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Review

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Expression of Flavonoid 3′,5′-Hydroxylase and Acetolactate Synthase Genes in Transgenic Carnation: Assessing the Safety of a Nonfood Plant

Stephen F. Chandler,[†] Michael Senior,[†] Noriko Nakamura,[§] Shinzo Tsuda,[§] and Yoshikazu Tanaka*[,§]

[†]School of Applied Sciences, RMIT University, P.O. Box 71, Bundoora, VIC 3083, Australia

[§]Institute for Plant Science, Suntory Business Expert Limited, Mishima, Osaka 618-8503, Japan

ABSTRACT: For 16 years, genetically modified flowers of carnation (*Dianthus caryophyllus*) have been sold to the floristry industry. The transgenic carnation carries a herbicide tolerance gene (a mutant gene encoding acetolactate synthase (ALS)) and has been modified to produce delphinidin-based anthocyanins in flowers, which conventionally bred carnation cannot produce. The modified flower color has been achieved by introduction of a gene encoding flavonoid 3′,5′-hydroxylase (F3′5′H). Transgenic carnation flowers are produced in South America and are primarily distributed to North America, Europe, and Japan. Although a nonfood crop, the release of the genetically modified carnation varieties required an environmental risk impact assessment and an assessment of the potential for any increased risk of harm to human or animal health compared to conventionally bred carnation. The results of the health safety assessment and the experimental studies that accompanied them are described in this review. The conclusion from the assessments has been that the release of genetically modified carnation varieties which express *F3′5′H* and *ALS* genes and which accumulate delphinidin-based anthocyanins do not pose an increased risk of harm to human or animal health.

KEYWORDS: *carnation, delphinidin, flavonoid 3,′5′-hydroxylase, acetolactate synthase, risk assessment*

INTRODUCTION

Genetically modified (GM) varieties of soybean, canola, and corn now dominant production of these crops in North America and represent a significant part of the market in dozens of other countries. In parallel with these developments in agriculture, research in ornamentals also occurred, and in 1996 the first genetically modified ornamental, a cut flower variety of carnation (*Dianthus caryophyllus*), was released in Australia. Since then, other varieties of transgenic carnation and a variety of rose have also been released.[1,2] The commercial release of GM plant varieties was only possible after regulatory approval was obtained, which was in turn dependent on an assessment of the potential for increased risk to the environment and human and animal health.

Legislation and policy governing the commercialization of transgenic crops vary by country, and different countries require different amounts of information, but all have in common the need for these risk assessments. Many detailed studies relating to such risk assessment have been published for the food crops, but to date the ornamentals have received less attention. In the European Union (EU), the import and sale of cut flowers of transgenic carnation are assessed under directive 2001/18/EC. In addition to extensive literature studies, comprehensive molecular analysis is required, including determination of copy numbers, integration patterns, flanking sequences around integration sites, and establishment of line-specific detection protocols. Similar sets of analysis as well as field trial data are required in Japan. In the United States and Canada, imported cut flowers of color-modified transgenic carnation varieties have been determined to be nonregulated, or exempt, products. Although research on the environmental risk associated with the release of transgenic carnation has been published elsewhere,[1,3−5] the results of the risk assessment applied to the potential impact of the inserted genes and to the delphinidin-based anthocyanins accumulation resulting from the genetic modification have not been published. Although carnations are not food, it is possible petals could sometimes be utilized as edible decoration or garnish and so be consumed. An assessment of the potential health risks associated with such occasional consumption is therefore encouraged by regulators and is also important from the viewpoint of corporate social responsibility. The safety assessments that were carried out on transgenic carnation are summarized here with the expectation that the information may be useful to others contemplating the commercial release of nonfood plants.

DESCRIPTION OF GENETIC MODIFICATION IN CARNATION

Anthocyanins and Flower Color. Yellow and orange flowers normally contain carotenoids and sometimes betalains. Pelargonidin-, cyanidin-, and delphinidin-based anthocyanins tend to confer, respectively, brick-red, red/magenta, and violet/blue shades of color when they accumulate in flowers. The difference in chemical structure between the three anthocyanidins pelargonidin, cyanidin, and delphinidin lies in the number of hydroxyl groups on the B-ring. Pelargonidin has a

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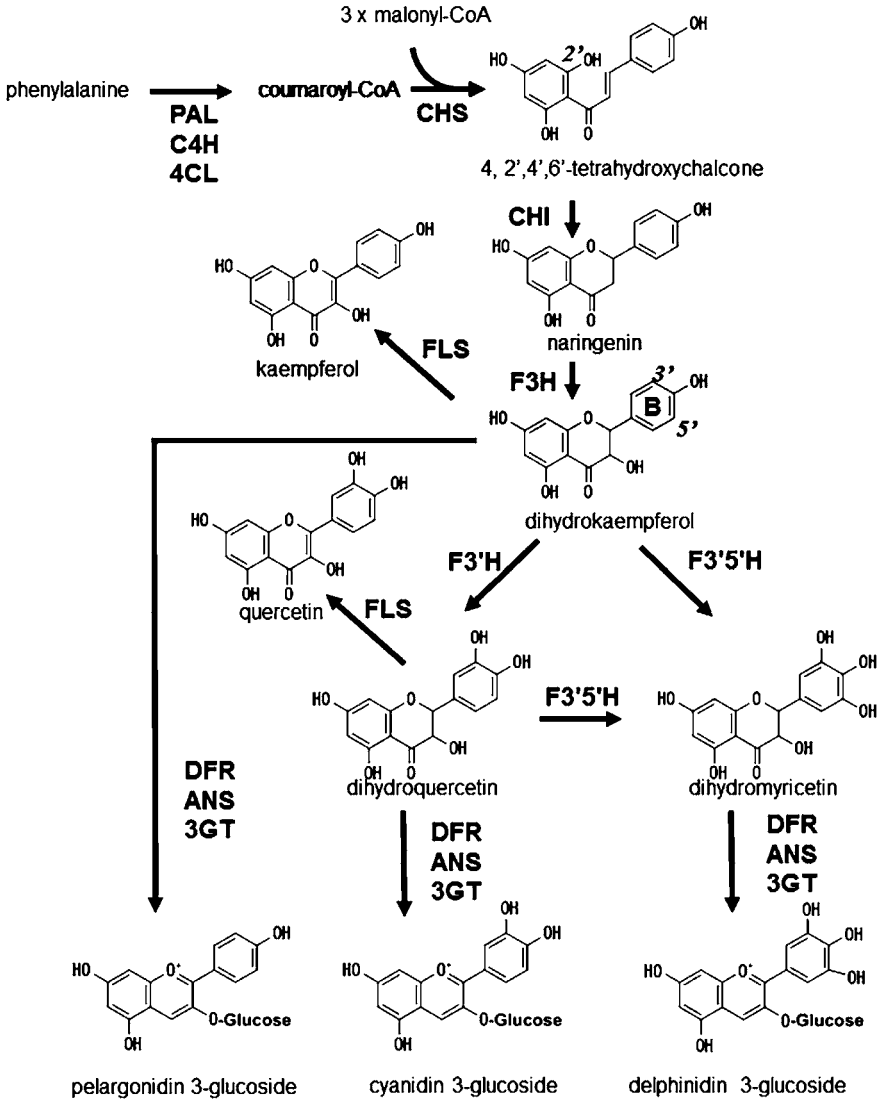

Figure 1. Anthocyanin biosynthesis pathway. Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; FLS, flavonol synthase; 3GT, UDP-glucose:anthocyanidin 3-*O*-glucosyltransferase.

single hydroxyl side group, cyanidin two, and delphinidin three. This relatively small difference confers the different colors to the anthocyanins[2] and so different colors to flowers. Nontransgenic carnation comes in a wide range of colors, due to the relative amount and concentration of the cyanidin- or pelargonidin-based anthocyanins, yellow chalcone glucoside, and colorless flavonols. Nontransgenic carnation does not contain the anthocyanidin delphinidin.

Anthocyanin Biosynthesis Pathway. The anthocyanin biosynthesis pathway is a branch of the phenylpropanoid pathway. The anthocyanin biosynthesis pathway is shown in Figure 1. An early critical enzyme is chalcone synthase, which catalyzes the biosynthesis of 4,2′,4′,6′-tetrahydroxychalcone (Figure 1). This compound is converted to the dihydroflavonol dihydrokaempferol (DHK) by chalcone isomerase and flavanone 3-hydroxylase or to yellow tetrahydroxychalcone 2′-glucoside by glucosyltransferases. DHK can then be hydroxylated at the 3′-position by the enzyme flavonoid 3′-hydroxylase (F3′H) to produce dihydroquercetin (DHQ), or at both the 3′- and 5′-positions by the enzyme flavonoid 3′,5′-hydroxylase (F3′5′H) to produce dihydromyricetin (DHM) (Figure 1). The colorless dihydroflavonols (DHK, DHM, or DHQ) are then subsequently converted to the colored and first anthocyanins, anthocyanidin 3-glucosides, by the enzymes dihydroflavonol 4-reductase, anthocyanidin synthase, and anthocyanidin 3-glucosyltransferase (Figure 1), with DHK being converted to the brick-red pelargonidin-based anthocyanins, DHQ being converted to the red cyanidin-based anthocyanins, and DHM being converted to the purple-violet-blue delphinidin-based anthocyanins (Figure 1). The activity of F3′5′H is therefore necessary for biosynthesis of the delphinidin-based anthocyanins responsible for mauve, violet, or blue flowers. F3′5′H does not occur in carnation, as the gene encoding the F3′5′H enzyme is not present in this species. The gene is also absent in other very important cut flowers, such as rose, lily, and chrysanthemum.

Delphinidin-Based Anthocyanins in Transgenic Carnation. To date, 10 transgenic carnation varieties have been released commercially and more are in development. Accumulation of delphinidin-based anthocyanins occurs in

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Table 1. Concentrations of Anthocyanidins Measured in Flowers of 10 Varieties of Transgenic Carnation[a]

commercial name (variety)	unique identifier	anthocyanidin (mg/g fresh weight petal)	delphinidin (%)
FLORIGENE Moonique	IFD-19907-9	3.40	90
FLORIGENE Moonvelvet	IFD-26407-2	2.87	88
FLORIGENE Moonvista	FLO-40685-2	1.79	99
FLORIGENE Moonshade	FLO-40619-8	0.60	94
FLORIGENE Moonberry	IFD-25958-3	0.54	81
FLORIGENE Moonshadow	FLO-11363-1	0.34	94
FLORIGENE Moonpearl	IFD-25947-1	0.17	61
FLORIGENE Moonlite	FLO-40644-6	0.10	75
FLORIGENE Moonaqua	FLO-40689-6	0.07	74
FLORIGENE Moondust	FLO-07442-4	0.04	100

[a]Anthocyanidin concentration is in hydrolyzed petal extract and is expressed in mg/g fresh weight petal tissue. The percentage of delphinidin is the amount of delphinidin as a percentage of all anthocyanidins. The unique identifiers allocated to each variety are for the purpose of registration on the Biosafety Clearing House (http://bch.cbd.int/).

the flowers of all these lines due to the expression of the *F3′5′H* gene, which is present in all of the transformation vectors used to generate these lines.[2] Table 1 provides information on the level of delphinidin found in each of the varieties that had been commercialized by the end of 2012. The color of each variety is phenotypically stable, a prerequisite for marketing of the range of colors available in the pool of transgenic varieties. Although there is a nearly 100-fold range in concentration in delphinidin, in each case it is the dominant anthocyanidin present (Table 1). Delphinidin-based pigments accumulate only in the petals of the flowers and have not been observed in other tissues of the transgenic flowers and plants, such as stems, nodes, leaves, and roots. The primary forms of the delphinidin-based anthocyanin in transgenic carnation are delphinidin 3-(6″-*O*-4-malyl-glucosyl)-5-glucoside, delphinidin 3-(6″-*O*-4-malyl-glucosyl)-5-(6″-*O*-1-malyl-glucoside), and delphinidin 3,5-diglucoside-6″-*O*-4, 6″-*O*-1-cyclic-malyl diester.[6]

Flavonoid 3′,5′-Hydroxylase. F3′5′H is a cytochrome P450 type monooxygenase, and F3′5′H and F3′H belong to the same family of the cytochrome P450 superfamily.[2] The gene encoding the protein was first isolated from petunia[7] and has since been isolated from several other plant species.[2] In the transgenic carnation varieties, the species of origin have primarily been petunia and *Viola* (pansy) species.[2] The F3′5′H protein is located in the microsomal fraction.[8] The protein has not been purified.

Acetolactate Synthase (Acetohydroxyacid Synthase) Selectable Marker Gene. An inserted *Su*RB genetic element encoding a mutated acetolactate synthase (*ALS*) gene (*Su*RB) is also present in all transformation vectors used to generate the transgenic carnation varieties. This gene confers resistance to the sulfonylurea type herbicide chlorsulfuron, which is used as a selection agent during the in vitro isolation of the transgenic carnation. The sulfonylureas are one class of ALS-inhibiting herbicides.[9,10] The function of *Su*RB is only to enable selection in vitro, and the herbicide tolerance trait conferred on the transgenic carnation is not exploited during cultivation. In tobacco there are two unlinked *ALS* loci, *Su*RA and *Su*RB.[11,12] The mutation of the *Su*RB gene that has been used as the selectable marker is the gene *S4-Hra*. This mutation was derived from a line (S4) isolated from a mutagenized haploid cell culture[9,13] selected on the sulfonylurea herbicide chlorsulfuron. A diploid herbicide-resistant plant homozygous for a single dominant gene was regenerated, which was then subjected to further selection leading to the double mutant line S4-Hra.[13] Lee et al.[14] characterized and cloned the *S4-Hra* mutant of the *Su*RB gene, showing there were three nucleotide substitutions in the S4-Hra line when compared to the wild type.

COMMON USE OF THE PARENTAL ORGANISM, CARNATION

Knowledge of the use of the parental organism by humans is essential to establish a benchmark for what is considered normal safe use, from a health and environmental risk assessment perspective. Aside from the primary use of the plant and flowers for decorative purposes, there are occasional reports of the use of carnation and other *Dianthus* species[15,16] in traditional cosmetics[17] and in medicine.[18,19] Carnation is not a basic foodstuff. Bussman et al.[19] assessed a range of medicinal medicines from Peru for toxicity, including *Dianthus caryophyllus*. Aqueous and ethanol extracts were shown to be nontoxic in a brine shrimp lethality assay. Allergic reactions to carnation are very rare, given billions of carnation flowers are produced globally per annum. There are a couple of reports of mild contact dermatitis[20,21] and two reports in the literature of allergenicity in Spanish flower farm workers exposed to carnation.[22,23] In one of these studies an allergic response was obtained in a susceptible worker using carnation extract,[23] but other studies indicated that spider mites associated with the carnation were the cause of the response.[24,25] Vidal and Polo[26] carried out control experiments to eliminate the possibility that the allergy was caused by *Tetranychus urticae* (two-spotted spider mite), demonstrating in some individuals that prolonged handling of fresh flowers may cause a mild allergic reaction. Baur[27] has included carnation and its associated mites in a comprehensive list of potential allergens and irritants in the workplace. Eugenol, which is a constituent of the carnation oil used by some dentists, has also been reported to cause contact dermatitis.[28]

RISK ASSESSMENT PROCESS

The key principles we have used in assessing whether any potential health risks could be associated with the release of transgenic carnation have been comparison to nontransgenic isogenic varieties and establishment of a baseline of knowledge of both the conventional, nontransgenic organism and the genes tht have been used for transformation. The results of these studies are required for regulators to be able to identify possible risks, assess their probability of occurring, and assess their potential impact.

RISK ASSESSMENT; GENE PRODUCTS

An assessment of any potential risk associated with the proteins derived from the introduced genes was primarily made by bioinformatic analysis and an analysis of the scientific literature.

Table 2. Animal Feeding Studies in Which Seed and Extracts from Transgenic Plants Containing Modified *ALS* Genes Have Been Compared to Appropriate Nontransgenic Control Lines

ref	test animal	source	characterization	conclusion	*ALS* gene or protein
61	rat	soybean	body weight, clinical signs, mortality, sensory response, clinical pathology, organ weights, gross pathology, microscopic pathology	no biologically relevant adverse effects	*gm-hra*
62	rat	maize	body weight, ophthalmology, neurobehavioral evaluation, clinical pathology, gross and anatomical pathology	no biologically relevant adverse effects	*zm-hra*
63	rat	soybean	body weight, organ weights, clinical pathology, gross necropsy and histopathology	no substantial alteration to composition of soybeans. no adverse effects on nutritional or safety status	*csr 1-2*
64	rat	soybean	body weight, clinical signs, mortality, toxicity, sensory response, clinical pathology, organ weights, gross pathology, clinical pathology	no biologically relevant adverse effects	*gm-hra*
34	mice	acute toxicity test	body weight and lesions at necropsy	no significant differences	purified protein encoded by *gm-hra* gene administered at 463 mg/kg body weight
34	mice	repeated dose toxicity assay	body weight, clinical and ophthalmological observations, motor activity, clinical chemistry response, histopathological observations	no biologically relevant adverse effects	purified protein encoded by *gm-hra* gene administered in food at 1000 mg/kg body weight
65	chicken	maize and soybean	weight and egg production, cracking	no significant differences	*zm-hra, gm-hra*
66	chicken	maize and soybean	weight gain, feed intake, carcass yield	no significant differences	*zm-hra, gm-hra*
67	chicken	soybean	weight and egg production and quality, cracking	no significant differences	*gm-hra*

Flavonoid 3′,5′-Hydroxylase Bioinformatic Analysis. The F3′5′H protein is found in all plants producing delphinidin. This includes several raw foods containing high levels of delphinidin, such as blueberries and cranberries. Bioinformatic analysis showed that for the F3′5′H protein (derived from viola or petunia) in the transgenic carnation, highest homology was observed with F3′5′H's from other plant species. Lower level identities were observed with various F3′H's (Figure 1) from plants. There was no homology to known toxic proteins or allergens. A search of the Faarp protein database of known allergens (http://www.allergenonline.org) with the protein sequence of the F3′5′H's used in the transgenic carnation showed no significant full-length alignments (maximum similarity = 30%), no 35% or greater similarities in 80-mer sliding window analysis, and no 8 amino acid sequence matches. These data suggest the F3′5′H proteins are not allergens.

Acetolactate Synthase Bioinformatic Analysis. In plants, ALS catalyzes the biosynthesis of the branched chain amino acids leucine, valine, and isoleucine.[29,30] The enzyme is therefore present in all plant species. ALS is also found in bacteria, and although not present in mammals, a homologue of bacterial ALS has been identified in humans.[31] The reviews by Duggleby and Pang[32] and Duggleby et al.[33] provide an overview of ALS function in both micro-organisms and plants. Bioinformatic analysis was undertaken to provide up-to-date searches of the SwissProt database using the translated sequences of the mutant *Sur*B coding regions contained in the T-DNA of the transformation vectors used to generate the transgenic carnation. The translated amino acid sequence (the longest open reading frame with an AUG start codon and a stop codon) derived from the nucleic acid sequence was used together with the search program BLAST (Basic Local Alignment Search Tool) 2.2.27 located within the NCBI (The National Centre for Biotechnology Information) Web site (http://www.ncbi.nlm.nih.gov). Highest identities were observed with ALS proteins from various plant species. No homology to known toxic proteins or allergens was found, using the same databases used for the F3′5′H analysis. Mathesius et al.[34] showed in a BLASTP search that the protein encoded by the *gm-hra* gene (a modified soybean *ALS* gene with mutations analogous to those of *Su*RB) did not have sequence similarity to known toxic proteins. They also isolated and purified the gm-hra protein and measured the toxicity of the protein in an acute toxicity assay in mice. The study showed the gm-hra protein was not toxic.[34] Single amino acid substitution in ALS conferring herbicide resistance is found in several food crops and weed species and occurs naturally in bacteria. Humans eating plant food would have been exposed to a wide variety of enzymes very similar (including the same amino acid change) to the protein encoded by *S4-Hra*.

Potential for Allergenicity. The ALS and F3′5′H proteins have not been identified as allergens despite the presence of the ALS protein in all plants. Mathesius et al.[34] showed that the purified gm-hra protein was quickly degraded by the digestive enzymes pepsin and pancreatin. The same authors demonstrated that enzymatic activity was lost at temperatures above 50 °C. These physical characteristics are not usually associated with a protein food allergen, which would normally be resistant to degradation.[35] ALS mutations similar to *S4-Hra* are now present in many food crops[36] and in the pollen of weed species. Whereas there is no literature we are aware of reporting studies of the allergenicity of delphinidin, reactions to anthocyanin

Table 3. Evidence of Anticancer Effects of Delphinidin and Delphinidin Derivatives

ref	chemical tested	observed effect	in vitro system
68	delphinidin as pure anthocyanidin (up to 240 μM)	suppression of NF-κB pathway, arresting cell cycle	cultured human colon cancer cells
69	delphinidin as pure anthocyanidin (up to 100 μM)	inhibition of glyoxalase I; maximum inhibition at 10 μM	in vitro enzymatic assay
70	delphinidin as pure anthocyanidin (up to 100 μmol/L)	inhibition of cell growth	human prostate cancer PC3 cells
71	delphinidin as pure anthocyanidin (40 μM)	inhibition of epidermal growth factor receptor in cancer cells	human breast cancer cells
72	delphinidin as anthocyanidin (100 μM)	cytotoxicity	cultured human colorectal carcinoma cells
57	delphinidin as anthocyanidin (50 μM) and delphinidin 3-glucoside	suppression of plasminogen activation, through inhibition of expression of both urokinase-type plasminogen activator receptor (uPAR) and the low-density lipoprotein receptor-based protein (LRP) and strong inhibition of cell migration	cultured human glioblastoma cell line U-87
73	delphinidin, at a concentration of 0.01 g/L	inhibited vascular endothelial growth factor-induced proliferation by blocking cell cycle in G0/G1 phase	cultured bovine aortic endothelial cells
74	delphinidin as anthocyanidin (10 and 20 mg/kg bw)	micronuclei in polychromatic erythrocytes induced by cyclophosphamid	mice feeding study

containing foods have been studied because most people are exposed to at least some anthocyanin in the course of their regular diet. A recent review states that there are no reports of allergic reaction to either grape skin extract or grape color extract, both of which are widely used food colorants[37] and contain delphinidin.

Animal Feeding Studies. The *gm-hra* gene, a modified soybean *ALS* gene, has mutations analogous to those of *SuR*B, and transgenic plants containing *gm-hra* have been studied in several animal feeding studies (Table 2), along with similar genes. The results of these studies, summarized in Table 2, suggest the protein derived from *gm-hra* poses no harm to animals.[34]

RISK ASSESSMENT; DELPHINIDIN

Delphinidin-related anthocyanins are widely distributed in nature, and there is extensive scientific literature in which these compounds have been studied.

Delphinidin in Other Ornamental Plants. Aside from transgenic carnation and rose, delphinidin is found in most plant species with violet, mauve, or and blue flowers. Examples of widely grown ornamental plants that contain delphinidin-based anthocyanins include *Agapanthus*, cyclamen, *Hydrangea*, verbena, *Petunia*, *Delphinium*, *Lobelia*, freesia, pansy, and *Hyacinth*.[38] The concentration of delphinidin in these species encompasses the range seen in transgenic carnation (Table 1).

Delphinidin in Foods. Delphinidin is found in many unprocessed and processed foods. Rich sources of delphinidin-based anthocyanins include red grapes, black currants, egg plant (aubergine), blueberry, elderberry, and bilberry.

The maximum concentration of delphinidin we have measured in transgenic carnation (Table 1) is approximately 3.5 mg delphinidin/g fresh weight (FW) petal. This is approximately the concentration of delphinidin in blueberry, for example, which may have up to 5 mg anthocyanin/g FW and 40−60% of the anthocyanidin present as delphinidin.[39] The majority of transgenic carnation varieties that have been commercialized have much lower concentrations of delphinidin (Table 1). A dietary survey by Wu et al.[40] estimated that the daily intake of anthocyanins in the U.S. population was 12.5 mg/day/person, with cyanidin-based anthocyanins, delphinidin-based anthocyanins, and malvidin (3′,5′-dimethyldelphinidin)-based anthocyanins contributing approximately 45, 21, and 15%, respectively, of total anthocyanin intake. A review of the dietary intake of flavonoids, including anthocyanidins, in other countries suggests the U.S. intake is similar to other countries of the world but may be lower than those where berry consumption is high. For example, Heinonen[41] has estimated Finns eat approximately 80 mg anthocyanin/day. Anthocyanins are likely to be present in large amounts in some individual diets because of the high concentration of anthocyanins in some food sources. For example, 100 g of berries may have approximately 500 mg of anthocyanins, translating to 100−300 mg anthocyanin/serving.[42] The average daily estimated consumption of delphinidin as the anthocyanidin, from commonly consumed fruits and vegetables in the American diet, has been estimated at 2.5 mg/day[40] and in France at 1.6 mg/day.[43] A recent culinary trend is the use of edible flowers, typically to garnish meals.[44] It is not possible therefore to rule out that petals from the transgenic carnation may be used by some individuals for this purpose and that they may be deliberately consumed as part of this meal. However, it is reasonable to expect just a few petals would be used, which would be comparable to a small portion of a fruit containing delphinidin.

Delphinidin and Health. Major health benefits attributed to anthocyanin consumption include improved cardiovascular health and improved treatment of infection. Other properties of anthocyanins, including antiviral and anticancer roles, are further described by Lila.[45] Several reviews summarize the scientific rationale for the positive effects of polyphenols and flavonoids, including anthocyanins, on health.[41,42,46−49] Tomato genetically modified for enhanced anthocyanin accumulation was found to enhance the life span of cancer-susceptible mice.[50,51] Several studies have compared the relative health-related efficacy of the major anthocyanins and have shown that delphinidin or delphinidin-based anthocyanins have greater activity than other anthocyanins.[52−57] Table 3 summarizes observations made in studies that have attributed an anticancer role to delphinidin, and Table 4 summarizes studies in which delphinidin has been associated with aspects of improved cardiovascular health. Delphinidin-based anthocyanins are absorbed relatively quickly, and although they are primarily not excreted,[40] they disappear from circulation within 6−8 h.[47]

ALLELOPATHY STUDIES

To complement the literature review and bioinformatic studies, any potential increase in allelopathic properties associated with the genetic modification in the transgenic carnation was assessed. In these studies lettuce seed germination rate and seedling growth were used as the biological indicator, and comparison was made between a number of transgenic lines

Table 4. Evidence of Positive Effects of Delphinidin and Delphinidin Derivatives on Cardiovascular Health

ref	chemical tested	observed effect	in vitro system	health effect
75	delphinidin 3-glucoside (up to 150 μM)	neutralize oxidative stress induced by oxidized low-density lipoprotein	cultured porcine aorticendothelial cells	improved cardiovascular protection
76	delphinidin 3-*O*-sambubioside (2 mg/mL)	inhibition of angiotension I converting enzyme	in vitro enzymatic assay	antihypertension
77	delphinidin 3-(*p*-coumaroylrutinoside)-5-glucoside from eggplant (10−200 μM)	angiogenesis inhibitor-reducing growth rate of blood vessel cells	human umbilical vein endothelial cells in vitro	anticancer and Improved cardiovascular protection
55	delphinidin as pure anthocyanidin (100 μM)	suppression of excretion of the vasoconstrictor endothelin and stimulation of the vasodilator nitric oxide (NO)	cultured human umbilical vein endothelial cells	beneficial effects on the cardiovascular system
54	delphinidin as anthocyanidin	inhibition of expression of cyclooxygenase-2 (COX-2) blocking nitrogen-activated protein kinase pathways	lipopolysaccharide (LPS)-activated murine macrophage RAW264 cells	anti-inflammatory
78	delphinidin as anthocyanidin (up to 50 μmol/L)	reduced loss of viability of low-density lipoprotein as a result of oxidation	cultured human umbilical vein endothelial cells	cardiovascular protection
52	delphinidin as anthocyanidin (30 μM)	4-fold inhibition of endothelin-1 synthesis	cultured bovine aortic endothelial cells	cardiovascular protection against atherosclerosis
53	delphinidin as anthocyanidin (10 μM)	complete inhibition of H_2O_2-induced lipid peroxidation	rat brain homogenate	antioxidant activity

and, as controls, the nontransgenic parental line they were derived from.

Two sets of experiments were carried out. In the first, seed was germinated in used soil from which either control or transgenic plants were grown, as a measure of any potential difference in allelopathic compounds excreted into the soil. In the second, seed was germinated in fresh soil to which was added ground leaf of either the control or transgenic plant. The results, summarized in Table 5, showed that in all tests there was no significant difference in either germination rate or seedling growth rate in the paired comparison of transgenic line and its parental control.

MUTAGENICITY AND TOXICITY STUDIES

In an additional complement to the literature review, mutagenicity tests (Ames test) and toxicity tests (animal feeding study) were undertaken, using several varieties of the transgenic carnation. In these tests comparison was made to isogenic controls comprising the parental variety the transgenic line was derived from.

Ames Tests. An Ames test was carried out following the recommendations of Maron and Ames[58] and according to the Organization for Economic Co-operation and Development (OECD) guideline 471.[59] The Ames test was carried out with *Salmonella typhimurium* strains TA97, TA98, TA100, and TA102 provided by the National Institute of Health Sciences, Japan, grown with or without a metabolic activator, rat liver microsomal fraction (S9 mix). TA97 and TA98 are frame shift strains, and strains TA100 and TA102 are base change strains. Mutagenicity was assayed by revertant count. The results, shown in Tables 6 and 7, show there was no mutagenic effect of either control (the parental variety the transgenic lines were derived from) or transgenic carnation petal extract on the number of revertants per plate, with or without rat liver microsomal fraction. All positive control mutagens were effective. As the two varieties chosen for the test have a relatively high concentration of delphinidin (Table 1), it may be concluded that extracts from the GM carnations have no enhanced potential for causing mutagenic activity as measured in an Ames/*Salmonella* test when compared to the non-transgenic controls.

Animal Feeding Studies. An acute toxicity test was carried out with mice fed orally with extracts of petal from nontransgenic (the parent variety used for transformation) or transgenic (five different lines) carnation or water only. The transgenic lines selected for test had either a relatively high or relatively low concentration of delphinidin (Table 1). The procedure used was approved by the Ministry of Health, Labour and Welfare, Japan. Replication, dosage, and duration are consistent with EPA guidelines,[60] which are themselves consistent with OECD chemical testing guideline 420. Body weight was measured immediately before administration of the materials and regularly thereafter. All mice were killed by cervical vertebrae dislocation after 14 days of the administration and were observed macroscopically. Weight gain results are shown in Figure 2. There was no significant difference in body weight at 14 days between any of the seven test groups in the experiment. No apparent abnormalities were observed in any groups throughout the experimental period, and no abnormalities were observed in either control or test group during autopsy. As no significant change to body weight was detected (Figure 2) it was concluded that consumption of the petal extract exerted no toxic effects. When measured in terms of

Table 5. Summary of Allelopathy Assay Using Lettuce Seed Germination[a]

transgenic line	germination rate (%)		mean of shoot FW (mg)	
	control	test variety	control	test variety
(A) Germination in Used Soil				
FLORIGENE Moonvelvet	87	95	109 ± 43	121 ± 20
FLORIGENE Moonberry	87	98	110 ± 42	101 ± 20
FLORIGENE Moonlite	96	99	151 ± 18	158 ± 12
FLORIGENE Moonshade	99	95	98 ± 5	96 ± 6
FLORIGENE Moondust	94	93	56 ± 16	80 ± 23
FLORIGENE Moonshadow	98	98	96 ± 11	82 ± 18
(B) Germination in Soil Supplemented with Ground Leaf Material				
FLORIGENE Moonvelvet	97	96	106 ± 43	125 ± 16
FLORIGENE Moonberry	97	96	107 ± 24	106 ± 4
FLORIGENE Moonlite	97	98	109 ± 10	107 ± 9
FLORIGENE Moonshade	96	98	61 ± 9	58 ± 7
FLORIGENE Moondust	95	96	100 ± 18	103 ± 10
FLORIGENE Moonshadow	96	96	101 ± 13	94 ± 15

[a]Two sets of experiments were carried out with (A) used soil and (B) soil supplemented with carnation leaf. In these experiments 50 or 100 seeds were planted for each treatment and germination and shoot fresh weight measured 14 days after planting.

Table 6. Mutagenicity Testing of the Transgenic Carnation Line FLORIGENE Moonvista (FLO-40685-1) and the Variety It Was Derived from (Control) in the *Salmonella*/Microsome Assay (Tester Strains TA100, TA102, TA97, and TA98) in the Absence (−S9) and in the Presence (+S9) of an Extrinsic Metabolic System (S9 Mixture)[a]

	TA100		TA102		TA97		TA98	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
control (mg/plate)								
0	190	139	323	345	158	148	32	38
2.5	209	157	319	333	123	152	32	37
5	205	145	341	360	164	170	29	41
FLORIGENE Moonvista (FLO-40685-1) (mg/plate)								
0	190	139	323	345	158	148	32	38
2.5	175	132	319	368	173	156	35	35
5	217	154	325	337	164	169	31	46

[a]Average number of revertants per plate is shown. In this method 0.1 mL of sample (50 mg of frozen petal ground in 1 mL of water) plus 0.5 mL of S9 mix (or 0.1 M sodium phosphate buffer (pH 7.4) as control) was mixed with 0.1 mL of overnight bacterial culture. After incubation at 37 °C for 2 days, the number of revertants was counted. Samples giving revertants >200% of the control (the number of spontaneous revertants) were regarded as positive. Positive control for TA100, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) (0.02 μg/plate), benzo(*a*)pyrene (B(*a*)P) (5 μg/plate); for TA102, 70% *tert*-butyl hydroperoxide solution (0.5 μg/plate), 2-aminoanthracene (10 μg/plate); for TA97, AF-2 (0.1 μg/plate), 2-aminofluorene (5 μg/plate); for TA98, AF-2 (0.1 μg/plate), (B(*a*)P) (5 μg/plate).

Table 7. Mutagenicity Testing of the Transgenic Carnation Line FLORIGENE Moonvelvet (IFD-26407-2) and the Variety It Was Derived from (Control) in the *Salmonella*/Microsome Assay (Tester Strains TA100, TA102, TA97, and TA98) in the Absence (−S9) and in the Presence (+S9) of an Extrinsic Metabolic System (S9 Mixture)[a]

	TA100		TA102		TA97		TA98	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
control (mg/plate)								
0	149	158	263	279	119	147	32	37
2.5	159	163	238	284	118	142	28	38
5	163	189	271	267	129	152	30	40
FLORIGENE Moonvelvet (IFD-26407-2) (mg/plate)								
0	149	158	263	279	119	147	32	37
2.5	170	154	282	271	134	144	31	34
5	174	163	252	287	132	141	36	40

[a]Average number of revertants per plate is shown. Methods are as described in the footnote to Table 6.

gain from initial (day 0) body weight, the mice fed the transgenic carnation, which contains the anthocyanin delphinidin, actually showed a slight increase in body weight (32%) compared to control carnation (28%) or water treatments (28%). For "calibration" to potential human consumption, a typical fresh flower of transgenic carnation weighs approximately 6 g. On a purely weight basis the test carried out represents the consumption of about 20 flowers by a 60 kg human.

CONCLUDING REMARKS

All varieties of transgenic carnation that have been commercialized to date and which are in the product development phase have two inserted genetic elements in common. These are a mutated *ALS* gene from tobacco, used for in vitro selection, and a *F3′5′H* gene utilized to modify the anthocyanin biosynthesis pathway in flowers. All varieties also accumulate, uniquely for the species carnation, delphinidin-based anthocyanins. Tests for any potential adverse effects on human or animal health showed that extracts from the transgenic carnation were as benign as those from the nonparental control, regardless of how much delphinidin was present in the transgenic lines tested. These results were expected after consideration of information in the scientific literature review and bioinformatics databases, which suggested that neither the gene products nor delphinidin-based anthocyanins would be likely to have any negative impacts, given the ubiquitous presence of ALS in food plants and the presence of F3′5′H in the many ornamental and food plants that contain delphinidin-based anthocyanins. The color-modified transgenic carnations have a history of safe use, reinforcing the belief that they are likely to pose no greater risk to health than nontransgenic carnation. The flowers have been produced on a commercial scale, at various times, in The Netherlands, Australia, Japan, Colombia, and Ecuador. Virtually

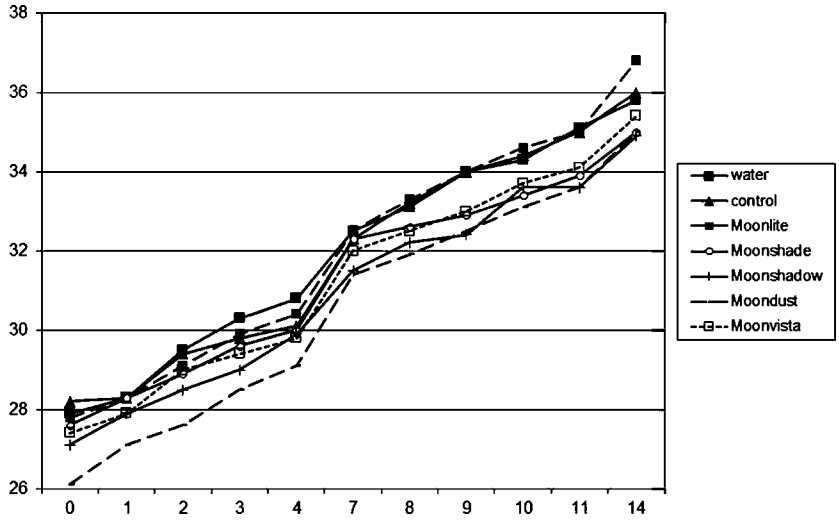

Figure 2. Acute oral toxicity test in mice. Data show average body weight, in grams, for 14 days of feeding. Petals of control (nontransgenic) and transgenic carnation were frozen in liquid nitrogen and ground in distilled water. Two grams of petal was dissolved in 10 mL of distilled water, after which extracts were filtered through a 0.45 μm membrane. The solution was administered orally at a dose level of 2 g/kg to 4-week-old ICR male mice (SPF) purchased from CLEA Japan Inc. Mice were held in polyisopentene cages, five mice in a cage, in an air-conditioned room (temperature of 23.5 ± 2.0 °C, humidity of 55 ± 5%, lighting of 12 h/day). Food (CE-2, CLEA Japan Inc.) and water were taken freely. Mice were divided into two groups of five each for each treatment.

all production is now based in Colombia and Ecuador. None of the growers of transgenic carnation flowers have reported any incidents of dermatological or allergic reactions from handling the more than 180 million flowers that have now been distributed to the florist industry worldwide.

AUTHOR INFORMATION

Corresponding Author

*Phone: +81-75-962-8807. Fax: +81-75-962-3791. E-mail: Yoshikazu_Tanaka@suntory.co.jp.

Notes

The authors declare no competing financial interest.

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