Ishihara Sangyo Kaisha, LTD. Petition for Determination of Nonregulated Status for Moth Orchid (*Phalaenopsis* spp. Hybrid) Containing the Flavonoid 3'5'-Hydroxylase Gene of Asiatic Dayflower (*Commelina communis*) Event ISK311NR-4

OECD Unique Identifier: ISK-311NR-4

Draft Plant Pest Risk Assessment

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1 INTRODUCTION

In March 2025, Ishihara Sangyo Kaisha, LTD. (ISK) submitted a petition (ISK 2025) to the U.S. Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS) requesting a determination of nonregulated status for genetically engineered moth orchid (*Phalaenopsis* spp. hybrid) (referred to as 311NR Phalaenopsis; OECD Unique Identifier ISK-311NR-4), and any progeny derived from them, under Title 7 of the Code of Federal Regulations part 340 (7 CFR 340). APHIS assigned number 25-062-01p to the petition. 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. 7701 *et seq.*). 311NR Phalaenopsis has been genetically engineered to express the flavonoid 3' 5'-hydroxylase enzyme from *Commelina communis* allowing the plants to produce delphinidin-based anthocyanins resulting in blue-purple colored flowers. APHIS conducted this plant pest risk assessment (PPRA) to determine whether 311NR Phalaenopsis is unlikely to pose a greater plant pest risk than the nonmodified recipient organism from which it was derived.

APHIS regulations in part 340 regulate the introduction (importation, interstate movement, or release into the environment) of certain organisms and products altered or produced through genetic engineering. A genetically engineered organism is considered a "regulated article" under part 340 if the donor organism, recipient organism, or vector or vector agent belongs to any genera or taxa designated in § 340.2 and meets the definition of 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 as defined in § 340.1. A genetically engineered plant (modified plant) that meets the definition of a regulated article is no longer subject to the regulatory requirements of part 340 when APHIS determines that it is unlikely to pose a greater plant pest risk than the nonmodified plant from which it was derived.

ISK produced 311NR Phalaenopsis by genetically modifying Wedding Promenade PP3387 Phalaenopsis using a plant pest vector, *Agrobacterium tumefaciens*, to insert specific genetic sequences (ISK 2025). 311NR Phalaenopsis contains the 35S promoter and terminator from Cauliflower Mosaic Virus and left and right T-DNA border sequences and NOS (nopaline synthase) terminator from *A. tumefaciens*, which are plant pests, along with other sequences. Therefore, 311NR Phalaenopsis is considered a regulated article under APHIS regulations in part 340.

APHIS assessed the potential for increased plant pest risk associated with the unconfined release of 311NR Phalaenopsis and its progeny relative to the nonmodified plant and other appropriate comparators. APHIS regulations in § 340.6(c) specify the information needed in a petition for nonregulated status. The PPRA considered information submitted by the applicant and publicly available information related to the genetic and phenotypic differences between 311NR Phalaenopsis and its comparator(s), including: expression of the gene product, new enzymes, or changes to plant metabolism; plant pest risk characteristics; disease and pest susceptibilities; indirect plant pest effects on other agricultural products; effects of the regulated article on nontarget organisms beneficial to agriculture; weediness of the regulated article; impact on the weediness of any other plant with which it can interbreed; changes to agricultural or cultivation practices that may impact diseases and pests of plants; transfer of genetic information to organisms with which it cannot interbreed; and any other information which the administrator believes to be relevant to a determination.

311NR Phalaenopsis may also undergo regulatory review by other U.S. agencies. Under the Coordinated Framework, the oversight of biotechnology-derived plants rests with the Department of Agriculture (USDA), the Food and Drug Administration (FDA), and the U.S. Environmental Protection Agency (EPA) (51 FR 23302; 57 FR 6753; Biotechnology WG 2017). Biotechnology-derived plants are subject to review by one or more of these agencies depending on their characteristics and intended use. FDA has primary responsibility for ensuring the safety of human food and animal feed (Biotechnology WG 2017). EPA regulates pesticides, including plants with plant-incorporated protectants (pesticides intended to be produced and used in a living plant), to ensure public safety (Biotechnology WG 2017).

2 BACKGROUND

Purpose of the modification

Ishihara Sangyo Kaisha, Ltd (ISK) developed 311NR Phalaenopsis using genetic engineering techniques to produce a blue-purple flower color (ISK 2025). The comparator Phalaenopsis Wedding Promenade PP3387 produces a red-purple flower (ISK 2025). The genus Phalaenopsis, commonly known as moth orchids due to their flower shape resembling a moth in flight, is one of the most popular groups of orchids worldwide. Their flowers come in a wide range of colors and patterns, including white, yellow, and purple (red-purple, purple, purple-violet, violet and violet-blue) (Liang et al. 2020). Violet-blue orchids are more difficult to breed because there are few native species with blue flowers (Liang et al. 2020).

The genetically engineered 311NR Phalaenopsis will offer consumers an additional choice to purchase a Phalaenopsis that produces blue-purple flowers. Currently, it is possible to purchase

blue colored *Phalaenopsis* spp. hybrids plants that have been created by the injection of dye into white flowers.

Biology of the comparator plant

Phalaenopsis is the most popular cultivated orchid worldwide. It is an economically important horticultural crop cultivated either as an ornamental or for cut flowers (Tiwari et al. 2024). The genus *Phalaenopsis* Blume (*Orchidaceae*) includes around 80 accepted species and nine natural hybrids (POWO 2024). Wedding Promenade is the cultivar from which 311NR Phalaenopsis was developed (ISK 2025). Wedding Promenade is a commercial hybrid of multiple species in the subgenus *Phalaenopsis* with the following genetic makeup: 50% *Phalaenopsis equestris*, 18% *P. amabilis*, 13% *P. schilleriana*, 10% *P. amabilis* subsp. amabilis, 5% *P. Aphrodite*, 3% *P. sanderiana*, and 0.5% *P. stuartiana* (OrchidRoots 2025).

Phalaenopsis species grow naturally in evergreen forests of tropical and subtropical Asia as epiphytes, typically on tree branches and trunks. These environments are characterized by consistently humid, warm, and relatively shaded conditions (Zuk 1997; Christenson 2001). Growth of Phalaenopsis in cultivation is slow, and it can take one to two years from germination for plants to flower under optimal temperature conditions (Van Tongerlo et al. 2021).

Phalaenopsis orchids occur in the United States in cultivation and are commercially produced in greenhouses. They can be grown on trees and in gardens in Hawaii, Florida, Puerto Rico, and parts of California (American Orchid Society 2024). There is no evidence that Phalaenopsis volunteers or escapes from indoor cultivation locations like greenhouses or from gardens in these locations (APHIS 2003).

In their natural habitat, Phalaenopsis orchids normally reproduce by insect pollination, commonly bees and potentially other insects such as wasps and moths, whereas in cultivation they are hand pollinated to create hybrids (Arditti 1980; Ray and Vendrame 2015; Ackerman et al. 2023). The reproductive structure in orchids consists of a central structure known as the column which houses both the anther and stigma, and the labellum (or lip) right below the column that acts as a landing area for pollinators. As insects move up the lip they come into contact with the pollen which in orchids is known as pollinia (Ray and Vendrame 2015). Fertilization occurs months after the flowers have been pollinated and fruits may take a year or more to ripen (Arditti 1980). If pollination works, pollen tubes develop and grow inside the stigma and the ovary of the flower will form seed capsules filled with thousands of seeds.

Orchid seeds rarely germinate on their own as they lack the nutrient-rich endosperm and rely on mycorrhizal fungi for water and nutrients (Ray and Vendrame 2015).

3 DESCRIPTION OF INSERTED GENETIC MATERIAL, ITS EXPRESSION PROFILE, GENE PRODUCT, AND CHANGES TO PLANT METABOLISM AND PHENOTYPE

APHIS assessed genetic, metabolic, and phenotypic changes to the plant known or plausibly expected to occur due to the genetic modification. This information is used in this risk assessment to evaluate the potential for 311NR Phalaenopsis to pose a greater plant pest risk than the unmodified comparator by reviewing the known and potential differences related to pest and disease impacts, weediness of the modified plant, changes to weediness of sexually compatible relatives, harm to nontarget organisms beneficial to agriculture, changes to agricultural and cultivation practices, and impacts from transfer of genetic information to organisms with which the modified plant cannot interbreed.

Description of the genetic modification and inheritance of the inserted DNA

To inform the evaluation of potential hazards resulting from the genetic modification and potential routes of exposure related to the inserted DNA and its 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 311NR Phalaenopsis; 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 inserted copies. APHIS also assessed data presented in the petition regarding whether the genetic modification results in the expression of new genes, proteins, enzymes, or changes in plant metabolism or composition in 311NR Phalaenopsis relative to the comparator. This assessment includes a consideration of the expressed flavonoid 3', 5'-hydroxylase (*F3'5'H*) of Asiatic dayflower (*Commelina communis*) (*CcF3'5'H*) and Hygromycin B phosphotransferase (*hpt*) from *Escherichia coli*. Additionally, it examines any observed or anticipated effects on the composition of 311NR Phalaenopsis, including relevant physiological changes of 311NR Phalaenopsis relative to the comparator plant.

Agrobacterium mediated transformation method was used to create the genetically modified 311NR Phalaenopsis

The petitioner used the Agrobacterium-mediated transformation method to create the genetically modified 311NR Phalaenopsis. Protocorm-like bodies of the comparator Phalaenopsis PP3387 were inoculated by co-culturing with *Agrobacterium tumefaciens*

containing expression vector *pBIH-35S-CcF3'5'H* (Figure 1) (ISK 2025). Protocorms are the small, rounded tuber-like bodies formed by germinating orchid seeds, and protocorm-like bodies are similar in structure to protocorms but are formed by tissue culture of explants and/or from callus in vitro (Paek et al. 2011).

Figure 1. (re-created from ISK 2025) Map of the T-DNA construct inserted into genetically modified 311NR Phalaenopsis. T-DNA contains right T-DNA border (RB), promoter of cauliflower mosaic virus 35S RNA (CaMV35S, P35S), Hygromycin B phosphotransferase from *Escherichia coli* (*hpt*), Terminator of 3' terminal untranslated region of the nopaline synthase gene from Ti plasmid (TNOS), flavonoid 3', 5'-hydroxylase (*F3'5'H*) of Asiatic dayflower (*C. communis*) (*CcF3'5'H*), Terminator of the 3' terminal untranslated region of 35S RNA of the cauliflower mosaic virus (T35S) and left T-DNA border.

Disarmed A. tumefaciens was used for transformation and was eliminated from the 311NR Phalaenopsis

The plasmid *pBIH-35S-CcF3'5'H* used for the development of 311NR Phalaenopsis was constructed based on the binary vector pBI121 (Chen et al. 2003). Components other than the T-DNA region in *pBIH-35S-CcF3'5'H*, called vector backbone, consists of non-T-DNA region of a binary vector Bin19 (Frisch et al. 1995). The construct used for the transformation does not contain any tumorigenic DNA encoding for oncogenes (Figure 1).

Southern analysis experimental results confirmed the absence of vector backbone in genetically modified 311NR Phalaenopsis (page #29-30, ISK 2025). PCR analysis of the aminoglycoside phosphotransferase III (*aphA-3*) gene, which is in the non-T-DNA region of plasmid *pBIH-35S-CcF3'5'H*, confirmed the absence of vector backbone and *A. tumefaciens* (page #36-37, ISK 2025). Furthermore, ISK used a colony formation assay to confirm the absence of live *A. tumefaciens,* which is a plant pest, in 311NR Phalaenopsis (page # 38-40, ISK 2025). Southern and PCR analyses demonstrated the absence of any unintended DNA in the 311NR Phalaenopsis.

Inserted DNA components in the 311NR Phalaenopsis

ISK stably integrated the genes of interest and their regulatory elements within the left and right borders of T-DNA into the genome of Wedding Promenade PP3387 Phalaenopsis (Figure 1). They modified 311NR Phalaenopsis to constitutively express Flavonoid 3', 5'-hydroxylase (*F3'5'H*) from *C. communis* (Asiatic dayflower) and Hygromycin B phosphotransferase (Hpt) from *E. coli* (Gritz and Davies 1983) as a selection marker. They employed the cauliflower

mosaic virus 35S RNA (CaMV35S, P35S) promoter to drive the expression of both genes, while the 3' terminal untranslated region of the 35S RNA (T35S) and of the nopaline synthase gene from Ti plasmid (TNOS) terminator terminated the transcription of the *CcF3'5'H* and *hpt* genes, respectively.

The petitioner placed the omega leader, a 5' terminal untranslated region from the tobacco mosaic virus (TMV) omega sequence (Gallie 2002), upstream of the *F3'5'H* gene to enhance its translation (ISK 2025). Furthermore, some small synthetic intervening sequences (15 to 80 nucleotides) are present in between the genetic elements in the T-DNA. These intervening sequences are not known to code any protein or form any regulatory elements. They are synthetic sequences used in DNA cloning and there is no other apparent function known for these synthetic sequences.

Inserted DNA is a single copy, intact and stably integrated

ISK confirmed the insertion and stability of the genetic elements listed above by conducting a detailed molecular characterization of the inserted T-DNA to determine the number of insertions within the plant genome, integrity of the insert, stable genetic inheritance of the inserted cassettes, and the absence of plasmid backbone sequences in 311NR Phalaenopsis (ISK 2025). PCR analysis and Southern analysis confirmed the presence of inserted DNA, which encompassed only one copy of the inserted genes *CcF3'5'H* and *hpt*, and was stably integrated in the genome of modified 311NR Phalaenopsis (ISK 2025). ISK analyzed and confirmed the stable integration of inserted DNA in the T0 clonal plants by testing the presence and functionality of the *hpt* marker gene, which confers hygromycin resistance, in the clones (ISK 2025). These analyses confirmed that the inserted DNA in 311NR Phalaenopsis is intact, exists in a single copy and is stably integrated in all seedling clones (Gelvin 2017; ISK 2025). Given that the transgenic DNA is stably integrated into the genome of 311NR Phalaenopsis, APHIS expects its stable inheritance across generations.

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

CcF3'5'H and hpt genes are expressed in flowers, leaves, and roots in 311NR Phalaenopsis

The petitioner analyzed the expression of the *CcF3'5'H* and *hpt* genes in the flower petals and the leaf tissues of the genetically modified lines using RT-PCR (ISK 2025). The results showed the constitutive expression of both *F3'5'H* and *hpt* genes in the flower petals and leaf tissues. Both genes are under the control of CaMV35S promoter. CaMV35S is a constitutive promoter known to be active in roots, leaves, and flowers in monocot plants (Terada and Shimamoto

1990). ISK did not analyze the complete expression pattern of the *CcF3'5'H* and *hpt* genes in 311NR Phalaenopsis; they reported expression in the leaves and flowers in their petition, but these genes are plausibly expressed constitutively in the root tissues too because of the CaMV35S promoter. The petitioner has shown that the genetically modified plants were able to grow roots in hygromycin-containing media plates, supporting APHIS' expectation that the genes are also expressed in root tissues.

F3'5'H is involved in the delphinidin-based anthocyanin biosynthesis leading to the production of blue-purple colored flower

The petition has explained the role of *F3'5'H* in delphinidin-based anthocyanin biosynthesis (ISK 2025). Flower color is determined by the production of pigments, namely anthocyanins, terpenoids, and betalains (Tanaka et al. 2008). Anthocyanins are a class of flavonoids that contribute to flower color in the orange/red and violet/blue spectrum (Katsumoto et al. 2007; Tanaka et al. 2008; Liang et al. 2020). Flavonoids are widely distributed among plants and comprise 19 different types of anthocyanins. These pigments comprise a skeleton of 15 carbon atoms (C6-C3-C6) containing two phenolic rings (A, B) and one pyran (C) ring (Husain et al. 2022). The color generated by anthocyanins depends greatly on the number of hydroxyl groups on the B-ring of each molecule. The more hydroxyl groups present, the deeper the blue color produced (Tanaka et al. 2008). Based on the number of hydroxyl groups, three different types of anthocyanins are known (Figure 2) (Katsumoto et al. 2007). Pelargonidin-based anthocyanins possess one hydroxy group in their B-ring, producing an orange-red color in flower petals. Cyanidin-based anthocyanins possess two hydroxy groups in their B-ring, producing a red-purple color in flower petals. Delphinidin-based anthocyanins possess three hydroxy groups in their B-ring, producing a violet-blue color in flower petals.

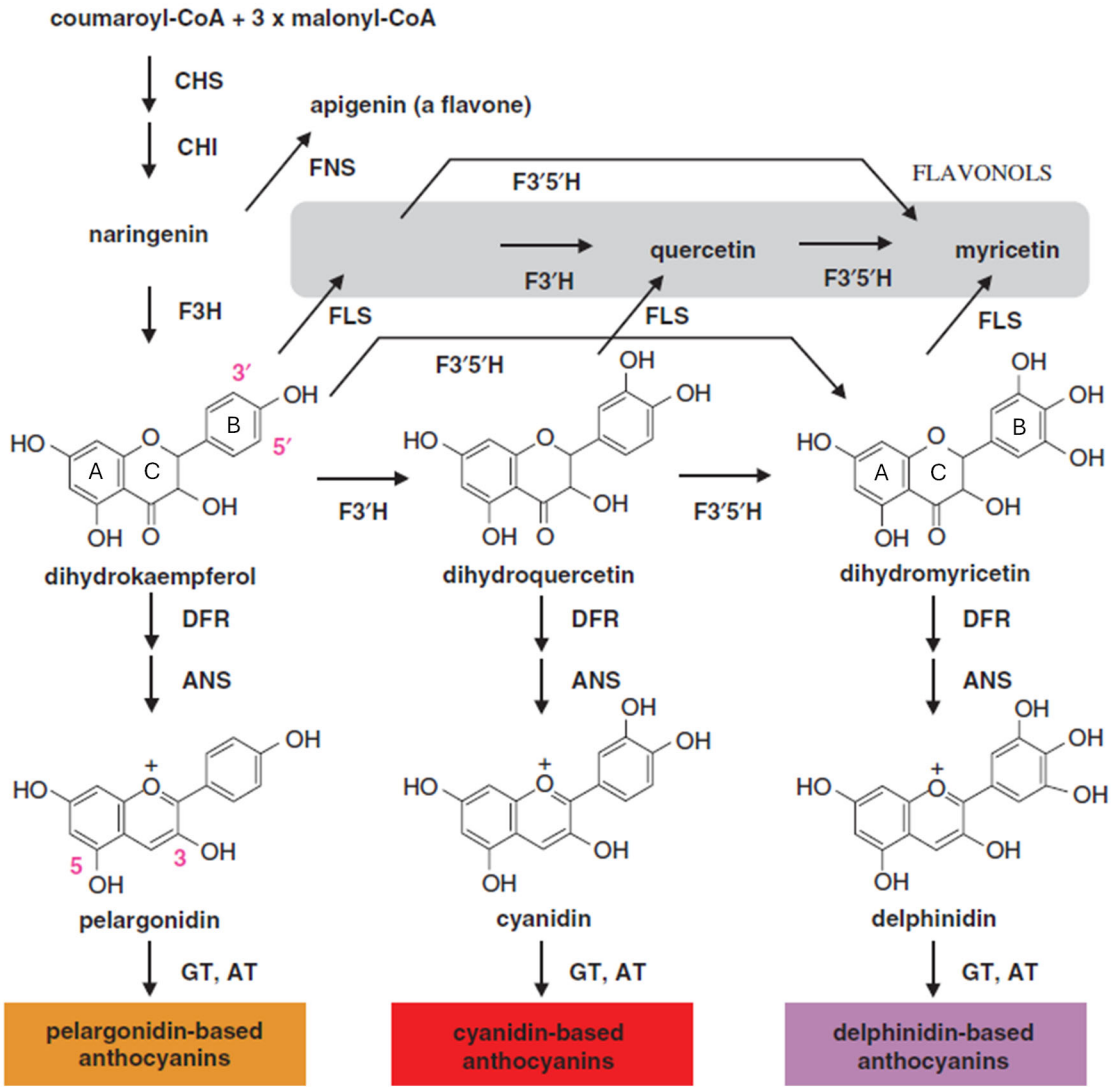

Figure 2 (Katsumoto et al. 2007). Generalized flavonoid biosynthetic pathway relevant to flower color. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; GT, anthocyanidin glucosyltransferase; AT, anthocyanin acyltransferase. Flavanols comprise two phenolic rings (A, B) and one pyran (C) ring (Husain et al. 2022).

Analysis of the anthocyanins revealed that cyanidin is the sole anthocyanin compound in Phalaenopsis of different colors (Liang et al. 2020). In many plants, a functional F3'5'H enzyme typically leads to the production of blue or purple flowers, provided all other anthocyanin pathway enzymes are active. The red flower color observed in some Phalaenopsis orchids suggests the presence of all other necessary pathway enzymes. Furthermore, evolutionary

trends in angiosperms indicate that the shift from blue to red flowers due to the loss of *F3'5'H* function is more frequent than a change from red to blue (Tanaka and Brugliera 2013).

The F3'5'H and delphinidin anthocyanin are found naturally in many plants

The petitioner has explained that the insertion of the *CcF3'5'H* gene in the modified 311NR Phalaenopsis flower produces dihydromyricetin, which is then converted to delphinidin. The accumulation of delphinidin produces the intended blue-purple color phenotype in 311NR Phalaenopsis flowers. The *F3'5'H* gene and its catalytic product delphinidin are found naturally in many ornamental and food plants, most often resulting in blue colors, including *C. communis* (donor plant of *CcF3'5'H* of 311NR Phalaenopsis) (ISK 2025, Page # 18), *Petunia hybrida* (petunia) (Toguri et al. 1993), *Catharanthus roseus* (Madagascar periwinkle), *Vitis vinifera* (grape) (Bogs et al. 2005), *Campanula medium* (Canterbury bells), *Solanum tuberosum* (potato), *Solanum melongena* (eggplant) (Chapple 1998), and many others (Olsen et al. 2010).

Hygromycin B phosphotransferase (hpt) was used as a selection marker

ISK has constitutively expressed the Hygromycin B phosphotransferase gene (*hpt*) from *E. coli* in the 311NR Phalaenopsis as a selectable marker. Hygromycin B, an aminoglycoside antibiotic, inhibits protein synthesis by binding and causing conformational changes in the ribosome (Borovinskaya et al. 2008). Hygromycin B phosphotransferase (Hpt) protein is a kinase that catalyzes the transfer of the phosphate group of ATP to hygromycin B, which is then inactivated and loses its growth inhibitory activity (Rao et al. 1983). Accordingly, hygromycin B can be used to select transformed plants. The activity of Hpt shows high specificity to compounds of hygromycin B-like aminoglycoside antibiotics (EFSA 2004).

The production of Hpt in plant cells is only relevant in the presence of a suitable substrate. Presence of hygromycin B in the environment is a highly unlikely situation due to the limited clinical and veterinary use of hygromycin B (OGTR 2024). Unless plants encounter hygromycin B in nature, no other phenotypic effects are expected due to the absence of a substrate for Hpt enzyme activity.

Hpt does not have any significant similarity with known allergen proteins and does not trigger any immunoreaction (Lu et al. 2007), and is not toxic to animals (Zhuo et al. 2009). APHIS has previously reviewed genetically modified plants containing the *hpt* gene as a selection marker and found no plausible pathway to risk associated with the expression of this gene (APHIS-USDA 2005). Overall, there are no plausible pathways for the Hpt protein to interfere with biological processes in plants and to cause any risk associated with plant pests.

Changes to plant phenotype

The overexpression of the *CcF3'5'H* in 311NR Phalaenopsis produced blue-purple-colored flowers instead of the red-purple color of the nonmodified comparator Wedding Promenade PP3387 (ISK2025). The overexpression of Phalaenopsis *PhF3'5'H* in petunia deepened the flower color and increased anthocyanin content by approximately 70% (Lou et al. 2023) indicating that 311NR Phalaenopsis might also have an increased anthocyanin content in addition to the production of delphinidin anthocyanin. Any increase in total anthocyanin is likely to be modest, as elevated delphinidin levels, may be offset by reduction in cyanidin content, as predicted from the anthocyanin biosynthesis pathway (Figure 1) and evident from a shift in anthocyanin composition resulting in the change of flower color from red purple to blue purple. Anthocyanins are well-known antioxidants and play a role in plant biotic and abiotic stresses (Yang et al. 2016; Li and Ahammed 2023) suggesting the potential for a slight increase in stress tolerance of the modified plant.

Increased expression of flavonoid biosynthesis pathway genes, including *PmF3'5'H* in *Pinus massoniana*, was correlated to increased insect resistance (Yang et al. 2016) and delphinidin is also known to suppress the germination of *Fusarium solani* spores (Kraft 1977). Anthocyanins are known to alleviate oxidative stress during drought by decreasing the reactive oxygen species including H_2O_2 and O_2^- (Jan et al. 2024), suggesting that increased anthocyanins might increase drought tolerance in 311NR Phalaenopsis. While direct studies on the overexpression of F3'5'H in drought tolerance are not available, overexpression of the pearl millet PgF3H gene, a key enzyme in the same pathway of flavonoid synthesis, increased flavonol and anthocyanin accumulation, and enhanced tolerance to water-deficit stress in Arabidopsis (Shivhare et al. 2025). This indicates that overexpression of *F3'5'H* in Phalaenopsis might enhance the drought tolerance in 311NR Phalaenopsis. However, as any increase in total anthocyanin levels within 311NR Phalaenopsis is expected to be modest, the corresponding enhancement in drought tolerance is also likely to be modest. Furthermore, Phalaenopsis orchids are predominantly cultivated indoors or in controlled greenhouse environments and face multiple constraints, including slow growth rates, reliance on specific substrates, dependence on mycorrhizal fungi for germination, sensitivity to cold temperatures, and reliance on pollinators for reproduction. Consequently, any modest enhancement in the drought tolerance of 311NR Phalaenopsis is likely to have an insignificant impact.

Anthocyanins confer plant tolerance to cold stress by scavenging reactive oxygen species and enhancing photosynthetic efficiency during cold stress (Li et al. 2025) suggesting that 311NR Phalaenopsis might have an increased cold tolerance due to a potential increase in anthocyanin content. The petitioner has analyzed the cold and heat stress tolerance of the 311NR

Phalaenopsis (page # 44-49, ISK 2025). They observed no changes in the cold tolerance, while the heat tolerance of modified plants was lower than that of comparator plants. Given that ISK did not observe any significant changes to cold tolerance, and observed lower heat tolerance than comparator plants, APHIS expects no increase in survival or greater geographical distribution of this modified plant.

The overexpression of *PhF3'5'H* in *Phalaenopsis* increased the number of branches and flowers (Lou et al. 2023). This indicates that the overexpression of *CcF3'5'H* in Phalaenopsis could lead to morphological changes in 311NR Phalaenopsis. APHIS assessed ISK's field trial data for morphological and growth characteristics (page # 41-50, ISK 2025). While some characteristics did not show any statistically significant difference between the nonmodified and genetically modified lines, the size of the largest leaf showed a statistically significant decrease in the genetically modified 311NR Phalaenopsis (ISK 2025). Although the petitioner did not observe an increase in branching, they observed subtle somaclonal variations in morphology including petal shape between different transformation events. However, the 311NR Phalaenopsis line selected for this petition displayed minimal variation in morphological characters (Page # 14, ISK 2025).

4 POTENTIAL PLANT PEST AND DISEASE IMPACTS

The modified 311NR Phalaenopsis overexpressing the flavonoid 3'5'hydroxylase (*F3'5'H*) gene, may exhibit an increased total anthocyanin content, along with higher levels of delphinidin-based anthocyanin (Lou et al. 2023). Anthocyanins are well-known antioxidants that play a crucial role in plant responses to biotic stresses (Yang et al. 2016; Li and Ahammed 2023). The *F3'5'H* gene, along with delphinidin levels, is upregulated in response to different biotic stressors such as pests, defense hormones, and viral infections. Delphinidin has been shown to inhibit fungal sporulation *in vitro* (Kraft 1977), indicating its potential to confer resistance to the plant pathogens and contribute to plant protection against diseases.

Furthermore, enhanced expression of three genes involved in flavonoid biosynthesis pathway, *PmF3H*, *PmC4H* and *PmF3'5'H* were observed against leaf eating pests in *P. massoniana* (Yang et al. 2016). Both the *F3'5'H* gene and delphinidin levels have been upregulated in response to biotic stressors such as pests, defense hormones and viral infections. Overall, these findings imply that the *F3'5'H* gene could be a stress responsive gene and may play a role in enhancing Phalaenopsis defense responses, potentially leading to increased disease resistance in the modified 311NR Phalaenopsis (Kraft 1977; Gutha et al. 2010; Yang et al. 2016; Zhou et al. 2016;

Negi et al. 2023). Although an increase in delphinidin might marginally enhance the insect resistance of the 311NR Phalaenopsis, this improvement may not be significant, as it may require the coordinated overexpression of additional genes.

The other introduced gene in the modified 311NR Phalaenopsis is the hygromycin B phosphotransferase (*hpt*) gene. This gene is not known to pose plant pest risk. Hygromycin B is the second most frequently used antibiotic selectable marker after kanamycin and ranks the second most frequently employed in transgenic plants under field trials in the United States (Miki and McHugh 2004). The Hpt enzyme activity is highly specific to hygromycin B-like compounds, such as aminoglycoside antibiotics (Daigle et al. 1999), and it is rarely reported to have any effects beyond those anticipated. It was used as a selectable marker in the tissue culture of Phalaenopsis plants.

Based on APHIS' review of the petition and publicly available information, none of the phenotype changes that are known or plausibly expected to occur for the modified 311NR Phalaenopsis expressing the *F3'5'H* gene due to the genetic modification are expected to result in increased risk from plant pest and disease impacts relative to the comparator plants. This conclusion is based on an earlier assessment by USDA-APHIS of a modified, blue-colored rose containing delphinidin, its precursor biochemicals, and the catalytic enzyme F3'5'H. The evaluation found that these modifications did not alter the disease and pest susceptibilities of the modified rose relative to its nonmodified comparator plant (APHIS 2011). Orchids in the United States need human assistance to thrive outside their natural cultivation areas, typically in greenhouses, which significantly minimizes exposure risks. Moreover, if anything, expression of *CcF3'5'H* would increase disease resistance in 311NR Phalaenopsis.

Therefore, APHIS concludes that the modified 311NR Phalaenopsis is unlikely to alter potential plant pest and disease impacts or lead to greater plant pest risk than the nonmodified comparator.

5 POTENTIAL IMPACTS ON NONTARGET ORGANISMS BENEFICIAL TO AGRICULTURE

Flower colors play a crucial role in attracting insects for pollination. Consequently, flower color changes could influence the pollination behavior of insects. The insertion of the *F3'5'H* gene into the comparator orchid background results in blue-purple flower color (section 3). In the wild, Phalaenopsis flowers are mainly pollinated by bees and potentially other hymenoptera and lepidoptera insects, such as wasps, moths, and butterflies (Ackerman et al. 2023). Research has shown that some pollinator insects like honeybees and bumblebees prefer shorter-

wavelength blue colors (Dyer et al. 2020) and blue flowers offer higher rewards more frequently (Giurfa et al. 1995); therefore, the new blue-purple color of the modified orchid could potentially attract additional nontarget beneficial pollinators known to pollinate Phalaenopsis. Although increased levels of anthocyanins have been linked to increased insect resistance, there is no evidence showing delphinidin producing flowers are toxic to pollinators. Moreover, some phytochemicals are known to have a defensive role against insects but are beneficial for pollinators (Stevenson 2020) and anthocyanin-derived polyphenols are major phytonutrients of honey (Jibril et al. 2019). The *F3'5'H* gene is not known to increase any known plant defense-related metabolite to date. In addition, Phalaenopsis orchids are cultivated indoors in the United States, greatly diminishing any potential exposure to wild pollinators. It is also worth mentioning that bees, wasps, and other hymenopteran insects (that could potentially pollinate Phalaenopsis) do not rely on orchids as their main source of food; they all pollinate hundreds of wild plants and crops (Hristov et al. 2020) hence greatly diminishing any potential exposure of pollinators to a single food source. Finally, since Phalaenopsis orchids are predominantly cultivated in greenhouses or indoors, the exposure to nontarget beneficial organisms, such as pollinators, is significantly reduced. Therefore, changing the flower color to blue purple is unlikely to impact nontarget beneficial pollinators.

After reviewing the petition and available public information, APHIS concludes that none of the expected or known changes in the phenotype of the modified 311NR Phalaenopsis is likely to increase the risk to beneficial nontarget organisms in agriculture compared to the nonmodified plants; therefore, the modified 311NR Phalaenopsis is not expected to pose a greater pest risk than the nonmodified counterpart.

6 POTENTIAL FOR INCREASED WEEDINESS OF THE MODIFIED PLANT

APHIS evaluated the characteristics of the modified plant that could influence weediness or the ability to manage the plant as a weed. The comparator Wedding Promenade PP3387 is not regulated as a noxious weed in the United States (APHIS 2010; USDA-AMS 2024). *Phalaenopsis* spp. are slow-growing plants that typically take around three years to reach reproductive age (Sauleda 1976). Their seeds usually do not germinate independently; they require human assistance or specific mycorrhizal fungi that provide essential carbon resources (Chamara et al. 2024). Phalaenopsis grows naturally in humid and warm evergreen forests of tropical and subtropical forests of Asia (Zuk 1997; Christenson 2001), suggesting it is unfit to grow in most of the United States. As a result, these orchids are unlikely to establish themselves outside of indoor cultivated areas in the United States without human assistance. With human assistance they could be cultivated in gardens in Hawaii, Florida, Puerto Rico, and parts of California

(American Orchid Society 2024; Fairchild Tropical Botanic Garden 2024). Additionally, orchids possess ecological traits that further limit their growth and spread, such as their unique interactions with pollinators and their reliance on mycorrhizal fungi for seedling development and sometimes even into adulthood (Arditti 1980; Chamara et al. 2024). No reports of invasiveness of *Phalaenopsis* spp. have been observed in key references recognized as standards (Holm et al. 1977; Reed 1977; Holm et al. 1979). Moreover, decades of permitted importation have not led to any documented cases of *Phalaenopsis* spp. becoming invasive in the United States, including Hawaii and Florida (APHIS 2003). The PLANTS database (NRCS accessed 2025) and other weed reporting services (APHIS 2010; USDA-AMS 2024) do not include reports of imported moth orchids as invasive or having weed potential (APHIS 2003).

APHIS conducted a further assessment of the potential for increased survival and geographical distribution of modified Phalaenopsis that might contribute to the weediness relative to the nonmodified plants. As described in section 3, 311NR Phalaenopsis may have an increased anthocyanin content in addition to the production of delphinidin anthocyanin. Anthocyanins are known to play a role in plant biotic and abiotic stresses by scavenging reactive oxygen species, including H_2O_2 and O_2^- (Yang et al. 2016; Li and Ahammed 2023; Jan et al. 2024). The constitutive overexpression of *CcF3'5'H* gene may slightly increase plant resistance to diseases and insect herbivores, drought, and cold stress.

Epiphytic orchids, including *Phalaenopsis* spp., already have some drought adaptive characteristics such as rapid water uptake by the velamen radicum, succulent leaves for water storage, and leaves with thick cell walls, cuticles, and sunken stomata to minimize water loss (Zhang et al. 2018). Velamen radicum comprises dead cells covering the entire root except the tip and assists in the absorption of moisture and nutrients from the surrounding humid atmosphere. Although elevated delphinidin levels may enhance drought tolerance in 311NR Phalaenopsis, the actual effect is likely to be minimal. Orchids, especially as epiphytes, are naturally adapted to tolerate environmental stresses such as water-limiting conditions. Nevertheless, where phalaenopsis currently can be cultivated outdoors, these plants are not expected to survive independently due to multiple additional limitations, as discussed earlier. Moreover, when grown outdoors in Hawaii, Florida, Puerto Rico, and parts of California, these orchids are generally grown in containers or on tree trunks, and are not grown or able to grow in open agricultural fields (American Orchid Society 2024; Fairchild Tropical Botanic Garden 2024). Therefore, concerns regarding drought and cold tolerance do not significantly impact the spread or invasiveness of this orchid.

Similarly, anthocyanins might increase plant tolerance to cold stress by scavenging reactive oxygen species and enhancing photosynthetic efficiency during cold stress (Li et al. 2025), suggesting a plausible increase in the cold tolerance of the genetically modified plant. When APHIS evaluated the ISK's experimental results on cold and heat tolerance in the modified plants, they observed no changes in cold tolerance, but there was a decrease in heat tolerance (ISK2025).

Finally, APHIS evaluated the ISK's data on the morphological and growth characteristics of the 311NR Phalaenopsis. Their results indicate that 311NR Phalaenopsis is shorter, with fewer inflorescences and a smaller leaf but a slightly larger flower, suggesting decreased competitiveness relative to the nonmodified plant. ISK observed no difference for traits that would affect weediness, such as seed production by self-pollination.

Overall, while the genetically modified 311NR Phalaenopsis may exhibit subtle phenotypic changes compared to non-modified Phalaenopsis, these differences are not expected to enhance survival, reproduction, geographical distribution, or increase weediness. Phalaenopsis orchids face numerous limitations including slow growth, epiphytic nature, specific substrate requirements, dependence on mycorrhizae to germinate, intolerance to cold and arid conditions, and reliance on pollinators for reproduction. Furthermore, the observed decrease in heat tolerance further diminishes the modified plant's potential for weediness. Even if the genetic modification resulted in a slight increase in stress tolerance, it is not expected to increase weediness due to these multiple limiting factors. Therefore, APHIS concludes that 311NR Phalaenopsis is unlikely to have enhanced weediness that would lead to greater plant pest risk than its the nonmodified comparator.

7 POTENTIAL IMPACTS ON THE WEEDINESS OF OTHER PLANTS WITH WHICH THE MODIFIED PLANT CAN INTERBREED

Gene flow is a natural biological process with significant evolutionary importance. A number of angiosperm taxa are believed to be derived from hybridization or introgression between closely related taxa (Grant 1981; Rieseberg and Wendel 1993; Mallet 2005; Hegde et al. 2006; Stull et al. 2023) and even in the existing floras, the occurrence of hybridization or introgression is reported to be widespread (Rieseberg and Wendel 1993; Hegde et al. 2006). 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 other crops (Ellstrand et al. 1999).

APHIS assessed the potential for gene flow from 311NR Phalaenopsis to increase the weediness of sexually compatible relatives by evaluating: 1) the potential for hybridization and introgression from the modified crop event to sexually compatible relatives, including cultivated, wild, and weedy free-living relatives in the United States and its territories, and 2) the potential for phenotypes imparted by the genetic modification to increase weediness of relatives in the cases when gene flow may plausibly occur.

Potential for gene flow, and gene introgression

APHIS did not find any reports of natural intergeneric hybrids between *Phalaenopsis* spp. and other genera in the literature. However, within the Phalaenopsis genus it would be possible for gene flow to occur. However, this is highly unlikely. Firstly, pollen-mediated gene flow is more likely to occur over short to intermediate distances, usually less than one hundred meters (Zhang et al. 2019). Additionally, in the U.S., orchids do not typically reproduce without human assistance and are mostly cultivated in greenhouses. Outdoor cultivation of Phalaenopsis happens only in a few states, such as Florida, Hawaii, Puerto Rico, and parts of California, where orchids can be grown outdoors or on trees. Therefore, the likelihood of gene flow from the modified Phalaenopsis is very low, and the genetic modification is not expected to alter the potential for gene flow and introgression that would lead to greater plant pest risk than the nonmodified comparator.

Potential for enhanced weediness of recipients after hybridization and/or introgression

As noted above, gene flow is a natural biological process and is not an adverse effect per se. The identification of potential adverse effects on the environment resulting from the gene flow from a modified donor plant to a sexually compatible recipient plant should be informed by the characteristics of the donor species and the trait and phenotype of the modified plant, and of the potential receiving environment(s) including the characteristics of the recipient species (OECD 2023).

APHIS has determined that none of the plausible changes in phenotype that may occur in the modified 311NR Phalaenopsis are expected to result in increased weediness (as described in section 6). Even in the few U.S. states where Phalaenopsis can be grown outdoors (Florida, Hawaii, Puerto Rico, and parts of California), there is no evidence that it has naturalized after being introduced. Additionally, the change in flower color to blue is not expected to change the weediness of the modified plant (see section 6). Thus, even if gene flow or introgression occurs from the modified plant into other Phalaenopsis orchids, it is unlikely to enhance the weediness of other nonmodified Phalaenopsis orchids. Therefore, APHIS concludes that the modified 311NR Phalaenopsis is unlikely to enhance weediness of recipient orchids after hybridization

and/or introgression, which would lead to greater plant pest risk than the nonmodified comparator.

8 POTENTIAL CHANGES TO AGRICULTURAL OR CULTIVATION PRACTICES

Phalaenopsis species typically do not grow without intentional human assistance and are mainly cultivated in greenhouses across the Unites states. APHIS did not identify any significant changes to agricultural or cultivation practices resulting from the adoption of a modified 311NR Phalaenopsis that carries and expresses the flavonoid 3'5'-hydroxylase (*F3'5'H*) gene from *C. communis,* even if it is cultivated outside greenhouse conditions. Based on APHIS' review of the petition and publicly available information, the modified 311NR Phalaenopsis is not expected to cause changes to agriculture or cultivation practices, including pesticide applications, tillage, irrigation, harvesting, or any other pest management practices. Therefore, APHIS concludes that the modified 311NR Phalaenopsis is unlikely to alter agriculture or cultivation practices that would lead to greater plant pest risk than the nonmodified comparator.

9 POTENTIAL IMPACTS FROM TRANSFER OF GENETIC INFORMATION TO ORGANISMS WITH WHICH THE MODIFIED PLANT CANNOT INTERBREED

APHIS examined the potential for the genetic material inserted into 311NR Phalaenopsis to be horizontally transferred without sexual reproduction to other organisms and whether such an event could lead 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), also referred to as lateral gene transfer, between unrelated organisms, has been intensively studied since 1928, and the issue gained extra attention with the release of plants modified through genetic engineering into the environment (Wickell and Li 2020; Keeling 2024). The processes involved in HGT are understood and include mechanisms of DNA and RNA uptake (Pradhan et al. 2024). The potential risks associated with stable HGT from modified organisms have been reviewed (Keese 2008; Philips et al. 2022). APHIS considered the possibility of horizontal gene transfer from 311NR Phalaenopsis to parasitic plants, viruses, and other organisms in the surrounding environment including bacteria, fungi, and invertebrates.

Potential for horizontal gene transfer to parasitic plants

Plant-to-plant HGT is known to occur over evolutionary timescales (Wickell and Li 2020; Chen et al. 2021) but is unlikely over contemporary timescales. It is more likely when plant cells are in close physical contact, such as between a parasitic plant and its host, or between cells of two

plants in a graft junction, but genetic changes beyond the site where the cells are in contact are rarely observed (Bock 2010). Therefore, heritable instances of HGT are even less likely because these changes are not generally transmitted to germline cells (Bock 2010). *Phalaenopsis* spp. are not parasitic or known to serve as a host to any parasitic plants, nor is 311NR Phalaenopsis likely to be grafted with other plants, so even limited transfer by those mechanisms is not expected.

Transfers between mitochondrial genomes is the most prevalent form of HGT between plants, with some parasitic species having as much as 40% of their mitochondrial genomes originating from the mitochondrial genomes of host plant species (Davis and Xi 2015), although the transfer of nuclear genes between plants has also been demonstrated (Aubin et al. 2021). In 311NR Phalaenopsis, the DNA sequences were inserted into the nuclear genome, not the mitochondrial genome.

The known examples of HGT between non-parasitic plant species usually involve transposons, which have unique properties that enable their integration into nuclear genomes (Bock 2010; Gao et al. 2014). The genetic modification in 311NR Phalaenopsis is not associated with an intact transposon, so transfer by this mechanism is not expected.

Overall, HGT from *Phalaenopsis* spp. to other plants is extremely unlikely and the genetic modification is not expected to alter this likelihood. The genetic modification in 311NR Phalaenopsis is located in the nuclear genome and lacks transposon machinery to facilitate gene transfer. In addition, there is no indication that the inserted genes could change the rate of HGT. Therefore, APHIS concludes that 311NR Phalaenopsis is unlikely to cause increased risk related to HGT to parasitic plants.

Potential for horizontal gene transfer to viruses

HGT among viruses is a common mode of viral evolution but this is typically limited to viruses present within mixed infections and most commonly relies on homologous recombination (Keese 2008). Viral acquisition of non-viral genetic elements, including plant DNA sequences (Maori et al. 2007; Catoni et al. 2018), is rare despite a long history of host-virus association (Keese 2008). Furthermore, selection is a major barrier to viruses acquiring heterologous sequences (Keese 2008). Therefore, HGT of the inserted plant and bacterial genes from 311NR Phalaenopsis to viruses is considered unlikely.

Recombination between virus-derived sequences in a genetically modified plant and infecting viruses could possibly occur (Philips et al. 2022). 311NR Phalaenopsis contains noncoding

regions from the promoter and terminator of cauliflower mosaic virus 35S RNA (ISK 2025). These small sequences regulate the expression of the inserted genes. However, the presence of viral sequences in modified plants is unlikely to increase viral recombination or risk from horizontal transfer beyond what already occurs among viruses in mixed infections and from the broad range of viral sequences already present in plant genomes (Keese 2008). In addition, non-viral genes are unlikely to affect the function of plant viruses even if the presence of a viral promoter in the transgenic insert affected the rate of HGT involving such sequences. Despite over three decades of use, viral acquisition of these types of sequences from transgenic insertions has not been reported. Therefore, APHIS concludes that the potential for the emergence of a novel or more harmful plant viral pathogen through HGT in 311NR Phalaenopsis is considered extremely unlikely.

Potential for horizontal gene transfer to other organisms

Overall, the probability of HGT from plants to other organisms, such as bacteria, fungi, or invertebrates, is extremely low. Functional and selective barriers to HGT increase as organisms become more distantly related, contributing to the very low frequency of HGT from plants to other taxa (Keese 2008; Philips et al. 2022). As sequencing tools continue to improve, an increasing number of species across taxa have well-resolved, annotated genomes. Despite these advancements, relatively few incidents of HGT from plants have been reported.

Bacteria-to-bacteria HGT is a common mechanism of ongoing evolution in bacteria (Hall et al. 2017) but reports of potential plant-to-bacteria HGT (Miyashita et al. 2013; Nikolaidis et al. 2013; Haimlich et al. 2024) are comparatively rare (Philips et al. 2022). Although HGT from a genetically modified plant to bacteria has been observed to occur under optimized experimental settings (Kay et al. 2002), HGT of the inserted DNA from genetically modified plants to bacteria in the environment is considered unlikely (Philips et al. 2022; OGTR 2024) . Some consider there to be an increased risk from the use of bacteria-derived antibiotic marker genes, however, the presence of antibiotic resistance genes in modified plants is unlikely to increase the level of antibiotic-resistant bacteria beyond what is already present in the environment (Hily et al. 2018; Philips et al. 2022).

A small number of HGT events from plants to fungi have been reported (Richards et al. 2009; Nikolaidis et al. 2013; Li et al. 2018) but these are also considered rare. Despite the high diversity of invertebrates and close association with plants, HGT of plant genes has been observed very few times out of the over one million described species of insects (Gilbert and Maumus 2023), rotifers (Boschetti et al. 2012), and nematodes (Paganini et al. 2012; Ko et al. 2024). Considering that bacteria, fungi, and invertebrates have been co-evolving with plants for

millions of years, the scarcity of examples further supports that horizontal gene transfer from plants to these organisms is an extremely infrequent event and therefore is unlikely. In addition, the genetic modification is not expected to alter this likelihood. Therefore, APHIS concludes that HGT from 311NR Phalaenopsis to other organisms is also unlikely.

Based on the above analysis, APHIS concludes that HGT of the genetic material inserted into 311NR Phalaenopsis 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.

10 CONCLUSIONS

APHIS has reviewed the information submitted in the petition, supporting documents, public literature, and other relevant information to assess the potential for increased plant pest risk of the 311NR Phalaenopsis compared to the nonmodified plant from which it was derived. APHIS assessed the potential for the 311NR Phalaenopsis to pose greater risks in the following categories: plant pest and disease impacts, increased weediness of the modified plant, increased weediness of sexually compatible relatives, harm to nontarget organisms, changes to agricultural and cultivation practices, and impacts from the transfer of genetic information to organisms with which the modified plant cannot interbreed. Based on the APHIS' Plant Pest Risk Assessment, APHIS concludes that 311NR Phalaenopsis is unlikely to pose a greater plant pest risk than the nonmodified recipient organism from which it was derived based on the following findings.

- Based on information in petition and public literature about genetic modifications, 311NR Phalaenopsis is expected to express a plant-derived F3'5'H enzyme for purple blue flower color and a bacterial-derived Hpt enzyme as a selectable marker to identify modified plants during transformation. Both enzymes are expected to express in the flower petals, leaf and root tissues. No other significant phenotypic changes are expected based on the function of genetic modification.
- No plant pest risk was identified from the transformation process or the insertion of new genetic material in 311NR Phalaenopsis because the *Agrobacterium tumefaciens* transformation vector was disarmed, and the inserted plant pest sequences do not cause disease or create an infectious agent.
- A potential for enhanced resistance to plant pests was identified based on the function of the expressed genes in 311NR Phalaenopsis. Therefore, increased risk related to plant pest and disease susceptibility is not expected.
- Exposure to 311NR Phalaenopsis is unlikely to have any adverse impact on organisms beneficial to agriculture. It is not their only sole source of food, and a change of flower color to blue purple is unlikely to stop pollinator insects from visiting the modified orchid.

Phalaenopsis orchids are predominantly grown in greenhouses or indoors, greatly reducing exposure to the nontarget beneficial organisms, such as pollinators.

- 311NR Phalaenopsis is unlikely to have enhanced weediness that would lead to greater plant pest risk. Phalaenopsis does not occur outside of cultivation due to multiple limiting factors, including slow growth, epiphytic nature, specific substrate requirements, requirement of mycorrhizae to germinate, intolerance to cold and arid climates, and reliance on pollinators for reproduction, and the insertion of the *F3'5'H* gene is not expected to increase the weediness.
- No natural hybridization between Phalaenopsis and other genera is known. Gene flow within *Phalaenopsis* genus is possible, but it is unlikely due to limited pollen dispersal and the need for human-assisted reproduction. Outdoor cultivation is rare and limited to specific U.S. regions. Thus, the modified 311NR Phalaenopsis is not expected to increase the weed risk potential of other species.
- Introduction of 311NR Phalaenopsis is not expected to cause changes to agriculture or cultivation practices, including pesticide applications, tillage, irrigation, harvesting, or any other pest management practices.
- Horizontal gene transfer (HGT) from 311NR Phalaenopsis to other organisms is extremely 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.

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