ISHIHARA SANGYO KAISHA, LTD.

Bernadette Juarez
Deputy Administrator
United States Department of Agriculture
Animal and Plant Health Inspection Services
Biotechnology Regulatory Services

RECEIVED
By Joseph Tangredi at 1:26 pm, May 06, 2025

April 9, 2025

Dear Ms. Juarez,

On behalf of Ishihara Sangyo Kaisha, Limited, we submit the following petition for determination of nonregulated status for Phalaenopsis ISK-311NR-4.

PETITION FOR DETERMINATION OF NONREGULATED STATUS

The undersigned submits this petition under 7 CFR § 340.6 to request that the Administrator make a determination that the article, ISK-311NR-4 phalaenopsis (311NR) not be regulated under 7 CFR part 340.

T. Kozuka — April 9, 2025

Petitioner — Date

A. *Statement of Grounds*

Based on the information contained in this petition, the petitioner asserts that the ISK-311NR should not be regulated under 7 CFR part 340

The 311NR phalaenopsis presented in this petition was developed using genetic engineering techniques to produce a blue-purple flower color.

311NR phalaenopsis was generated using *Agrobacterium*-mediated transformation with pBIH-35S-CcF3'5'H containing the *CcF3'5'H* and *hpt* genes. Molecular characterization of 311NR phalaenopsis by Southern analysis confirmed that a single, intact DNA insert was inserted into the genome.

The potential environmental impact of the introduction of 311NR phalaenopsis considered two primary areas: the potential for 311NR phalaenopsis to become weedy or invasive; and the potential for gene flow to sexually compatible wild relatives. The 311NR phalaenopsis does not exhibit characteristics that would indicate it is any more likely than non-genetically engineered phalaenopsis to become a weed or plant pest.

ISHIHARA SANGYO KAISHA, LTD.

The data and information contained herein supports the conclusion that 311NR phalaenopsis is unlikely to pose a greater plant pest risk than conventional, non-genetically engineered phalaenopsis and is not otherwise deleterious to the environment. Therefore, we request that APHIS grant the request for a determination of nonregulated status for 311NR phalaenopsis, 311NR phalaenopsis progeny, and any crosses of 311NR phalaenopsis with other nonregulated phalaenopsis.

Unfavorable information: NONE

B. *Certification*

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner which are unfavorable to the petition.

T. Kezuka April 9, 2025

Petitioner Date

Tomoaki Kezuka
General Manager, Bluish Ornamental Flower Division
Biosciences Business Headquarters, ISHIHARA SANGYO KAISHA, LTD.
3-15, Edobori 1-Chome, Nishi-Ku Osaka, 550-0002, Japan
E-mail: t-kezuka@iskweb.co.jp

Petition for Determination of Nonregulated Status for Blue-purple Phalaenopsis ISK-311NR-4

Submitting Company:
Ishihara Sangyo Kaisha, Limited
Biosciences Business Headquarters, ISHIHARA SANGYO KAISHA, LTD.
3-15, Edobori 1-Chome, Nishi-Ku Osaka, 550-0002, Japan

Prepared by:
North Hill Group

OECD Identifier: ISK-311NR-4

Updated Submission Date: April 9, 2025

DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

Release of Information

Ishihara Sangyo Kaisha, Limited (ISK) is submitting information in this document for review by the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) Biotechnology Regulatory Services (BRS) as part of the Petition process established under USDA's regulations at 7 CFR §340 which implements its authority under the Plant Protection Act, 7 U.S.C. § 7701 *et seq*. ISK does not waive any rights to prevent release to any third party. If USDA were to receive a request under the Freedom of Information Act (FOIA), 5 U.S.C. section 522, seeking all of some of the information provided in this submission, ISK expects that USDA will provide ISK with a copy of the material proposed to be released in advance of the release of any document(s) or information, and the opportunity to object to the release of any information based on appropriate legal grounds.

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Abbreviations, Acronyms, and Definitions

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311NR ..... *Phalaenopsis event ISK-311NR-4*

A

A. tumefaciens ..... *Agrobacterium tumefaciens*
ANS ..... *Anthocyanidin synthase*
aphA-3 ..... *Aminoglycoside phosphotransferase type III*
APHIS ..... *Animal and Plant Health Inspection Service*
AT ..... *Anthocyanin acyltransferase*

B

BRS ..... *Biotechnology Regulatory Services*

C

CaMV ..... *Cauliflower mosaic virus*
CBI ..... *Confidential Business Information*
cDNA ..... *Complementary DNA*
CHI ..... *Chalcone isomerase*
CHS ..... Chalcone synthase

D

DFR ..... *Dihydroflavonol reductase*

E

E. coli ..... *Escherichia coli*

F

F3'5H ..... *Flavonoid 3', 5' hydroxylase*
F3'H ..... *Flavonone 3-hydroxylase*
F3H ..... *Flavanone 3-hydroxylase*
FOIA ..... *Freedom of Information Act*

G

GE ..... *Genetically engineered*
GT ..... *Anthocyanidin glucosyltransferase*

I

ISK ..... *Ishihara Sangyo Kaisha, Limited*

K

kb ..... *Kilobases*

L

LB ..... *Luria-Bertain*

P

PCR ..... *Polymerase chain reaction*
PLB ..... *Protocorm like bodies*

R

R. radiobacter ..... *Rhizobium radiobacter*

S

S. faecalis ..... *Streptococcus faecalis*

T

TMV ..... *Tobacco Mosaic Virus*

U

USDA ..... *United States Department of Agriculture*
UTR ..... *Untranslated region*

µ

µg ..... *Micrograms*

Executive Summary

Ishihara Sangyo Kaisha, Limited (ISK) is submitting a Petition for Determination of Nonregulated Status for blue-purple flower color phalaenopsis event ISK-311NR-4, hereafter referred to as 311NR. ISK requests a determination from USDA Animal and Plant Health Inspection Service (APHIS) that 311NR phalaenopsis, 311NR phalaenopsis progeny, and any crosses of 311NR phalaenopsis with other nonregulated phalaenopsis no longer be considered regulated articles under 7 CFR § 340.

311NR phalaenopsis was developed by ISK using genetic engineering techniques to produce a blue-purple flower color.

311NR phalaenopsis was generated using *Agrobacterium*-mediated transformation with pBIH-35S-CcF3'5'H containing the *CcF3'5'H* and *hpt* genes. Molecular characterization of 311NR phalaenopsis by Southern analysis confirmed that a single, intact DNA insert was inserted into the genome.

The potential environmental impact of the introduction of 311NR phalaenopsis considered two primary areas: the potential for 311NR phalaenopsis to become weedy or invasive; and the potential for gene flow to sexually compatible wild relatives. Therefore, 311NR phalaenopsis does not exhibit characteristics that would indicate it is any more likely than non-genetically engineered phalaenopsis to become a weed or plant pest.

The data and information contained herein supports the conclusion that 311NR phalaenopsis is unlikely to pose a greater plant pest risk than conventional, non-genetically engineered phalaenopsis and is not otherwise deleterious to the environment. Therefore, ISK requests that APHIS grant the request for a determination of nonregulated status for 311NR phalaenopsis, 311NR phalaenopsis progeny, and any crosses of 311NR phalaenopsis with other nonregulated phalaenopsis.

No known information is available which would be unfavorable to this petition.

I. Rationale for the Development of 311NR Phalaenopsis

I.A. Basis for the Request for a Determination of Nonregulated Status under 7 CFR § 340.6.

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. 7701-7772), to prevent the introduction or dissemination of plant pests into or within the United States. 7 CFR § 340 regulates the introduction of organisms altered or produced through genetic engineering which are plant pests or for which there is a reason to believe are plant pests. The APHIS regulations at 7 CFR § 340.6 provide that an applicant may petition APHIS to evaluate submitted data on the genetically engineered (GE) plant to determine that a regulated article does not present a plant pest risk and therefore should no longer be regulated.

Ishihara Sangyo Kaisha, Limited (ISK) is submitting data for genetically engineered phalaenopsis with blue-purple flower color ISK-311NR-4 (311NR) and requests a determination from USDA-APHIS that 311NR phalaenopsis, its progeny, and any crosses with other nonregulated phalaenopsis no longer be considered regulated articles under 7 CFR § 340.

I.B. Rationale for the Development of 311NR Phalaenopsis

The 311NR phalaenopsis has been genetically engineered to produce a blue-purple flower. This trait was introduced using *Agrobacterium tumefaciens* (*A. tumefaciens)* mediated transformation with plasmid pBIH-35S-CcF3’5’H (Figure 3).

The comparator phalaenopsis plant is Wedding Promenade PP3387 (hereafter referred to as PP3387 phalaenopsis) which produces a red-purple flower. The genetically engineered 311NR phalaenopsis will offer an additional choice of a plant that produces a blue-purple flower.

I.C. Prior Environmental Release and Submissions to Other Regulatory Agencies

In accordance with Japan's law governing the use of genetically engineered plants, the application for 311NR phalaenopsis was submitted on May 17, 2020 and approved on March 3, 2021.

As of the date of this submission, 311NR phalaenopsis is cultivated only in Japan. Commercial cultivation has occurred at two greenhouses in Japan, totaling approximately 10,000 plants in 2024.

No other submissions have been made on behalf of 311NR phalaenopsis in any other geographies.

I.D. Phalaenopsis Cultivation in the United States and Usage

Phalaenopsis are popular in the United States for commercial cultivation and as houseplants and make up one-third of the total wholesale value of potted flowering crops. It is estimated that the United States produces approximately 36 million plants per year at a value of $300,000,000. (FloraCulture International 2023). Phalaenopsis orchids are commonly cultivated in warmer climates but are also successfully grown as houseplants in cooler climates.

Phalaenopsis orchids are sold as both potted plants and cut flowers. In 2023, California was the U.S. leader in overall floriculture production (which includes orchids), closely followed by Michigan, Pennsylvania, New York, and Florida (United States Department of Agriculture 2024).

II. The Biology of Phalaenopsis

II.A. Phalaenopsis as a Horticultural Plant

The genus *Phalaenopsis*, commonly known as moth orchids, is one of the most popular groups of orchids worldwide. Native primarily to Southeast Asia, *Phalaenopsis* species are not indigenous to North America but have been widely cultivated and hybridized for both commercial and hobbyist purposes (Runkle, et al. 2007) (Hsiao, et al. 2011). Their biology and growth habits in North America are closely tied to controlled environments such as greenhouses and indoor gardening, where they have become staples of ornamental horticulture.

Phalaenopsis orchids are classified within the family Orchidaceae and comprise about 70 species, along with countless hybrids developed for their large flowers, vibrant colors, and prolonged blooming periods (Teoh 2016). Morphologically, these orchids are characterized by thick, fleshy, elliptical leaves that are dark green and serve as their primary photosynthetic structure. Their roots are thick and covered with velamen, aiding in water absorption and protection, while their flowers, which resemble a moth in flight, come in a wide range of colors and patterns, including white, pink, purple, and yellow. As monopodial plants, they grow from a single stem that elongates over time (American Orchid Society n.d.).

In their native tropical and subtropical forests, *Phalaenopsis* orchids are epiphytes, growing on trees with roots exposed to air and moisture. In North America, however, they are cultivated primarily as houseplants or in greenhouses. Their adaptability to low-light conditions and ability to thrive indoors make them a popular choice among gardeners and hobbyists (Brickell 1997). These orchids are well-suited to environments with temperatures between 65–75°F (18–24°C), indirect sunlight, and humidity levels of 50-70%, which can be maintained with humidifiers or water trays. They require well-draining substrates like bark or sphagnum moss to replicate their natural epiphytic conditions (Teoh 2016) (American Orchid Society n.d.).

Reproduction in *Phalaenopsis* orchids occurs naturally through insect pollination, often by moths in their native habitats (Ray and Vendrame 2015) (American Orchid Society n.d.). In cultivation, they are typically hand-pollinated to create hybrids. While wild *Phalaenopsis* reproduce through seeds, North American growers often propagate them through tissue culture or by encouraging the development of keiki (plantlets) on flowering stems (American Orchid Society n.d.). These orchids usually bloom once a year, though hybrids may flower multiple times under optimal conditions.

Common pests affecting *Phalaenopsis* orchids in North America include mealybugs, spider mites, and scale insects, while diseases such as root rot, bacterial blight, and fungal infections can result from overwatering, high humidity, or stagnant air (Brickell 1997). Despite these challenges, *Phalaenopsis* orchids have become a cornerstone of the floriculture industry. They are highly valued for their beauty and ease of care, making them a favorite among plant enthusiasts.

Although *Phalaenopsis* orchids are not endangered, wild populations in their native habitats face threats from habitat destruction and overcollection. Conservation efforts focus on sustainable cultivation and the development of hybrids that are more resilient to pests and diseases (Fay 2018). In North America, *Phalaenopsis* orchids play a significant role not only in the ornamental plant market but also in education, where they are used to teach plant biology due to their unique reproductive and ecological traits. Their enduring popularity and adaptability ensure that they remain an integral part of horticulture in North America.

The *Phalaenopsis* orchid has a distinctive and intricate flower structure (Figure 1). Each flower consists of three sepals and three petals, with the sepals being petal-like in appearance. The dorsal sepal is positioned at the top, while the two lateral sepals are located on either side, forming part of the floral display. The petals include two lateral petals, and a uniquely modified petal called the labellum or lip. The lateral petals resemble the sepals in shape and color, enhancing the symmetry of the flower, while the labellum, positioned at the bottom of the flower, serves as a landing platform for pollinators. The labellum is often intricately shaped and brightly colored with patterns that attract pollinators, and it may feature calluses or ridges to guide them toward the central reproductive column (Dressler 1993).

The column is the central structure of the flower, housing both the stamens (male) and pistil (female). It is a fused organ that includes the anther cap, which protects the pollinia (waxy masses of pollen), and the sticky stigma, which captures pollen during pollination. Below the column is the ovary, located between the flower's pedicel (stalk) and the column. The ovary develops into a seed capsule after successful pollination. *Phalaenopsis* flowers exhibit zygomorphy, meaning they are bilaterally symmetrical and can be divided into two mirror-image halves. Additionally, the flowers undergo resupination during development, twisting 180 degrees so that the labellum is positioned downward to facilitate pollination (Hsiao, et al. 2011). This specialized structure, coupled with the plant's vibrant colors and patterns, makes

Phalaenopsis orchids highly effective at attracting pollinators, particularly moths, and highly desirable as ornamental plants (Hsiao, et al. 2011) (Ray and Vendrame 2015).

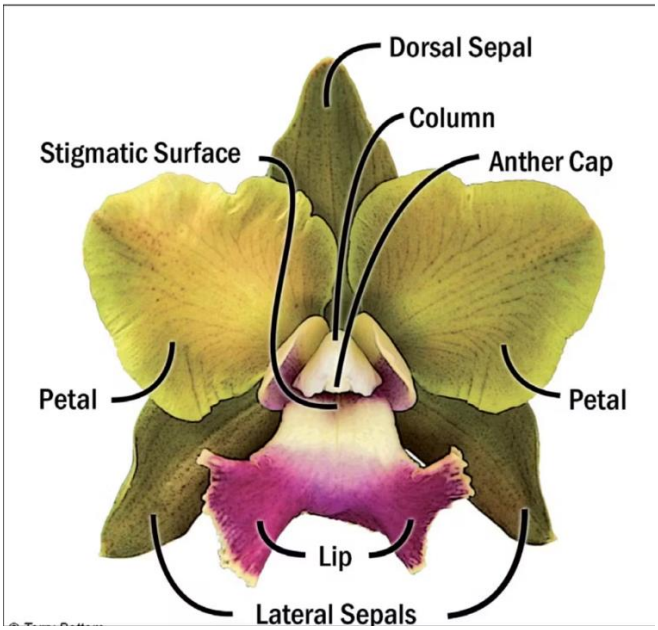


Figure 1. Enlarged View of *Phalaenopsis* Flower

Figure taken from (Bottom 2024).

II.B. Description of the Non-Transformed Recipient Plant

II.C. Recipient Phalaenopsis Line

Wedding Promenade PP3387 phalaenopsis (also referred to as PP3387 phalaenopsis) is the non-transformed wild-type line used for creation of the genetically engineered 311NR phalaenopsis line. PP3387 phalaenopsis has a medium flower size and red-purple flowers in a raceme. Its arched stalk can reach 60cm height. Flower size is 6 – 7 cm, and each inflorescence has 10 – 15 flowers.

III. Method of Development of 311NR Phalaenopsis

III.A. Description of Transformation, Selection, and Breeding Method

III.A.1. Transformation

The 311NR phalaenopsis was created by *Agrobacterium*-mediated transformation with plasmid pBIH-35S-CcF3’5’H (Figure 3).

Protocorm like bodies (PLB) of comparator line PP3387 were co-cultured with *Agrobacterium*

tumefaciens (*A. tumefaciens*) containing expression vector pBIH-35S-CcF3'5'H (Figure 3). Annotation of the genetic components contained in plasmid pBIH-35S-CcF3'5'H are presented in Table 2. The inserted expression vector (pBIH-35S-CcF3'5'H) is located between the right and left borders and is marked with connected arrows shown in Figure 3.

PLB of PP3387 phalaenopsis were co-cultured with *A. tumefaciens* containing expression vector pBIH-35S-CcF3'5'H. PLBs were then transferred to PLB culture medium with hygromycin B (for selection) and meropenem (for removal of *Agrobacterium*). Forty-five strains of hygromycin-resistant cells were obtained from transformation.

Transformation of PLB of PP3387 phalaenopsis was performed in 2008 in Japan. Thirty-two plants were obtained from 45 regenerated strains of transformed PLB. Plants were cultivated in a containment greenhouse and net-house. Two lines of blue-purple *Phalaenopsis* (lines 311 and 164) were obtained in 2012. The presence of the T-DNA region from plasmid pBIH-35S-CcF3'5'H in these two lines has been confirmed via PCR (see section V.C). Lines 311 and 164 showed somaclonal variation in petal shape as previously reported in Wedding Promenade (Tokuhara 1998). Of these lines, *Phalaenopsis* line 311 had lesser petal variation (petals slightly transformed into lip) and was selected and advanced for analysis of the insert and its genetic elements.

From 2012, clone seedlings of *Phalaenopsis* line 311 from the tissue culture of the axillary bud of scapes were prepared, and 37 potted plants were obtained. In 2015, these plants produced flowers with three different shapes of petals: 1) 311WL: petals slightly transform into the flower lip similar to line 311 (19 plants), 2) 311TL: petals completely transform into the flower lip (10 plants) and 3) 311NR: produced normal shape of petals (2 plants). Information on biological diversity risk assessment was collected by using these three lines in a net-house.

Southern blot analysis for the insertion and junction sequence to 311WL, 311TL, and 311NR plants showed the same result as line 311 plants. In addition, an identical result was confirmed in these plants and line 311 via PCR analysis using a primer set which was designed targeting the insertion and the junction sequence.

Based on these results, it is concluded that the 31 plants used for the assessment are all from the line 311, and variation of petals were due to somaclonal variation.

Line 311NR was selected to advance based on its normal petal shape, and clone plants of the line 311NR were used for collection of information for a biological diversity risk assessment at a confined field and net-house.

In this petition, it is defined that "the line 311" is the first plant of 311 series, and 311NR, 311WL and 311TL are three different clone plants obtained from the line 311. These four lines used for studies in the dossier are all T0 generation. The scope of this application is 311NR phalaenopsis, clone plants of 311NR, and all crossbreed progeny derived from 311NR.

The breeding diagram of 311NR phalaenopsis is shown in Figure 2. Generations used in each experiment described in this document are presented in Table 1.

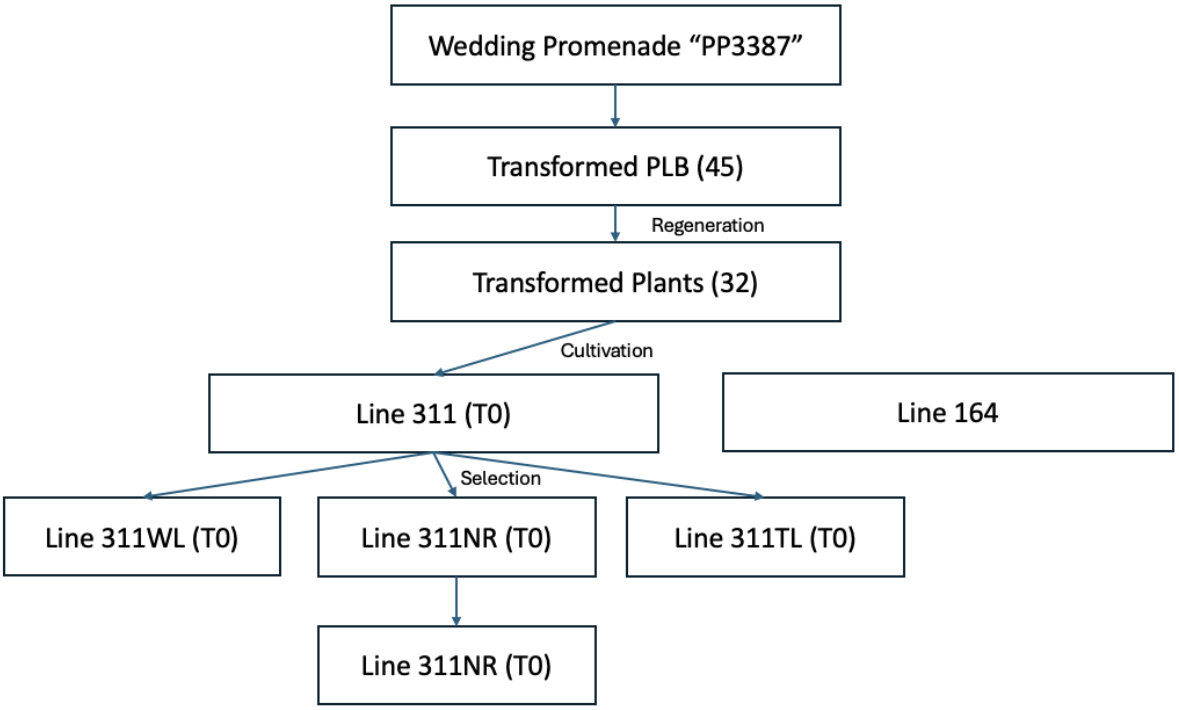


Figure 2. Breeding Diagram of 311NR Phalaenopsis

Table 1. Generations Used for 311NR Phalaenopsis Data Collection

Generation	Experiment
T0	Structure of the insert and genetic elements, absence of Agrobacterium in flower, leaf, and root via PCR, confined field trial in net-house, absence of *Agrobacterium* on cultivation media.
F1	N/A

III.A.2. Selection

Agrobacterium cells used for transformation were removed using culture medium supplemented with meropenem. Additionally, polymerase chain reaction (PCR) analysis targeting the aminoglycoside phosphotransferase III (*aphA-3*) gene which is located in the non T-DNA region of the pBIH-35S-CcF3'5'H vector. DNA was extracted from flower, leaf, and root of the T0 generation of 311NR phalaenopsis for PCR analysis. PCR results showed no presence of the *aphA-3* gene and, therefore, no presence of *Agrobacterium* backbone DNA. Additionally, growth of *Agrobacterium* was not shown during culture and cultivation of PLB on media formulated to encourage *Agrobacterium* growth.

III.B. Selection of Comparators for 311NR Phalaenopsis

Wedding Promenade PP3387 phalaenopsis (also referred to as PP3387 phalaenopsis) is the non-transformed wild-type line used for creation of the genetically engineered 311NR line. The PP3387 line is used in the experiments described in this petition as the non-GE comparator for 311NR phalaenopsis.

IV. Donor Genes and Regulatory Sequences

The genetic information including the annotation of the genetic material inserted, construct component donor information and function of the inserted genetic material (Table 2) for 311NR phalaenopsis are provided below.

IV.A. DNA Used in Transformation

PLBs of recipient line PP3387 phalaenopsis were co-cultured with *A. tumefaciens* containing expression vector pBIH-35S-CcF3'5'H (Figure 3). Annotation of the genetic components contained in plasmid pBIH-35S-CcF3'5'H are presented in Table 2. The inserted expression vector (pBIH-35S-CcF3'5'H) is located between the right and left borders and is marked with connected arrows shown in Figure 3.

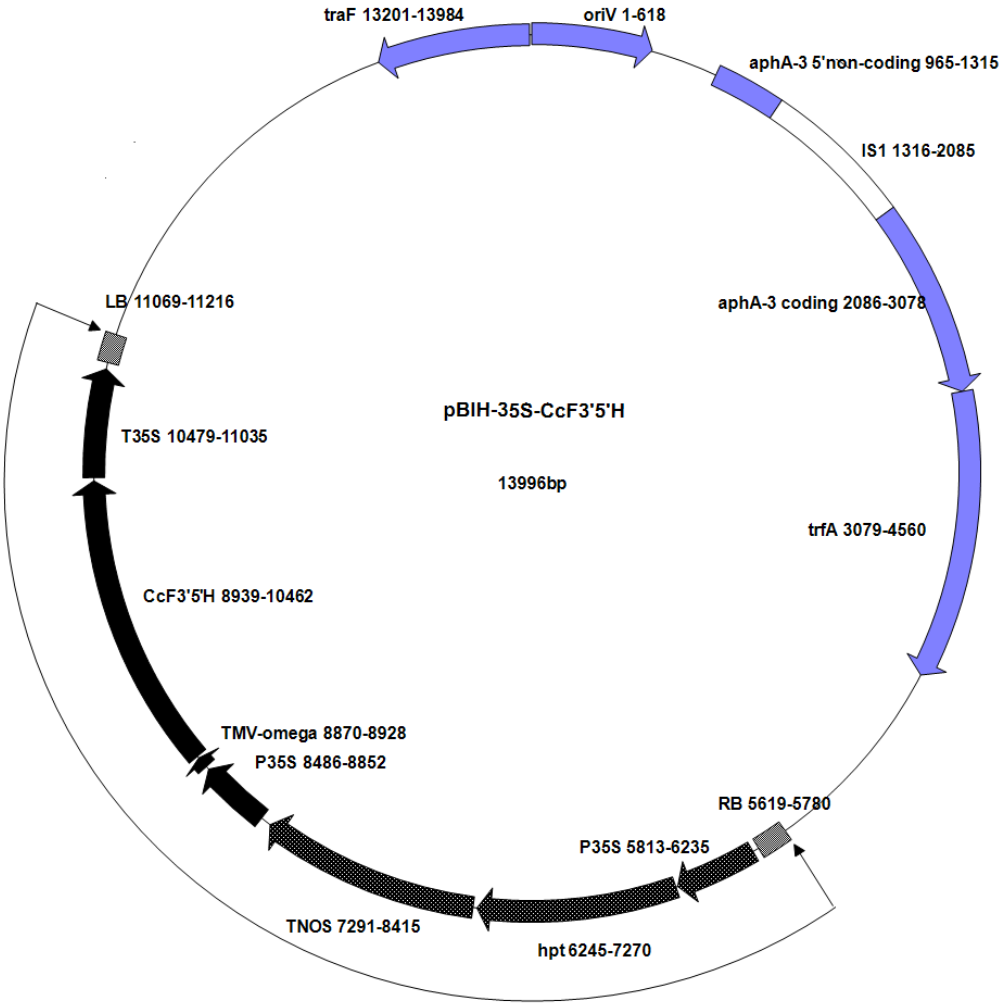


Figure 3. Map of Plasmid Containing Expression Vector pBIH-35S-CcF3’5’H

Numbers to the right of each genetic component in Figure 3 represent the base pair position of the component from the first nucleotide of origin. Genetic element base pair locations are also included in the annotations of each genetic element presented in Table 2.

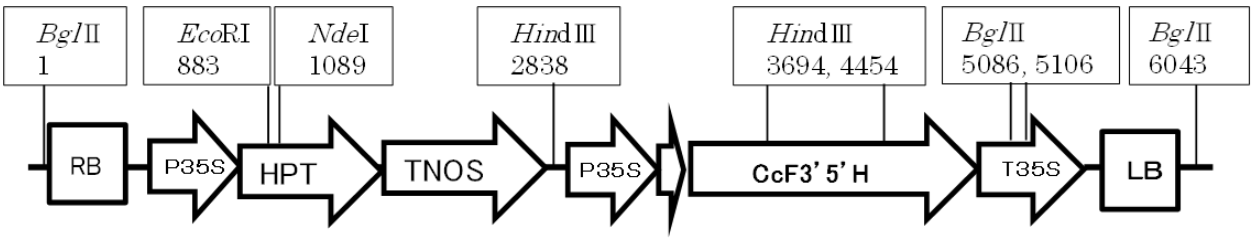


Figure 4. Structure of the T-DNA Region in pBIH-35S-CcF3'5'H

The numbers below the restriction enzymes indicate positions of each restriction enzyme recognitions site when the first base of the *Bgl*II in the right border sequence of the T-DNA region is set to bp 1.

IV.A.1. Information on Vectors Used to Produce 311NR Phalaenopsis

IV.A.1.(a) Plasmid Information

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 (Figure 3) consists of non T-DNA region of pBI121 which is non T-DNA region of a binary vector Bin 19 (Frisch, et al. 1995). However, the direction of the T-DNA region in pBIH-35S-CcF3'5'H is reversed at two *Bgl*II sites (Figure 4) compared to that in pBI121 and Bin 19 (Frisch, et al. 1995). The Non T-DNA region in Bin 19 was constructed based on the vector pRK252 and aminoglycoside phosphotransferase III gene (*aphA-3*) of *Streptcoccus faecalis* (Hasnain, Manavathu and Leung 1985).

Plasmid RK2 was initially isolated from *Klebsiella pneumoniae* though it was isolated from various gram-negative bacteria.

The number of bases of pBIH-35S-CcF3'5'H used to create 311NR phalaenopsis is 13,996 bp.

IV.A.1.(b) Function of the *CcF3'5'H* Gene in 311NR Phalaenopsis

The intended phenotype is a Blue-purple flower which is produced by the insertion and expression of the *Commelina communis* flavonoid 3'5'-hydroxylase (F3'5'H) gene in 311NR phalaenopsis.

Additionally, 311NR phalaenopsis contains the hygromycin B phosphotransferase (*hpt*) gene. The *hpt* gene confers resistance to antibiotic hygromycin B and was used as a selectable marker.

The F3'5'H gene was inserted into 311NR phalaenopsis to produce a blue-purple flower color. The production of the blue-purple phenotype via use of the F3'5'H enzyme in the flavonoid pathway is described below.

It is well known that flower color is determined by the production of pigments, namely anthocyanins, terpenoids, and betalains. Anthocyanins are a class of flavonoids which play a role in flower color and contribute to flower color in the orange/red and violet/blue spectrum (Katsumoto, et al. 2007) (Tanaka, Sasaki and Ohmiya 2008) (Liang, et al. 2020). Terpenoids are synthesized in chloroplasts and contribute to photosynthesis. Betalains are yellow to red pigments derived from tyrosine and are not as common as anthocyanins. All three pigment classes are visual and attractive to pollinators and can provide plant protection from visible and UV light (Tanaka, Sasaki and Ohmiya 2008).

Flavonoids are widely distributed among plants and contain 19 different types of anthocyanins. Six major anthocyanins are pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin. The color generated by anthocyanins depends greatly on the number of hydroxyl groups on the B-ring of each molecule. The larger number of hydroxyl groups, the bluer the color produced (Tanaka, Sasaki and Ohmiya 2008).

The structure of anthocyanins allows for the production of a wide range of flower colors from orange-red to violet-blue. Variation of anthocyanins is determined in the flavonoid pathway (Figure 5) by the absence or presence of the activity of flavonoid 3'-hydroxylase (F3'H), and flavonoid 3'5'-hydroxylase (F3'5'H). Based on these variations, the following outcomes are possible:

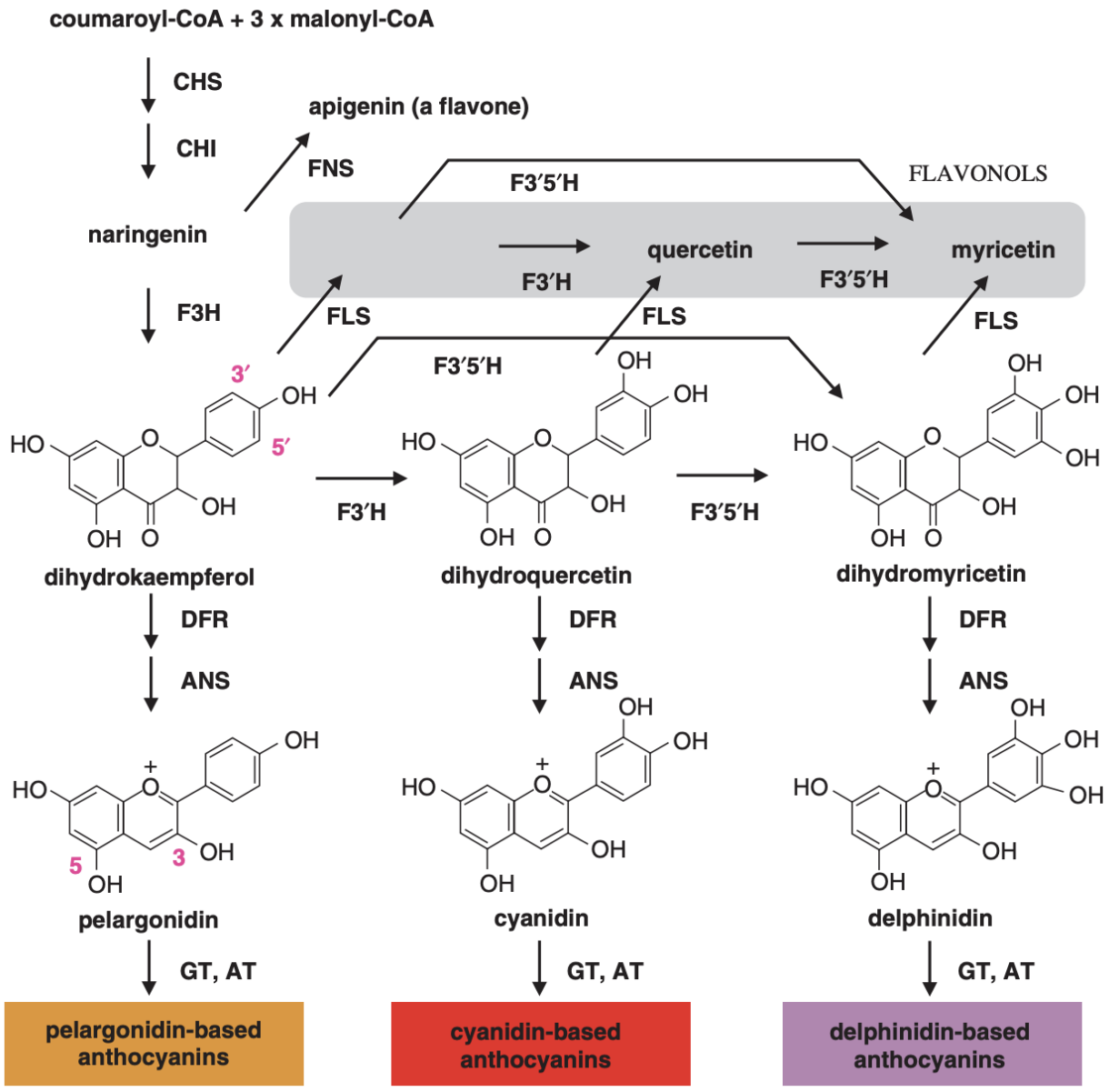
1. Pelargonidin-based anthocyanins: An orange-red color is the flower petal phenotype if pelargonidin aglycones are produced. The B-ring of pelargonidin possesses one hydroxy group. Pelargonidin aglycones are accumulated with the absence of both F3'H and F3'5'H.
2. Cyanidin-based anthocyanins: A red-purple color is the flower petal phenotype if cyanidin aglycones are produced. The B-ring of cyanidin possesses two hydroxy groups. Cyanidin aglycones are accumulated in the presence of F3'H.
3. Delphinidin-based anthocyanins: A violet-blue color is the flower petal phenotype if delphinidin aglycones are produced. The B-ring of delphinidin possesses three hydroxy groups. Delphinidin aglycone are accumulated in the presence of F3'5'H and the presence or absence of F3'H (Hsiao, et al. 2011) (Tanaka, Sasaki and Ohmiya 2008) (Zhou, et al. 2021) (Liang, et al. 2020).

Figure 5 shows the flavonoid biosynthetic pathways necessary to produce pelargonidin, cyanidin, and delphinidin-based anthocyanins. A description of the flavonoid pathway and the role of the F3'5'H enzyme in determining flower color is described below.

Anthocyanins are water soluble pigments that determine flower color and are synthesized through flavonoid metabolism (Figure 5) in the following steps (Katsumoto, et al. 2007):

1. In the first committed step of the flavonoid pathway, chalcone synthase (CHS) produces chalcones, which are precursors for all flavonoid classes.
2. The conversion of chalcones to naringenins is catalyzed by chalcone isomerase (CHI)
3. Naringenin is then converted to dihydrokaempferol by flavanone 3-hydroxylase (F3H).

4. Dihydrokaempferol is a major branch point in the production of flavonoids. Three endpoints are possible via several biosynthetic pathways:
    a. Production of pelargonidin-based anthocyanins
    b. Production of cyanidin-based anthocyanins
    c. Production of delphinidin-based anthocyanins
5. Production of pelargonidin-based anthocyanins proceeds in the absence of flavonoid 3'-hydroxylase (F3'H). Dihydrokaempferol is converted to pelargonidin via several enzymatic steps using dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS). Pelargonidin-based anthocyanins are then generated from pelargonidin via several enzymatic steps involving anthocyanidin glucosyltransferase (GT), and anthocyanin acyltransferase (AT). The production of pelargonidin-based anthocyanins produces an orange-red color phenotype.
6. Production of cyanidin-based anthocyanins proceed in the presence of F3'H and the absence of F3'5'H. The F3'H enzyme converts dihydrokaempferol to dihydroquercetin. Dihydroquercetin is then converted to cyanidin via several enzymatic steps using DFR and ANS. Cyanidin-based anthocyanins are then generated from cyanidin via several enzymatic steps involving GT and AT. Cyanidin-based anthocyanins produce a red-purple color phenotype.
7. Production of delphinidin-based anthocyanins occurs in the presence of the F3'5'H enzyme via one of two pathways. One is dependent on the presence of the F3'H enzyme, and the second is independent of the presence of the F3'H enzyme. As stated earlier, the accumulation of delphinidin-based anthocyanins produces a violet-blue phenotype.
    a. In the presence of the F3'H enzyme:
        i. Dihydrokaempferol is converted to dihydroquercetin.
        ii. Dihydroquercetin is then converted to dihydromyricetin by F3'5'H.
        iii. Dihydromyricetin is converted to delphinidin through several enzymatic steps using DFR and ANS.
        iv. Delphinidin-based anthocyanins are produced from delphinidin via several enzymatic steps using GT and AT.
    b. In the absence of the F3'H enzyme:
        i. Dihydrokaempferol is converted to dihydromyricetin using the F3'5'H enzyme.
        ii. Dihydromyricetin is converted to delphinidin via several enzymatic steps using DFR and ANS.
        iii. Delphinidin-based anthocyanins are produced via several enzymatic steps using GT and AT.



Katsumoto et al., 2007
Abbreviations: CHS – chalcone synthase, CHI – chalcone isomerase, FLS – flavonol synthase, F3H – flavanone 3-hydroxylase, F3'H – flavonoid 3'-hydroxylase, F3'5'H – flavonoid 3'5'-hydroxylase, DFR – dihydroflavonol reductase, ANS – anthocyanidin synthase, 3GT – 3-O-glucosyltransferase, AT – anthocyanin acyltransferase.

Figure 5. Anthocyanin Biosynthetic Pathway

The production of violet-blue color in *Phalaenopsis* orchids has been previously studied and those studies have shown that plants with white flowers do not express F3'5'H (Liang, et al. 2020). Additionally, these plants did not accumulate delphinidin. This provides further evidence that the expression of a F3'5'H gene in 311NR phalaenopsis flowers is expected to produce the violet-blue phenotype.

The insertion of the F3'5'H gene in the genetically modified 311NR phalaenopsis flower produces dihyrdromyricetin, which is then converted to delphinidin. The accumulation of delphinidin produces the intended violet-blue color phenotype in 311NR phalaenopsis flowers.

IV.A.1.(c) Function of the *hpt* Gene in 311NR Phalaenopsis

Hygromycin B phosphotransferase (*hpt*) is an enzyme that phosphorylates hygromycin B and confers hygromycin resistance. The *hpt* gene is used as a dominant selectable marker in 311NR phalaenopsis and has been previously used in GE bacteria, fungi, plants, insects, and mammalian cells (Smulian, et al. 2007). The activity of *hpt* is known to have high specificity to compounds of hygromycin B-like aminoglycoside antibiotics (Daigle, et al. 1999). The use of the *hpt* gene has rarely been reported to result in effects outside of the expected use and has not been associated with insertional mutagenesis.

Hygromycin B which is an aminoglycoside antibiotic, interferes with the recognition of aminoacyl tRNA and the translocation of peptidyl tRNA on ribosomes in prokaryotic and eukaryotic organisms, causing mRNA misreading and inhibition of protein synthesis, thereby inhibiting growth (Cabanas, Vazquez and Modolell 1978). Many plants, including *Phalaenopsis*, are also sensitive to hygromycin B. Hygromycin B phosphotransferase is a kinase that catalyzes the transfer of the phosphate group of ATP to hygromycin B. Phosphorylated hygromycin B loses its growth inhibitory activity (Rao, et al. 1983).

PLBs (tissue cultures called protocorm-like bodies) of PP3387 phalaenopsis which is the host of 311NR phalaenopsis are also sensitive to hygromycin B. On the other hand, PLBs of recombinant plants expressing hygromycin B phosphotransferase are resistant to hygromycin B. Therefore, PLBs of recombinant plants can be selected by culturing them on medium containing hygromycin B.

IV.A.1.(d) Other Gene Function Information

The *ori*V gene from the RK2 plasmid and the *trfA* gene are elements necessary to for the function of the origin of replication, which contributes to the replication and maintenance of the plasmid in *E. coli* and *Agrobacterium*. The *traF* gene from the RK2 plasmid has a function required for plasmid transfer, but this function was not used for the development of 311NR phalaenopsis.

The aminoglycoside phosphotransferase III (*aphA-3*) gene of *Streptococcus faecalis* confers resistance to kanamycin. This resistance was used as a selection marker gene for the construction vector in *E. coli* and *Agrobacterium*. Additionally, transposase 1 (*IS1*) is inserted between 5' terminal untranslated region and coding sequence of the *aphA-3* gene.

IV.A.2. Method for Preparing Living Modified Organisms

The position, direction, and restriction enzyme sites of the constituent elements of the donor nucleic acids in plasmid pBIH-35S-CcF3'5'H is shown in Figure 4. Sequence introduced into plant

genome is 5,391 bp from middle of RB to middle of LB. Table 2 lists the genetic elements of the plasmid.

Table 2. Genetic Elements of pBIH-35S-CcF3'5'H

Genetic Element	Location in the Plasmid Vector	Function (Reference)
OriV	1-618	Origin of replication from plasmid RK2 providing replication and maintenance of the plasmid in broader hosts including *E. coli* and *R. radiobacter*.
Intervening Sequence	619-964	Synthetic
aphA-3 5' non-coding	965-1315	5' terminal untranslated region of aminoglycoside phosphotransferase type III (*aphA-3*)from *Streptococcus faecalis* (*S. faecalis*)
IS1	1316-2085	Transposase 1 of *E. coli*.
aphA-3 coding	2086-3078	Coding sequence of neo gene from *S. faecalis* encoding aminoglycoside phosphotransferase type III that phosphorylases and inactivates kanamycin. The *aphA-3* coding sequence was used as a selection marker in *E. coli* and *R. radiobacter*.
trfA	3079-4560	Sequence from plasmid RK2 necessary for *oriV*.
Intervening Sequence	4561-5618	Synthetic
RB	5619-5780	DNA fragment containing the right border region derived from *R. radiobacter*. Part of RB is not inserted into the transformation plant (Barker, et al. 1983).
Intervening Sequence	5781-5812	Synthetic
P35S	5813-6235	Promoter of the 35S RNA of the cauliflower mosaic virus (CaMV). The CaMV genome is a circular double-stranded DNA which contains the genes necessary for expression in 311NR phalaenopsis. The 35S promoter confers constitutive expression across growth stages. (Mitsuhara, et al. 1996)
Intervening Sequence	6236-6244	Synthetic
hpt	6245-7270	Hygromycin B phosphotransferase of *Escherichia coli* (*E. coli*), which phosphorylates and inactivates hygromycin B. Plant cells expressing the phosphotransferase have resistance to hygromycin B. (Gritz and Davies 1983).
Intervening Sequence	7271-7290	Synthetic
TNOS	7291-8415	Terminator of 3' terminal untranslated region of the nopaline synthase gene from Ti plasmid of *Rhizobium radiobacter* (*R. radiobacter*) encoding nopaline synthase. *TNOS* contains a poly A sequence and terminates upstream elements. (Bevan, Flavell and Chilton 1983).
Intervening Sequence	8416-8485	Synthetic
P35S	8486-8852	Promoter of the 35S RNA of the cauliflower mosaic virus (CaMV). The CaMV genome is a circular double-stranded DNA which contains the genes necessary for expression in 311NR phalaenopsis. The 35S promoter confers constitutive expression across growth stages. (Mitsuhara, et al. 1996)
Intervening Sequence	8853-8869	Synthetic
TMV-omega	8870-8928	5' terminal untranslated region of Tobacco Mosaic Virus (TMV) omega sequence. The omega leader mRNA with the 5' untranslated region (UTR) enhances translation. (Gallie and Walbot 1992).
Intervening Sequence	8929-8938	Synthetic
CcF3'5'H	8939-10462	Complementary DNA (cDNA) of flavonoid 3', 5'-hydroxylase (F3'5'H) of Asiatic dayflower (*Commelina communis* L.). The enzyme encoded by cDNA hydroxylates positions 3' and 5' in the B ring of dihydroflavonol, and converts naringenin or eriodyctiol to 5, 7, 3', 4', 5'-pentahydorxyflavanone, or converts dihydrokaempferol dihydroquercetin to dihydromyricetin (Holton and Cornish 1995) which is the gene of interest in 311NR phalaenopsis.
Intervening Sequence	10463-10478	Synthetic
T35S	10479-11035	Terminator of the 3' terminal untranslated region of *35S RNA* of the cauliflower mosaic virus (CaMV), which contains a polyA sequence and terminates transcription of upstream elements. (Pietrzak, et al. 1986).
Intervening Sequence	11036-11068	Synthetic
LB	11069-11216	DNA fragment containing the left border region derived from *R. radiobacter*. Part of the left border is not inserted into the transformation plant (Barker, et al. 1983).
Intervening Sequence	11217-13200	Synthetic
traF	13201-13984	Sequence from plasmid RK2 for plasmid transposition
Intervening Sequence	13985-13996	Synthetic

IV.B. Identity and Source of the *CcF3'5'H* and *hpt* Gene Cassettes in Plasmid pBIH-35S-CcF3'5'H

IV.B.1. Source of the *CcF3'5'H* Gene

***Commelina communis*: Donor of the *CcF3'5'H* Gene**

Order: Commelinales
Family: Commelinaceae
Genus: *Commelina*
Species: *C. communis*

Commelina communis is commonly known as the Asiatic Dayflower. It is a fast-growing herbaceous summer annual of the Spiderwort family (Commelinaceae). It is native throughout East Asia and northern parts of Southeast Asia and is considered a weed in many parts of the world, especially in temperate and subtropical regions. *Commelina communis* can be found throughout the eastern and central United States and is common in states with warm, humid climates.

IV.B.2. Source of the *hpt* Gene

***Escherichia coli*: Donor of the *hpt* Gene**

Class: Gammaproteobacteria
Order: Enterobacterales
Family: Enterobacteriaceae
Genus: *Escherichia*
Species: *E. coli*

Escherichia coli (*E. coli*) is a gram-negative, facultative anaerobic, rod-shaped coliform bacterium. It is commonly found in the lower intestine of warm-blooded organisms. A good portion of *E. coli* strains are part of the normal microbiota of the gut and are mostly harmless or can even be beneficial to humans.

V. Genetic Characterization of 311NR Phalaenopsis

V.A. Molecular Analysis Overview

Molecular characterization of genetically engineered events determines the insertion copy number, integrity of the insertion, and absence of plasmid DNA unintended for integration. 311NR phalaenopsis plants were characterized by Southern analysis to determine the number of insertions within the plant genome, integrity of the insert, confirm the stable genetic inheritance of the inserted cassettes, and to confirm the absence of plasmid backbone sequences. Sequence information is provided in Appendix 5. Sequence Information.

V.B. Southern Analysis, Copy Number, Integrity, Absence of Vector Backbone Sequence

V.B.1. Southern Blot Analysis for Copy Number

Southern blot analysis was performed to confirm the presence and copy number of insertions in the genomic DNA from leaf tissue of original line 311 phalaenopsis (Figure 2). Southern blot methods for copy number are presented in Appendix 2. Materials and Methods for Southern Blot Analysis.

Two Southern blots were produced in this experiment. Blot A (Figure 6) shows results using the *hpt* gene-specific probe. Blot B (Figure 6) shows results using the *CcF3'5'H* gene-specific probe. Location of probes and restriction sites used in Southern blot analysis of copy number are indicated in Figure 7.

A single band of 5-6 kilobases (kb) in size was detected in blots of *EcoRI*-digested DNA from the genomic DNA of the original 311 phalaenopsis line and a single band of 7-8 kb was detected in blots of *NdeI*-digested DNA, whether using the *hpt* gene specific probe or a *CcF3'5'H* gene specific probe (Figure 6). Therefore, it is determined that one copy of the *hpt* gene and one copy of the *CcF3'5'H* gene were inserted into the 311 phalaenopsis line.

Location of probes and restriction sites used in Southern analysis are indicated in Figure 7.

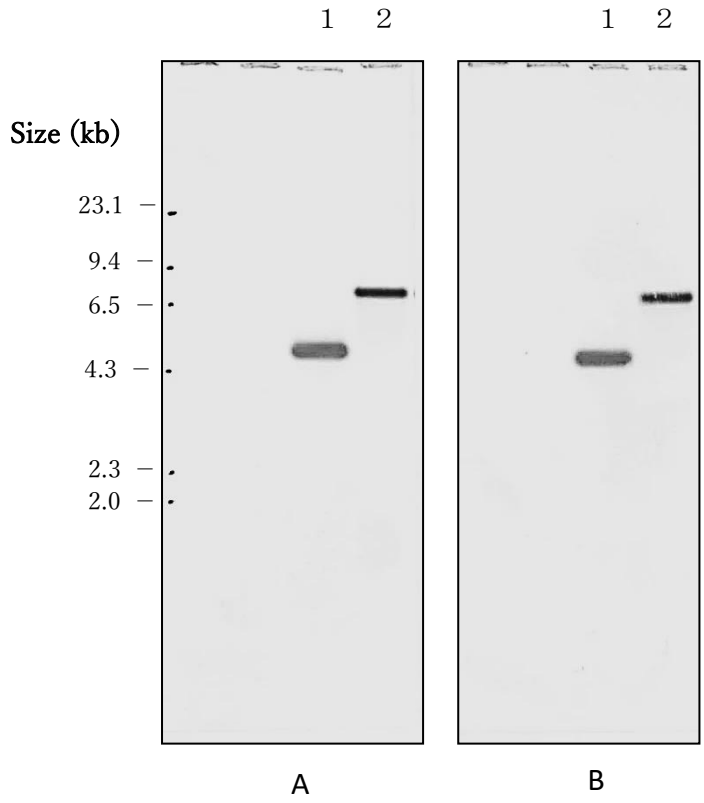


Figure 6. Southern Blot Analysis of 311NR Phalaenopsis

Figure 6A shows results of Southern blot analysis with the *hpt* gene-specific probe with DIG label. Figure 6B shows results of Southern blot analysis with the *CcF3'5'H* gene-specific probe with DIG label. Lane 1 in Figures 6A and 6B were run with *Eco*RI-digested DNA. Lane 2 in Figures 6A and 6B were run with *Nde*I-digested DNA.

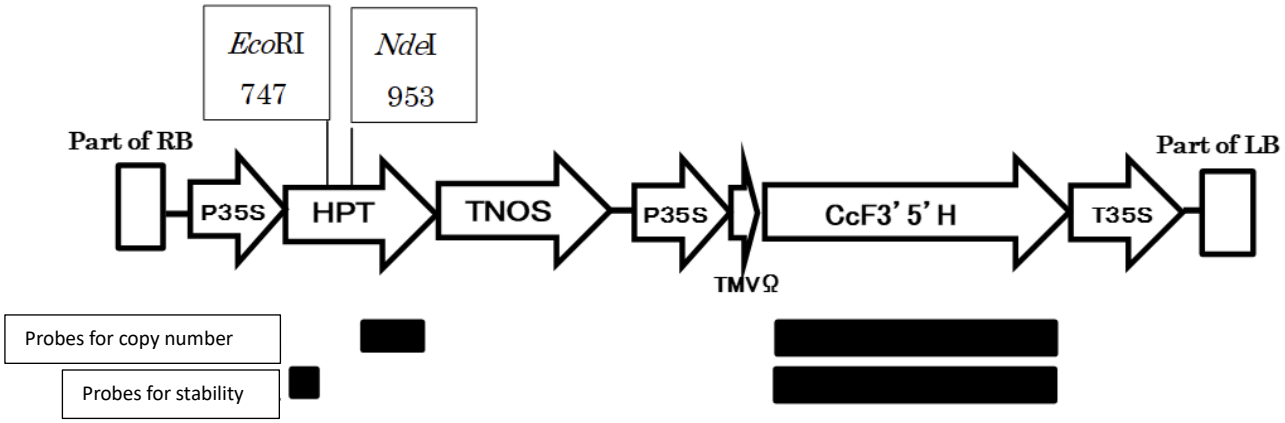


Figure 7. Locations of Probes and Restriction Sites in 311NR Insert

Numbers below restriction enzyme names represent the positions of each restriction site when the nucleotide at the 5' terminal end of the insert (at the right border) is set to position 1. The total length of the insert is 5,391 bp. Black bars indicated "probes for copy number' are probes used to determine copy number. Black bars indicated "probes for stability" were used to determine stability of the insert across 3 clones of the 311 line.

Based on the results of this analysis, one copy of the *hpt* gene and one copy of the *CcF3'5'H* gene were inserted into the original 311 phalaenopsis line.

V.B.2. Southern Blot Analysis for Generational Stability of the Insert in 311NR Phalaenopsis

Three clone seedlings (311WL, 311NR, and 311TL) developed from the original line 311 (see Figure 2) were used for Southern blot analysis of generational stability of the insert. Southern blot analysis was performed using DNA extracted from leaf samples of the three clone seedlings to confirm presence and copy number of the insert for comparison to the original 311 line. Methods of analysis are presented in Appendix 2. Materials and Methods for Southern Blot Analysis.

Study A: *CcF3'5'H* gene specific probe analysis of line 311 clones
A single band of 5-6 kb in size was detected in Southern blots of the three clones (311WL, 311NR, and 311TL). This is the same band detected in the blot of the *CcF3'5'H* gene-specific probe from line 311 (see Figure 6B).

Study B: *hpt* gene specific probe analysis of line 311 clones
A single band of 10-15 kb in size was detected in a Southern blot of line 311 using the *hpt* gene-specific probe. A single band of the same size (10-15 kb) was also detected in blots of all three clone seedlings (311WL, 311NR, and 311TL). No band was detected in the PP3387 phalaenopsis non-GE control using any probes.

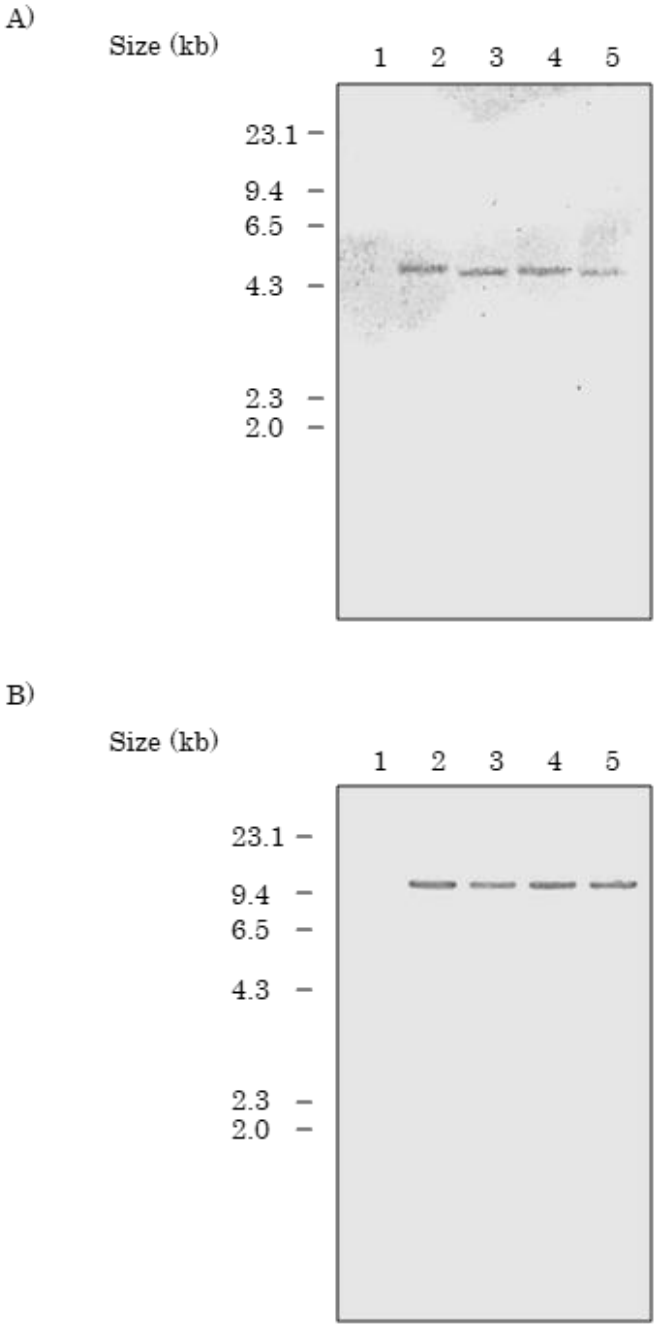


Figure 8. Southern Blot Analysis of Line 311 and Clone Seedlings

Lane	Sample
1	PP3387 control
2	Line 311
3	Clone 311WL
4	Clone 311NR
5	Clone 311TL

Figure 8A shows results of the *CcF3'5'H* gene-specific probe with a DIG label. Figure 8B shows results of the *hpt* gene-specific probe with a DIG label.

Based on the results presented in this section, the insert in line 311 is stably inherited and maintains its structure and copy number across three clone seedlings (311WL, 311NR, and 311TL) developed from line 311.

V.B.3. Southern Blot Analysis for Confirmation of Absence of Vector Backbone Sequence

Southern blot analysis was performed to confirm the absence of vector backbone sequence in the genomic DNA of a leaf sample of line 311.

Probes used in the analysis are presented in Figure 9. Please see Appendix 2. Materials and Methods for Southern Blot Analysis for details regarding this experiment.

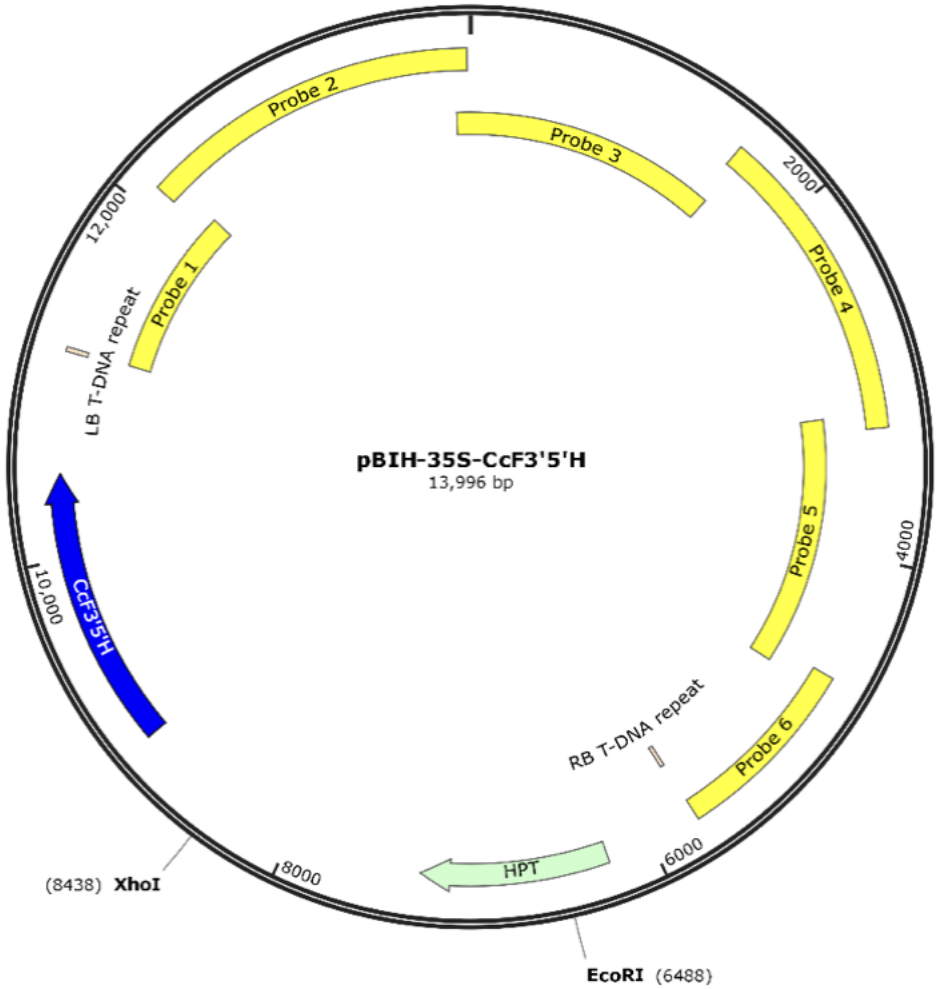


Figure 9. Location of Probes Used for Backbone Southern Blot Analysis of *Phalaenopsis* Line 311

A single band was detected in blots with each probe when plasmid pBIH-35S-CcF3’5’H was used as a template. No bands were observed in any blot with line 311 genomic DNA (Figure 10).

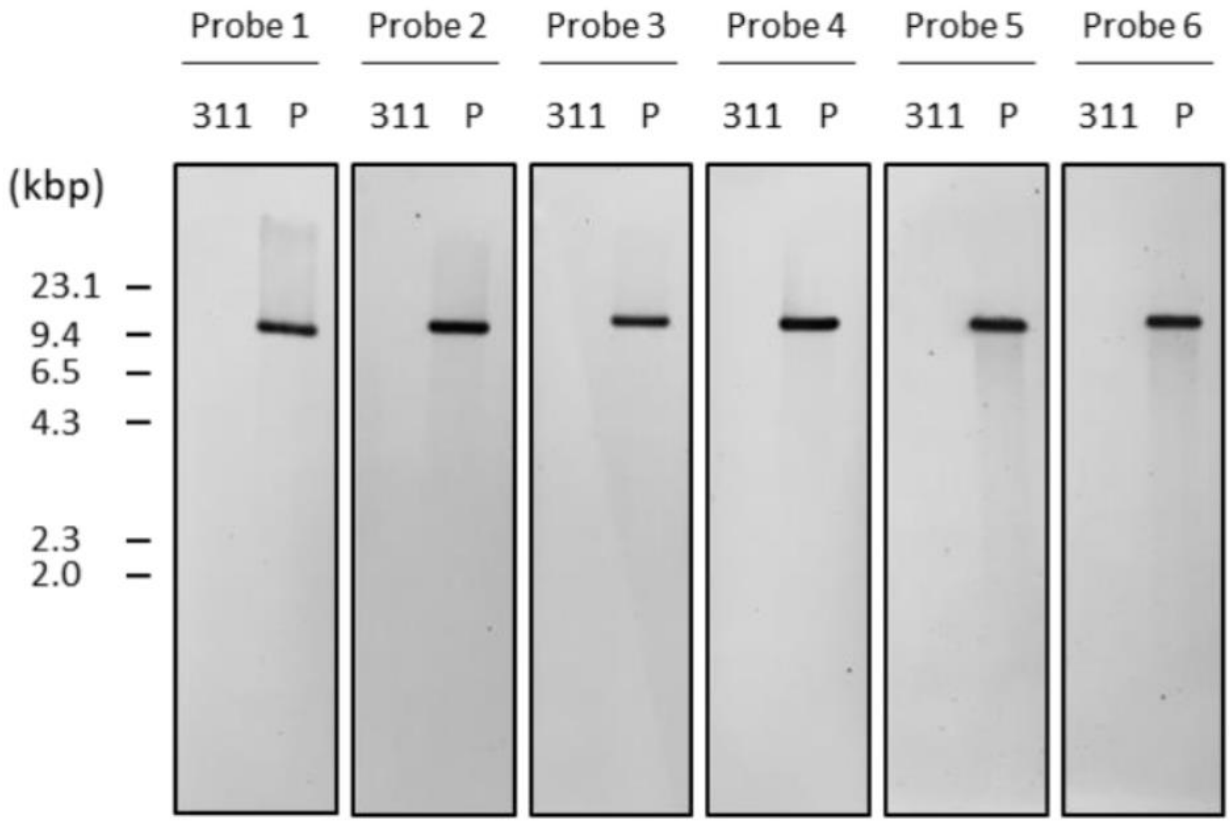


Figure 10. Southern Blot Analysis for Confirmation of Absence of Backbone Sequence in Line 311

Lane ID	Sample
311	Genomic DNA of Line 311
P	pBIH-35S-CcF3’5’H Plasmid DNA

Based on the results of this experiment, line 311 does not contain *Agrobacterium* backbone sequence from plasmid pBIH-35S-CcF3’5’H used for transformation.

V.C. Polymerase Chain Reaction Analysis

V.C.1. PCR Analysis for Presence/Absence of Transgenes

PCR analysis was performed to confirm presence or absence of transgenes in genomic DNA extracted from flower, leaf, and root samples of line 311. See Appendix 1. PCR Methods for details regarding methods used for PCR analysis.

Figure 11 shows results of the PCR analysis of the *CcF3'5'H* and *hpt* genes in flower, leaf, and root tissues of 311NR phalaenopsis. Phalaenopsis chalcone isomerase (CHI) was used as a positive control for assay quality control. The non-GE comparator PP3387 was used as a negative control.

PCR analysis confirmed the presence of transgenes *CcF3'5'H* and *hpt* in flower, leaf, and root samples of line 311.

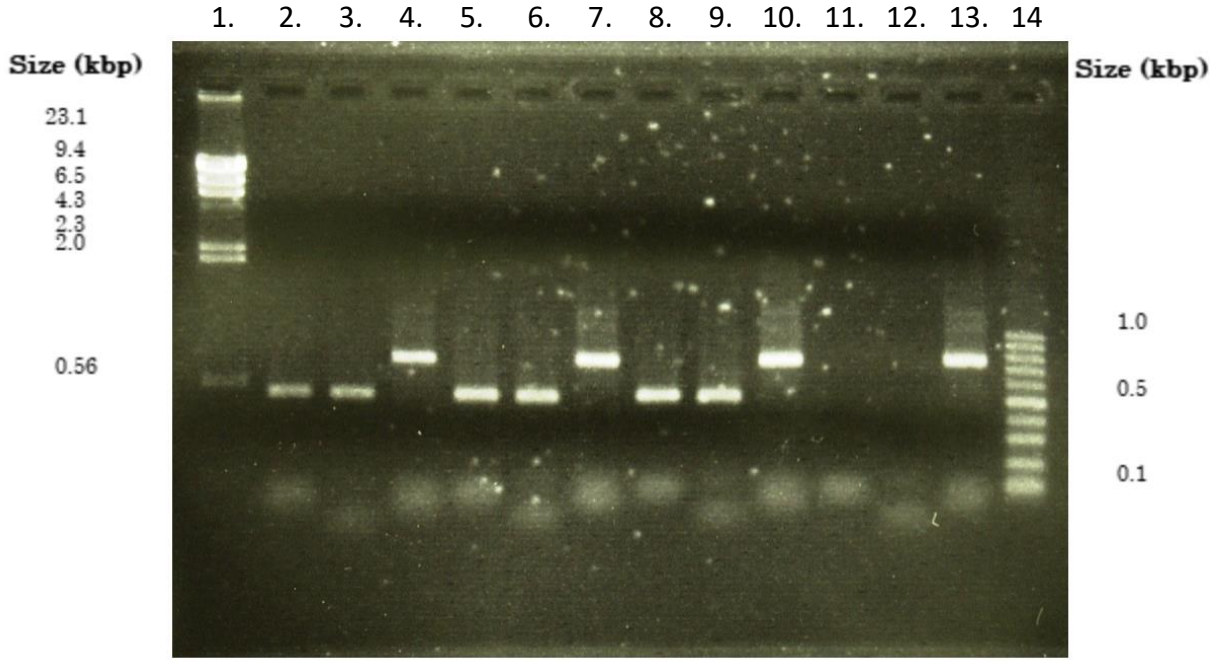


Figure 11. PCR for Transgenes in the Flower, Leaf, and Root Tissues of Original Line 311

Lane	Tissue	Gene
1		Gamma DNA/*Hind*III marker
2	Flower (Sepal)	*CcF3'5'H*
3	Flower (Sepal)	*hpt*
4	Flower (Sepal)	Phalaenopsis chalcone CHI
5	Leaf	*CcF3'5'H*
6	Leaf	*hpt*
7	Leaf	Phalaenopsis chalcone CHI
8	Root	*CcF3'5'H*
9	Root	*hpt*
10	Root	Phalaenopsis chalcone CHI
11	PP3387 (control) Leaf	*CcF3'5'H*
12	PP3387 (control) Leaf	*hpt*
13	PP3387 (control) Leaf	Phalaenopsis chalcone CHI
14		100-bp DNA Ladder

V.C.2. RT-PCR Analysis for Stability of Gene Expression in Petal and Leaf Tissues

Expression of the *CcF3'5'H* and *hpt* genes in petal and leaf tissues of original line 311 and three clone seedlings (311WL, 311NR, and 311TL) was measured using RT-PCR.

Figure 12 shows the results of the RT- PCR analysis of the *CcF3'5'H* and *hpt* genes in petal tissues and Figure 13 shows the results of RT-PCR analysis of the *CcF3'5'H* and *hpt* genes in leaf tissues of 311WL, 311NR, and 311TL phalaenopsis. Phalaenopsis chalcone isomerase (CHI) was used as a positive control for assay quality control. The non-GE comparator PP3387 was used as a negative control.

RT-PCR method is presented in Appendix 1. PCR Methods.

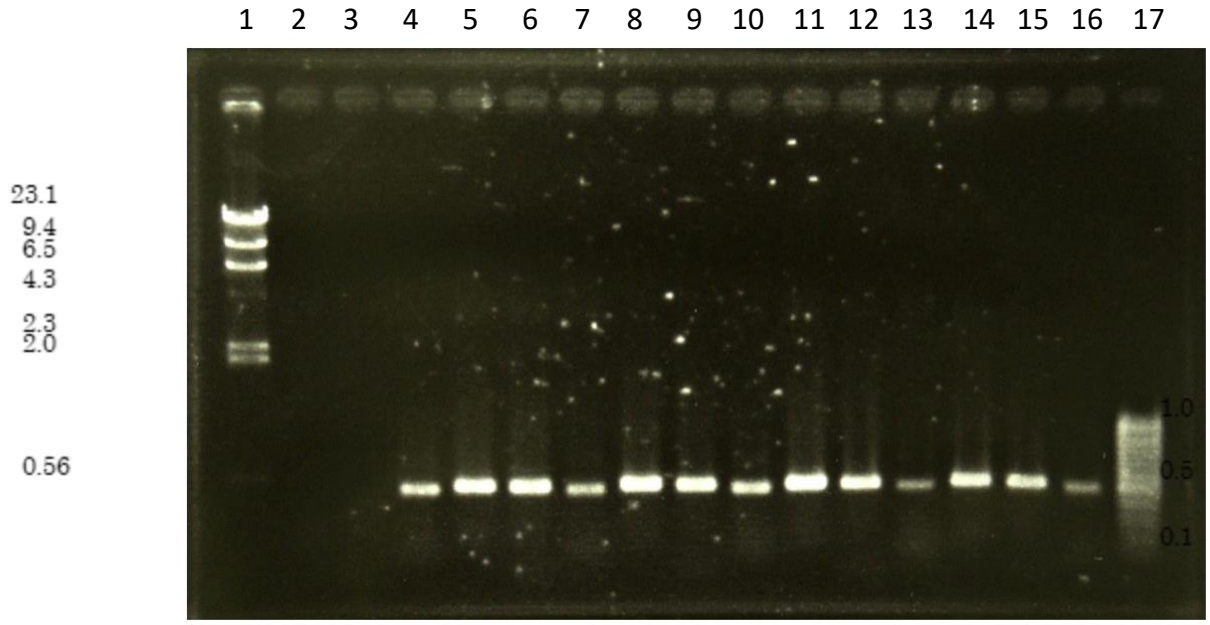


Figure 12. RT-PCR Analysis of Expression of Transgenes in Petals of *Phalaenopsis Line* 311 Clones 311WL, 311NR, and 311TL

Lane	Sample	Gene
1	Gamma DNA/HindIII Marker	
2	PP3387 Petal	*CcF3'5'H*
3	PP3387 Petal	*hpt*
4	PP3387 Petal	Phalaenopsis CHI
5	Line 311 Petal	*CcF3'5'H*
6	Line 311 Petal	*hpt*
7	Line 311 Petal	Phalaenopsis CHI
8	311WL Petal	*CcF3'5'H*
9	311WL Petal	*hpt*
10	311WL Petal	Phalaenopsis CHI
11	311NR Petal	*CcF3'5'H*
12	311NR Petal	*hpt*
13	311NR Petal	Phalaenopsis CHI
14	311TL Petal	*CcF3'5'H*
15	311TL Petal	*hpt*
16	311TL Petal	Phalaenopsis CHI
17	100-bp DNA Ladder	

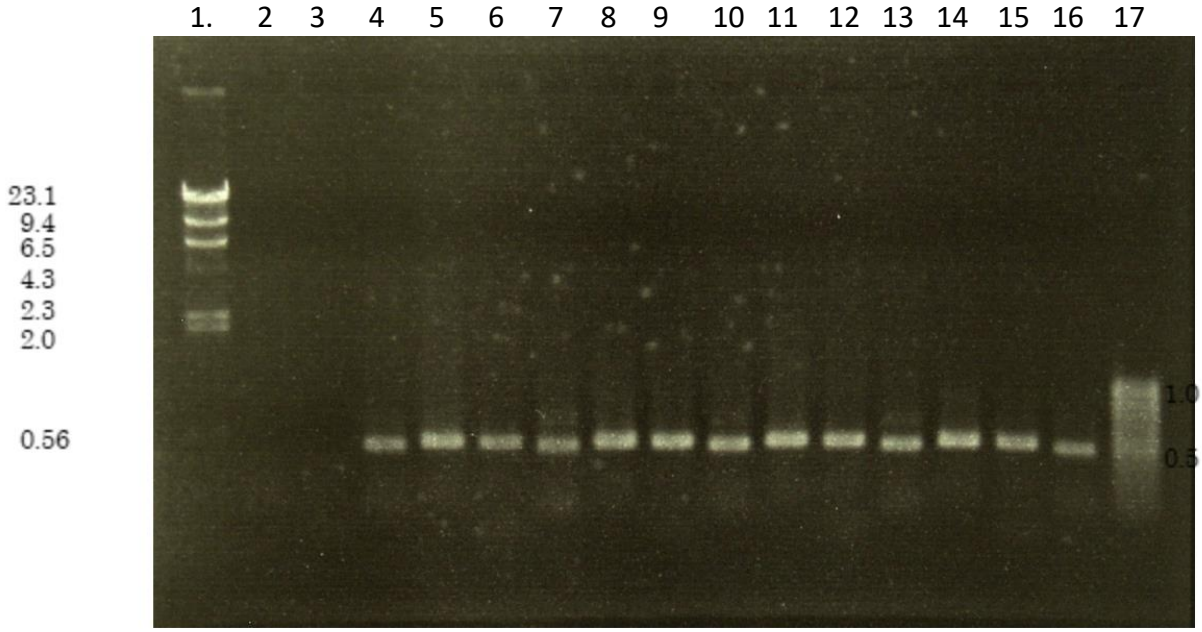


Figure 13. RT-PCR Analysis of Expression of Transgenes in Leaf of *Phalaenopsis* Line 311 Clones 311WL, 311NR, and 311TL

Lane	Sample	Gene
1	Gamma DNA/HindIII Marker	
2	PP3387 Leaf	*CcF3'5'H*
3	PP3387 Leaf	*hpt*
4	PP3387 Leaf	Phalaenopsis CHI
5	Line 311 Leaf	*CcF3'5'H*
6	Line 311 Leaf	*hpt*
7	Line 311 Leaf	Phalaenopsis CHI
8	311WL Leaf	*CcF3'5'H*
9	311WL Leaf	*hpt*
10	311WL Leaf	Phalaenopsis CHI
11	311NR Leaf	*CcF3'5'H*
12	311NR Leaf	*hpt*
13	311NR Leaf	Phalaenopsis CHI
14	311TL Leaf	*CcF3'5'H*
15	311TL Leaf	*hpt*
16	311TL Leaf	Phalaenopsis CHI
17	100-bp DNA Ladder	

Based on the results of the RT-PCR analysis, the expression of the *CcF3'5'H* and *hpt* genes are confirmed in petal and leaf tissues of line 311.

V.D. Resistance to hygromycin B in Protocorm Like Bodies of 311NR Phalaenopsis

Protocorm like bodies (PLBs) of 311NR phalaenopsis and the PP3387 phalaenopsis were cultivated on agar medium containing hygromycin B to determine resistance to hygromycin B for 10 weeks at 25°C.

Please see Appendix 3. Materials and Methods for Determination of Hygromycin Resistance in PLBs for details regarding this experiment.

The PLBs of the non-GE comparator PP3387 began to die after 4 weeks, and all PLBs died after 7 weeks of cultivation.

The PLBs of the 311NR line grew throughout the 10-week experiment. 9 PLBs produced new buds and grew into a clump (Figure 14).

Based on the results of this experiment, it was confirmed that 311NR phalaenopsis stably expresses hygromycin B phosphotransferase even after clone reproduction through tissue culture.

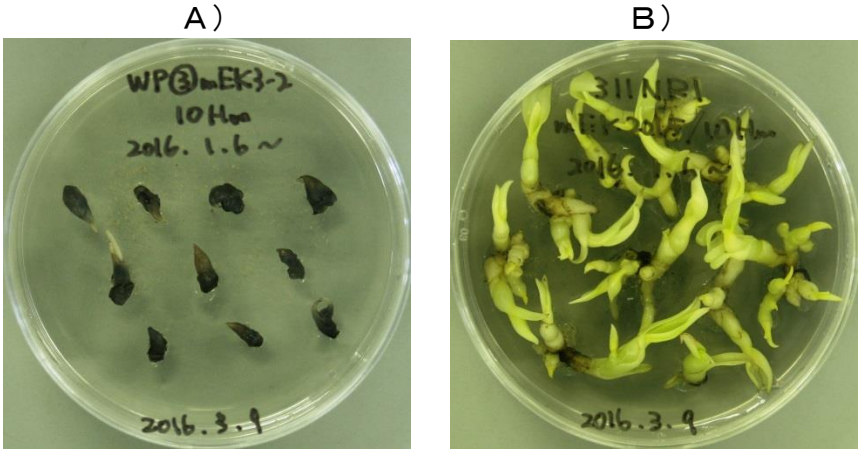


Figure 14. PLBs on agar medium with hygromycin B after 10 weeks of cultivation

Figure 14A shows PLBs from the PP3387 host plant.
Figure 14B shows PLBs from 311NR phalaenopsis.

V.E. Confirmation of the Absence of *Agrobacterium tumefaciens* in 311NR Phalaenopsis

V.E.1. Southern Blot Analysis of Line 311 to Determine Absence of *Agrobacterium*

Southern blot analysis was performed to confirm the absence of *Agrobacterium* vector backbone in a genomic DNA sample obtained from leaf tissue of line 311 (Figure 10).

Please see Appendix 2. Materials and Methods for Southern Blot Analysis for details regarding this experiment.

Southern blot analysis showed that line 311 does not contain *Agrobacterium* backbone sequence from plasmid pBIH-35S-CcF3'5'H used for transformation (Figure 10).

V.E.2. PCR Analysis of Line 311 to Determine Absence of *Agrobacterium*

PCR analysis was used to confirm the absence of *Agrobacterium* used for the transformation of original line 311. Analysis targeted the aminoglycoside phosphotransferase III (*aphA-3*) gene which is in the non T-DNA region of plasmid pBIH-35S-CcF3'5'H (Figure 3). DNA was extracted from flower, leaf, and root tissue of the T0 generation of line 311 (Figure 2).

Please see Appendix 1. PCR Methods for details regarding analysis.

PCR analysis confirmed that the same expected sizes of amplicons (565 bp) were observed in plasmid DNA of pBIH-35S-CcF3'5'H but not in flower, leaf and root tissue of the line 311 (Figure 15). Based on the results of this experiment, the *Agrobacterium* used for transformation is absent in flower, leaf and root tissue of line 311.

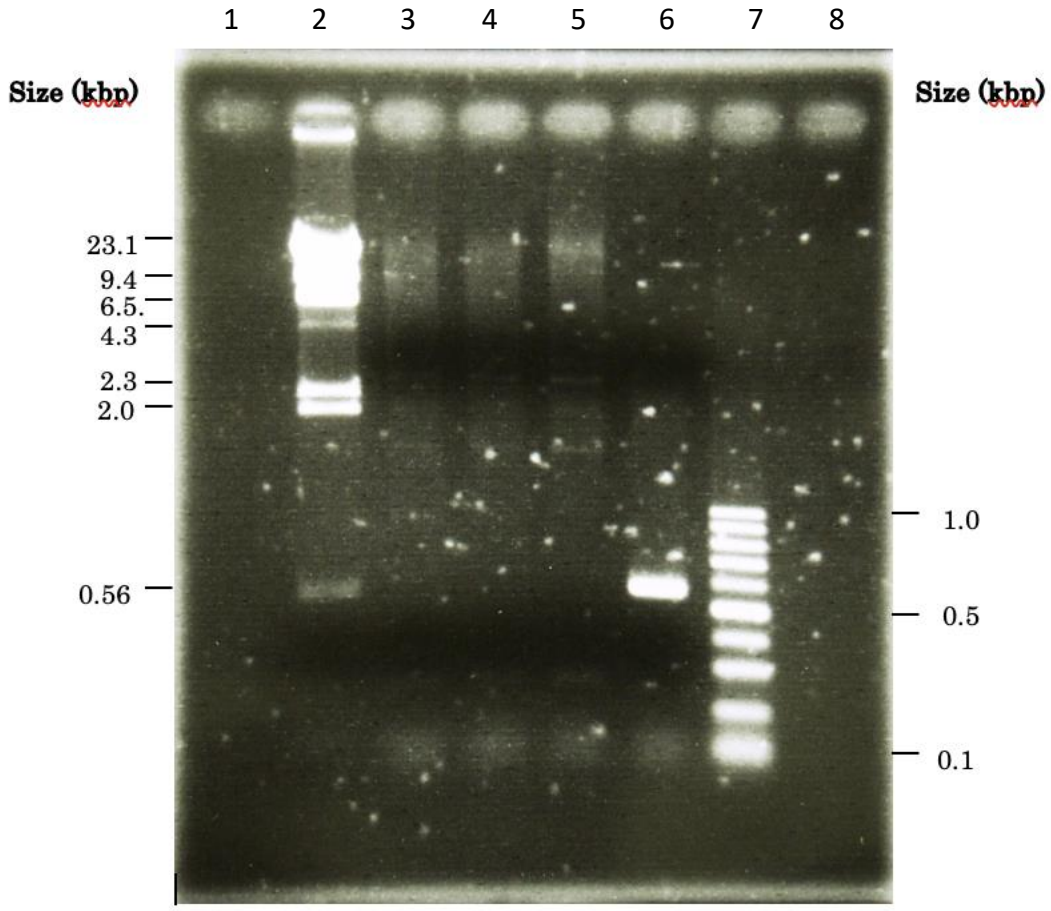


Figure 15. *Agrobacterium* PCR Results Using *aphA-3* Gene Probe

Lane	Sample
1	(empty)
2	Gamma DNA/*Hind*III marker
3	Flower (sepal) of line 311
4	Leaf of line 311
5	Root of line 311
6	pBIH-35S-CcF3’5’H
7	100-bp DNA ladder
8	(empty)

V.E.3. Colony Formation Assay for Confirmation of Absence of *Agrobacterium tumefaciens* in Line 311NR

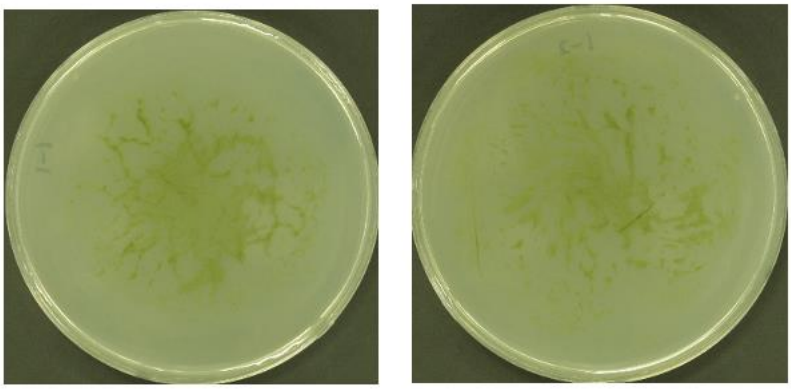
Seedlings of the 311NR phalaenopsis line were tested for the presence of *Agrobacterium tumefaciens* using a colony formation assay on agar plates.

Seedlings of 311NR phalaenopsis were ground and spread on Luria-Bertani (LB) agar media. Number of *Agrobacterium* colonies were counted after incubation at 28°C for 3 days.

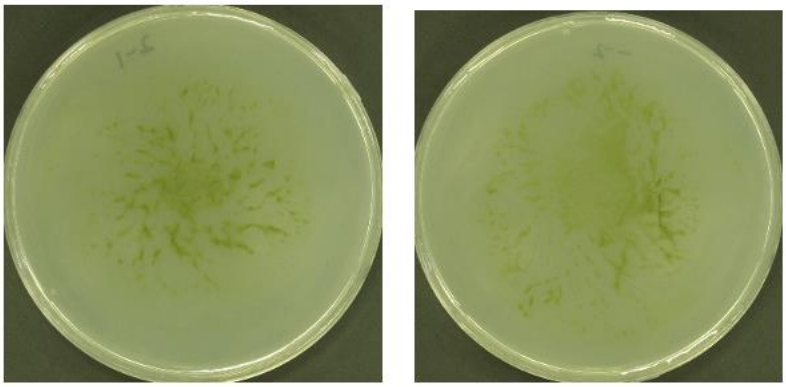
Methods of analysis are presented in Appendix 4. Materials and Methods for Determination of Agrobacterium Presence Using Agar Plates.

A sample of the *Agrobacterium* line used for transformation of the 311 line was used as a positive control.

311NR No. 1



311NR No. 2



311NR No. 3

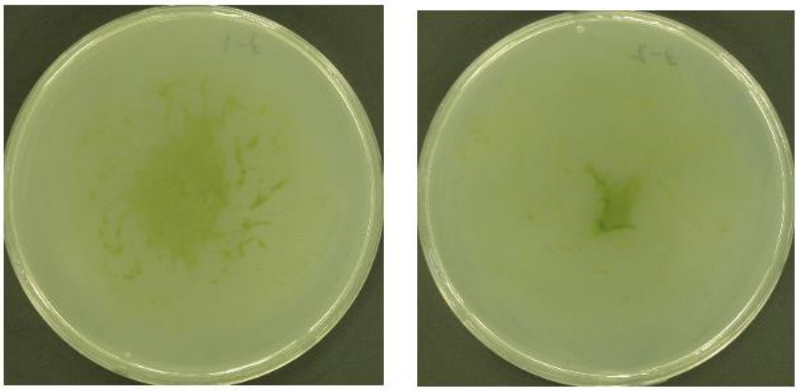


Figure 16. 311NR Phalaenopsis on LB Agar

Figure Legend: 311 NR No.1 – clone number 1; 311 NR No.2 – clone number 2; 311 NR No.3 – clone number 3. All clones were prepared from a 311 NR seedling.

After 3 days of incubation, no colonies were observed on the LB media plates (Figure 16). The positive control sample did show growth after 3 days, indicating a viable experiment (Figure 17).

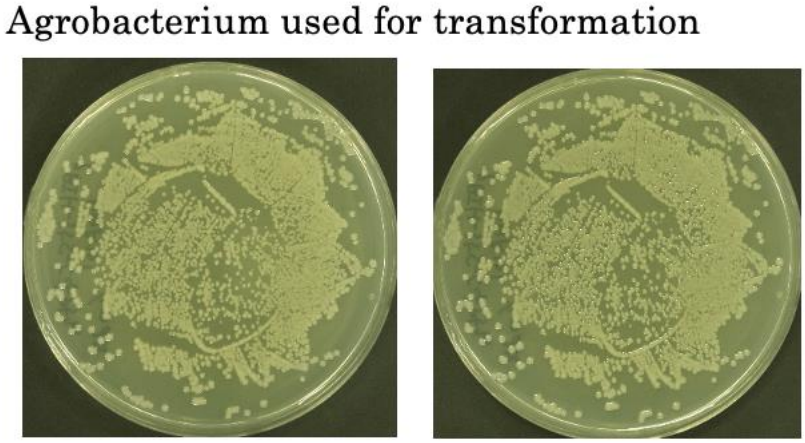


Figure 17. *Agrobacterium* on LB Agar

Based on the results of this experiment, the 311NR line does not contain residual *Agrobacterium* from transformation.

V.F. Conclusions on the Molecular Characterization and Genetic Stability of 311NR Phalaenopsis

Southern blot, PCR, RT-PCR, and colony formation assays were conducted to characterize the insertion in 311NR phalaenopsis.

Southern blot confirmed that a single intact pBIH-35S-CcF3'5'H T-DNA was inserted into the 311 phalaenopsis line genome and the integrity of the inserted DNA was maintained across three line 311 clones (311WL, 311NR, 311TL). Southern blots and a colony formation assay confirmed no plasmid backbone sequences were incorporated into line 311 and clone 311NR.

PCR analysis confirmed the presence of the *CcF3'5'H* and *hpt* genes in line 311. RT-PCR showed expression of the *CcF3'5'H* and *hpt* genes in line 311 clones 311WL, 311NR, and 311TL.

Together, these analyses demonstrate the presence of a single, intact stable T-DNA insertion with no plasmid backbone sequences.

VI. Morphological, Growth Characteristics and Ecological Observations

Morphological, growth characteristics, and ecological observations were evaluated to assess the comparability of 311NR phalaenopsis to the PP3387 non-GE comparator phalaenopsis. These

evaluations form the basis to determine whether 311NR phalaenopsis is comparable to a conventional (non-GE) phalaenopsis and is therefore no more likely to pose a plant pest risk.

VI.A. Morphological and Growth Characteristic Field Trial

A study was conducted in a confined field trial location at the Tsukuba Plant Innovation Research Center (T-PIRC), University of Tsukuba (Tsukuba, Ibaraki, Japan) from March to June 2019. The study utilized 25 comparator (PP3387) plants and 17 311NR phalaenopsis plants. Table 3 presents the characteristics and how they were measured in the field trial.

Table 3. Characteristics Measured in Phalaenopsis Confined Field Trial

Characteristic	Measurement
Date of flowering	Date when 50% of plants blooming at first inflorescence.
Plant length	Taken at date of flowering. Plant length from ground to top of inflorescence.
Number of inflorescences	Measured at date of flowering
Shape of inflorescences	Measured at date of flowering
Number of flowers per inflorescence	Measured at date of flowering
Length and width of largest leaf	Measured at date of flowering
Flower width (front view)	Measured at date of flowering
Flower length (front view)	Measured at date of flowering
Number of pollinia	Measured at date of flowering
Size of pollinia	Measured at date of flowering

VI.A.1. Date of Flowering

Table 4 shows date of flowering data for the non-GE comparator PP3387 and the 311NR phalaenopsis plants.

No difference in date of flowering is observed for PP3387 and 311NR phalaenopsis.

Table 4. Date of Flowering for PP3387 and 311NR Phalaenopsis

Plant	Date of Flowering
PP3387	May 1 – June 5, 2019
311NR	April 22 – June 10, 2019

VI.A.2. Plant Length

Table 5 shows results from measurement of plant length for PP3387 and 311NR phalaenopsis. The mean values in plant length of 311NR phalaenopsis were statistically significantly shorter than plant length of the comparator PP3387 (student t-test, $p = 0.05$).

Table 5. Plant Length for PP3387 and 311NR Phalaenopsis

Plant	Plant Length (cm)
PP3387	52.2 ± 8.6
311NR	39.3 ± 11.7*

*Statistically significant difference (Student t-test, p = 0.05)
The values for 311NR and PP3387 are shown as mean values ± standard deviation of 17 and 24 plants, respectively.

VI.A.3. Number of Inflorescences

Table 6 shows number of inflorescences in 311NR phalaenopsis and the PP3387 comparator. The number of inflorescences in 311NR phalaenopsis is statistically significantly smaller than that of the PP3387 comparator.

Table 6. Number of Inflorescences in PP3387 and 311NR Phalaenopsis

Plant/Number of Inflorescences	1	2	3	Mean*
PP3387	6 plants	17 plants	1 plant	1.8±0.5
311NR	16 plants	3 plants	0 plants	1.1±0.2*

*Statistically significant difference (Student t-test, p = 0.05)
The values for 311NR and PP3387 are shown as mean value ± standard deviation of 17 and 24 plants, respectively.

VI.A.4. Shape of Inflorescences and Number of Flowers per Inflorescence

Table 7 shows results from evaluation of the shape of inflorescence and number of flowers per inflorescence for 311NR and PP3387 comparator.

No statistically significant difference in shape or number of flowers per inflorescence was observed between 311NR phalaenopsis and the comparator PP3387.

Table 7. Shape of Inflorescences and Number of Flowers per Inflorescence in PP3387 and 311NR Phalaenopsis

Plant	Shape of Inflorescence	Number of Flowers per Inflorescence
PP3387	Raceme or panicle	16.1 ±6.3
311NR	Raceme or panicle	17.3 ±5.7

Values are shown as mean values ± standard deviation calculated from 17 plants of 311NR and 24 plants of PP3387. Statistical analysis was conducted using Student's t-test with p = 0.05.

VI.A.5. Length and Width of the Largest Leaf

Table 8 shows results from the measurement of the length and width of the largest leaf of 311NR phalaenopsis and PP3387 comparator plants.

The size of the largest leaf (length and width) of 311NR phalaenopsis was statistically significantly smaller than that of the PP3387 comparator (Welch t-test for length, Student's t-test for width, p = 0.05).

Table 8. Length and Width of Largest Leaf for PP3387 and 311NR

Plant	Largest Leaf	
	Length (cm)	Width (cm)
PP3387	20.7±2.3	7.1±0.4
311NR	19.0±1.2*	6.7±0.5*

*Statistically significant difference (Welch t-test for length, Student's t-test for width, p = 0.05)
Values are shown as mean values ± standard deviation calculated from 17 plants of 311NR and 24 plants of PP3387.

VI.A.6. Flower Width and Length

Table 9 shows the flower width and length (as viewed from the front of the plant) for the PP3387 comparator and 311NR phalaenopsis plants.

Flower width and flower length (as viewed from the front of the plant) of 311NR phalaenopsis was statistically larger than PP3387 phalaenopsis.

Table 9. Flower Width and Length of PP3387 and 311NR Phalaenopsis

Plant	Flower	
	Length (cm)	Width (cm)
PP3387	6.1±0.3	5.9±0.3
311NR	6.5±0.4*	6.2±0.5

*Statistically significant difference with PP3387 (Welch t-test for length, Student's t-test for width, p = 0.05)
Values are shown as mean values ± standard deviation calculated from 17 plants of 311NR and 24 plants of PP3387.

VI.A.7. Number and Size of Pollinia

Table 10 shows the number and size of pollinia for the PP3387 comparator and 311NR phalaenopsis plants.

All flowers of the PP3387 plants had two pollinia. The 311NR plant had sixteen flowers with two pollinia and one flower with four pollinia. No statistically significant difference was observed between PP3387 and 311NR for number of pollinia.

No statistically significant difference was observed between PP3387 and 311NR for size of pollinia.

Table 10. Number and Size of Pollinia of PP3387 and 311NR Phalaenopsis

Plant	Pollinia	
	Number of Pollinia	Diameter (mm)
PP3387	2.0±0.0	0.92±0.07
311NR	2.1±0.5	0.96±0.05

The values for number of pollinia are shown as mean values ± standard deviation calculated from 17 plants of 311NR and 25 plants of PP3387. The values for pollinia diameter are shown as mean values ± standard deviation calculated from 15 plants of 311NR and 24 plants of PP3387. Statistical analysis was conducted by Student's t-test with $p = 0.05$.

VI.B. Ecological Evaluations

VI.B.1. Seed Production Ability by Self-Pollination

Table 11 shows results from evaluation of the ability to produce seed by self-pollination for 311NR phalaenopsis and the comparator PP3387 phalaenopsis.

A study was conducted in a confined field in T-PIRC, University of Tsukuba (Tsukuba, Ibaraki, Japan) from May to June in 2019. Artificial self-pollination was performed for 5 plants of PP3387 and 311NR phalaenopsis. Flowers (seed capsules) were observed until plant senescence.

Self-pollinated flowers of PP3387 and 311NR plants were considered senescent 2 weeks after pollination. No seed capsules developed as a result of artificial self-pollination.

Table 11. Number of Self-Pollinated Flowers for PP3387 and 311NR Phalaenopsis

Plant	Number of self-pollinated flowers	Number of senescent flowers at 2 weeks after pollination
PP3387	25	25
311NR	25	25

No difference in self-pollinated flowers was observed for PP3387 and 311NR phalaenopsis.

VI.B.2. Seedling Cold Tolerance

Cold tolerance of seedling plants of 311NR phalaenopsis and comparator PP3387 was investigated in a confined field trial study at T-PRIC, University of Tsukuba (Tsukuba, Ibaraki, Japan) from December 2018 to February 2019.

Five potted seedlings of PP3387 and 311NR phalaenopsis were used for this study and were grown at the central research institute of Ishihara Sangyo Kaisha, Ltd. (Kusatsu, Shiga, Japan). After 10 days of acclimation in a grass house, seedlings were transferred to a confined field and observed from December 10, 2018, through February 27, 2019.

Photographs were taken on February 27 and analyzed for plant cold tolerance. Temperature and humidity were recorded during trial (Figure 19).

Leaves of PP3387 and 311NR became soft and wilted and changed from bright green to dark green color after 3 days in the confined field trial conditions. After 15 days, leaves were fading in color and leaves were wrinkled (Figure 18(b)). After 23 days, leaves had lost further color and base color of leaves became brown in nature (Figure 18(c)). Leaves were completely senescent after 51 days (Figure 18(d)). Complete plant senescence was determined at 79 days (Figure 18(e)).

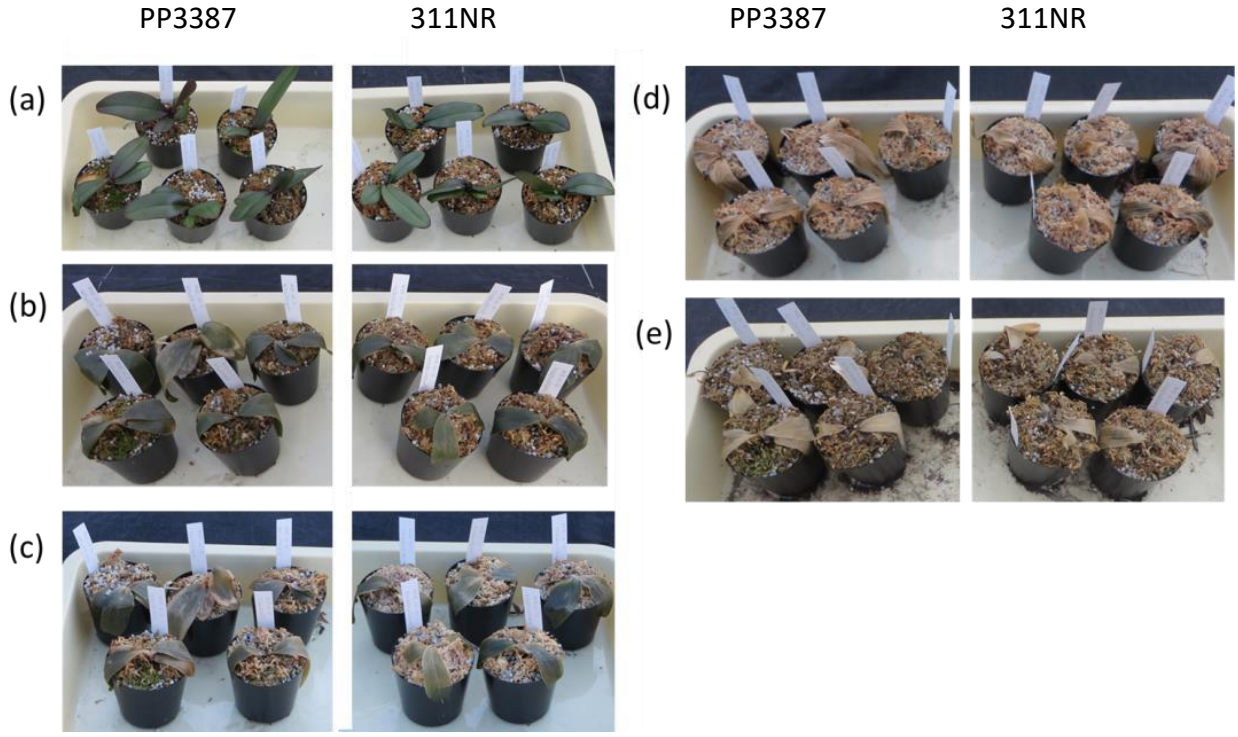


Figure 18. Seedlings of PP3387 and 311NR in the Confined Field Trial for Cold Tolerance

(a) Day 1, (b) Day 15, (c) Day 23, (d) Day 51, (e) Day 79

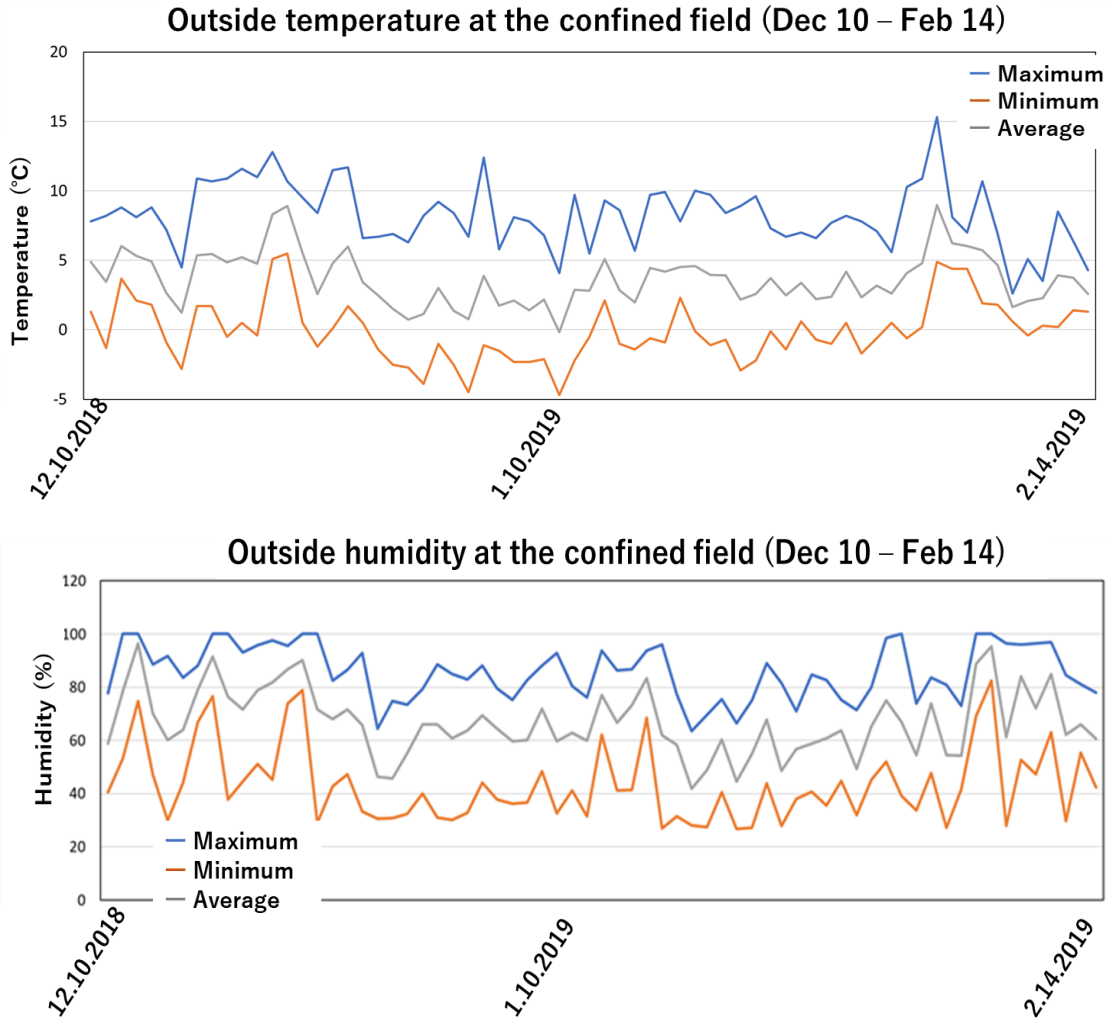


Figure 19. Ambient Temperature and Humidity During Cold Tolerance Field Trial

There was no difference observed in damage and growth inhibition between PP3387 and 311NR phalaenopsis during the study. Therefore, it is not likely that there is any difference between 311NR and PP3387 phalaenopsis in cold tolerance.

VI.B.3. Seedling Heat Tolerance

Heat tolerance of seedling plants of 311NR phalaenopsis and comparator PP3387 was investigated in a confined field trial study at T-PRIC, University of Tsukuba (Tsukuba, Ibaraki, Japan) from June to September 2019.

Five potted seedlings of PP3387 and 311NR phalaenopsis were used for this study and were grown at the central research institute of Ishihara Sangyo Kaisha, Ltd. (Kusatsu, Shiga, Japan). After 5 days of acclimation in a grass house, seedlings were transferred to a confined field and observed from June 25 through September 1, 2019.

Photographs were taken on regular intervals and ended by September 1. Photos were analyzed for plant heat tolerance (Figure 22). Temperature and humidity were recorded during the trial (Figures 20 and 21).

Seedlings were grown in a glass house during the month of July as higher than average rain occurred during that month. In early July, temperature averaged 20°C which is not high enough to evaluate heat tolerance (Figure 20). After July 24, the temperature average rose above 30°C and seedlings were moved outside for observation (Figure 21).

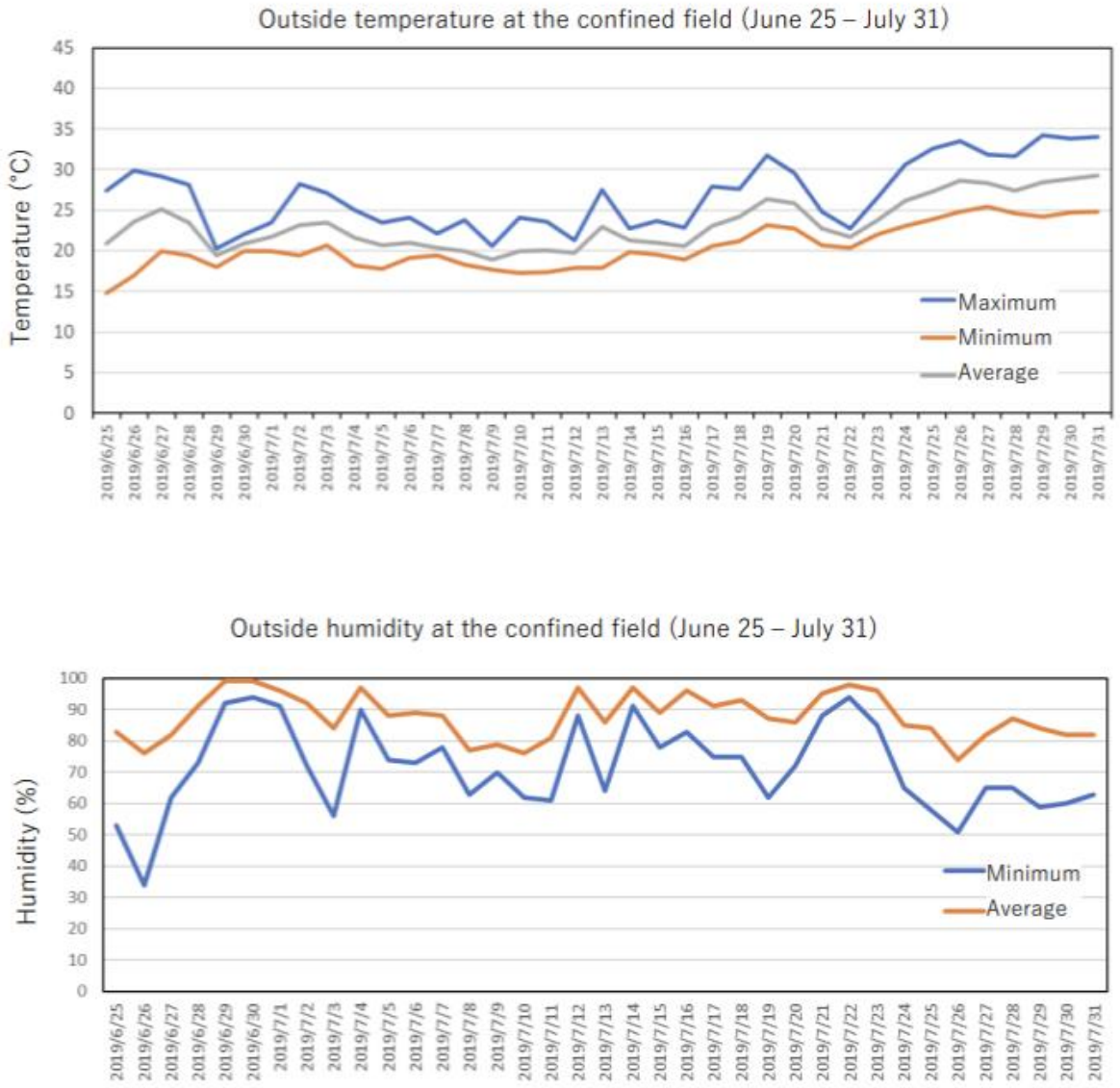


Figure 20. Temperature and Humidity Measurements at Field Trial Site (June 25 – July 31)

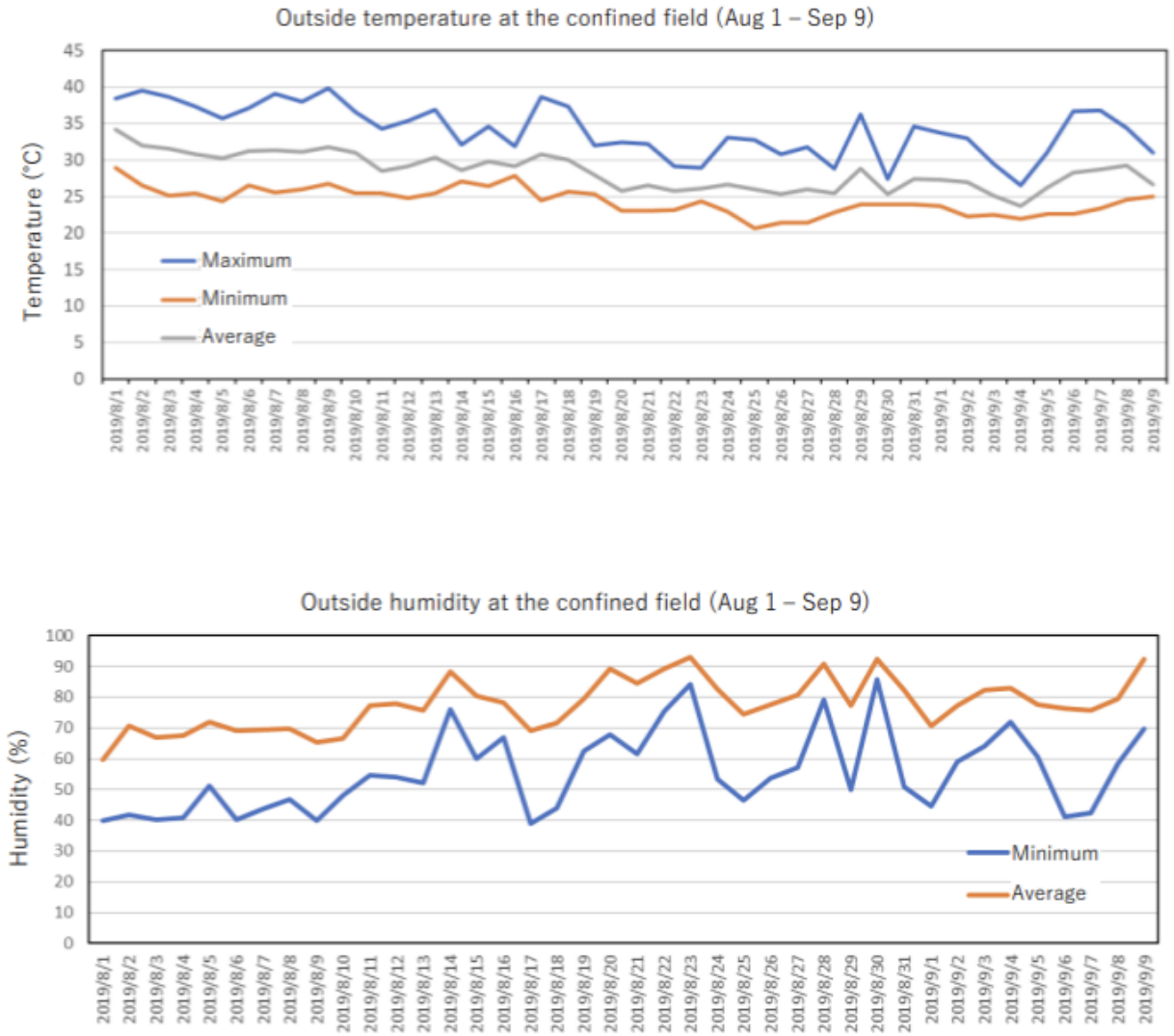


Figure 21. Temperature and Humidity Measurements at Field Trial Site (August 1 – September 9)

Heat damage such as leaf yellowing was observed in PP3387 and 311NR at 37 days (approximately 1 week of heat treatment) after initiation of the study. At day 65, the majority of 311NR phalaenopsis plants were senescent. The PP3387 plants tolerated heat treatment throughout the study, with yellowing beginning at day 37 and continuing through day 65 (Figure 22).

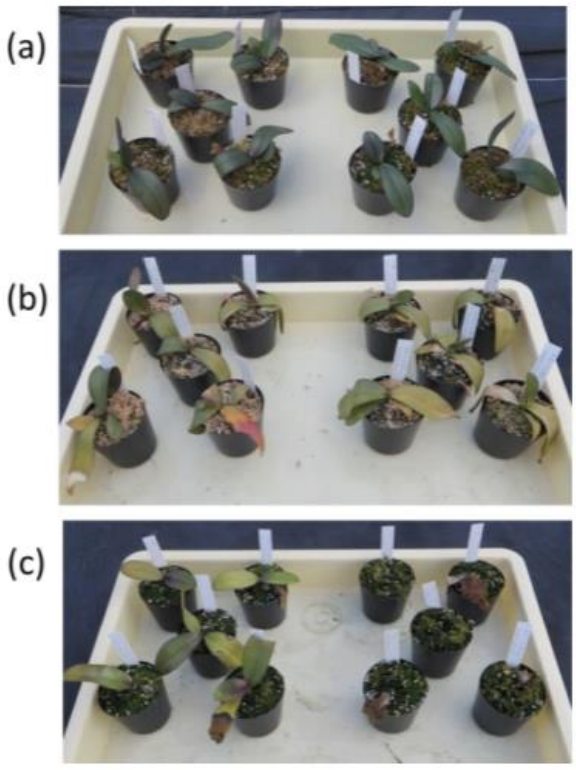


Figure 22. Seedlings of PP3387 and 311NR Phalaenopsis in Heat Treatment Experiment

Picture (a) – June 25, 2019 (Day 1), (b) August 1, 2019 (Day 37), and (c) August 29, 2019 (day 65)

Based on the results of this study, it is concluded that the heat tolerance of 311NR phalaenopsis seedlings is lower than the control PP3387 plants. It is possible that somaclonal variation is contributing to this difference.

VI.C. Conclusions on Morphological and Growth Characteristics, and Ecological Observations.

VI.C.1. Morphological and Growth Characteristics

The morphological and growth characteristics of 311NR phalaenopsis was investigated by comparison to the comparator PP3387 line. Date of flowering, plant length, number of inflorescences, shape and number of flowers per inflorescence, flower width, and flower length observations were collected and statistically analyzed.

No statistical differences were observed in date of flowering, number of flowers per inflorescence, number of pollinia, and size of pollinia for PP3387 and 311NR phalaenopsis.

Statistical differences were observed in plant length (311NR is shorter), number of inflorescences (311NR has fewer), size of the largest leaf (311NR is smaller), and flower length and width (311NR is larger).

Taken overall, it is unlikely that the statistically different morphology observations have an effect that would cause 311NR phalaenopsis to have altered disease, unintended pest susceptibilities, or to become weedy or invasive. The differences observed are small and most are due to smaller plant and leaf size – two traits that are not directly connected to weediness characteristics.

VI.C.2. Ecological Observations

Ecological observations of 311NR phalaenopsis were collected and compared to the comparator PP3387 line. Self-pollination capacity, cold tolerance, and heat tolerance were tested in controlled field trials.

No statistical differences were noted in seed capsule development and cold tolerance. As statistically different result occurred in the heat tolerance study.

Taken overall, it is unlikely that the statistically different ecological observations have an effect that would cause 311NR phalaenopsis to have altered disease, unintended pest susceptibilities, or to become weedy or invasive. The difference observed in the heat tolerance indicates that the 311NR phalaenopsis is less likely to survive elevated temperature making it less likely to become weedy.

VII. Potential Environmental Impact of the Introduction of 311NR Phalaenopsis

VII.A. Potential for 311NR Phalaenopsis to Have Altered Disease and Unintended Pest Susceptibilities or to Become Weedy or Invasive

Weedy plants have been characterized by a variety of ecological, reproductive, and morphological traits. These traits typically enable them to thrive in disturbed environments. Increased reproductive capacity, producing large numbers of seeds, and easy seed dispersal are traits associated with weediness (Morishita n.d.). This trait was not observed in the comparison of self-pollination of 311NR phalaenopsis and the PP3387 comparator plant.

The ability to adapt in response to environmental changes is also a characteristic of weedy plants (Morishita n.d.). Adjustment of growth patterns, reproductive methods, and other strategies have been noted in weedy plants. The 311NR phalaenopsis did not differ in cold tolerance when compared to the PP3387 comparator, indicating no increased ability to compete with non-GE phalaenopsis. In the case of heat tolerance, 311NR showed less tolerance to increased temperature – an indicator that it is not likely to grow in hot environments.

VII.B. Potential for Gene Flow Between 311NR Phalaenopsis and Sexually Compatible Wild Relatives

Phalaenopsis orchids are not native to the United States. *Phalaenopsis* is primarily native to Southeast Asia, including the Philippines, Indonesia, Taiwan, Malaysia, and parts of China. They prefer tropical and subtropical climates and often grow as epiphytes in humid forests.

Crossbreeding *Phalaenopsis* orchids with orchids native to the United States is unlikely due to genetic and reproductive barriers.

Phalaenopsis belongs to the subtribe of Aeridinae, while many United States native orchids belong to different subtribes such as Cypripedioideae. The disparity in taxonomy between *Phalaenopsis* and orchids native to the U.S. makes intergeneric hybridization a challenge (Orchidboard 2025).

Additionally, orchids have specialized pollination mechanisms. Hybridization is more likely among closely related species with overlapping flowering periods and similar (or compatible) pollination strategies. In the case of hybridization of *Phalaenopsis* with U.S. native orchids, these factors are not expected to align, preventing natural hybridization.

Horticulturists have successfully created hybrids within the *Phalaenopsis* genus and closely related species. Crossing *Phalaenopsis* with distantly related U.S. native orchids has not been documented and would likely face significant genetic compatibility issues. It was demonstrated in the self-pollination experiment in this document that developing viable seed pods is difficult (Table 11).

Unless closely related, using similar pollination strategies, and in the right environment, it is unlikely that 311NR phalaenopsis will outcross to native United States orchids.

VII.C. Phalaenopsis 311NR and Pollinators

In the wild, Phalaenopsis flowers are primarily pollinated by bees and wasps (in the order of Hymenoptera), but pollination by organisms in the orders of Lepidoptera, Diptera, Coleoptera have also been observed (Ray and Vendrame 2015). Flower color is involved, but orchids, in general, have evolved forms of deception to attract pollinators such as physical appearance of insect mating partners and/or chemical cues (Ray and Vendrame 2015). Due to these adapted phenotypes, a change in flower color is unlikely to deter pollination by Hymenopteran insects.

Bees and wasps do not rely on orchids as their only source of food. Insects in the order Hymenoptera are known to pollinate over 100 commercial crops in North America (including almonds vegetables, fruits and soybeans) and many wild plants (Hristov et al., 2020). A change in flower color is not likely to cause any harm to the feeding potential to Hymenopterian insects.

VIII. Adverse Consequences of Introduction

The information presented in this petition demonstrates that 311NR phalaenopsis is unlikely to pose a plant pest risk as compared to non-GE phalaenopsis. The analysis of molecular data confirmed the insertion of one copy of the pBIH-35S-CcF3'5'H T-DNA, containing the *CcF3'5'H* and *hpt* genes. The pBIH-35S-CcF3'5'H T-DNA is intact and is stably integrated into the 311NR genome.

Measurement of morphological, growth, and ecological characteristics showed no likely difference when compared to non-GE Phalaenopsis.

The data and information presented herein supports the conclusion that 311NR phalaenopsis does not present a plant pest risk and is not otherwise deleterious to the environment. Therefore, ISK requests that APHIS grant the request for a determination of nonregulated status for 311NR phalaenopsis, 311NR phalaenopsis progeny, and any crosses of 311NR phalaenopsis with other nonregulated phalaenopsis.

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Appendices

Appendix 1. PCR Methods

PCR Identification of Genes of Interest

DNA was extracted from flower (sepal), leaf and root tissue of the line 311 by DNeasy® Plant Mini Kit (QIAGEN). Also, DNA of leaf sample was extracted from non-GE Wedding Promenade PP3387 which is host plant of 311NR as a negative control. PCR was performed by *TaKaRa Ex Taq*® HS (TaKaRa) for extracted DNA as template to amplify transgenes (*F3'5'H* gene and *htp* gene) and endogenous gene (Phalaenopsis *CHI* gene).

PCR reaction components:	25 mM TAPS buffer, pH 9.3
（total 10 µl）	50 mM KCl
	2 mM $MgCl_2$
	0.1 mM DTT
	0.2 mM dNTP (each)
	0.5 µM primer 1
	0.5 µM primer 2
	2 ng genomic DNA
	0.25 units TaKaRa Ex Taq HS™

PCR conditions:	98℃ 2 min	
	98℃ 10 sec, 55℃ 30 sec, 72℃ 1 min	30 cycles
	72℃ 7 min	
	4℃	

PCR amplicon was separated by agarose gel electrophoresis and stained by ethidium bromide (EtBr). A primer set used for the PCR analysis is described below.

Commelina communis F3'5'H gene
CF35H F3 ： 5’-AGTGAACCCAACAATACTTCAC-3’
CF35H R1 ： 5’-CACAGTCTTTTGAAGTGCGATTC-3’

E. coli hpt gene
HPT F2 ： 5’-AGGCTCTCGATGAGCTGATG-3’
HPT R1 ： 5’-GGCATCTACTCTATTCCTTTG-3’

Phalaenopsis chalcone *CHI* gene
PCHI F3 ： 5’-AAGCTCACTGGTTCGGTTGAG-3’
PCHI R2 ： 5’-AACTTGGCTTTCTCCACATCC-3’

F3’5’H: flavonoid 3', 5'-hydroxylase
HPT: hygromycin B phosphotransferase
CHI: chalcone isomerase

RT-PCR Analysis of Gene Expression in Petals and Leaf

RNA was extracted using RNeasy® Plant Mini Kit (QIAGEN) from petal and leaf samples of the Wedding Promenade PP3387 line, the line 311, and three clone seedlings (311WL, 311NR and 311TL).

cDNA was synthesized from 500 ng of the extracted RNA extracted from each sample by PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa) using oligo dT as primer.

Using 1 µl of each of the cDNAs as a template, PCR was performed using TaKaRa Ex Taq HSTM (TaKaRa) under the following conditions to amplify the transgene (*CcF3'5'H* gene and *hpt* gene) and the endogenous gene (Phalaenopsis *CHI* gene).

PCR reaction: 98℃ 2 min
98℃ 10 sec, 55℃ 30 sec, 72℃ 1 min, 25 cycles
72℃ 7 min
4℃

PCR amplicon was separated by agarose gel electrophoresis and stained by ethidium bromide (EtBr). A primer set used for the PCR analysis is described below.

CcF3'5'H gene
CF35H F3 : 5’-AGTGAACCCAACAATACTTCAC-3’
CF35H R1 : 5’-CACAGTCTTTTGAAGTGCGATTC-3’

hpt gene
HPT F2 : 5’-AGGCTCTCGATGAGCTGATG-3’
HPT R1 : 5’-GGCATCTACTCTATTCCTTTG-3’

Phalaenopsis *CHI* gene
PCHI F3 : 5’-AAGCTCACTGGTTCGGTTGAG-3’
PCHI R2 : 5’-AACTTGGCTTTCTCCACATCC-3’

F3’5’H: flavonoid 3', 5'-hydroxylase
HPT: hygromycin B phosphotransferase
CHI: chalcone isomerase

Materials and Methods for PCR Analysis to Confirm Absence of Agrobacterium Backbone DNA

DNA was extracted from flower (sepal), leaf and root of the line 311 by DNeasy® Plant Mini Kit (QIAGEN). PCR was performed by *TaKaRa Ex Taq*® HS (TaKaRa) for extracted DNA as template to amplify aminoglycoside phosphotransferase III (*aphA-3*) gene which is non T-DNA region of pBIH-35S-CcF3'5'H. Plasmid DNA of pBIH-35S-CcF3'5'H was also used for the PCR amplification as control material.

PCR reaction components: (total 10 µl)	25 mM TAPS buffer, pH 9.3 50 mM KCl 2 mM $MgCl_2$ 0.1 mM DTT 0.2 mM dNTP (each) 0.5 µM APH F1 0.5 µM APH R1 50 ng genomic DNA 0.25 units *TaKaRa Ex Taq*® HS

PCR conditions:	98℃ 2 min	
	98℃ 10 sec, 55℃ 30 sec, 72℃ 1 min	30 cycles
	72℃ 7 min	
	4℃	

PCR amplicon was separated by agarose gel electrophoresis and stained by ethidium bromide (EtBr). A primer set used for the PCR analysis is described below.

APH F1：5'-TGCAAGGAACAGTGAATTGG-3'

APH R1：5'-AGTTTTCGCAATCCACATCG-3'

Appendix 2. Materials and Methods for Southern Blot Analysis

Southern Blots for Copy Number

CcF3'5'H and *hpt* Gene Southern Blot Methods

DNA was extracted from the leaf sample of the line 311 by DNeasy® Plant Mini Kit (QIAGEN). 15 µg of extracted DNA was separated by electrophoresis with 1% agarose gel after digestion of DNA by *Eco*RI or *Nde*I. DNA in the agarose gel was transferred to the membrane after denaturing and neutralization. After pre-hybridization in DIG Easy Hyb (Roche), the DIG labeled probe (3' terminal of *Nde*I in *hpt* gene or *CcF3'5'H* gene; Figure 7) which was generated by PCR DIG probe synthesis kit (Roche) was added, and the membrane was incubated for 16 hours at 42℃. The membrane was washed twice for 5 minutes at room temperature and twice for 15 minutes at 60℃ in 2xSSC solution with 0.1% SDS. Fluorescence signal was detected according to the operating procedures of the DIG Nucleic Acid Detection Kit (Roche).

Sequence of Analysis

First, hybridization, washing, and detection were performed using an the *hpt* gene-specific probe, followed by detachment of the probe from the membrane and subsequent Southern hybridization using an *CcF3'5'H* gene-specific probe.

Southern Blots for Generational Stability

DNA was extracted using DNeasy® Plant Mini Kit (QIAGEN) from leaf samples of Wedding Promenade PP3387, the line 311, 311WL that has the same flower mutation as line 311, 311NR which has the reverse mutation and a normal flower shape and 311TL which has petals that are transformed into the flower lip.

20 µg of extracted each DNA sample was separated by electrophoresis with 1% agarose gel after digestion of DNA by *Eco*RI. DNA in the agarose gel was transferred to the membrane after denaturing and neutralization. Two blotted membranes were prepared for each sample. After pre-hybridization in DIG Easy Hyb (Roche), the DIG labeled probe (*CcF3'5'H* gene or 5' terminal of *Eco*RI site in *hpt* gene; Figure 7) which was generated by PCR DIG probe synthesis kit (Roche) was added, and the membrane was incubated for 16 hours at 42℃. Subsequent process followed in same manner as study A.

Southern Blots for Absence of Agrobacterium Backbone

DNA was extracted from the leaf sample of line 311 by DNeasy® Plant Maxi Kit (QIAGEN). 20 µg of extracted DNA was separated by electrophoresis with 1% agarose gel after digestion of DNA by *Eco*RI. 0.1 ng of DNA from pBIH-35S-CcF3'5'H was digested by *Eco*RI and separated by electrophoresis as a control material.

DNA in the agarose gel was transferred to the membrane after depurination, denaturing and neutralization. Six membranes were prepared in the same manner.

After pre-hybridization in DIG Easy Hyb (Roche), the DIG labeled probe (Probe 1 ~ 6 (Frisch, et al. 1995) which was generated by PCR DIG probe synthesis kit (Roche) was added, and the membrane was incubated for 16 hours at 42℃. The membrane was washed twice for 5 minutes at room temperature and twice for 15 minutes at 60℃ in 2xSSC solution with 0.1% SDS. Fluorescence signal was detected according to the operating procedures of the DIG Nucleic Acid Detection Kit (Roche).

Appendix 3. Materials and Methods for Determination of Hygromycin Resistance in PLBs

A study was conducted to determine hygromycin resistance in 311NR and control PLBs at the Central Research institute of Ishihara Sangyo Kaisha, Ltd. (Kusatsu, Shiga, Japan) in January to March 2016.

Ten PLBs from 311NR phalaenopsis and the host plant (PP3387), respectively, were cultivated on Hyponex agar media with 10 mg/l of hygromycin, 0.1 mg/l of naphthalen acetic acid and 1 mg/l of benzyl adenine at 25℃ under dark condition. Cultivation media was replaced every week, and PLBs were cultivated for 10 weeks.

Appendix 4. Materials and Methods for Determination of Agrobacterium Presence Using Agar Plates

A study was conducted at Central research institute of Ishihara Sangyo Kaisha, Ltd. (Kusatsu, Shiga, Japan) in February 2016.

Leaf samples collected from three 311NR seedlings in flask (0.36~0.56 g) was ground with LB media (0.2 ml/0.1 g leaf sample) in a mortar. After grinding, each sample was filtered using a nylon membrane filter with pore size of 100 micrometer.

Next, 0.4 ml of filtered sample liquid was spread on two LB agar media plates.

Presence or absence of colonies were confirmed at 3 days after incubation at 28℃.

LB medium contained yeast extract (5 g/l), tryptone (10 g/l) and sodium chloride (10 g/l). 13 g/l of agar was added to the agar medium.

Appendix 5. Sequence Information

```
        10         20         30         40         50         60
CCGGGCTGGTTGCCCTCGCCGCTGGGCTGGCGGCCGTCTATGGCCCTGCAAACGCGCCAG
Ori V
        70         80         90        100        110        120
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       130        140        150        160        170        180
AAAACTTGGCCCTCACTGACAGATGAGGGGCGGACGTTGACACTTGAGGGGCCGACTCAC

       190        200        210        220        230        240
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       250        260        270        280        290        300
CAGCCTCGCAAATCGGCGAAAACGCCTGATTTTACGCGAGTTTCCCACAGATGATGTGGA

       310        320        330        340        350        360
CAAGCCTGGGGATAAGTGCCCTGCGGTATTGACACTTGAGGGGCGCGACTACTGACAGAT

       370        380        390        400        410        420
GAGGGGCGCGATCCTTGACACTTGAGGGGCAGAGTGCTGACAGATGAGGGGCGCACCTAT

       430        440        450        460        470        480
TGACATTTGAGGGGCTGTCCACAGGCAGAAAATCCAGCATTTGCAAGGGTTTCCGCCCGT

       490        500        510        520        530        540
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       550        560        570        580        590        600
TTTTTAACCAGGGCTGCGCCCTGTGCGCGTGACCGCGCACGCCGAAGGGGGGTGCCCCCC

       610        620        630        640        650        660
CTTCTCGAACCCTCCCGGCCCGCTAACGCGGGCCTCCCATCCCCCCAGGGGCTGCGCCCC

       670        680        690        700        710        720
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       730        740        750        760        770        780
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790 800 810 820 830 840
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850 860 870 880 890 900
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910 920 930 940 950 960
GCAATTTTTACCTTGGGCATTCTTGGCATAGTGGTCGCGGGTGCCGTGCTCGTGTTCGGG

970 980 990 1000 1010 1020
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aphA-3 5' non-coding

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IS1

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1870 1880 1890 1900 1910 1920
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1930 1940 1950 1960 1970 1980
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1990 2000 2010 2020 2030 2040
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aphA-3 coding

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2170 2180 2190 2200 2210 2220
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2230 2240 2250 2260 2270 2280
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/ right border
Bg/II

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6250 6260 6270 6280 6290 6300
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hpt

```
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eAspSerValSerAspLeuMetGlnLeuSerGluGlyGluGluSerArgAlaPheSerPh

      6370       6380       6390       6400       6410       6420
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      6430       6440       6450       6460       6470       6480
AGATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGA
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      6490       6500       6510       6520       6530       6540
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pIleGlyGluPheSerGluSerLeuThrTyrCysIleSerArgArgAlaGlnGlyValTh

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      6670       6680       6690       6700       6710       6720
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      6730       6740       6750       6760       6770       6780
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      6790       6800       6810       6820       6830       6840
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      6850       6860       6870       6880       6890       6900
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6970 6980 6990 7000 7010 7020
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7090 7100 7110 7120 7130 7140
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7150 7160 7170 7180 7190 7200
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7270 7280 7290 7300 7310 7320
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7450 7460 7470 7480 7490 7500
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7570 7580 7590 7600 7610 7620
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7630 7640 7650 7660 7670 7680
CAACTGGCAAATCATCCAGCGTGATTGGTAACTTCAGTTCCAGCGACTTGATTCGTTTTG

7690 7700 7710 7720 7730 7740
GTGCTACCCACGTTTTCAATAAGGACGAGATGGTGGAGTAAAGAAGGAGTGCGTCGAAGC

7750 7760 7770 7780 7790 7800
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7810 7820 7830 7840 7850 7860
GATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATG

7870 7880 7890 7900 7910 7920
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7930 7940 7950 7960 7970 7980
CGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATC

7990 8000 8010 8020 8030 8040
TATGTTACTAGATCGATCAAACTTCGGTACTGTGTAATGACGATGAGCAATCGAGAGGCT

8050 8060 8070 8080 8090 8100
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8110 8120 8130 8140 8150 8160
GCTCATGCCGACAGGCATAACTTAGATATTCGCGGGCTATTCCCACTAATTCGTCCTGCT

8170 8180 8190 8200 8210 8220
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8230 8240 8250 8260 8270 8280
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8290 8300 8310 8320 8330 8340
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8350 8360 8370 8380 8390 8400
GCAAGTCCTTTCTGTGCTCGTCGGAAATTCCTCTCCTGTCAGACGGTCGTGCGCATGTCT

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      8410       8420       8430       8440       8450       8460
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      8470       8480       8490       8500       8510       8520
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                          P35S

      8530       8540       8550       8560       8570       8580
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      8590       8600       8610       8620       8630       8640
AAAGGACAGTAGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGG

      8650       8660       8670       8680       8690       8700
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      8710       8720       8730       8740       8750       8760
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      8770       8780       8790       8800       8810       8820
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      8830       8840       8850       8860       8870       8880
TATAAGGAAGTTCATTTCATTTGGAGAGGACAGGCTTCTTGAGATCCTTCAACAATTACC
                                                  TMV-omega

      8890       8900       8910       8920       8930       8940
AACAACAACAAACAACAAACAACATTACAATTACTATTTACAATTACAGTCGGGATTTAT
                                                        Me
                                                      CcF3'5'H

      8950       8960       8970       8980       8990       9000
GGTACCCCTTACGTACCTTGCATGTCTCCTCCTCCCCTTCCTCCTCCACCACCTCCTCCT
tValProLeuThrTyrLeuAlaCysLeuLeuLeuProPheLeuLeuHisHisLeuLeuLe

      9010       9020       9030       9040       9050       9060
CCTCCATCGCCGACGTCGACTCCCCCCCGGTCCCCTCGGCTTCCCCATCCTAGGCTCCCT
uLeuHisArgArgArgArgLeuProProGlyProLeuGlyPheProIleLeuGlySerLe
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9070 9080 9090 9100 9110 9120
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9130 9140 9150 9160 9170 9180
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9190 9200 9210 9220 9230 9240
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9250 9260 9270 9280 9290 9300
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9370 9380 9390 9400 9410 9420
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9430 9440 9450 9460 9470 9480
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9670 9680 9690 9700 9710 9720
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9970 9980 9990 10000 10010 10020
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10690 10700 10710 10720 10730 10740
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/ /
Bg/II *Bg*/II

10750 10760 10770 10780 10790 10800
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11050 11060 11070 11080 11090 11100
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left border

11110 11120 11130 11140 11150 11160
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11170 11180 11190 11200 11210 11220
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11290 11300 11310 11320 11330 11340
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11530 11540 11550 11560 11570 11580
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11650 11660 11670 11680 11690 11700
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/
Bg/II

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traF

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13870 13880 13890 13900 13910 13920
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13990
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