellers in the market before it became known that they were GE-petunias (Bashandy and Teeri 2017; Haselmair-Gosch et al. 2018; Westhoff 2019). Although there are no reports of negative effects on human health or environment from any particular type of petunia, it is illegal to import, move interstate, or release into the environment without an APHIS authorization per 7 CFR part 340. Since the discovery of unauthorized GE petunia in the market, USDA has worked with breeders to screen suspected GE petunias using CaMV 35S primers and asked the growers and sellers to destroy their inventory of a total of 124 varieties, which are listed on the APHIS Website (USDA-APHIS 2017b). However, these 124 GE varieties are not necessarily only A1-DFR petunia created by Meyer et al. in the 1980s. This is because the sequences from the CaMV 35S promoter alone but not those from the expressed flower color gene A1-DFR were used for screening. Additionally, petunias with flavonoid-3', 5'-hydroxylase gene were also detected by CaMV35S promoter screening in the European market (Yukihisa et al. 1999; COGEM 2017; ZKBS 2017). Regardless of the likely original events of A1-DFR petunia or any other sources of GE petunia, the scope of this PPRA analyzes the 23 A1-DFR events listed in the Westhoff petition (2019).

C. Description of Inserted Genetic Material, Its Inheritance and Expression, Gene Products and Changes to Plant Metabolism

To inform the potential hazards resulting from the genetic modification and potential routes of exposure related to the inserted DNAs and their expression products, APHIS assessed data and information presented in the petition related to: the transformation process; the source of the inserted genetic material and its function in both the donor organism and the GE crop event; and the integrity, stability and mode of inheritance of the inserted genetic material through sexual or asexual reproduction based on the location of the insertion (e.g., nucleus or organelle) and the number of loci inserted.

APHIS also assessed data presented in the petition on whether the genetic modification results in the expression of new genes, proteins or enzymes, or causes changes in plant metabolism or composition in A1-DFR petunia relative to the parental control variety and other petunia comparator varieties. The assessment encompasses consideration of both the transformation vector p35A1, the transformation method, and the integrated segments identified in the Westhoff petition and scientific publications.

This information is used later in this risk assessment to evaluate whether there is any potential for plant pest vectors or sequences to cause disease or greater plant pest risks in

the GE crop event; or for expression of inserted DNAs, new proteins or enzymes, or changes in metabolism that would affect plant pests or diseases, beneficial organisms, weediness, agricultural practices that impact pests or diseases or their management, or plant pest risks through horizontal gene flow.

Description of the genetic modification and inheritance of inserted DNA

A1-DFR petunia was developed by transforming protoplasts of petunia with DNA of plasmid p35A1 and involved no live plant pathogen (Hain et al. 1985; Meyer et al. 1987).

Plasmid Vector p35A1 as integrated into A1-DFR petunia

The transformation vector p34A1 is ~8 kb with the following elements integrated into the parental petunia line RL01 from 5' to 3' end (single underlined is A1-DFR cassette and double underlines is *nptII* cassette): truncated beta-lactamase sequence, 35S promoter from CaMV (cauliflower mosaic virus), dihydroflavonol 4-reductase (A1) gene from *Zea mays*, the 35S terminator from CaMV, an EcoR I site + lacUV5 fragment from *Escherichia coli (E. coli)*, nopaline synthase promoter (*pnos*) from *A. tumefaciens*, neomycin phosphotransferase (*npt II*) gene from *E. coli*, and octopine synthase (*ocs*) poly A terminator region from *A. tumefaciens* (Meyer et al. 1987).

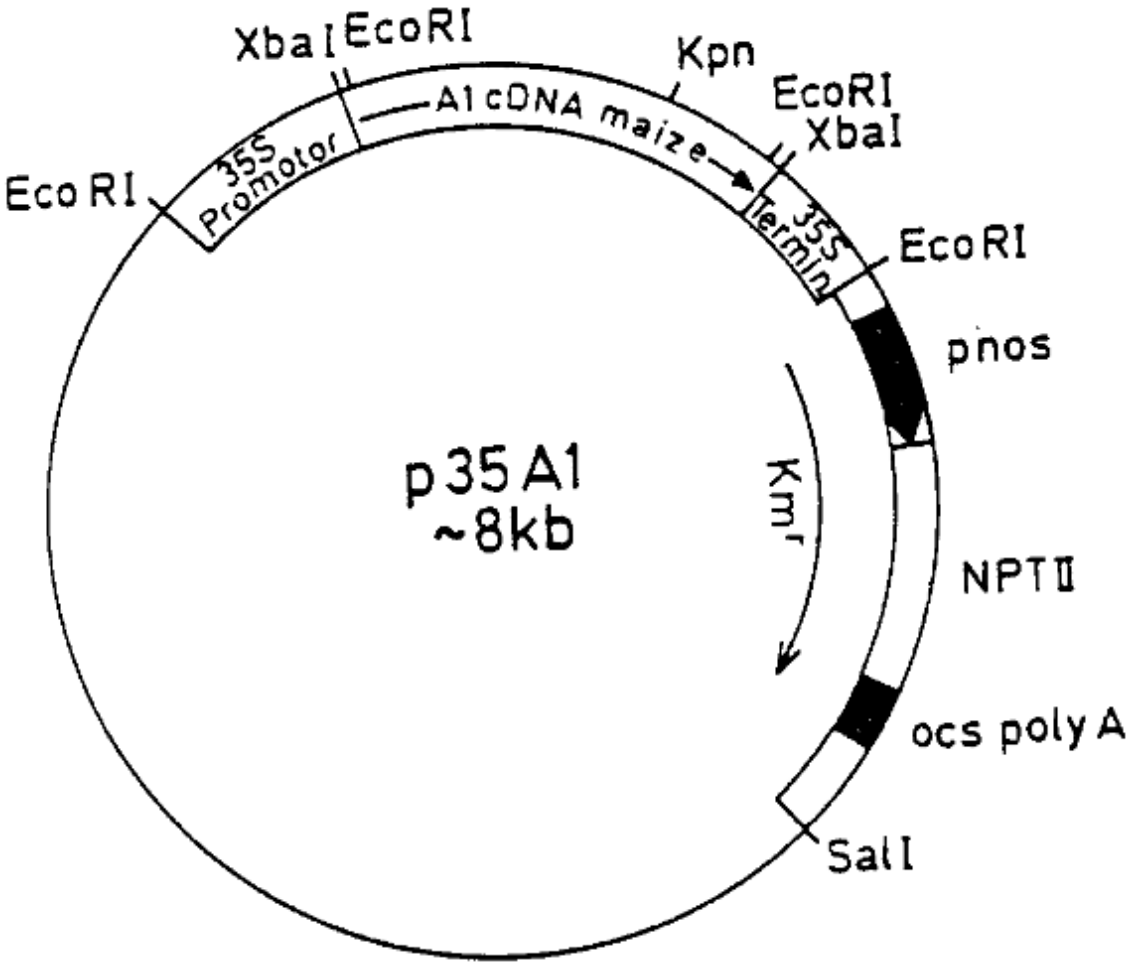

Figure 1. The elements of plasmid p35A1. CaMV35S promoter drives a 1320 base pair (bp) cDNA clone of a type 2 A1 gene of *Zea mays*, whose transcription is terminated by 35S terminator. The *Eco*RI restriction sites were filled in and *Xba*I-linkers were attached, which restored the filled in *Eco*RI sites. The *Xba*I fragment was cloned into the unique *Xba*I site of plasmid pCKanI, where it is located between 35S promoter and terminator sequence of CaMV. An *Eco*RI site + lacUV5 fragment was located between 35 terminator and the nopaline synthase gene promoter (*pnos*). The lacUV5 promoter is a mutant of the lactose operon promoter from *E. coli* and also functions as an efficient transcription terminator (Bogosian and Kane 1987). *Pnos* drives *nptII* gene expression to allow for the selection of transformants by kanamycin. The expression of the *nptII* gene is terminated by *ocs* poly (A), taken from Westhoff (2019).

- The gene of interest is driven by CaMV35S, a constitutive promoter used to provide high levels of gene expression. The maize A1 gene encodes dihydroflavonol 4-reductase which catalyzes the conversion of dihydrokaempferol into leucopelargonidin before being converted into the pelargonidin pigment. The transcript is terminated by the 35S terminator.

- The selectable marker cassette contains the nopaline synthase (*nos*) gene promoter and *ocs* poly (A) terminator from *A. tumefaciens*. The *nptII* gene from *E. coli* encodes aminoglycoside 3'-phosphotransferase (denoted *aph*(3')-II or NPTII) enzyme, which catalyzes the addition of phosphate to and the deactivation of kanamycin. This will allow the transformed protoplasts to survive in tissue culture media containing kanamycin.
- p35A1 was constructed from pLGV1103 which is an *E. coli* plasmid cloning vector and contains no plant pest sequence(Velten et al. 1984; Hain et al. 1985; Standford Univ. 1997).
- A sequence segment of a non-viral transposable element named Cin4-1(Schwarz-Sommer et al. 1987) was detected at the 3' end of the A1 DFR sequence. The Cin4-1 sequence is a natural gene of maize and does not add any functional advantage with respect to DFR activity (Haselmair-Gosch et al. 2018).

Among the above transgene elements, the CaMV35S promoter and terminator are derived from a plant pest (7CFR §340.2) cauliflower mosaic virus; the *pnos* promoter and the *ocs* terminator are derived from *A. tumefaciens*, also a plant pest. None of the genetic elements are known to cause plant diseases and have regulatory functions. The above information also supports the conclusion that the vector sequence is not derived from plant pests and therefore not relevant to plant pest risk.

Characteristics, Stability, and Inheritance of the Introduced DNA

Westhoff has provided data and literature references to characterize the inserted transgene DNA in A1-DFR petunias with a combination of methods, including Southern and Northern blot analysis, PCR, and DNA sequencing.

Southern blot analysis, recloning of the integrated A1 gene, determination of its allelic site in RL01 and sequencing of the plant-plasmid junctions, as well as the parental allelic site, revealed that only one copy of plasmid p35A1 DNA had integrated into 235/1-17. It also indicated that part of the ampicillin resistance gene and the origin of replication of the plasmid were deleted. While part of the plasmid is deleted, 215 bp of the host DNA has also been lost at the site of integration (Meyer et al. 1992). While the Meyer 1992 paper did not provide detailed information about the function and chromosome location of the deleted 215 bp host sequence, the research paper did not mention any phenotype that could potentially be attributed to such a deletion. Pröls and Meyer (1992) characterized the integration site of transgene DNA for events 235/1-16, 235/1-17, and 235/1-24 to gain an understanding of flanking regions. Probing poly(A)$^+$ RNA from leaves of parental RL01 with DNA sequences flanking the integration sites of the three events did not reveal any transcripts detectable by Northern blot analysis (Pröls and Meyer 1992). Further, deletion does not pose a new plant pest risk since no new plant pest sequence is introduced. Therefore, event 235/1-17 contains only the A1-DFR gene and the *nptII* gene from p35A1 plasmid driven in a functional state and the insertion is unlikely to interrupt a normally functional gene in petunia.

F_1 progeny from the crosses between GE petunia and a conventional breeding line were not orange, due to the presence of dominant allele from the conventional line. Orange

flower color plants were found in F_2 populations after those recombinant offspring were self-pollinated, which indicates the maize dihydroflavonol 4-reductase (A1) gene segregates and follows Mendelian inheritance. The stability of the transgene integration in the A1-DFR petunia genome (event 235/1-17) was demonstrated by Mayer et al. (1992). Heterozygous transformant 235/1-17 went through backcross, selfing, vegetative regeneration, and backcross again to obtain seeds. Out of 31000 plants produced, only four white flowering plants derived from the same capsule were shown to be mutants in which part of the A1-DFR gene had been deleted. The estimated mutation rate for those constant white plants is $7x10^{-5}$ (Meyer et al. 1992).

Southern blot of event 235/1-24 also indicates that one A1 cDNA copy integrated at one genomic locus without obvious rearrangements or deletions (Linn et al. 1990; Westhoff 2019). This vector construct has been maintained in the unintentional GE petunia varieties discovered in 2017 as the one used to obtain the original transformants by Dr. Mayer (Meyer et al. 1987; Bashandy and Teeri 2017; Haselmair-Gosch et al. 2018; Westhoff 2019).

Expression of inserted DNA, changes in gene expression, new proteins or metabolism, and toxicity and allergenicity

A1-DFR petunias express two recombinant proteins, i.e., the maize A1-DFR protein and the bacterial NPTII protein.

A1-DFR protein

The transcript for the maize dihydroflavonol 4-reductase gene was detected in the transgenic petunia event 235/1-15 but not in the non-transgenic petunia; the proper function of the gene is revealed by the spectrophotometric peak of 512 nm and the formation of brick red-colored flowers (Meyer et al. 1987; Westhoff 2019). Northern blot analysis of A1 gene activity in events 16 (235/1-16/2), 17 (235/1-17), 24 (235/1-24) suggests that the differences in pelargonidin production resulted from the differential transcription of the A1 gene and that the *nptII* gene is also expressed at corresponding levels (Pröls and Meyer 1992).

The A1-DFR gene is derived from maize and is naturally expressed in different genotypes and tissues (Bernhardt et al. 1998). The amino acid sequence coded by the A1-DFR gene transformed into petunia is nearly identical (99%, with one change in position 22 out of 357 amino acids) to hypothetical protein Zm00014a_011529 in maize (NCBI 2019; Westhoff 2019). Maize kernel and stalk have a long history of safe consumption by people and animals as food and feed, and maize flowers are regularly visited by insect pollinators without ill effects. In Australia, GE carnations containing petunia DFR (a homolog of maize A1-DFR) were approved for commercial uncontained releases (OGTR 2004) after being assessed as being of negligible risk to people and the environment. Thus, the maize A1-DFR protein is not expected to be toxic or allergenic to people or other organisms, including insect pollinators (OGTR 2004; Westhoff 2019).

NPTII Protein

The *nptII* gene encodes an enzyme that confers resistance to kanamycin, which was used as a selectable marker during A1-DFR petunia development. Rooting assays revealed that the *nptII* activity in the events 16, 17, and 24 corresponded to the differences observed for A1-DFR activity (Pröls and Meyer 1992). The *nptII* gene is the most frequently used selectable marker gene for generating transgenic plants and it is found in many crops currently approved for commercial production, including corn, potato, oilseeds, tomato, papaya, petunia, flax, and chicory (Miki and McHugh 2004; Goldstein et al. 2005; USDA-APHIS 2017). The food, feed and environmental safety of the NPTII protein has been evaluated extensively in both the peer-reviewed literature and by regulatory authorities of different countries, with no reports of adverse effects such as toxicity or allergenicity on humans, animals or the environment (FDA 1998; Miki and McHugh 2004; Goldstein et al. 2005).

Metabolism composition Analysis

Flavonoids are ubiquitous in angiosperms with extremely diverse chemical structure, color, and biological function. Anthocyanidins and their corresponding glycosides (anthocyanins), including pelargonidin, are flower and fruit pigments. Flavonols, the co-pigments of anthocyanidins and anthocyanins, may play a role in UV protection, disease resistance, and/or hormone signaling (Dudek et al. 2016). The A1-DFR genetic modification in petunia results in the biosynthesis of anthocyanin only in the petals of the flower. This is because the substrates of the pathway (required for pelargonidin biosynthesis in the transgenic plants) are only produced in flowers (Westhoff 2019). Pelargonidin content was tested in the petal and leaf parts of petunia. While some petunia varieties revealed the presence of high levels of pelargonidin, indicating expression of the A1-DFR gene, no pelargonidin was detected in the leaf parts of the GE petunia varieties and non-GE petunias (Westhoff 2019). Pelargonidin production in flower as a result of A1-DFR modification is not expected to incur any plant pest or disease risks.

The A1-DFR gene increased pelargonidin by about 10 fold in flowers when compared with parental line RL01; the amount of cyanidin and delphinidin was also increased by the genetic modification but to a smaller degree. However, the increase in anthocyanin production in the transgenic plants resulted in a corresponding molar decrease in flavonol accumulation (Griesbach 1993). The plant defense related metabolites lignin and scopoletin are also derived from phenylalanine, like flavonol and anthocyanin (Schenke et al. 2011). However, their biosynthetic pathway is much upstream of flavonol and anthocyanin synthesis. A1-DFR modification is also unlikely to change lignin and scopoletin in flower or other parts of a petunia plant.

D. Potential Plant Pest and Disease Impacts

APHIS assessed whether potential plant pest or disease impacts are likely to result from the transformation process, from DNA sequences from plant pests, or from any other expression products, new enzymes, proteins or changes in plant metabolic products in

A1-DFR petunia that are known or anticipated to cause disease symptoms or to affect plant pests or diseases or plant defense responses (as identified from the previous section). APHIS also assessed whether A1-DFR petunia is likely to have significantly increased disease and pest susceptibility based on greenhouse and garden observations. Impacts or changes are assessed to determine if they would (1) affect the new GE crop and/or result in a significant introduction or spread of a damaging pest or disease to other plants; (2) result in the introduction, spread, and/or creation of a new disease; and/or (3) result in a significant exacerbation of a pest or disease for which APHIS has a control program. Any increase in pest or disease susceptibility is evaluated with respect to the context of currently cultivated varieties, the ability to manage the pest or disease, and the potential impact on agriculture.

Plant Protection and Quarantine (PPQ) is an APHIS program that safeguards agriculture and natural resources from the entry, establishment, and spread of animal and plant pests and noxious weeds into the United States of America; and supports trade and exports of U.S. agricultural products. PPQ responds to many new introductions of plant pests to eradicate, suppress, or contain them through various programs in cooperation with state departments of agriculture and other government agencies. These may be emergency or longer term domestic programs that target a specific pest. A variety of insect, plant disease, mollusk, nematode or weed programs exist (USDA-PPQ 2017); however, none of these programs specifically target pests of petunia.

Petunia itself is not a plant pest in the United States (7 CFR part 340; USDA-NRCS 2019). The inserted DNA elements in the GE petunias that are derived from plant pests do not result in the production of infectious agents or disease symptoms in plants. The protoplast transformation process involves no live plant pathogen and the backbone of the plasmid used for transformation is not derived from a plant pest. In addition, the portions of the inserted genetic material derived from plant pests, i.e., the promoter and terminator sequences from Cauliflower mosaic virus and *A. tumefaciens*, do not result in the production of infectious agents or disease symptoms in plants. The introduced genetic elements are thereby not expected to impart any new plant pest or disease risk even though some of these construct components are derived from plant pathogens. As reasoned in the previous section, the metabolic changes caused by the expression of the A1-DFR gene are unlikely to change the plant defense response to plant pests.

The maize A1-DFR allele had been spread into most petunia breeding programs in many countries, likely by unintentional integration before the discovery of the unauthorized petunia in 2017. The petunia lines involved in those breeding programs are of very variable phenotypes, i.e., different habits from upright mounding to trailing, different flower sizes and different flower colors and color patterns. As discussed in the previous section, the production of pelargonidin in A1-DFR petunia is not expected to alter plant insect or disease susceptibility. Horticultural trials showed no difference between GE and non-GE petunias for all evaluated traits (Westhoff 2019). The traits evaluated were: flower quantity and quality, vegetative vigor, flower number, plant height and width, flowering quantity and total performance in the early and later summer, and the general impression evaluation by a group of horticulture specialists. GE and non-GE petunias are

so similar that the identification of GE petunia is impossible other than by traits of orange flower and genetic or chemical tests (Westhoff 2019). Pests such as slugs, thrips, and aphids have the same preference for modified and non-modified petunia (Westhoff (2019.) Differences in mildew disease susceptibility are known to exist between varieties but not with regard to GE and non-GE group (Westhoff 2019). Therefore, the genetic modification does not have any effect on pests and diseases (Westhoff 2019). During the history (up to 7 years) of handling of GE petunias by Westhoff, the varieties that were found to be genetically engineered did not appear different from other conventional varieties for traits such as heat, cold, flood, disease or insect tolerance, attractiveness to pollinators, or flowering time (Westhoff 2019).

Unintended alterations of the scent by A1-DFR modification cannot be excluded as the scent of GE petunia has not been evaluated in detail. Floral herbivores (beetle and cricket) could be influenced by altered scent profiles of transgenic petunia (Kessler et al. 2008). As a result, there might be a theoretical chance for the A1-DFR gene to change petunia pest risk indirectly, i.e., making GE flowers more resistant or susceptible to insect damage. However, since most commercial petunias are produced under controlled environmental conditions and with necessary pest management and then are used by consumers in small volumes, it is hard to imagine any change in pest risk that will have a wide effect. Moreover, Westhoff has not noticed any difference including scent between the GE and non-GE petunias in their many years of observation; there have never been any complaints about problems in that regard during the long history of commercial distribution of unintended GE petunia (Westhoff 2019). Thus there is no evidence to support the hypothesis that the plant pest risk of GE petunia might be changed by altering flower scent or other factors.

E. Potential Impacts on Non-target Organisms Beneficial to Agriculture

A1-DFR petunia is not engineered for pest resistance; it is engineered for altered flower color through the production of pelargonidin. Thus there is no 'non-target' species for this GE petunia. APHIS assessed whether exposure or consumption of A1-DFR petunia would have a direct or indirect adverse impact on non-target species beneficial to agriculture. Organisms considered were representatives of the species associated with the production of the GE crop in the agricultural environment. The assessment includes an analysis of data and information on A1-DFR petunia compared to the non-GE varieties for any relevant changes in the phenotype or substances produced that may be novel or significantly altered in amounts. Such substances will be evaluated for their impacts on organisms beneficial to agriculture, and/or on any beneficial organisms associated with the plants.

As described above in Section C, neither of the proteins expressed by the inserted genetic material in A1-DFR petunia is toxic. The *nptII* gene and its products were previously evaluated, and no identifiable human and environmental safety issues for the use of the *nptII* gene in genetically engineered plants and plant products were identified (Nap et al. 1992; Miki and McHugh 2004; USDA-APHIS 2017). As for the A1-DFR gene, it originates from maize, which is a staple food for the human. The gene product,

pelargonidin, is a common anthocyanin that occurs in several food plants and is the main pigment in strawberry (Lopez and Runkle 2005; Zamora-Ros et al. 2011). Humans or animals consuming these foods, therefore, consume the A1-DFR protein, pelargonidin, and pelargonidin derivatives. Anthocyanins as a group have a very low acute toxicity and there are no reports of adverse health effects from usual human consumption (Burton-Freeman et al. 2016). As an ornamental crop, petunia is not intended to be used as food or feed; thus, there is no dietary exposure pathway for humans or livestock.

No pelargonidin was detected in leaves of GE petunia varieties and non-GE petunias (Westhoff 2019). The anthocyanin biosynthesis pathway substrates required for pelargonidin biosynthesis in the A1-DFR transgenic plants are only produced in flower. The genetic modification results in the biosynthesis of anthocyanin only in the petals of the flower and there is no known effect on herbivores as mentioned above. Also, as mentioned in section D, no differences were observed for susceptibility to slugs, thrips, and aphids between GE and non-GE varieties.

A1-DFR petunia is not expected to negatively impact non-target organisms beneficial to agriculture, either from the expression of the A1-DFR color trait gene or from the *nptII* selectable marker gene. There is no observed difference between GE and non-GE petunias varieties other than the flower color. The production method of petunia in greenhouses by vegetative propagation and commercial use as an ornamental plant makes it less likely that beneficial organisms would rely on petunia to play a beneficial role in agriculture outside the small-scale or controlled environment. Therefore, APHIS concludes that the GE petunias are unlikely to have any adverse impacts on non-target organisms beneficial to agriculture.

F. Potential for Enhanced Weediness of A1-DFR Petunia

APHIS assessed whether the GE petunia is likely to become weedier (i.e., more prevalent, competitive, damaging or difficult-to-control in situations where it is not wanted) than the non-GE petunias. The assessment includes (1) consideration of the weediness of the plant including the situations in which garden volunteers or feral populations are considered weeds, and (2) an evaluation of the GE petunia compared to non-GE petunias evaluated under field (and/or lab) conditions characteristic for the regions of the United States where A1-DFR petunia is intended to be grown. The characteristics for the evaluation of the GE petunias are related to the establishment, competitiveness, reproduction, survival, persistence and/or spread, features that could influence weediness and the ability to manage the crop as a weed. For this crop, such characteristics include seed dormancy and germination, disease and pest susceptibility, tolerance to stresses including cold and drought, and competitive ability. The assessment also considers whether the engineered trait affects methods of control for the crop in situations where it is managed as a weed or volunteer in subsequent crops or feral populations.

(1) Consideration of the weediness of the plant

Petunia x *hybrida* is not known for its invasiveness; even in the southeastern U.S. where the climate is most similar to the habitat of the parental lineages of *Petunia* x *hybrida* (subtropical South America), it is not listed as an invasive plant (Miller et al. 2011). Nor has *Petunia* x *hybrida* been listed as a plant that influences plant communities in Florida (FLEPPC 2007). Since the warm climate of Florida does not favor the uncontrolled development of petunia, it can be expected that in other US states, there is even less likelihood of a negative impact due to petunia, which is not cold hardy. In Australia, *Petunia* x *hybrida* has naturalized in New South Wales and Queensland, but naturalized petunia is not considered a weed of national concern (OGTR 2017). OGTR concludes that "…unwanted petunias could be minor weeds in gardens, but probably only if the gardens are in a warm climate and frequently watered" (OGTR 2017).

(2) General comparison of A1-DFR petunia to non-GE petunias.

All transgenic *Petunia* x *hybrida* plants that originated from the two events (235/1-15 and 235/1-17) studied by Oud et al. (1995) had a normal appearance when compared to non-transgenic control plants, which makes sense as the trait is intended to alter the petal color, but not the growth or spread of the plant. The marker gene is also not expected to change the plant ecology in substantive ways. The substrate specificity of the marker gene aminoglycoside-3'-phosphotransferase II suggests that no new metabolic products will be created in the GE petunias under field conditions. This gene does not confer a selective advantage on the GE plants under field conditions, as aminoglycoside antibiotics such as kanamycin and neomycin do not prevail in the soil in concentrations detrimental to plants. The ecological impact of the use of the *nptII* gene in crops has been extensively reviewed, with the conclusion that kanamycin resistance would not lead to enhanced weediness of a *nptII* gene-expressing plant (Nap et al. 1992; Miki and McHugh 2004).

(2a) Comparison of A1-DFR petunia to non-GE petunias with regard to seed dormancy and germination

Although the production of flavonoids in the seeds may be associated with dormancy breaking in the seed, seed dormancy is also determined by many other characters such as hormone levels or sensitivity (Finkelstein et al. 2008). There is no evidence that altering flavonoid pathways in the petals leads to changes in dormancy and germination.

(2b) Comparison of A1-DFR petunia to the non-GE petunias with regards to disease and pest susceptibility

Although the production of flavonoids in the leaves may be associated with protection against UV light and defense against pathogens (Gill and Tuteja 2010), the metabolic change as a result of the expression of the A1-DFR gene is unlikely to changes plant defense response to plant pests (see section C). There is also no

evidence that altering flavonoid pathways in the petals leads to changes in disease and pest susceptibility in A1-DFR petunias (Westhoff 2019).

(2c) Comparison of A1-DFR petunia to non-GE petunias with regards to tolerance to stresses including cold and drought.

Plant association with microbes can lead to stress tolerance, e.g., in cases where the production of flavonoids in the roots may be associated with root nodulating bacteria (Subramanian et al. 2007). Nonetheless, there is no evidence that altering flavonoid pathways in the petals leads to changes in response to stressful soil conditions.

Anthocyanin is known to provide abiotic tolerance and reduced accumulation of anthocyanin and proanthocyanidin was shown to slow the recovery from cold stress in purple sweet potato (*Ipomoea batatas* Lam. cv. Ayamurasaki) (Wang et al. 2013). However, an increase of total anthocyanidin was seen in the RL01 genetic background, a mutant line that cannot produce the normal amount of cyanidin and delphinidin (Meyer et al. 1987; Griesbach 1993). As mentioned earlier, RL01 is the genetic background in which A1-DFR was introduced. The total amount of anthocyanidins in GE petunia lines is within the range of other non-GE lines (Haselmair-Gosch et al. 2018). In other words, while the stress tolerance change is possible when comparing A1-DFR petunias with the RL01 parental line, such observed changes are not beyond the variation of normal petunia varieties.

Petunia x *hybrida* is tolerant of early frosts and light freezes. On mature plants, cold temperatures delay the flowering and ripening of seed pods (Vandenbussche et al. 2016). When non-acclimated *Petunia* x *hybrida* was exposed to 5°C conditions, they were severely injured, experiencing 3-4 times as much electrolyte leakage as non-chilled controls (Pennycooke et al. 2005). Cold acclimated plants, on the other hand, can tolerate -2°C with minimal electrolyte leakage (Pennycooke et al. 2003). Pennycooke et al. (2003) were able to increase the frost tolerance from -4°C of the wildtype to -6°C to -8°C in GE petunias by suppressing the enzyme β-galactosidase. However, since the A1-DFR modification is only expressed in the petals and the change in anthocyanin content is within the range of normal petunias, A1-DFR modification is unlikely to extend the range of petunia into colder regions.

Petunia is also tolerant of mildly reduced watering in garden conditions. Although 25% saturated soil led to a 50% reduction in flower production, petunia was still able to survive in the reduced water conditions and was able to increase its water use efficiency (Blanusa et al. 2009). In fact, as described in the next section, petunia appeared to compete better for water than impatiens plants (Blanusa et al. 2009). Using the same reasoning as above, A1-DFR modification is unlikely to further reduce petunia's water requirement to expand its range to regions with low precipitation (Stehmann et al. 2009).

There is also no evidence that A1-DFR modification leads to changes in drought and cold tolerances in A1-DFR petunias (Westhoff 2019).

(2d) Comparison of A1-DFR petunia to non-GE petunias with regards to competitive ability.

As petunia plants require cultivation and escapes are only found in gardens and not in natural habitats (SEINet 2018), *Petunia* x *hybrida* is not documented as a competitive plant. When petunias are in a container with reduced watering conditions, intraspecific competition from other petunia plants led to more marked reductions in height, dry weight, number of flowers, and number of buds compared to when petunia experienced interspecific competition from impatiens plants. Since the A1-DFR petunia is not known to have altered root growth or altered above-ground plant architecture (altered height, altered leaf size, etc.) (Westhoff 2019), it does not seem plausible that the trait would change the competitive ability of the plant.

(2e) Comparison of A1-DFR petunia to non-GE petunias with regards to methods of control

As the A1-DFR trait is not associated with herbicide resistance, the trait is not expected to make the plant more difficult to control chemically. Secondly, since A1-DFR trait isn't likely to change the abiotic stress tolerance beyond the normal range of commercial petunia varieties (see section 2c), the trait is not expected to change the degree to which the GE petunia is able to persist in the environment or the degree to which it is able to evade control. Moreover, because the A1-DFR trait does not change the plant's susceptibility to biotic stress (Westhoff 2019), it is not expected to change the plant's susceptibility to biological control (2b). Taken together, the evidence suggests that the A1-DFR petunia is not different from non-GE petunias with regards to methods of control or the plant's persistence in the environment in the absence of direct control efforts.

In summary, these results show that the engineered traits do not confer phenotypic or ecological characteristics resulting in a selective advantage in terms of better survival and reproduction for A1-DFR petunia compared to non-GE petunias. Consequently, A1-DFR petunia is no more likely to become a weed than conventional varieties.

G. Potential Impacts on the Weediness of Any Other Plants with which A1-DFR Petunia Can Interbreed

Gene flow is a natural biological process with significant evolutionary importance; plant breeders sometimes also artificially introgress traits from wild relatives into crop plants to develop new cultivars (Khoury et al. 2013). However, gene flow from crops to wild relatives has the potential to enhance the weediness of wild relatives, as observed in rice, sorghum, sunflower and a few other crops (Ellstrand et al. 1999). This topic is covered

in two sections: 1) the potential for gene flow, hybridization and introgression from the GE crop event to sexually compatible relatives in the United States and its territories, and 2) the potential for the phenotypic changes observed in the engineered plants to alter the weediness of those taxa.

Potential for gene flow, hybridization and gene introgression

The reproductive biology and pollination characteristics of petunia are well known and have previously been described. Petunia has a perfect flower and is predominately self-pollinating; relatively few pollinators visit *Petunia x hybrida* (Corbet et al. 2001). A few prerequisites for successful gene flow are: i) presence of sexually compatible relatives; ii) sympatric distribution of GE and its sexually compatible taxa; and iii) overlapping phenology between GE and its sexually compatible taxa. These prerequisites are all met with A1-DFR petunia, because other *Petunia* x *hybrida* hybrids are expected to be grown in the neighborhoods where A1-DFR petunia is grown, and flowering during the same period. Since hybrids between *Petunia* species are diploid and generally fertile (Vandenbussche et al. 2016), we assume that within species hybridization between ornamental varieties is plausible. APHIS considers it likely that pollen from GE petunias will fertilize non-GE petunias that occur in the same neighborhoods.

A1-DFR petunia is also capable of hybridizing with wild relatives. In the U.S. there are 2 other species of *Petunia* that have been reported from habitats obviously outside of cultivation (SEINet 2018). They are *P. axillaris* and *P. integrifolia*, and the putative progenitor plants (USDA ARS NGRS 2018). Further, the genus *Petunia* Juss. is incompatible with genus *Calibrachoa* with chromosome number difference despite the fact that the two were classified as from the same genus before 1990 due to botanical similarities (Wijsman 1990; Stehmann and Semir 1997).

In summary, A1-DFR petunia may hybridize with cultivated, wild or feral petunias. However, the introduced genetic material in A1-DFR petunia does not cause any major changes in the phenotype of petunia plants other than the intended color change in the petals.

Potential for enhanced weediness of recipients after gene flow and/or introgression

As discussed above in Section F "*Potential for Enhanced Weediness of A1-DFR Petunia*", the expression of the integrated genetic materials in A1-DFR petunia does not confer or enhance weedy characteristics of cultivated petunia. If gene flow and/or introgression from A1-DFR petunia to its sexually compatible species occur, the introduced genetic material is unlikely to cause enhanced weediness of the recipient plants. Thus, APHIS has determined that any adverse consequences of gene flow and/or introgression from A1-DFR petunia to wild relatives or weedy species in the U.S. and its territories are highly unlikely.

H. Potential Changes to Agriculture or Cultivation Practices

APHIS assessed whether significant changes to agricultural or cultivation practices from the adoption of the A1-DFR petunia are likely to impact plant diseases or pests or their management, including any APHIS control programs. This includes consideration of any changes in pesticide applications, tillage, irrigation, harvesting, etc. as they relate to plant pests and diseases.

As mentioned in sections D-G, there is no observed agronomic, disease and insect resistance, weediness, or any other phenotypic differences between A1-DFR petunia and other conventional varieties (Westhoff 2019). Therefore, A1-DFR petunia does not require any change in crop and pest management practices. The GE petunia will be propagated by a limited number of experienced growers inside greenhouses. Consumers place flowering petunia on the balcony and isolated beds. Among consumers who like to grow from seed, very few harvest seeds or taking cuttings (Westhoff 2019) and are therefore of very small scale. The propagation and cultivation of GE petunias do not require any practices that are different from those of non-GE conventional petunias and will not interfere with the current horticultural and field crop operations.

A1-DFR petunias have been handled just like any other petunias during its past presence on the market without being known as GE organisms. APHIS could not identify any significant changes to agricultural or cultivation practices (e.g., herbicide and pesticide applications, flower propagation and usage, irrigation and other management practices, etc.) from the adoption of A1-DFR petunia; therefore, no impact on plant diseases or pests or their management is likely to occur.

I. Potential Impacts from Transfer of Genetic Information to Organisms with which A1-DFR Petunia Cannot Interbreed

APHIS examined the potential for the new genetic material inserted into A1-DFR petunia to be horizontally transferred without sexual reproduction to other organisms and whether such an event could lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants. Horizontal gene transfer (HGT) between unrelated organisms is one of the most intensively studied fields in the biosciences since 1940, and the issue gained extra attention with the release of transgenic plants into the environment (Dröge et al. 1998). Potential risks from stable HGT from GE organisms to other organisms without reproduction or human intervention were reviewed (Keese 2008; Nicolia et al. 2014). Mechanisms of HGT include conjugation, transformation and transduction, and other diverse mechanisms of DNA and RNA uptake and recombination and rearrangement, most notably through viruses and mobile genetic elements. HGT has been a major contributor to the spread of antibiotic resistance amongst pathogenic bacteria; the emergence of increased virulence in bacteria, eukaryotes and viruses; and, in the long run, to major transitions in evolution.

Potential for horizontal gene transfer to bacteria, fungi, or invertebrates
A1-DFR petunia has five genetic elements from bacteria, including the beta-lactamase gene (partial), the selectable marker gene *nptII,* and the lacUV5 fragment (regulatory) from *E. coli*, as well as the *pnos* promoter and the *ocs* terminator (both regulatory) from *A. tumefaciens*. Horizontal gene transfer and expression of DNA from a plant species to bacterial, fungal or invertebrate species is unlikely to occur based on the following literature review.

Although there are many opportunities for plants to directly interact with fungi and bacteria (e.g., as commensals, symbionts, parasites, pathogens, decomposers, or in the guts of herbivores) and with invertebrates as plant pests, there are almost no evolutionary examples of HGT from eukaryotes to bacteria or from plants to fungi or invertebrates. Examples of HGT between eukaryotes and fungi primarily involve gene acquisition or transfer by fungi to or from other distantly related fungi or bacteria (van den Eede et al. 2004; Keeling and Palmer 2008; Keese 2008) and HGT between plants and fungi is extremely rare (Richardson and Palmer 2007). Examples of HGT between plants and invertebrates are also extremely rare, and most examples of HGT in insects involve the acquisition of genes from their pathogens or endosymbionts (Keese 2008; Zhu et al. 2011; Acuna et al. 2012).

Horizontal gene transfer and expression of DNA from a plant species to bacteria is extremely low and unlikely to occur (Conner et al. 2003; van den Eede et al. 2004; Keese 2008; EFSA 2009). The genomes (or parts thereof) of many bacteria that are closely associated with plants, including *Agrobacterium* and *Rhizobium*, have been sequenced; there is no evidence that these organisms contain genes derived from plants (Wood et al. 2001; Kaneko et al. 2002). Experiments in the field have not shown any evidence of horizontal gene transfer from plants to bacteria (Badosa et al. 2004; Wagner et al. 2007; Demanèche et al. 2008; Isaza et al. 2011; Ma et al. 2011; Nicolia et al. 2014). In cases where sequencing data implied that HGT occurred, these events are often discussed on an evolutionary time scale (Koonin et al. 2001; Brown 2003; Soucy et al. 2015). Additionally, transgene DNA promoters and coding sequences are optimized for plant expression, not prokaryotic bacterial expression. Thus even if HGT occurred, proteins corresponding to the transgenes are not likely to be produced. Finally, the *nptII* gene would be widely spread in soil bacteria and Enterobacteriaceae; HGT from GE plants to other organisms, if they did occur at all, would be negligible and will not change the population of natural antibiotic resistant bacteria already present in the soil (D'Costa et al. 2006; ZKBS 2008; EFSA 2009; Nicolia et al. 2014). FDA (1998) and the European Food Safety Authority (EFSA 2009) have also evaluated HGT from the use of antibiotic resistance marker genes and concluded that the likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of humans or animals is very rare or remote (FDA 1998; EFSA 2009). Therefore, APHIS agrees with Westhoff (2019) that the presence of the *nptII* gene in the genome of the GE petunias is unlikely to affect the spread of this antibiotic resistance gene in the environment. Nor does APHIS believe that the probability of the horizontal transfer of other A1-DFR construct components will be high enough to increase plant pest risk.

Potential for horizontal gene transfer to viruses

APHIS also considers whether the horizontal transfer of DNA from the GE plant to plant viruses is likely to occur and would lead to the creation or selection of plant viruses that are more virulent or have a broader host range. This issue has been considered before by other science review panels and government regulatory bodies (EPA-FIFRA-SAP 2006; Keese 2008). HGT is not unusual among plant viruses; however, this is generally limited to exchange between viruses present in the same host organism in mixed infections, and most commonly involves homologous recombination, relying on sequence similarity at the point of crossover (Keese 2008).

Virus sequences engineered into plants have been shown to be able to transfer into infecting or challenge viruses, including both DNA viruses (e.g., geminiviruses which replicate in the nucleus) (Frischmuth and Stanley 1998) and RNA viruses which typically replicate in the cytoplasm (Borja et al. 1999; Adair and Kearney 2000). However, those studies only demonstrated homologous recombination that restored defective viruses under ideal laboratory conditions; the crop field virus load may only permit a lower recombination frequency and is not likely to create selective pressures favoring the recombinant (Borja et al. 1999; Adair and Kearney 2000). Virus populations with recombination between virus transgene integrated into plants and the genes of related natural virus are similar to recombinants found in mixed infections of the same virus in nontransgenic plants, indicating that there was no novel recombination mechanism with regards to transgenic plants; and no increased risk is expected over what is expected from mixed infections (Fuchs and Gonsalves 2007; Keese 2008; Turturo et al. 2008).

Non-homologous recombination in HGT among viruses or between virus transgenes and infecting viruses can occur, but frequently results in gene deletions and nonviable viruses; the recombinants detected were very similar to those in nature; deep sequencing of recombinant virus populations in transgenic and nontransgenic tobacco plants infected with cucumber mosaic virus identified no novel recombinants of biosafety concern (Morroni et al. 2013). Depending on the particular virus and sequences involved, various hot-spots for recombination have been found in both coding and noncoding regions, and strategies implemented in the design of transgenes to avoid recombination have been suggested.

Over at least 8-10 years in field tests of GE crops or during commercial production in the United States of deregulated virus resistant plum, squash, or papaya engineered with genes from viruses, no recombinant or undesirable viruses with new properties have been detected (EPA-FIFRA-SAP 2006; Fuchs and Gonsalves 2007). Current scientific knowledge does not indicate that transgenic plants using virus components present biosafety concerns (Ilardi 2014). A1-DFR petunia was engineered with the 35S promoter and terminator from the cauliflower mosaic virus. Both the 35S promoter and terminator are non-coding regulatory sequences and have a long history of safe use. HGT from A1-DFR petunias to the virus population is highly unlikely.

Potential for horizontal gene transfer to parasitic plants

Although there are reports of HGT between vascular plants based on phylogenetic study (Manavella et al. 2006; Davis and Xi 2015), evidence for HGT from plants to other plants is mostly limited to two specific scenarios: (1) exchange of genes between a parasitic plant and its host (Yoshida et al. 2010); and (2) exchange of genes between cells of two plants living in close proximity, such as in a graft junction. In both cases, this type of HGT requires physical contact between the two plants. Yoshida reported HGT also occurred before the speciation of the parasitic plant purple witchweed (*Striga. hermonthica*) and related cowpea witchweed (*S. gesnerioides*) from their common ancestor millions of years ago. Furthermore, *S. hermonthica* is not found in the U.S. and *S. asiatica*, another related parasite of cereal crops, is only present in North Carolina and South Carolina (USDA-NRCS 2013).

Most cases of HGT in plants involve the transfer of mitochondrial genomes, which are primarily maternally inherited in plants (Barr et al. 2005), to other mitochondrial genomes, and most involve parasitic plants and their hosts (Richardson and Palmer 2007). Even though the more recent studies (Xi et al. 2012; Xi et al. 2013) reported relatively higher rates of HGT, it is still limited to mitochondrial DNA. For A1-DFR petunia, the DNA sequences were inserted into the nuclear genome, not the mitochondrial genome. Further, even if the petunia plants were infected by a parasitic plant or were naturally grafted to another plant, there is a very low probability that HGT could result in the other plant acquiring DNA from A1-DFR petunia. In both scenarios, this newly introduced DNA would likely reside in somatic cells, and with little chance of reaching the germ cells, this introduced DNA could not persist in subsequent generations unless the recipient plant reproduced asexually from the affected cells. The scenario is also less likely to occur as petunia production and usage are mostly in well a managed horticultural environment that usually don't have parasitic plants (Westhoff 2019)

Based on the above analysis APHIS therefore concludes that HGT of the new genetic material inserted into the GE petunia to other organisms is highly unlikely, and is not expected to lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

J. Conclusion

APHIS has reviewed the information submitted in the petition (Westhoff 2019), supporting documents, and other relevant information to assess the plant pest risk of the A1-DFR petunias compared to the unmodified commercial varieties and the original line from which they were derived. APHIS concludes that the A1-DFR petunia is unlikely to pose a greater plant pest risk than the unmodified organism based on the following findings.

- No plant pest risk was identified from the transformation process or the insertion of new genetic material in A1-DFR petunia. The direct gene transfer protocol used involved no plant pests. The only plant pest sequences in the inserted genetic

material are non-coding sequences from Cauliflower mosaic virus and *A. tumefaciens*. They are promoters and terminators that are not expected to cause plant disease risk. The other vector components are not of plant pest origin. No introduced genetic material will create an infectious agent or otherwise confer any plant pest characteristic to A1-DFR petunias.

- The expression of the maize A1-DFR gene in petunia is unlikely to change plant pest resistance. No difference was observed in plant susceptibility to diseases and insect pests in A1-DFR petunias compared to the unmodified varieties. Observed agronomic or horticultural traits also did not reveal any differences that would indicate that A1-DFR petunias will become more susceptible to pests or diseases as the indirect result of genetic engineering. Therefore, no plant pest effects are expected from A1-DFR petunia varieties. They will not impact other agricultural products or APHIS pest control programs.
- Exposure to A1-DFR petunias is unlikely to have any adverse impacts on organisms beneficial to agriculture based on the analysis of phenotypic data, past observation, and pattern of the petunia production and usage.
- The expression of the maize A1-DFR gene in petunia is unlikely to change stress tolerance. A1-DFR petunias are no more likely to become a weed than conventional petunia varieties based on their observed agronomic characteristics, weediness potential of the crop, and current management practices available to control A1-DFR petunia as a weed. Volunteers and feral populations of A1-DFR petunia are unlikely to occur and can be managed using a variety of currently available methods and herbicides.
- A1-DFR petunias are not expected to increase the weed risk potential of other species with which they can interbreed in the U.S. or its territories. Hybridization and/or introgression of inserted genes from A1-DFR petunias to other sexually compatible relatives with which they can interbreed is not likely to occur. A1-DFR petunias do not confer or enhance the weedy characteristics of cultivated petunias.
- Significant changes to agricultural or cultivation practices (e.g., herbicide or pesticide applications, irrigation, etc.) from the adoption of A1-DFR petunia were not known in the past and are not expected to be identified in the future.
- HGT of the new genetic material inserted into A1-DFR petunias to other organisms is highly unlikely and is not expected to lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

K. References

7 CFR part 340. 2003. *Introduction of Organisms and Products Altered or Produced through Genetic Engineering Which are Plant Pests or Which There is Reason to Believe are Plant Pests*. Retrieved from https://www.govinfo.gov/content/pkg/CFR-2003-title7-vol5/pdf/CFR-2003-title7-vol5-part340.pdf

51 FR 23302. *Coordinated Framework for Regulation of Biotechnology*. Retrieved from regulations.gov

57 FR 22984. *Statement of Policy: Foods Derived from New Plant Varieties*. Retrieved from regulations.gov

Acuna R, Padilla BE, Florez-Ramos CP, Rubio JD, Herrera JC, Benavides P, Lee SJ, Yeats TH, Egan AN, Doyle JJ, and Rose JK. 2012. *Adaptive horizontal transfer of a bacterial gene to an invasive insect pest of coffee*. PNAS 109, pp. 4197-4202. Retrieved from http://www.pnas.org/content/early/2012/02/17/1121190109.abstract

Adair TL and Kearney CM. 2000. *Recombination between a 3-kilobase tobacco mosaic virus transgene and a homologous viral construct in the restoration of viral and nonviral genes*. Archieves of Virology 145, pp. 1867–1883. Retrieved from https://link.springer.com/article/10.1007/s007050070062

All-America Selections. 2018. *All-America Selections Winners - Petunia*. Retrieved from https://all-americaselections.org/search-results/keyword/petunia/search-in/product/cat-in/all/search-other/product

Badosa E, Moreno C, and Montesinos E. 2004. *Lack of detection of ampicillin resistance gene transfer from Bt176 transgenic corn to culturable bacteria under field conditions*. FEMS Microbiology Ecology 48, pp. 169-178. Retrieved from http://dx.doi.org/10.1016/j.femsec.2004.01.005 Last accessed 9/28/2017.

Barr CM, Neiman M, and Taylor DR. 2005. *Inheritance and recombination of mitochondrial genomes in plants, fungi and animals*. New Phytologist 168, pp. 39-50; DOI: 10.1111/j.1469-8137.2005.01492.x. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1469-8137.2005.01492.x/pdf Last accessed Aug 2014.

Bashandy H and Teeri TH. 2017. *Genetically engineered orange petunias on the market*. Planta 246, pp. 277-280. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/28647812

Bernhardt J, Stich K, Schwarz-Sommer Z, Saedler H, and Wienand U. 1998. *Molecular analysis of a second functional A1 gene (dihydroflavonol 4-reductase) in Zea mays*. The Plant Journal 14, pp. 483–488.

Blanusa T, Vysini E, and Cameron RW. 2009. *Growth and flowering of Petunia and Impatiens: Effects of competition and reduced water content within a container*. HortScience 44, pp. 1302-1307.

Bogosian G and Kane JF. 1987. *Nucleotide sequence of the EcoRI fragment frnm pLJ3 baring two tandem lacUV5 promoters*. Nucleic Acids Research 15, pp. 7185. Last accessed https://www.ncbi.nlm.nih.gov/pmc/articles/PMC306209/pdf/nar00261-0415.pdf.

Borja M, Rubio T, Scholthof HB, and Jackson AO. 1999. *Restoration of Wild-Type Virus by Double Recombination of Tombusvirus Mutants with a Host Transgene*. Molecular Plant-Microbe Interactions 12, pp. 153-162. Retrieved from https://apsjournals.apsnet.org/doi/abs/10.1094/MPMI.1999.12.2.153

Brown JR. 2003. *Ancient horizontal gene transfer*. Nature Reviews Genetics 4, pp. 121-132; doi:110.1038/nrg1000. Retrieved from http://www.nature.com/nrg/journal/v4/n2/full/nrg1000.html Last accessed Aug 2014.

Burton-Freeman B, Sandhu A, and Edirisinghe I. 2016. *Anthocyanins*, pp. 489-500. Last accessed 08/16/2019.

COGEM. 2017. *Update on unauthorised genetically modified garden petunia varieties*. Bilthoven, The Netherlands. Commission on Genetic Modification.

Conner AJ, Glare TR, and Nap J-P. 2003. *The release of genetically modified crops into the environment*. The Plant Journal 33, pp. 19-46. Retrieved from http://dx.doi.org/10.1046/j.0960-7412.2002.001607.x

Corbet SA, Bee J, Dasmahapatra K, Gale S, Gorringe E, La Ferla B, Moorhouse T, Trevail A, Van Bergen Y, and Vorontsova M. 2001. *Native or exotic? Double or single? Evaluating plants for pollinator-friendly gardens*. Annals of Botany 87, pp. 219-232.

Costa Farms. 2018. *Petunia*. Retrieved from http://www.costafarms.com/plants/petunia

Craib E. 2016. *1890s (mostly) Petunia History by L.H. Bailey and Others, and One Amazing Color Plate*. Retrieved from https://horticultural-history.blogspot.com/2016/08/1890s-mostly-petunia-history-by-lh.html

D'Costa VM, McGrann KM, Hughes DW, and Wright GD. 2006. *Sampling the Antibiotic Resistome*. Science 311, pp. 374-377.

Davis CC and Xi Z. 2015. *Horizontal gene transfer in parasitic plants*. Curr Opin Plant Biol 26, pp. 14-19. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/26051213

Demanèche S, Sanguin H, Poté J, Navarro E, Bernillon D, Mavingui P, Wildi W, Vogel TM, and Simonet P. 2008. *Antibiotic-resistant soil bacteria in transgenic plant fields*. Proceedings of the National Academy of Sciences 105, pp. 3957-3962. Retrieved from http://www.pnas.org/content/105/10/3957.abstract

Dröge M, Puhler A, and Selbitschka W. 1998. *Horizontal gene transfer as a biosafety issue: A natural phenomenon of public concern*. Journal of Biotechnology 64, pp. 75-90.

Dudek B, Warskulat A-C, and Schneider B. 2016. *The Occurrence of Flavonoids and Related Compounds in Flower Sections of Papaver nudicaule*. Plants (Basel) 5, pp. 28. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/27338493 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4931408/

EFSA. 2009. *Consolidated presentation of the joint Scientific Opinion of the GMO and BIOHAZ Panels on the "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants" and the Scientific Opinion of the GMO Panel on "Consequences of the Opinion on the Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants on Previous EFSA Assessments of Individual GM Plants*. The European Food Safety Authority Journal 7, pp. 1-107. Retrieved from http://www.efsa.europa.eu/en/efsajournal/doc/1108.pdf Last accessed Aug 2014.

Ellstrand NC, Prentice HC, and Hancock JF. 1999. *Gene flow and introgression from domesticated plants into their wild relatives*. Annual Review of Ecology and Systematics 30, pp. 539-563; DOI: 510.1146/annurev.ecolsys.1130.1141.1539. Retrieved from http://www.annualreviews.org/doi/abs/10.1146%2Fannurev.ecolsys.30.1.539 Last accessed Aug 2014.

EPA-FIFRA-SAP. 2006. *Plant Incorporated Protectants Based on Virus Coat Protein Genes: Science Issues Asosciated with the Proposed Rule*. Retrieved from

https://archive.epa.gov/scipoly/sap/meetings/web/pdf/minutes1205.pdf Last accessed 09/28/2017.

Falcone Ferreyra ML, Rius SP, and Casati P. 2012. *Flavonoids: biosynthesis, biological functions, and biotechnological applications*. Front Plant Sci 3, pp. 222. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23060891

FDA. 1998. *Draft Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants*. Food and Drug Administration. Retrieved from https://pdfs.semanticscholar.org/ab10/e9819a9677963006b86a6a3ad61495de8419.pdf Last accessed 06/26/2019.

Finkelstein R, Reeves W, Ariizumi T, and Steber C. 2008. *Molecular aspects of seed dormancy*. Annu. Rev. Plant Biol. 59, pp. 387-415.

FLEPPC. 2007. Florida Exotic Pest Plant Council: *Invasive plant species list*. Retrieved from http://www.fleppc.org/list/2007/07list.htm

Frischmuth T and Stanley J. 1998. *Recombination between viral DNA and the transgenic coat protein gene of African cassava mosaic geminivirus*. Journal of General Virology 79, pp. 1265-1271. Retrieved from http://vir.sgmjournals.org/content/79/5/1265.full.pdf+html Last accessed Aug 2014.

Fuchs M and Gonsalves D. 2007. *Safety of virus-resistant transgenic plants two decades after their introduction: Lessons from realistic field risk assessment studies*. Annual Review of Phytopathology 45:, pp. 173-202; doi:110.1146/annurev.phyto.1145.062806.094434. Retrieved from http://www.annualreviews.org/doi/full/10.1146/annurev.phyto.45.062806.094434?url_ver=Z39.88-2003 Last accessed Aug 2014.

Gill SS and Tuteja N. 2010. *Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants*. Plant physiology and biochemistry 48, pp. 909-930.

Goldstein DA, Tinland B, Gilbertson LA, Staub JM, Bannon GA, Goodman RE, McCoy RL, and Silvanovich A. 2005. *Human safety and genetically modified plants: a review of antibiotic resistance markers and future transformation selection technologies*. J Appl Microbiol 99, pp. 7-23. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15960661

Griesbach RJ. 1993. *Characterization of the Flavonoids from Petunia ×hybrida Flowers Expressing the A1 Gene of Zea mays*. HORTSCIENCE 28, pp. 659-660. Retrieved from https://pdfs.semanticscholar.org/d7dc/86ed408b49cdedf1ab5f46a98a2ab487435c.pdf Last accessed 10/16/2019.

Hain R, Stabel P, Czernilofsky AP, Steinbiß HH, Herrera-Estrella L, and Schell J. 1985. *Uptake, integration, expression and genetic transmission of a selectable chimaeric gene by plant protoplasts*. Molecular and General Genetics MGG 199, pp. 161-168. Retrieved from https://link.springer.com/content/pdf/10.1007%2FBF00330254.pdf

Haselmair-Gosch C, Miosic S, Nitarska D, Roth BL, Walliser B, Paltram R, Lucaciu RC, Eidenberger L, Rattei T, Olbricht K, Stich K, and Halbwirth H. 2018. *Great Cause-Small Effect: Undeclared Genetically Engineered Orange Petunias*

Harbor an Inefficient Dihydroflavonol 4-Reductase. Front Plant Sci 9, pp. 149. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/29541079

Ilardi V. 2014. *Could Transgenic Plants Expressing Virus-Derived Sequences Create New Routes for Virus Evolution?* Biosafety 3. Retrieved from https://www.longdom.org/open-access/could-transgenic-plants-expressing-virus-derived-sequences-create-new-routes-for-virus-evolution-2167-0331.1000e151.pdf

Isaza L, Opelt K, Wagner T, Mattes E, Bieber E, Hatley E, Roth G, Sanjuán J, Fischer H, Sandermann H, Hartmann A, and D. E. 2011. *Lack of glyphosate resistance gene transfer from Roundup Ready ® soybean to Bradyrhizobium japonicum under field*. Zeitschrift fur Naturforschung 66. Last accessed 9/26/2017.

ITIS. 2019. *Integrated Taxonomic Information System On-line Database*. Retrieved from https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=30581#null Last accessed 10/3/2019.

Kaneko T, Nakamura Y, Sato S, Minamisawa K, Uchiumi T, Sasamoto S, Watanabe A, Idesawa K, Iriguchi M, Kawashima K, Kohara M, Matsumoto M, Shimpo S, Tsuruoka H, Wada T, Yamada M, and Tabata S. 2002. *Complete genomic sequence of nitrogen-fixing symbiotic bacterium Bradyrhizobium japonicum USDA110*. DNA Research 9, pp. 189–197. Retrieved from http://dnaresearch.oxfordjournals.org/content/9/6/189.long Last accessed Aug 2014.

Keeling PJ and Palmer JD. 2008. *Horizontal gene transfer in eukaryotic evolution*. Nature Reviews Genetics 9, pp. 605-618; doi:610.1038/nrg2386. Retrieved from http://www.nature.com/nrg/journal/v9/n8/full/nrg2386.html Last accessed Aug 2014.

Keese P. 2008. *Risks from GMOs due to horizontal gene transfer*. Environmental Biosafety Research 7, pp. 123-149; DOI: 110.1051/ebr:2008014. Retrieved from http://journals.cambridge.org/action/displayAbstract?fromPage=online&aid=8208895&fileId=S1635792208000146 Last accessed Dec 2014.

Kessler D, Gase K, and Baldwin IT. 2008. *Field experiments with transformed plants reveal the sense of floral scents*. Science 321, pp. 1200-1202.

Khoury CK, Greene S, Wiersema J, Maxted N, Jarvis A, and Struik PC. 2013. *An inventory of crop wild relatives of the United States*. Crop Science 53, pp. 1496; doi:1410.2135/cropsci2012.1410.0585. Retrieved from https://www.crops.org/publications/cs/abstracts/53/4/1496 Last accessed Aug 2014.

Koonin EV, Makarova KS, and Aravind L. 2001. *Horizonal gene transfer in prokaryotes: Quantification and classification*. Annual Review of Microbiology 55:, pp. 709-742. Retrieved from http://www.annualreviews.org/doi/full/10.1146/annurev.micro.55.1.709?url_ver=Z39.88-2003 Last accessed Aug 2014.

Linn F, Heidmann I, Saedler H, and Meyer P. 1990. *Epigenetic changes in the expression of the maize A1 gene in Petunia hybrida: role of numbers of integrated gene copies and state of methylation*. Molecular & general genetics: MGG 222, pp. 329-336.

Lopez RG and Runkle ES. 2005. *Daily light integral influences rooting and quality of petunia cuttings*.

Ma BL, Blackshaw RE, Roy J, and He T. 2011. *Investigation on gene transfer from genetically modified corn (Zea mays L.) plants to soil bacteria*. J Environ Sci Health B 46, pp. 590-599. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21722080

Manavella PA, Arce AL, Dezar CA, Bitton F, Renou JP, Crespi M, and Chan RL. 2006. *Cross-talk between ethylene and drought signalling pathways is mediated by the sunflower Hahb-4 transcription factor*. Plant J 48, pp. 125-137. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16972869

Meyer P, Heidmann I, Forkmann G, and Saedler H. 1987. *A new petunia flower colour generated by transformation of a mutant with a maize gene*. Nature 330, pp. 677.

Meyer P, Walgenbach E., Bussmann K, Hombrecher G, and Saedler H. 1985. *Synchronized Tobacco Protoplasts are Efficiently Transformed by DNA*. Mol Gen Genet 201, pp. 513-518.

Meyer P, Linn F, Heidmann I, Meyer H, Niedenhof I, and Saedler H. 1992. *Endogenous and environmental factors influence 35S promoter methylation of a maize A1 gene construct in transgenic petunia and its colour phenotype*. MGG Molecular & General Genetics 231, pp. 345-352.

Miki B and McHugh S. 2004. *Selectable marker genes in transgenic plants: applications, alternatives and biosafety*. Journal of Biotechnology 107, pp. 193-232. Retrieved from http://www.sciencedirect.com/science/article/pii/S0168165603002906

Miller R, Owens SJ, and Rørslett B. 2011. *Plants and colour: flowers and pollination*. Optics & Laser Technology 43, pp. 282-294.

Morroni M, Jacquemond M, and Tepfer M. 2013. *Deep sequencing of recombinant virus populations in transgenic and nontransgenic plants infected with Cucumber mosaic virus*. Molecular Plant-Microbe Interactions 26, pp. 801-811. Retrieved from http://dx.doi.org/10.1094/MPMI-02-13-0057-R Last accessed Aug 2014.

Nap J-P, Bijvoet J, and Stiekema WJ. 1992. *Biosafety of kanamycin-resistant transgenic plants*. Transgenic Research 1, pp. 239-249.

National Gardening Association. 2018. *Plants Database - Petunia search*. Retrieved from https://garden.org/plants/browse/plants/children/78235/?offset=&offset=520

NCBI. 2019. NCBI: *NCBI BLAST Zm00014a_011529 Zea mays*. Retrieved from https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_PWZ32460

Nicolia A, Manzo A, Veronesi F, and Rosellini D. 2014. *An overview of the last 10 years of genetically engineered crop safety research*. Crit Rev Biotechnol 34, pp. 77-88. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/24041244

OGTR. 2004. *Inclusion of dealings with GM carnation lines, modified for flower colour, on the GMO Register*. Australia Office of the Gene Technology Regulator. Retrieved from https://www.health.gov.au/internet/ogtr/publishing.nsf/Content/register-3/$FILE/reg001risk.pdf Last accessed 06/26/2019.

OGTR. 2017. *Inadvertent dealings with petunia genetically modified for altered flower colour*. Office of the Gene Technology Regulator. Retrieved from http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/3D63D298FC0BF7C

3CA25813200229C30/$File/GM%20petunia%20Risk%20Analysis.pdf Last accessed 06/26/2019.
Ohio State University. 2018. *OSU Pocket Gardener - Petunia*. Retrieved from https://hvp.osu.edu/pocketgardener/source/description/pe_brida.html Last accessed September 24, 2018.
Old Farmer's Almanac. n.d. *GROWING PETUNIAS*. Yankee Publishing. Retrieved from https://www.almanac.com/plant/petunias Last accessed 10/16/2019.
Oud JS, Schneiders H, Kool AJ, and van Grinsven MQ. 1995. *Breeding of transgenic orange Petunia hybrida varieties*. Euphytica 84, pp. 175-181.
Pennycooke JC, Jones ML, and Stushnoff C. 2003. *Down-regulating α-galactosidase enhances freezing tolerance in transgenic petunia*. Plant Physiology 133, pp. 901-909.
Pennycooke JC, Cox S, and Stushnoff C. 2005. *Relationship of cold acclimation, total phenolic content and antioxidant capacity with chilling tolerance in petunia (Petunia× hybrida)*. Environmental and Experimental Botany 53, pp. 225-232.
Pröls F and Meyer P. 1992. *The methylation patterns of chromosomal integration regions influence gene activity of transferred DNA in Petunia hybrida*. The Plant Journal 2, pp. 465-475. Retrieved from https://onlinelibrary.wiley.com/doi/abs/10.1046/j.1365-313X.1992.t01-20-00999.x Last accessed 12/14/2018.
Reck-Kortmann M, Silva-Arias GA, Segatto ALA, Mäder G, Bonatto SL, and de Freitas LB. 2014. *Multilocus phylogeny reconstruction: new insights into the evolutionary history of the genus Petunia*. Molecular phylogenetics and evolution 81, pp. 19-28.
Richardson AO and Palmer JD. 2007. *Horizontal gene transfer in plants*. Journal of Experimental Botany 58, pp. 1-9; doi:10.1093/jxb/erl1148. Retrieved from http://jxb.oxfordjournals.org/content/58/1/1.full.pdf+html Last accessed Aug 2014.
Schenke D, Böttcher C, and Scheel D. 2011. *Crosstalk between abiotic ultraviolet-B stress and biotic (flg22) stress signalling in Arabidopsis prevents flavonol accumulation in favor of pathogen defence compound production*. Plant, Cell & Environment 34, pp. 1849-1864. Retrieved from https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-3040.2011.02381.x
Schwarz-Sommer Z, Shepherd N, Tacke E, Gierl A, Rohde W, Leclercq L, Mattes M, Berndtgen R, Peterson PA, and Saedler H. 1987. *Influence of transposable elements on the structure and function of the Al gene of Zea mays*. The EMBO Journal 6, pp. 287-294.
SEINet. 2018. Southeast Environmental Information Network Herbarium Database Search: Petunia. : *Southeast* Retrieved from http://swbiodiversity.org/seinet/
Soucy SM, Huang J, and Gogarten JP. 2015. *Horizontal gene transfer: building the web of life*. Nature reviews. Genetics 16, pp. 472-482. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/26184597
Standford Univ. 1997. *Molecular Biology Vector Sequence Database*. Retrieved from http://genome-www.stanford.edu/vectordb/vector_pages/plasmid_pages/0-PDnotcomplete_plasmid.html

Stehmann JR and Semir J. 1997. *A New Species and New Combinations in Calibrachoa (Solanaceas)*. Novon 7, pp. 417-419.
Stehmann JR, Lorenz-Lemke AP, Freitas LB, and Semir J. 2009. The genus petunia. In: *Petunia* (Springer), pp. 1-28.
Subramanian S, Stacey G, and Yu O. 2007. *Distinct, crucial roles of flavonoids during legume nodulation*. Trends in plant science 12, pp. 282-285.
Swallowtail Garden Seeds. 2018. *Petunia Seeds*. Retrieved from https://www.swallowtailgardenseeds.com/annuals/petunia.html
Turturo C, Friscina A, Gaubert S, Jacquemond M, Thompson JR, and Tepfer M. 2008. *Evaluation of potential risks associated with recombination in transgenic plants expressing viral sequences*. Journal of General Virology 89:, pp. 327-335; DOI 310.1099/vir.1090.83339-83330. Retrieved from http://vir.sgmjournals.org/content/89/1/327.full.pdf+html Last accessed Aug 2014.
USDA-APHIS. 2017. *List of Deregulated GE crops*. Retrieved from https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-petitions/petitions/petition-status
USDA-NASS. 2015. *Census of Agriculture - 2014 Census of Horticultural Specialties; Table 3. Annual Bedding/Garden Plants Sold -- Total 2014*. Retrieved from https://www.nass.usda.gov/Publications/AgCensus/2012/Online_Resources/Census_of_Horticulture_Specialties/
USDA-NRCS. 2013. *Striga asiatica (L.) Kuntze, Asiatic witchweed*. Retrieved from http://plants.usda.gov/core/profile?symbol=STAS2 Last accessed 21 August 2013.
USDA-NRCS. 2018. *USDA Plants Database*. Retrieved from https://plants.sc.egov.usda.gov/core/profile?symbol=PETUN
USDA-NRCS. 2019. *Petunia ×atkinsiana D. Don ex Loudon [axillaris × integrifolia] petunia* Retrieved from https://plants.usda.gov/core/profile?symbol=PEAT7
USDA-PPQ. 2017. *Cotton Pest Programs*. USDA-PPQ. Retrieved from https://www.aphis.usda.gov/aphis/ourfocus/planthealth/plant-pest-and-disease-programs/pests-and-diseases/cotton-pests/ct_cotton_pests_home Last accessed August 04, 2017.
USDA ARS NGRS. 2018. *GRIN*. Retrieved from https://npgsweb.ars-grin.gov/gringlobal/taxonomydetail.aspx?id=462920
van den Eede G, Aarts H, Buhk HJ, Corthier G, Flint HJ, Hammes W, Jacobsen B, Midtvedt T, van der Vossen J, von Wright A, Wackernagel W, and Wilcks A. 2004. *The relevance of gene transfer to the safety of food and feed derived from genetically modified (GM) plants*. Food Chem Toxicol 42, pp. 1127-1156. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15123384
Vandenbussche M, Chambrier P, Rodrigues Bento S, and Morel P. 2016. *Petunia, your next supermodel?* Frontiers in plant science 7, pp. 72.
Velten J, Velten L, Hain R, and Schell J. 1984. *Isolation of a dual plant promoter fragment from the Ti plasmid of Agrobacterium tumefaciens*. The EMBO Joumal 3, pp. 2723-2730. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC557759/

Vite Greenhouses. 2018. *List of Petunias*. Retrieved from http://www.vitegreenhouses.com/PlantResults.asp?Query=52&Page=1

Wagner T, Arango Isaza LM, Grundmann S, Dörfler U, Schroll R, Schloter M, Hartmann A, Sandermann H, and Ernst D. 2007. *The Probability of a Horizontal Gene Transfer from Roundup Ready® Soybean to Root Symbiotic Bacteria: A Risk Assessment Study on the GSF Lysimeter Station*. Water, Air, & Soil Pollution: Focus 8, pp. 155-162. Last accessed 9/26/2017.

Wang H, Fan W, Li H, Yang J, Huang J, and Zhang P. 2013. *Functional Characterization of Dihydroflavonol-4-Reductase in Anthocyanin Biosynthesis of Purple Sweet Potato Underlies the Direct Evidence of Anthocyanins Function against Abiotic Stresses*. PLOS ONE 8, pp. e78484. Retrieved from https://doi.org/10.1371/journal.pone.0078484

Westhoff Vertriebsgesellschaft mbH. 2019. *Petition for the determination of non-regulated status for petunias containing the A1 gene of Maize (A1-DFR petunias)*. Submitted by C. Westhoff, CEO. Westhoff Vertriebsgesellschaft mbH. Germany.

Wijsman HJW. 1982. *On the Interrelationships of Certain Species of Petunia 1. Taxonomic Notes on the Parental Species of Petunia Hybrida*. Acta Bot. Neerl. 31, pp. 477-490.

Wijsman HJW. 1990. *On the inter-relationships of certain species of Petunia VI. New names for the species of Calibrachoa formerly included into Petunia (Solanaceae)*. Acta Bot. Neerl. 39, pp. 101-102.

Wood DW, Setubal JC, Kaul R, Monks DE, Kitajima JP, Okura VK, Zhou Y, Chen L, Wood GE, Almeida NF, Jr., Woo L, Chen Y, Paulsen IT, Eisen JA, Karp PD, Bovee D, Sr., Chapman P, Clendenning J, Deatherage G, Gillet W, Grant C, Kutyavin T, Levy R, Li MJ, McClelland E, Palmieri A, Raymond C, Rouse G, Saenphimmachak C, Wu Z, Romero P, Gordon D, Zhang S, Yoo H, Tao Y, Biddle P, Jung M, Krespan W, Perry M, Gordon-Kamm B, Liao L, Kim S, Hendrick C, Zhao ZY, Dolan M, Chumley F, Tingey SV, Tomb JF, Gordon MP, Olson MV, and Nester EW. 2001. *The genome of the natural genetic engineer Agrobacterium tumefaciens C58*. Science 294, pp. 2317-2323. Retrieved from http://www.sciencemag.org/content/294/5550/2317.long Last accessed September 26, 2017.

Xi Z, Wang Y, Bradley RK, Sugumaran M, Marx CJ, Rest JS, and Davis CC. 2013. *Massive mitochondrial gene transfer in a parasitic flowering plant clade*. PLoS Genet 9. Retrieved from http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003265

Xi ZX, Bradley RK, Wurdack KJ, Wong KM, Sugumaran M, Bomblies K, Rest JS, and Davis CC. 2012. *Horizontal transfer of expressed genes in a parasitic flowering plant*. BMC Genomics 13, pp. 227. Retrieved from http://www.biomedcentral.com/1471-2164/13/227

Yoshida S, Maruyama S, Nozaki H, and Shirasu K. 2010. *Horizontal gene transfer by the parasitic plant Striga hermonthica*. Science 328, pp. 1128; DOI:1110.1126/science.1187145. Retrieved from http://www.sciencemag.org/content/328/5982/1128.full Last accessed September 26, 2017.

Yukihisa S, Nakano-Shimadaa R, Ohbayashib M, Okinakac Y, Kiyokawad S, and Kikuchib Y. 1999. *Expression of chimeric P450 genes encoding £avonoid-3P,5P-hydroxylase in transgenic tobacco and petunia plants*. FEBS Letters 461, pp. 241-245.

Zamora-Ros R, Knaze V, Lujan-Barroso L, Slimani N, Romieu I, Touillaud M, Kaaks R, Teucher B, Mattiello A, Grioni S, Crowe F, Boeing H, Forster J, Quiros JR, Molina E, Huerta JM, Engeset D, Skeie G, Trichopoulou A, Dilis V, Tsiotas K, Peeters PH, Khaw KT, Wareham N, Bueno-de-Mesquita B, Ocke MC, Olsen A, Tjonneland A, Tumino R, Johansson G, Johansson I, Ardanaz E, Sacerdote C, Sonestedt E, Ericson U, Clavel-Chapelon F, Boutron-Ruault MC, Fagherazzi G, Salvini S, Amiano P, Riboli E, and Gonzalez CA. 2011. *Estimation of the intake of anthocyanidins and their food sources in the European Prospective Investigation into Cancer and Nutrition (EPIC) study*. Br J Nutr 106, pp. 1090-1099. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21481290

Zhu B, Lou MM, Xie GL, Zhang GQ, Zhou XP, Li B, and Jin GL. 2011. *Horizontal gene transfer in silkworm, Bombyx mori*. BMC genomics 12, pp. 248. Retrieved from http://www.biomedcentral.com/1471-2164/12/248

ZKBS. 2008. *Opinion of the Central Committee on Biological Safety (ZKBS) on the safety assessment of antibiotic resistance genes in the genome of genetically modified plants from December 2008*. Germany. Central Committee on Biological Safety. Retrieved from https://www.zkbsonline.de/ZKBS/EN/04_Allgemeine%20Stellungnahmen/allgemeine_stellungnahmen_node.html#doc8569998bodyText5 Last accessed 6/27/2019.

ZKBS. 2017. *Position statement of the ZKBS on the risk assessment of unauthorized genetically modified petunias*. Germany. Central Committee on Biological Safety. Retrieved from https://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/Ereignisse/Statement%20of%20ZKBS_GM%20petunias.pdf?__blob=publicationFile&v=2

Bashandy H and Teeri TH. 2017. *Genetically engineered orange petunias on the market*. Planta 246, pp. 277-280. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/28647812

Hain R, Stabel P, Czernilofsky AP, Steinbiß HH, Herrera-Estrella L, and Schell J. 1985. *Uptake, integration, expression and genetic transmission of a selectable chimaeric gene by plant protoplasts*. Molecular and General Genetics MGG 199, pp. 161-168. Retrieved from https://link.springer.com/content/pdf/10.1007%2FBF00330254.pdf

Haselmair-Gosch C, Miosic S, Nitarska D, Roth BL, Walliser B, Paltram R, Lucaciu RC, Eidenberger L, Rattei T, Olbricht K, Stich K, and Halbwirth H. 2018. *Great Cause-Small Effect: Undeclared Genetically Engineered Orange Petunias Harbor an Inefficient Dihydroflavonol 4-Reductase*. Front Plant Sci 9, pp. 149. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/29541079

Linn F, Heidmann I, Saedler H, and Meyer P. 1990. *Epigenetic changes in the expression of the maize A1 gene in Petunia hybrida: role of numbers of integrated gene*

copies and state of methylation. Molecular & general genetics: MGG 222, pp. 329-336.

Meyer P, Heidmann I, Forkmann G, and Saedler H. 1987. *A new petunia flower colour generated by transformation of a mutant with a maize gene*. Nature 330, pp. 677.

Oud JS, Schneiders H, Kool AJ, and van Grinsven MQ. 1995. *Breeding of transgenic orange Petunia hybrida varieties*. Euphytica 84, pp. 175-181.

Pröls F and Meyer P. 1992. *The methylation patterns of chromosomal integration regions influence gene activity of transferred DNA in Petunia hybrida*. The Plant Journal 2, pp. 465-475. Retrieved from https://onlinelibrary.wiley.com/doi/abs/10.1046/j.1365-313X.1992.t01-20-00999.x Last accessed 12/14/2018.

Schwarz-Sommer Z, Shepherd N, Tacke E, Gierl A, Rohde W, Leclercq L, Mattes M, Berndtgen R, Peterson PA, and Saedler H. 1987. *Influence of transposable elements on the structure and function of the Al gene of Zea mays*. The EMBO Journal 6, pp. 287-294.

Standford Univ. 1997. *Molecular Biology Vector Sequence Database*. Retrieved from http://genome-www.stanford.edu/vectordb/vector_pages/plasmid_pages/0-PDnotcomplete_plasmid.html

Velten J, Velten L, Hain R, and Schell J. 1984. *Isolation of a dual plant promoter fragment from the Ti plasmid of Agrobacterium tumefaciens*. The EMBO Joumal 3, pp. 2723-2730. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC557759/

Westhoff Vertriebsgesellschaft mbH. 2019. *Petition for the determination of non-regulated status for petunias containing the A1 gene of Maize (A1-DFR petunias)*. Submitted by C. Westhoff, CEO. Westhoff Vertriebsgesellschaft mbH. Germany.