Assessment of Plant Pest Risk for International Flower Developments Pty. Ltd. IFD-524Ø1-4 and IFD-529Ø1-9 *Rosa* x *hybrida* (rose) varieties

International Flower Developments Pty. Ltd. (IFD) (Victoria, Australia) has petitioned the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture for a determination that IFD-524Ø1-4 and IFD-529Ø1-9 roses (*Rosa* x *hybrida*) are unlikely to pose a plant pest risk and, therefore, should no longer be regulated articles under APHIS' regulations at 7 CFR part 340. APHIS administers 7 CFR part 340 under the authority of the plant pest provisions of the Plant Protection Act of 2000[1]. This plant pest risk assessment was conducted to determine whether IFD-524Ø1-4 and IFD-529Ø1-9 are unlikely to pose a plant pest risk.

History of Development of IFD-524Ø1-4 and IFD-529Ø1-9 *Rosa* x *hybrida* varieties

Working with a rose grower in the U.S., Florigene and IFD have been researching and developing two lines of genetically engineered roses since 2004. Both of these rose lines include added genes for flavonoid 3'-5' hydroxylase (from a black pansy, *Viola tricolor*) and anthocyanin 5-acyltransferase (from torenia, *Torenia hybrida*). These rose lines also contain the neomycin phosphotransferase gene (from the bacterium *Escherichia coli*) which was used for selection in the laboratory. Both of these rose lines have been approved for commercial use, including environmental release, in Japan (IFD 2010). One line (IFD-524Ø1-4) has also been approved for commercial use/environmental release in Australia (IFD 2010) (costs of the regulatory request for the other rose line and the small size of the Australian market led the company to only request approval for one line there). Addition of the *Viola* and *Torenia* genes alter the anthocyanin biosynthesis pathways and shunt some of these biochemicals toward production of the delphinidin-based anthocyanins, resulting in production of blue pigments in these rose lines. Production of these blue pigments alters the flower color of these rose lines (IFD 2010).

Description of added genes

Florigene's IFD-524Ø1-4 and IFD-529Ø1-9 roses (*Rosa* x *hybrida*) were produced using disarmed *Agrobacterium tumefaciens* (IFD 2010) and contain 3 transgene fragments (IFD 2010) from plasmid pSPB130 (IFD 2010):

(1) A nopaline synthase promoter/ neomycin phosphotransferase gene/ nopaline synthase terminator (NOS/NPT II/ NOS) fragment:

- The NOS promoter is from *Agrobacterium tumefaciens* and drives production of the *npt II* gene.

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

- The *npt II* gene (from *Escherichia coli*) results in production of neomycin phosphotransferase and confers tolerance to the antibiotic kanamycin. Kanamycin is used to select for transgenic tissues under laboratory conditions.
- The NOS terminator is from *Agrobacterium tumefaciens*.

(2) A cauliflower mosaic virus 35S promoter/ flavonoid 3', 5'- hydroxylase gene/ nopaline synthase fragment (CaMV35S/ F3'5'H/ NOS) fragment:

- The enhanced 35S promoter from cauliflower mosaic virus results in production of flavonoid 3', 5'- hydroxylase.
- The flavonoid 3', 5'-hydroxylase gene from *Viola tricolor* results in production of this biochemical.
- The NOS terminator is from *Agrobacterium tumefaciens*.

(3) A cauliflower mosaic virus 35S promoter/ anthocyanin 5-acyltransferase/ nopaline synthase fragment (CaMV35S/ 5AT/ NOS) fragment:

- The enhanced 35S promoter from cauliflower mosaic virus drives production of anthocyanin 5-acyltransferase.
- The anthocyanin 5-acyltransferase gene from *Torenia hybrida* results in production of this biochemical.
- The NOS terminator is from *Agrobacterium tumefaciens*.

Production of F3'5'H and 5AT enzymes shunts production of anthocyanins in these rose lines away from the cyanidin based anthocyanins (pink) and toward production of delphinidin based anthocyanins (blue) (IFD 2010).

Southern blots were used to analyze gene insertion from plasmid pSPB130 into the rose genome and to examine the integrity and expression of the inserted DNA. APHIS evaluated Florigene's Southern blot data and came to several conclusions regarding insertion of the various genes into these 2 rose lines. Based on gel quantitation analysis of DNA, there are between 1 and 4 copies of each of the genes (*npt II*, *F3'5'H*, and *5AT*) in both IFD-524Ø1-4 and IFD-529Ø1-9. Additionally, the Southern blots show the presence of multiple DNA bands (IFD 2010) indicating that it is also likely that some gene rearrangements have occurred. The DNA banding patterns does indicate that these genes were inserted differently in each of the two rose lines.

Florigene also provided Northern analyses documenting production of RNA from the inserted genes (IFD 2010). Each gene was expressed in the transgenic plants and there was no expression noted in the control non-transformed rose line.

Further, Florigene presented data on production of delphinidin and cyanidin in the petals of these roses. Both transgenic lines showed accumulation of delphinidin (a blue anthocyanin) and much decreased production of cyanidin (a red anthocyanin) (IFD 2010). The control non-transformed rose did not produce delphinidin but did produce cyanidin.

Plant Pest Risk Assessment

APHIS has prepared a Plant Pest Risk Assessment in response to a petition (APHIS No. 08-315-01p) from International Flower Developments Pty Ltd. APHIS regulation 7 CFR 340.6(c) (4) stipulates the information needed to be considered in a petition for nonregulated status. APHIS uses information submitted by the applicant related to plant pest risk characteristics, disease and pest susceptibilities, expression of the gene product, new enzymes, or changes to plant metabolism, weediness of the regulated article, any impacts on the weediness of any other plant with which it can interbreed, and the transfer of genetic information to organisms with which it cannot interbreed for IFD-524Ø1-4 and IFD-529Ø1-9 roses. Issues related to agricultural or cultivation practices and the effects of the regulated article on non-target organisms will be considered in the Environmental Assessment for these rose lines.

Based on information on the biology of rose (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/riskassessments-1, accessed 1/26/10), data presented in the Petition and scientific data relevant to a discussion of plant pest risk, APHIS has concluded the following regarding IFD-524Ø1-4 and IFD-529Ø1-9 roses:

Potential impacts of altered disease and pest susceptibilities

USDA-APHIS assessed whether IFD-524Ø1-4 and IFD-529Ø1-9 roses are likely to have significantly altered disease and pest susceptibilities. The assessment encompasses a consideration of introduced traits and interactions with pests and diseases.

Hybrid tea roses (*Rosa* x *hybrida*) are not considered plant pests in the United States. None of the gene sequences derived from the plant pests (*Agrobacterium* and cauliflower mosaic virus) that were incorporated into these rose lines result in the production of infectious agents or disease symptoms in plants, and so they are unlikely to pose a plant pest risk. The description of the genetic modifications, including genetic elements, expression of the gene products and their functions in these roses has been summarized above.

Cultivated hybrid tea roses are susceptible to numerous insect pests and diseases. Florigene has noted some of the major ones (IFD 2010). A variety of insecticides, miticides and other pesticides are commonly used on roses (http://urbanext.illinois.edu/roses/disease.cfm, accessed 1/25/10; http://www.ipm.ucdavis.edu/PMG/PESTNOTES/pn7466.html, accessed 1/25/10).

Florigene has contracted for growing these rose lines in greenhouses in California for over 4 years. The grower (Jackson and Perkins Wholesale, Inc.) has noted that these rose lines grow normally in all respects (IFD 2010). They further make note of the major pests of roses in California: two spotted spider mites, powdery mildew and downy mildew. They also indicate that all of these pests are controlled using common treatments and conventional protocols (IFD 2010). These rose lines have also been grown extensively in

Columbia under typical rose cultivation conditions there. The grower there indicates that both the transgenic and non-transgenic parental lines are susceptible to mildew but that there was no difference between the transgenic and non-transgenic plants (IFD 2010).

Finally, this information submitted by Florigene indicates that there are no significant differences in terms of pest or disease susceptibilities between these rose lines and their non-transgenic counterparts.

Potential impacts from new gene products, changes to plant metabolism or composition

As a point of reference, Figure 4 in the Petition (p. 22) presents the basic biochemical pathways for production of anthocyanins in plants.

New proteins produced in both IFD-524Ø1-4 and IFD-529Ø1-9 include the following:

1. Neomycin phosphotransferase (NPT II)
2. Flavonoid 3', 5'-hydroxylase (F3'5'H)
3. Anthocyanin 5- acyltransferase (5AT)

NPT II is a common protein found in a number of genetically engineered plants that have been widely planted across the U.S. and in other parts of the world. In every case, no issues related to health or environmental safety have been noted (APHIS petitions 04-317-01p, 04-264-01p, 01-137-01p, 01-206-02p, 01-206-01p, 95-352-01p, 96-051-01p, 95-045-01p, 94-308-01p) (USDA-APHIS 2011). NPT II confers tolerance to the antibiotic kanamycin and is used in a laboratory setting to select tissues transformed with the genes of interest.

Flavonoid 3', 5'-hydroxylase (F3'5'H) is an enzyme that is widely found in nature in plants producing anthocyanins, most often blue colors. This enzyme can be found in grapes (Bogs 2006), petunia (Toguri 1993), eggplant (*Solanum melongena*) (Chapple 1998), gentian, torenia, campanula and many other plants (Tanaka 2006). F3'5'H is in the cytochrome P450 family of enzymes (designated in the CYP75A subfamily) (Nelson 2009, http://drnelson.utmem.edu/CytochromeP450.html, accessed 1/26/10). Plant species lacking flavonoid 3'-5'-hydroxylase, such as non-engineered roses, do not make the blue delphinidin-based anthocyanins (Deng 2001).

Anthocyanin 5-acyltransferase (5AT) is also an enzyme that is widely found in nature in plants producing anthocyanins. In the anthocyanin biosynthesis pathways, this enzyme, as well as related anthocyanin acyltransferases, act to alter the biochemical structure of anthocyanin glucosides (AGS) (such as pelargonidin GS, cyanidin GS, and delphinidin GS) and make the resulting pigment (anthocyanin) more chemically stable in the plant cell (Nakayama et al 2003).

As described above, compositional assessment data supplied in the Petition regarding production of DNA, RNA and delphinidin supports the conclusion that IFD-524Ø1-4 and

IFD-529Ø1-9 roses contain introduced *f3'5'h* and *5at* genes from Florigene plasmid pSPB130 (IFD 2010).

Florigene also provided an assessment of delphinidins found in common foods and common flowers (IFD 2010). As noted in the tables, many foods, which are consumed widely, and ornamental flowers, which are grown widely, contain measurable quantities of delphinidin. None of the foods or ornamental plants noted are known to pose unique environmental risks because of the presence of delphinidin, its precursor biochemicals or catalytic enzymes (i.e., F3'5'H or 5AT) in the anthocyanin pathways. Specific data on toxicity and potential environmental effects of F3'5'H and 5AT is sparse but information on the chemistry of delphinidins and other anthocyanins is noted (Beheshti 2008; Vilanova 2009; Yu 2006). None of the documents identified or noted raise environmental concerns related to new gene products, changes in plant metabolism or plant composition.

Based on all the noted considerations, APHIS concludes that IFD-524Ø1-4 and IFD-529Ø1-9 roses pose no more of a plant pest risk from new gene products, changes to plant metabolism or composition than conventional hybrid tea roses.

Potential impacts from outcrossing of IFD-524Ø1-4 and IFD-529Ø1-9 roses to wild relatives

Florigene has described the biology of roses, including hybrid tea roses, in its Petition (IFD 2010). They note that the first hybrid tea roses were produced in France in 1867 and that since then, hundreds of new cultivars of hybrid tea roses have been introduced. They also note that cultivated rose is the most widely produced cut flower crop in the world and that over 60 million rose plants are planted each year just to meet demand for cut flower production. The Australian Office of Gene Technology Regulator (OGTR) has also produced an extensive document on the biology of *Rosa* x *hybrida* (http://www.health.gov.au/internet/ogtr/publishing.nsf/Content/rose-3/$FILE/biologyrose09.pdf, accessed 1/25/10) and completed a risk assessment of one of these rose lines (IFD-524Ø1-4) in 2009 (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dir090-4/$FILE/dir090rarmp.doc, accessed 1/25/10). They describe the taxonomy of roses, rose species, centers of diversity and domestication, development and commercialization of modern rose cultivars, cultivation practices, breeding work (including use of "sports" as well as irradiation induced mutations), plant morphology, plant development and reproduction, biochemistry, biotic and abiotic interactions, disease and insect pests and weediness. Most of the information in those documents is directly relevant to this application and plant pest risk assessment and is incorporated here by reference.

Florigene's description and analysis of IFD-524Ø1-4 and IFD-529Ø1-9 roses indicates that they are both L1 periclinal chimeras[2]. The significance of this is that the *f3'5'h* and *5at* genes introduced are only found and expressed in the epidermal tissues and cannot be

[2] An L-I periclinal chimera is a type of plant chimera in which the L-I cell layer has a different genetic make-up than the L-II and L-III cell layers. The L-I cell layer gives rise to epidermal tissues of a plant while the L-II and L-III layers give rise to reproductive tissues, vascular tissues and other internal plant tissues.

passed on to progeny through cross pollination (i.e., outcrossing). Florigene demonstrated this in the various pollination and *in situ* hybridization experiments they conducted during development of these lines (IFD 2010). Florigene analyzed over 100 seeds of hybrid pollinations with grandiflora and floribunda roses and found none that contained the IFD-524Ø1-4 and IFD-529Ø1-9 transgenes. They also analyzed 19 seeds from pollinations with wild rose (*Rosa multiflora*) and found none that contained the introduced genes. They also conducted specific RNA hybridization experiments using petal tissues of control and transgenic lines and identified stained cells only in the epidermis of the transgenic rose lines (IFD 2010).

In assessing the risk of gene introgression from IFD-524Ø1-4 and IFD-529Ø1-9 roses into its sexually compatible relatives, APHIS considers two primary issues: 1) the potential for gene flow and introgression and, 2) the potential impact of introgression.

Given the demonstrated L-I chimeric nature of these rose lines, the potential for gene flow and introgression into sexually compatible relatives of rose is essentially zero. Since gene flow cannot occur, any potential impact of introgression, therefore, is also zero.

Potential impacts based on the relative weediness of IFD-524Ø1-4 and IFD-529Ø1-9 rose.

APHIS assessed whether Florigene IFD-524Ø1-4 and IFD-529Ø1-9 roses are any more likely to become weeds than the non-transgenic recipient rose line, or other rose currently cultivated. The assessment encompasses a thorough consideration of the basic biology of rose and an evaluation of unique characteristics of these rose lines.

The biology and cultivation practices of hybrid tea rose have been well described (http://www.health.gov.au/internet/ogtr/publishing.nsf/Content/rose-3/$FILE/biologyrose09.pdf) and Florigene has also provided information on *Rosa x hybrida* cultivation, taxonomy, pollination, weediness and potential modes of gene flow (IFD 2010). Cultivated hybrid tea roses are all complex hybrids (IFD 2010) derived from breeding work done over centuries from more than 5 different rose species and numerous rounds of selection and cross hybridization at each step. Cut flower rose varieties are the most widely cultivated cut flower crop worldwide with sales of over 6 billion stems per year (IFD 2010). Florigene has noted that USDA lists over 100 species of *Rosa* in its Plants database (http://plants.usda.gov/). Only 3 of these *Rosa* species are common weeds in the U.S. (*Rosa arkansana, Rosa multiflora, and Rosa rugosa*) (http://plants.usda.gov/java/noxiousDriver#state) and *Rosa arkansana* is native to the U.S. None of the *Rosa* species are listed as Federal noxious weeds.

In the U.S., *Rosa* x *hybrida* is not listed as a weed in several major weed references (Crockett 1977; Holm et al. 1979; Muenscher 1980) nor is it listed as a noxious weed species by the U.S. Federal Government (http://www.aphis.usda.gov/plant_health/permits/organism/federal_noxious_weeds.shtml, accessed 12/22/09). As noted by Florigene, these roses also do not readily propagate by vegetative means (IFD 2010). Because of the L-I chimeric nature of these rose lines, they

also will not disperse the genes for *f3'5'h* and *5at* by pollen. As is typical of hybrid tea roses grown for cut flowers, the likelihood of moving genes around by seed is very small as these roses are cut and harvested long before hips or seeds would mature. Compared to the non-transgenic progenitor roses, these attributes are no different.

Weediness for the purposes of this part of the plant pest risk assessment is an attribute, which causes a crop to act as a weed due to the addition of genes, in comparison to the non-transgenic comparator. If the fitness of these rose lines improves in natural or agricultural ecosystems due to the inserted DNA, the potential for weediness could increase. The following analysis of the inserted DNA is intended to document that IFD-524Ø1-4 and IFD-529Ø1-9 roses have a negligible likelihood of increased weediness. As described previously, these rose lines differ only in the expression of the F3'5'H and 5AT enzymes which ultimately result in production of blue delphinidin-based anthocyanins.

Florigene collected data on numerous phenotypical features of these rose lines. Data collected related to plant height, flower stem length, flower height, flower diameter, petal length and width, number of pistils, number of stamens, and others (IFD 2010). While some of these characteristics showed statistical differences between the non-transgenic and transgenic lines, none of these characteristics stand out as ones that would provide a fitness advantage to these lines in an unmanaged situation. Florigene also collected data on pollen viability, pollen grain germination, and pollen diameter (IFD 2010). None of the data on pollen characteristics showed any statistical significance between the non-transgenic and transgenic lines.

None of the characteristics that APHIS considers in its assessment of weediness point to the potential for these rose lines to become more weedy or invasive than the non-transgenic comparator lines. APHIS concludes that introduction of these rose lines do no result in increased plant pest risk related to weediness.

Potential Impacts on Target and Non-target Organisms, Including Beneficial Organisms

Based on the data provided by the applicant and existing literature, APHIS evaluated the potential for deleterious effects or significant impacts of these rose lines on non-target or beneficial organisms.

The genes introduced into these rose lines result directly in production of the F3'5'H and 5AT proteins and indirectly in production of delphinidin, a blue pigment. Florigene notes that delphinidin and delphinidin derivatives are contained in many common foods in relatively large amounts (IFD 2010). Anyone or anything consuming these foods, therefore, consumes delphinidin as well as the F3'5'H and 5AT proteins required for its production. The 5AT protein is also found in foods containing other related anthocyanin pigments (IFD 2010). As noted by Florigene, the amount of delphinidin found in these rose lines is approximately 100 times less than that found in fresh blueberries (IFD 2010). Florigene also notes that, as a group, anthocyanins have a very low toxicity (IFD 2010; http://www.inchem.org/, accessed 1/25/10).

In addition to this lack of toxicity associated with these rose lines, Florigene notes that these roses will be grown in a limited number of locations under highly controlled conditions by experienced rose growers (IFD 2010). As such, exposure to organisms outside these conditions will be extremely limited.

This data submitted by the applicant indicates that the interactions between these rose lines and other roses, including the control lines, are similar. Considering all this, APHIS concludes that Florigene rose lines IFD-524Ø1-4 and IFD-529Ø1-9 are unlikely to pose safety risks to non-target or beneficial organisms.

Potential impacts from transferring genetic information from IFD-524Ø1-4 and IFD-529Ø1-9 rose to organisms with which it cannot interbreed.

APHIS examined the potential for the new genetic material inserted into these rose lines to be horizontally transferred to other organisms without sexual reproduction and whether such an event could lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of more virulent pathogens. First, many genomes (or parts thereof) have been sequenced from bacteria that are closely associated with plants including *Agrobacterium* and *Rhizobium* (Kaneko et al. 2000; Wood et al. 2001; Kaneko et al. 2002). There is no evidence that these organisms contain genes derived from plants. Second, in cases where review of sequence data implied that horizontal gene transfer occurred, these events are believed to occur on an evolutionary time scale on the order of millions of years (Koonin et al. 2001; Brown 2003). Third, transgene DNA promoters and coding sequences are optimized for plant expression, not prokaryotic bacterial expression. Thus even if horizontal gene transfer occurred, proteins corresponding to the transgenes are not likely to be produced. Fourth, the FDA has evaluated horizontal gene transfer from the use of antibiotic resistance marker genes, and concluded that the likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of humans or animals, or in the environment, is remote (Council for Biotechnology Information, 2001; http://vm.cfsan.fda.gov/~dms/opa-armg.html, accessed 1/26/10). Finally, a recent review of issues related to horizontal gene transfer concluded that this type of gene transfer is unlikely to occur and poses negligible risks to human health or the environment (Keese 2008). Therefore APHIS concludes that horizontal gene transfer is unlikely to occur and thus poses no significant environmental or plant pest risk.

Conclusion

APHIS has reviewed and conducted a plant pest risk assessment on Florigene's IFD-524Ø1-4 and IFD-529Ø1-9 roses. Due to the lack of plant pest risk from the inserted genetic material, the lack of weediness characteristics of these rose lines, the lack of atypical responses to disease or plant pests, the lack of deleterious effects on non-targets or beneficial organisms in the agro-ecosystem, and the lack of horizontal gene transfer, APHIS concludes that IFD-524Ø1-4 and IFD-529Ø1-9 roses are unlikely to pose a plant pest risk.

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