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KENMERK CGM/171213-01
ONDERWERP Advies 'nieuwe informatie over niet-toegelaten transgene petunia's'

Geachte mevrouw van Veldhoven-van der Meer,

De COGEM heeft enige tijd geleden, op basis van de toen beschikbare informatie, geadviseerd over illegale genetisch gemodificeerde (gg-) petunia's met een veranderde bloemkleur. Inmiddels is er meer informatie beschikbaar gekomen. De COGEM heeft de risico's van deze gg-petunia's opnieuw beoordeeld en deelt u het volgende mee.

Samenvatting:
De Finse autoriteiten hebben in het voorjaar van 2017 ontdekt dat er genetisch gemodificeerde (gg-)-petunia's werden verkocht, terwijl er geen vergunning voor de verkoop en/of teelt van dergelijke gg-petunia's is verstrekt. Deze gg-petunia's zijn ook in Nederland verkocht.
De COGEM heeft op basis van de gegevens die eerder dit jaar beschikbaar waren, geoordeeld dat de risico's van de gg-petunia's verwaarloosbaar klein zijn. Omdat toen nog niet bekend was welke sequenties de gg-petunia's exact bevatten, heeft de COGEM aangegeven dat zij opnieuw een advies uit zou brengen wanneer er meer informatie beschikbaar zou zijn. Inmiddels zijn de analyses van de gg-petunia's afgerond en blijken er twee verschillende typen gg-petunia's te zijn verkocht. Beide typen bevatten een gen dat de bloemkleur verandert, nl. het maïs *DFR* gen en het petunia *F3'5'H* gen. Hierdoor zijn de hoeveelheden en typen van de in bloemen aanwezige pigmenten (anthocyanen) veranderd. Verder bevatten de gg-petunia's het *nptII* antibioticumresistentiegen.
De COGEM heeft eerder geconcludeerd dat de aanwezigheid van het *nptII* gen in planten geen risico voor mens en milieu oplevert. Petunia's worden in Nederland veelvuldig als éénjarige perkplant verkocht. Petunia's zijn gevoelig voor vocht en vorst. Hoewel een enkele petuniaplant een milde winter kan overleven, zijn er geen aanwijzingen dat petunia zich in Nederland kan vestigen. Er is geen reden om te veronderstellen dat de gg-petunia's zich door de veranderingen in het type en de hoeveelheid van anthocyaan-pigmenten en de aanwezigheid van een antibioticumresistentiegen in Nederland zouden kunnen gaan vestigen. De COGEM is daarom van mening dat de risico's van de gg-petunia's met een veranderde bloemkleur verwaarloosbaar klein zijn.

De door de COGEM gehanteerde overwegingen en het hieruit voortvloeiende advies treft u hierbij aan als bijlage.

Hoogachtend,

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Update on unauthorised genetically modified garden petunia varieties

COGEM advice CGM/171213-01

Summary

Genetically modified (GM) garden petunias with an altered flower colour were detected in Finland in March 2017. They have since been detected in many other countries, including the Netherlands. No GM garden petunia varieties have been authorised for cultivation, import, distribution or retail in the European Union.

In May 2017, COGEM issued an advice on the illegal GM garden petunias. Based on the information that was available at that time, COGEM concluded that they pose a negligible risk to humans and the environment. COGEM committed itself to issue another advice as soon as more detailed information on the inserted transgenic elements would be available.

Molecular analyses that have been carried out since COGEM's previous advice indicate that two types of GM petunia varieties have been sold. One type expresses the DFR *gene from maize. These GM petunia varieties contain a* bla *gene fragment, followed by the 35S promoter, a* DFR *gene from maize, the 35S terminator, the nos promoter, the* nptII *gene and the ocs terminator. The second type expresses a gene encoding* F3'5'H *from petunia. These GM petunia varieties contain the nos promoter, the* nptII *gene, the nos terminator, the 35S CaMV promoter, a gene encoding* F3'5'H *from petunia, and the nos terminator.*

Both genes are involved in the biosynthesis of anthocyanins. Expression of these genes changes the type and amount of anthocyanin pigments present in the flowers and alters the flower colour. In addition, both types of GM petunias express a nptII *antibiotic resistance gene. COGEM has previously concluded that the presence of* nptII *antibiotic resistance genes in transgenic plants poses a negligible risk to the environment.*

The garden petunia is a so-called tender perennial, which is grown as an annual in many climate zones. It is sensitive to moisture and frost and only sporadically survives the winter. The garden petunia has no invasive or weedy characteristics and is not able to establish and form self-sustaining populations in Northwestern Europe.

The survival of garden petunias is predominantly determined by their sensitivity to cold and wet conditions. A different flower colour or resistance to certain antibiotics will not alter the sensitivity of the garden petunia to these conditions. Although an exceptional GM garden petunia or its progeny (a seedling) may survive the winter, it is unlikely that GM garden petunias will establish themselves in the Netherlands. Even though over a million of GM orange garden petunias have been sold, COGEM is not aware of any reports on feral petunia populations with orange flowers in Europe.

In view of the above, COGEM is of the opinion that GM garden petunias with an altered flower colour pose a negligible risk to humans and the environment.

1. Introduction

In the spring of 2017, the Finnish authorities alerted the Member States of the European Union and the European Commission that several genetically modified (GM) orange-coloured garden petunia varieties were present on the Finnish market. No GM garden petunia varieties have been authorised for cultivation, import, distribution or retail in the European Union.

After the alert of the Finnish authorities, the Dutch Human Environment and Transport Inspectorate (ILT) commissioned RIKILT to analyse suspect garden petunia varieties. RIKILT reported that several orange petunia varieties present on the Dutch market were genetically modified. As the GM petunia plants and seeds are illegal, the companies involved withdrew them from garden centers and nurseries.

ILT asked COGEM to advice on the destruction and possible impact of the unintentional release of the unauthorised GM petunias in the environment. Based on the information available on the transgenic traits introduced in the GM petunias (the altered flower colour and antibiotic resistance), COGEM concluded that the GM petunias pose a negligible risk to humans and the environment.

COGEM committed itself to issue an updated advice on the potential impact of the unauthorised GM petunias as soon as more detailed information on the inserted transgenic elements would become available.

2. GM petunias with altered flower colours

In 1987, *Petunia hybrida* was the first species which was genetically modified to change its flower colour.[1] Its large colourful flowers allow easy detection of changes in flower pigmentation. Since the first report, numerous GM petunias with altered flower colours have been developed (see Appendix A). The majority of these GM petunias were generated for research purposes, e.g. to study the biosynthesis of anthocyanins, the regulation of the anthocyanin pathway, the influence of pH and/or the presence of different anthocyanin pigments on flower colour, etc.

GM petunias have also been generated to expand petunias natural range of flower colours and assess their commercial potential. In China, a white pigmented GM petunia variety[2] is commercially available and has been cultivated since 1998.[3,4]

In the majority of GM petunias with modified flower colours, genes have been inserted that alter the type or amount of anthocyanins. Anthocyanins are pigments that confer a wide range of flower colours: pale yellow, orange, red, magenta, violet and blue.[5] The three main anthocyanins are cyanidin, delphinidin and pelargonidin. These anthocyanins are the basis for other types (over a hundred) of anthocyanins. The types of anthocyanins formed, the presence of co-pigments and metal-ions, and the pH of the vacuole where the anthocyanins accumulate, determine the colour of a flower.[6] Genetic modification of petunia yielded a spectrum of flower colours (white, pink, purple, orange, red, etc.).

3. Molecular analyses of the unauthorised GM petunias

At the time of COGEM's previous advice it was known, based on the analyses of the Finnish authorities and RIKILT, that there are GM petunia varieties which contain the transgenic promoter elements CaMV 35S and nos, the antibiotic resistance gene *nptII*, and the terminator elements CaMV 35S and ocs.[7]

Further analyses of the GM petunias have been carried out since by a.o. RIKILT, German authorities and Finnish researchers. Although it was initially thought that all GM petunias had orange flowers, it became clear that there were GM petunias with other flower colours on the market.[8] In addition, the molecular analyses carried out on several GM petunia varieties indicated that not all GM petunia varieties contain the same transgenic elements. There appear to be two different types of GM petunia varieties.

1. GM petunia varieties containing the DFR *gene from maize*

Sequence analyses indicate that one type contains the *DFR* gene from maize encoding the dihydroflavonol 4-reductase protein (also referred to as the *A1* gene).[9] The GM petunia varieties contain a *bla* gene fragment, followed by the 35S promoter, a *DFR* gene from maize, the 35S terminator, the nos promoter, the *nptII* gene and the ocs terminator.[9,10] The DFR-GM petunias were partially sequenced. Although there are no indications that the DFR-GM petunias contain other transgenic elements, based on the performed analyses the presence of vector fragments in other parts of the genome cannot be ruled out.

The insert present in these GM petunia varieties matches the p35A1 plasmid used by Meyer *et al.* (1987) to transform petunia protoplasts.[1,9] Two of twenty-three transformants, i.e. MPI-15 and MPI-17, were crossed to conventional *P. hybrida* varieties to investigate whether it was possible to obtain cultivars with commercial potential.[11,12] As part of the investigation, field trials were carried out in the USA. MPI-17 (also referred to as 235/1-17 or RL01-17) and progeny of this line was used to study flower pigmentation.[12,13,14] As part of these studies, field trials were carried out with progeny of the RL01-17 transformant.[15,16,17]

According to Bashandy & Teeri, the results of the analyses of two orange GM petunia varieties correspond to petunia line 235/1-17. This line has a single copy insert and a truncated *bla* gene.[15] However, according to RIKILT, the RL01-17 sequence does not contain the T-OCS primer binding site. As amplicons were generated using these primers, RIKILT concluded that the GM petunias varieties do not originate from the RL01-17 petunia line (pers. comm.). The exact origin of the GM petunia varieties is unknown, but the transgenic elements present in these varieties and their order, corresponds to the fragment of the p35A1 plasmid used by Meyer *et al.* (1987).[1]

2. GM petunia varieties containing a gene encoding F3'5'H *from petunia*

The other type of GM petunia varieties contains a gene encoding flavonoid-3'5'-hydroxylase (F3'5'H) from petunia.[8] The *F3'5'H* gene present in these GM petunia varieties could be identified by PCR amplification using P-35S and T-nos specific primers, and by determining its sequence. According to the German Central Committee on Biological Safety (ZKBS), the obtained F3'5'H nucleotide sequence was identical to that of the *F3'5'H* gene from *P. hybrida* which was used by Shimada *et al.* (1999) to generate GM petunias.[18]

Sequence characterisation of one of the unauthorised GM petunia varieties shows that they contain the P-nos promoter followed by the *nptII* gene, and the T-nos terminator, as well as the P-35S CaMV promoter, followed by a *F3'5'H* gene and the T-nos terminator.[19,20] The F3'5'H-GM petunias were partially sequenced. Although there are no indications that the F3'5'H-GM petunias contain other transgenic elements, based on the performed analyses the presence of vector fragments in other parts of the genome cannot be ruled out.

In summary, two types of GM petunia varieties appear to have been sold. The results from molecular analyses suggest that the GM petunia varieties originate from the petunia transformants generated by Meyer *et al.* (1987) and Shimada *et al.* (1999).[1,18] The transgenic elements present in the GM petunia varieties and their order corresponds to the constructs described in their publications. There is, however, currently no definite proof that the GM varieties are indeed derived from the GM lines described in these publications. Sequence comparisons between the inserts in the GM petunia varieties and the constructs used by Meyer and Shimada, could provide a decisive answer on the origin of the GM petunia varieties.

4. Characteristics of the garden petunia (*Petunia hybrida*)

4.1. The genus Petunia

The garden petunia (*Petunia hybrida* or *Petunia x atkinsiana*) belongs to the genus *Petunia* (family of the Solanaceae) and is native to South America (Brazil, Argentina and Uruguay).[21] *Petunia* species (*2n*=14) have a base chromosome number of x=7, unlike most species belonging to the Solanaceae which have a typical x=12 base chromosome number.[22]

The taxonomy of the genus *Petunia* has been subject to changes. Phylogenetic reconstruction of the 20 taxa in the genus revealed that it consists of two groups. The first group includes species (a.o. *Petunia integrifolia* and *Petunia inflata)* with purple flowers with a short corolla tube (except for *Petunia occidentalis* which has long corolla tubes). The species in this group are self-incompatible and most likely exclusively pollinated by bees. The species in the second group (a.o. *Petunia axillaris*) have long corolla tube flowers with different corolla shapes and colours. Most plants in this group are self-compatible (except for some *P. axillaris* lineages) and pollinated by moths, birds or bees.[21]

4.2. The garden petunia

The garden petunia is an ornamental plant and not used as food or feed. It is one of the most popular bedding plants throughout the world. In 2013, the garden petunia made the top 5 of bedding plants (11 million plants) sold by one of the largest international flowers and plants marketplaces of the world, Royal FloraHolland.[23,24]. Commercial varieties are either propagated vegetatively or by seed.[25]

Origin

The garden petunia presumably originates from interspecific crosses between species of the different groups within *Petunia,* i.e. between *P. axillaris* and species of the *P. integrifolia* clade (*P. integrifolia* ssp. *integrifolia*, *P. inflata* or *Petunia interior*).[22,26] The first hybrids were produced by European horticulturalists in the early nineteenth century (ca. 1830). Hybrids were probably produced multiple times from different accessions of the two parental clades.[22,26]

Biological characteristics of the garden petunia

The garden petunia is a so-called tender perennial, which is grown as an annual in many climate zones.[27] The garden petunia grows at daytime temperatures of 15°C to 25°C.[28] It does not like wet conditions[34] and is sensitive to frost,[29,30] but may survive mild winters. It has been reported that at -5°C 50% of petunia plants die due to freezing injury. Gradual cold acclimation from 15°C to 3°C can decrease the temperature at which 50% of the petunia plants die to -6°C.[31,32]

The garden petunia can be planted in gardens after the last spring frost[34] and blooms from spring to fall. The majority of petunia varieties are self-compatible, but self-incompatible petunia varieties exist as well.[33] Cross-pollination occurs via insect pollination.

Many garden petunia varieties easily set seeds. After the petunia flower falls to the ground, the calyx begins to swell and turns brown. After a couple of days, it splits open and the seeds fall out.[34] Each capsule may contain 60 to 200 seeds.[35] Petunia seeds are sensitive to cold, and humid conditions. Petunia seeds were unable to germinate after incubation at -4°C in a humid medium.[8]

Seeds from garden petunias can be collected and used to rear petunias the next growing season. Seeds germinate within 7 to 10 days (at 21°C to 29°C). Ten to twelve weeks after sowing, the plants can be planted in the garden.[34] Although it is feasible to rear garden petunias from self-collected seed, it is easier to grow them from transplants.[34]

Although garden petunias have been cultivated throughout Europe since the early nineteenth century, feral populations appear to be extremely rare. In some of the European flora, the garden petunia is mentioned as a casual or neophyte.[36,37,38,39] The garden petunia (*P. hybrida*) is mentioned as a rare species on the list of vascular plants of the Netherlands.[40] The garden petunia may reseed itself and seedlings are observed at locations were they have been planted.[41] In addition, there are a few exceptional reports of garden petunias that seem to have survived the winter in the Netherlands.[41]

Although there are occasional reports of *P. hybrida* in the Netherlands and other countries in Northwestern Europe,[37,38,40] it is most likely that these are reseeded garden petunias of which the majority will not survive wintertime. The garden petunia is not able to establish and form self-sustaining populations in Northwestern Europe.

4.3. Hybridisation

Within the genus Petunia

Besides *P. hybrida* other *Petunia* species, such as *P. integrifolia* (syn. *Petunia violacea*) and *P. axillaris* (syn. *Petunia nyctaginiflora*), are occasionally planted in gardens.[42,43,44,45] In Belgium and Germany, *P. integrifolia* is respectively classified as a casual neophyte[46] and a 'unbeständiger Neophyte',[47] i.e. a species that is observed but fails to maintain a population in the long term.

The species within the *Petunia* genus have preserved their intercrossing ability, at least under experimental conditions. *Petunia exserta* (at least when used as a seed parent) and *P. axillaris* subsp. *axillaris* are cross-compatible with all *Petunia* taxa that were investigated except *P. occidentalis.*[48,49]

Despite the lack of intrinsic barriers to crossing between most of the *Petunia* species, hybrids in nature are rare. Natural interspecific hybrids have only been described between *P. exserta* and *P. axillaris* ssp. *axillaris* and between two subspecies of *P. axillaris.*[21]

P. axillaris and *P. integrifolia* can be crossed artificially and produce fertile hybrids capable of setting abundant capsules filled with viable seeds.[50] Although there are at least four native populations in which *P. axillaris* and *P. integrifolia* occur together, no natural hybrids have been detected.[50]

With closely related genera

The genus most closely related to *Petunia* is *Calibrachoa*. Species from these two genera have different chromosome numbers: for *Petunia* $2n$=14 and for *Calibrachoa* $2n$=18. The species within *Calibrachoa* were initially classified as *Petunia* species, but were transferred to a separate genus later on. Over two hundred populations composed of *Petunia* and *Calibrachoa* species are known, but no intergeneric hybrids have been observed, even though the same insect species (*Leioproctus* subgen. *Hexantheda* sp.) acts as a pollinator for both *P. integrifolia* and *Calibrachoa heterophylla.*[50] Crossing experiments confirmed that the two genera cannot hybridise. *P. axillaris* and *P. integrifolia* have been crossed with *Calibrachoa parviflora* and *C. heterophylla*, but no capsules were formed.[50] *P. axillaris* nor *P. exserta* could be successfully crossed with any species of *Calibrachoa*.[48,49]

To summarize, the garden petunia is a common ornamental plant. It easily sets seeds and may form seedlings. Petunia plants and seeds are sensitive to frost and wet conditions, but in exceptional cases a petunia plant may survive mild winters in the Netherlands. The garden petunia is not able to establish and form self-sustaining populations in Northwestern Europe.
The pollen of garden petunias is distributed by insects. *Petunia* species may intercross under experimental conditions, but natural hybrids have only been observed between some species, which are also unable to form self-sustaining population in Northwestern Europe.

5. Transgenic traits introduced in the GM petunias

5.1 Anthocyanin biosynthesis genes

The two types of GM petunias both contain a gene involved in the biosynthesis of anthocyanins. The DFR-GM petunias express the *DFR* gene from maize and are now able to reduce dihydrokaempferol (DHK) into the orange-red pelargonidin, which is not naturally produced in petunia. Pelargonidin is, however, a regular anthocyanin synthesised in flowers of many other plant species such as carnation, morning glory and rose.[51]

The F3'5'H-GM petunias express a gene from petunia encoding flavonoid 3',5'hydroxylase (F3'5'H). F3'5'H is required for the synthesis of dihydromyricetin (DHM) which is a precursor of delphinidin. Delphinidin is a pigment with a blue to purple colour, and can be converted into two other pigments, i.e. petunidin and malvidin, which are purple in colour. There are many plant species, including petunia itself, of which the flowers normally produce delphinidin or delphinidin-derived pigments.

Anthocyanins such as pelargonidin and delphinidin provide colour to flowers and other plant parts. They attract pollinators and animals that disperse seeds, but also protect against the harmful effects of UV irradiation. In addition, anthocyanins are reported to provide antiviral and antimicrobial activities and to deter herbivores or inhibit growth of insect larvae.[52,53] Due to the expression of the *DFR* or *F3'5'H* gene, the amount of the different types of anthocyanin pigments present in the flowers of the GM petunias is altered.

As the colour of a flower is one of the traits attracting pollinators, an altered flower colour may influence the type of pollinator that visits a flower. A colour-mediated shift in the type of pollinator that is attracted to a flower, has been reported for *P. axillaris*. This species has white flowers and is pollinated by hawkmoths, bees and beetles. Introduction of the *AN2* gene in this species resulted in anthocyanin-pigmented flowers. Bumblebees preferred these anthocyanin-pigmented flowers over the white flowers of untransformed *P. axillaris* plants, whereas hawkmoths preferred the latter.[54]

Although the differently coloured flowers of the GM petunias might be preferred by different pollinators, *Petunia* flowers are usually visited by multiple pollinator species. It is therefore likely that the GM petunias will still be visited by pollinators and will be able to fertilise other garden petunia varieties.

5.2 Antibiotic resistance gene and gene fragments

Both types of GM petunias contain the *nptII* gene which confers resistance to aminoglycoside antibiotics, such as kanamycin and neomycin. The *nptII* gene is widely present in naturally occurring microorganisms. The chance of so-called horizontal gene transfer (HGT) between plants and bacteria is difficult to estimate, but the likelihood is extremely low. HGT has not been detected under field conditions.[55,56] The use of kanamycin and neomycine in clinical practice has been largely reduced, and is now mostly limited to veterinary practice. In 1998 and 2000, COGEM concluded that the presence of *nptII* genes in transgenic plants poses a negligible risk to humans and the environment.[57,58] In 2004, EFSA also concluded that use of the *nptII* antibiotic resistance gene as marker gene in GM plants does not pose a risk to the environment or to human and animal health.[59] EFSA reconfirmed its conclusion on the safe use of *nptII* genes in GM plants in a statement published in 2007.[60]

The DFR-GM petunias contain a small fragment of the *bla* gene (159bp). The *bla* gene confers resistance to ampicillin and is, under the control of prokaryotic regulatory elements, often introduced in vectors to allow selection of the bacteria that contain the vector. It is unlikely that a functional product will be produced from the small *bla* gene fragment present in the DFR-GM petunias. COGEM has previously concluded that the presence of a *bla* gene in transgenic plants poses a negligible risk to humans and the environment.[57,58]

6. Environmental risk assessment

Garden petunias are very popular bedding plants. Garden petunias are pollinated by insects and may reseed. Seedlings are observed at locations where garden petunias have been planted.

The garden petunia is a so-called tender perennial, which is grown as an annual in many climate zones. It is sensitive to moisture and frost and only sporadically survives the winter. The garden petunia has no invasive or weedy characteristics and is not able to establish and form self-sustaining populations in Northwestern Europe.

GM garden petunia varieties were sold in the Netherlands. These GM petunias have been modified with genes involved in the biosynthesis of anthocyanins resulting in an altered flower colour. In addition, they contain the *npt*II antibiotic resistance gene. COGEM has previously concluded that the presence of *nptII* antibiotic resistance genes in transgenic plants poses a negligible risk to the environment. The anthocyanin pigments present in the flowers of the GM garden petunias are produced by flowers of many other plant species such as carnation, morning glory and rose. In addition, they are present in the fruits of many plant species such as strawberry, grape and blueberries as well.[61] Therefore, COGEM is of the opinion that the GM garden petunias with altered types and levels anthocyanin pigments pose a negligible risk to other organisms.

The survival of garden petunias is predominantly determined by their sensitivity to cold and wet conditions. A different flower colour or resistance to certain antibiotics will not alter this. Although an exceptional GM garden petunia or its progeny (a seedling) may survive the winter, it is unlikely that GM garden petunias will establish themselves in the Netherlands. Since the beginning of this decade, over a million of GM petunias with orange flowers have been sold in Europe. Despite the large numbers of plants sold, there are no reports on feral petunia populations with orange flowers in Europe.

In view of the above, and based on the information currently available, COGEM is of the opinion that GM garden petunias with an altered flower colour pose a negligible risk to humans and the environment.

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Appendix A. GM petunias with altered flower colours

Numerous GM petunias with altered flower colours have been produced. In the USA, 88 applications concerning GM petunias have been filed. At least 23 entries concern GM petunias with (possible) altered flower colours.[1] In China, a white pigmented GM petunia variety[2] is commercially available and has been cultivated since 1998.[3,4]
The majority of the GM petunias were generated for research purposes, e.g. to study the biosynthesis of anthocyanins, the regulation of the anthocyanin pathway, the influence of pH and the presence of different anthocyanin pigments on flower colour, etc.

The biosynthesis of anthocyanins can be divided into the following three stages. The first stage (also known as the phenylpropanoid pathway) is the conversion of phenylalanine to 4-coumaroyl-CoA which involves several enzymes and is a step shared by many secondary metabolic pathways. The second stage is the key reaction in the metabolism of flavonoids: the formation of dihydroflavonols catalysed by chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), and flavonoid 3-hydroxylase (F3'H) or flavonoid 3', 5'hydroxylase (F3'5'H). The third stage is the formation of various anthocyanidins, catalysed by dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS). The anthocyanidins are then modified through a series of glycosylation and methylation steps catalyzed by UDP glucose-flavonoid glucosyltransferase (UFGT) and methyl transferase (MT) to form stable anthocyanins.[6]

GM petunias can be divided into different categories depending on the change in the amount and type of (anthocyanin) pigments formed:

1. Inhibition or reduction of the production of anthocyanin pigments

Petunias with a reduced or inhibited flower pigmentation were observed when genes from petunia itself (such as *CHS, F3H, F3'5'H* or *DFR*), a chimeric *chsA-uidA* or a mutated *CHS* gene from *Mazus japonicus* were introduced in sense or antisense orientations.[5,6,7,8,9,10,11,12,13,14,15,19] These genes encode enzymes that generate the precursors for anthocyanin pigments. In contrast to what was expected, introduction of the additional gene resulted in silencing or reduced expression of both the endogenous and the introduced gene. Pigmentation in these GM petunias was reduced across the entire flower resulting in white flowers, or locally yielding a variegated white-pigmented pattern (in sectors or in a ring).
Silencing of the *EFP* (*Enhancer of Flavonoid Production*) gene, encoding a type IV CHI protein, resulted in petunia flowers with pale colours due to the reduced amounts of anthocyanins and colourless flavonoids.[16]

2. Production of delphinidin or delphinidin-derived pigments

Petunias have been genetically altered to produce (more) delphinidin and delphinidin-based pigments (petunidin and malvidin), which have a blue to purple colour. A F3'5'H enzyme synthesizes the precursor of delphinidin, dihydromyricetin (DHM), and is required for the production of delphinidin pigments. To convert DHM into delphinidin, a DFR type enzyme must also be present. Genes encoding F3'5'H from different species, such as *Vinca major, Petunia hybrida,* cineraria, and *Phalaenopsis* (alone and combined with the *DFR* gene from *Hyacinthus orientalis*), have been introduced in petunia.[17,18,19,20,21] In addition, the *DFR* gene from *Gerbera* was introduced in a petunia line which lacked *DFR* activity but did contain the F3'5'H enzyme.[22] These modifications resulted in an altered flower colour due to the (increased) production of delphinidin pigments.

3. Production of cyanidin or cyanidin-derived pigments

Genetic modification has been used to increase the production of cyanidin and cyanidin-derived (peonidin) pigments, which confer a purple to red colour. High levels of cyanidin-based pigments are only obtained when F3'H is expressed but not F3'5'H and FLS. A F3'H enzyme is required for the production of cyanidin pigments, because it synthesizes dihydroquercetin (DHQ), a precursor of cyanidin. To convert DHQ to cyanidin, the DFR enzyme must also be produced. If F3'5'H is expressed in addition to F3'H, delphinidin (blue) pigments are produced as well, resulting in a lower amount of cyanidin (red) pigments. Similarly, expression of *FLS* would result in the accumulation of flavonols and a reduced amount of cyanidin and other anthocyanin pigments.[24]

Cyanidin-based pigments were produced in GM petunia lines in which F3'H genes from petunia or cineraria were introduced.[21,23] Introduction of the *DFR* gene from *Gerbera* in a petunia line which produced F3'H but lacked DFR activity also resulted in the production of cyanidin.[22] Introduction of several genes from other petunia lines (such as *F3'5'H, F3'5'H* combined with *FLS* or *FLS* and *AR-AT*) resulted in the suppression of these genes and redirected the anthocyanin pathway to produce cyanidin instead of delphinidin pigments.[11,24]

4. Production of pelargonidin pigments

Petunias do not naturally produce pelargonidin pigments, which have an orange to red colour, since the DFR enzyme from petunia is unable to reduce dihydrokaempferol (DHK), the precursor of pelargonidin.[25] There are several reports describing the introduction in petunia of *DFR* genes derived from other species, such as *Zea mays*, *Gerbera hybrida*, *Calibrachoa hybrida* and *Rosa hybrida* (alone, and combined with the *F3'H* gene from petunia). The flowers of these GM petunias were orange-red due to increased levels of pelargonidin pigments.[11,22,26,27,28,29,30,31,32,33]

5. Production of multiple anthocyanin pigments

Besides the above mentioned examples of GM petunias in which just one type of anthocyanin pigment was increased, genetic modification has also been used to increase different types of anthocyanin pigments simultaneously. *DFR* genes from *Agapanthus praecox* spp. *orientalis*, *Antirrhinum majus* or petunia were introduced in petunia lines recessive for the *dfr* gene, enabling the production of delphinidin and/or cyanidin pigments.[34,35,36]

6. Modification of the type of anthocyanin

The main types of anthocyanins: cyanidin, delphinidin and pelargonidin can be further modified with sugars, aliphatic acids, aromatic acids and methyl groups. These modifications influence the flower colour, e.g. aromatic acylation shifts the colour towards blue and methylation results in a slightly more reddish flower colour.[37] Methylation of cyanidin produces peonidin, whereas methylation of delphinidin produces petunidin and malvidin.[38,39]

Introduction of the *Methylation at Three2 (MT2)* or *Methylation at Five (MF2)* genes from petunia, or introduction of the *Anthocyanin-O-methyltransferase1 (VvAOMT1)* gene from *V. vinifera* in petunia lines which do not naturally express these genes either increased the level of peonidins and reduced the level of cyanidins, or increased the levels of petunidins and malvidins, while reducing the level of delphinidin, depending on the genetic background of the modified petunia lines.[40]

Suppression of the *3RT* gene encoding anthocyanidin 3-glucoside rhamnosyltransferase inhibited the synthesis of methylated anthocyanins and shifted the type of anthocyanins, for instance from malvidin to petunidin and delphinidin, which resulted in an altered flower colour.[11,41,42]

7. Redirection of the anthocyanin biosynthesis pathway

Genetic modification has also been used to redirect the biosynthesis pathway of anthocyanins, flavonols and flavones. Flavones and flavonols share precursors (i.e. flavanones and dihydroflavonols) with anthocyanin pigments. Flavonols and flavones are typically colourless, but can affect the colour of flowers as co-pigments by forming molecular complexes with anthocyanins.

Petunia flowers with reduced pigmentation were observed after introduction of the *FLS* gene encoding flavonol synthase from rose[11] and the introduction of the *FNSII* gene encoding flavone synthase from torenia.[11] The introduction of these genes increased the synthesis of respectively flavonols and flavones and led to a decrease in the production of anthocyanins.

Petunia flowers with increased pigmentation were observed after the introduction of the *FLS* gene from petunia in several petunia lines (alone or in combination with the *DFR* gene from *A. majus*). In some transformants, introduction of the *FLS* gene led to down-regulation of FLS, decreased flavonol and increased anthocyanin levels. Depending on the petunia line used for the transformation this resulted in redder or red purple flower colours.[11,13,36,43]

Petunia flowers with increased pigmentation were also observed when the *PhCCoAOMT1* gene involved in the synthesis of lignins and volatile phenylpropenes was silenced. Volatiles and anthocyanins share a common precursor (i.e. 4-coumaryl coenzyme A (CoA)). Reduced synthesis of volatiles and lignins led to the upregulation of R2R3 MYBs Purple Haze and Deep Purple resulting in activation of the anthocyanin pathway and increased synthesis of anthocyanins.[44]

Introduction of the Production of Anthocyanin Pigment1 (*Pap1*) *Myb* transcription factor from *Arabidopsis thaliana* in petunia resulted in increased levels of anthocyanin pigments and more fragrant flowers due to the simultaneous enhancement of the branches of the phenylpropanoid pathway producing colour and scent in flowers.[45]

8. Modification of the pH in the vacuole

Petunia has also been genetically modified with genes that indirectly influence flower colour. Silencing of the *PH5* gene, which encodes for a P_{3A}-H^+-ATPase and is required for vacuolar acidification, increases the pH of the vacuole. Anthocyanins are located in the vacuole, and their colour is affected by the vacuolar pH. When the vacuole is acidic, anthocyanins have a red colour, and when the pH is neutral or alkaline, anthocyanins are bluish.[37] In the GM petunia line in which *PH5* was silenced, the flower colour changed from red-purple to blue-purple.[46]

9. Production of chalcones yellow pigments (chalcones)

Genetic modification has also been used to alter the amount of another type of pigment, i.e. chalcones. Chalcones are yellow flavonoid pigments. Introduction of the chalcone reductase (*CHR*) gene from *Medicago sativa* led to the accumulation of 6'-deoxychalcones and a reduction in the amount of flavonols and anthocyanins resulting in pale yellow (instead of white) or pale purple (instead of deep purple) flowers.[47]

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