DISSERTATION

Breeding poinsettias with novel bract colouration by genome editing

ausgeführt zum Zwecke der Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften unter der Leitung von

Associate Prof. Dipl.-Ing. Dr.techn. Heidi Halbwirth

Insitut 166/6

Institut für Verfahrenstechnik, Umwelttechnik und technische Biowissenschaften

eingereicht an der Technischen Universität Wien

Fakultät für technische Chemie

von

MSc Daria Agata Nitarska

Martikelnummer 01576090

Wien, am

eigenhändige Unterschrift

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisor Associated Professor Dr Heidi Halbwirth for her invaluable supervision, continuous support and immense knowledge. Without her guidance and encouragement this thesis would have never been created.

I would like to offer my special thanks to European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 675657 Flower Power for founding my PhD project.

I would like to extend my sincere thanks to Prof. Thomas Debener for the insightful comments and suggestion, and for hosting me during my stay at the Leibniz University Hannover.
I would like also to thank Dr Robert Boehm for sharing his expertise in the topic of plants transformation and hosting me during my stay at Selecta Klemm GmbH & Co. KG.

Additionally, I would like to thank all other Flower Power project members: Dr Johannes Hadersdorfer, Dr Michael Neumüller, Prof. Thomas Rattei, Prof. Karl Stich, Dr Fritz Thümmler and Prof. Kristiina Wähälä for inspiring discussions during our meetings.

I would like to thank all other Flower Power PhD students: Martina Kolarek, Rares Calin Lucaciu, Carmen Stefanini, Vinicius Vilperte and Benjamin Walliser for support during my work and all the fun that we had during Flower Power meetings and seminars.

I also would like to thank all members of Research Group for Phytochemistry and Plant Biochemistry at TU Wien: Jürgen Greiner, Dr Christian Haselmair-Gosch, Dr Olly Hutabarat, Christoph Korpointner, Silvija Marinovic, Dr Christian Molitor, Renate Paltram, Barbara Roth, Christopher Schlosser, Philipp Waldhör and Julia Weissensteiner for all the help during my work in the lab, moral support and nice conversations in between of experiments.

Special thanks to Christopher Schlosser for proofreading of the thesis and language corrections and Dr Christian Haselmair-Gosch for all the valuable comments.

I would like to thank all members of Molecular Plant Breeding group at Leibniz University Hannover, especially Dr Annette Bartkiewicz, Dr Friederike Busse, Helena Domes, Klaus Dreier, Dr Juliane Geike, Dr Enzo Klein, Dr Ina Menz and Julia Schröter for all of the help during my stay in Hannover and relaxing launch breaks.

Acknowledgements

I would like to extend my thanks to Dr Maike Boxriker, Ann-Kathrin Pohl, Marcus Pohl, Emilia Polubok, Marika Takacs, Sabrina Wölki and all In Vitro Lab members at Selecta Klemm GmbH & Co. KG for all the support during my stay at the company.

Finally, I would like to thank my parents and my sister for all the encouragement and mental support in the bad and good moments during my work on this PhD thesis.

Abstract

Poinsettia (*Euphorbia pulcherrima*) commonly shows red bract colouration caused by the accumulation of cyanidin-type anthocyanins. Orange-red cultivars, which accumulate pelargonidin-type anthocyanins occur rarely, whereas blue poinsettias are not naturally present due to the lack of delphinidin formation. Breeding is an arduous process, and a lot of effort is necessary to obtain plants with desired traits. Some phenotypes are not possible to achieve by classical breeding approaches due to limitations in the genetic background of the plants. Molecular breeding techniques like genome editing can help to overcome those obstacles and obtain novel poinsettia varieties with orange or blue colour in a fast and efficient way. The colour of anthocyanin pigments depends on the B-ring hydroxylation pattern and ranges from orange pelargonidin (one hydroxyl group), dark red to pink cyanidin (two hydroxyl groups) and blue to violet delphinidin (three hydroxyl groups). Two factors are crucial for the establishment of the B-ring hydroxylation pattern. First, the presence or absence of flavonoid 3'-hydroxylase (F3'H) and flavonoid 3'5'-hydroxylase (F3'5'H) activity, and second, the substrate specificity of dihydroflavonol 4-reductase (DFR), which provides important intermediates in the formation of anthocyanins. In this thesis, the physiological background of colour formation was studied in petunia as a model plant and in red, and orange poinsettia cultivars. Based on this, a strategy for molecular breeding of orange and blue poinsettia was established. The investigations on the molecular background of pigment formation in orange petunia also resulted in the establishment of an event-specific PCR approach for the detection of genetically modified petunias, which had been undetected for several years on the market.

By silencing of *F3'H* in a red flowering cultivar with application of the CRISPR/Cas9 technique, orange-red flowering line was obtained, which accumulated prevalently pelargonidin-type anthocyanins. This is the first report of genome editing in poinsettia.

For breeding blue bract colouration, overexpression of *Cyclamen persicum F3'5'H* was performed in a cultivar with a high pelargonidin:cyanidin ratio. As a result, plants with a significantly higher delphinidin content were obtained, but the amounts were too low to result in the desired colour change. Thus, the thesis laid the foundation for future commercial breeding programmes for orange and blue colouration of poinsettia bracts with strong emphasis on the application of genome editing approaches.

Zusammenfassung

Die Brakteen des Weihnachtsstern (*Euphorbia pulcherrima*) haben üblicherweise eine leuchtend rote Farbe, die durch Akkumulation von Anthocyanen des Cyanidin-Typs verursacht wird. Orange-rote Sorten reichern hingegen Anthocyane vom Pelargonidin-Typ an, kommen jedoch äußerst selten vor, während blaue Weihnachtssterne, aufgrund der Unfähigkeit Delphinidine zu synthetisieren, in der Natur nicht vorkommen. Die Züchtung von Pflanzen mit spezifischen Merkmalen ist in der Regel ein langwieriger Prozess. Manche Eigenschaften können mit klassischen Züchtungsansätzen aufgrund von genetischen Einschränkungen nicht erreicht werden. Molekulare Züchtungstechniken wie Genome editing können helfen, diese Schranken zu überwinden und haben das Potential, relativ schnell und spezifisch neue Weihnachtssternsorten mit oranger oder sogar blauer Farbe zu erhalten. Die Farbe der Anthocyane hängt vom B-Ring-Hydroxylierungsmuster ab und reicht von orange (Pelargonidin, eine Hydroxylgruppe) über dunkelrot bis rosa (Cyanidin, zwei Hydroxylgruppen) und blau bis violett (Delphinidin, drei Hydroxylgruppen). Zwei Faktoren sind entscheidend für die Etablierung des B-Ring-Hydroxylierungsmusters. Zunächst das Vorhandensein von Flavonoid-3'-Hydroxylase-Aktivität (F3'H) und Flavonoid-3'5'-Hydroxylase-Aktivität (F3'5'H) und außerdem die Substratspezifität der Dihydroflavonol-4-Reduktase (DFR), die wichtige Zwischenprodukte bei der Bildung von Anthocyanen herstellt. In dieser Arbeit wurde der molekulare Hintergrund der Farbbildung bei der Modellpflanze Petunie sowie bei roten und orangefarbenen Weihnachtssternsorten untersucht. Auf dieser Grundlage wurde eine Strategie für die molekulare Züchtung von orangefarbenen und blauen Weihnachtssternen entwickelt. Die Untersuchungen zum molekularen Hintergrund der Pigmentbildung bei orangefarbenen Petunien führten auch zur Etablierung eines Event-spezifischen PCR-Ansatzes zum Nachweis von gentechnisch veränderten Petuniensorten, die mehrere Jahre unerkannt auf dem Markt waren.

Durch Ausschaltung der *F3'H* in einer rot blühenden Sorte unter Verwendung der CRISPR / Cas9-Technik wurde eine orangerote Linie erhalten, die überwiegend Anthocyane des Pelargonidin-Typs akkumuliert. Dies ist die erste Anwendung von CRISP/Cas9 bei der Züchtung von neuen Weihnachtssternsorten.

Zur Züchtung von blaublühenden Brakteen wurde eine Überexpression der *F3'5'H* von *Cyclamen persicum* in einer Sorte mit einem hohen Pelargonidin:Cyanidin-Verhältnis durchgeführt. Das Ergebnis waren Pflanzen mit einem signifikant höheren Delphinidingehalt, aber die gebildeten Mengen waren zu gering, um die gewünschte Farbänderung zu erzielen. Diese Arbeit legte somit den Grundstein für zukünftige kommerzielle Züchtungsprogramme zur Orange- und Blaufärbung von Weihnachtssternbrakteen mit starkem Schwerpunkt auf der Anwendung von Genome editing.

Table of Contents

1 Introductory chapter

1.1 Introduction and problem statement

1.1.1 Poinsettia

The winter-flowering *Euphorbia pulcherrima* (poinsettia or Christmas star) belongs to the most economically important potted ornamental plants in North America, Europe, Asia and Australia, especially during the Christmas season. In the US, Christmas Star accounts for over 17% of potted plant sales at US$153 million in 2019 [1], with most being sold during the lead-up to Christmas.

In the EU, around 110 million plants were sold in 2015, 35 million of those in Germany alone. Annual German sales are consistently over 30 million, amounting to €105 million in 2018, with about 90% of poinsettia turn around taking place in just six weeks of the year, peaking in the 48th calendar week, coinciding with the first week of Christmas[1]. According to AMI Statistik, poinsettia is consistently the second most popular potted flowering plant, with 10-11% market share, second only to potted orchids with over 30%[1].

Botanically, the plant is known as *Euphorbia pulcherrima,* a name given in 1834 by the German botanist Wilenow and meaning 'very beautiful' Euphorbia. The more colloquial name, poinsettia, comes from the US ambassador of Mexico, Joel Roberts Poinsett, who was reportedly the first to bring the plant to the US [2].

Fig. 1 Poinsettia flowering in Mediterranean garden. Photographs taken by Heidi Halbwirth.

[1]https://taspo.de/kategorien/poinsettien-geschaeft-nur-was-fuer-die-grossen/#:~:text=Deutsche%20geben%20rund%20110%20Millionen%20Euro%20f%C3%BCr%20Poinsettien%20aus&text=Laut%20AMI%2DStatistik%20belegt%20er,110%20Millionen%20Euro%20pro%20Jahr.

Poinsettia belongs to the *Euphorbiaceae* or Spurge family, which is the fifth-largest flowering plant family [3], comprising about 7,500 species [4]. The genus *Euphorbia* is the largest of the angiosperm genera, currently having around 2,000 recognised species [5]. Poinsettia is a diploid plant with chromosome number 2n=28 [6]. Many species in this class produce a milky sap or latex.

The well-known Christmas Star was not always as ubiquitous and estimated. Poinsettias are perennial flowering plants that were once considered weeds. They are native to the tropical forests of southern Mexico and Central America, where they grow in the wild. They are not frost-tolerant but will grow outdoors in temperate coastal climates. Wild specimens are found as shrubs or small trees of up to 3 meters height, with long internodes, a few stems, and narrow leaves and bracts [7, 8].

The original poinsettia native to central America looked very different to the plant known around the world today, its modern shape, size and colour all being the results of years of progressive breeding efforts (Fig. 1, Fig. 2). Nowadays, poinsettias have large bracts, often in various shapes and colours, and are compact plants with well-developed branching [7, 8].

Poinsettias are well known for their intense red so-called flowers. This is, however a popular misconception. The actual flowers of poinsettia, or *cyathia,* are contained within the small yellow-green globes in the centre of the large brilliant red floral bracts. The bracts escort the relatively small and unimpressive reproductive structures and - as flowers - serve the function of attracting pollinators. Phylogenetically, they are leaves that change colour from green to red, thus also changing their function from photosynthesis, providing assimilates for growth, towards pollinator attraction [9-11]. The deep red colouration of poinsettia bracts is induced by the shortening days during the winter months.

Fig. 2 Diversity of poinsettia cultivars. Photographs taken by Heidi Halbwirth.

Traditionally, the mass market prefers intense scarlet or dark-red colouration of the bracts [2], accounting for around 80% of market share. However, in recent years an industry based investigation of consumer preferences has led to a redefinition of target markets. In particular, it has been found that consumer tastes are much more varied than previously assumed and that unique or 'odd-ball' varieties can fetch much higher prices and greatly expand demand on the consumer market [12]. These unusually coloured varieties are particularly popular in the lead up to the Christmas season, thus significantly expanding the market season for this plant. This realisation and a more consumer oriented approach to breeding programs have led to a considerable increase in the variety of poinsettia being developed for the market [12]. There are more than 100 varieties of poinsettia available today. Poinsettia come in colours like the traditional red, as well as white, pink, burgundy, salmon, yellow, marbled and speckled (Fig.2).

In recent years a new trend has further boosted sales, known as the "Painted Poinsettias" (Fig.3). As the name suggests, people have taken to adding even more colour and variety to the poinsettia range, by painting their bracts with specially conceived sprays [12]. This trend has the potential to further expand the plant's commercial season. Some of the more exotic possibilities that arise from this technique include the 2020 trend colour indigo, which would otherwise not be possible, or even combination pots containing multiple different colours.

Fig. 3 Painted poinsettia. Picture source: http://www.danschantz.com/christmas/painted-poinsettias/

1.1.2 Petunia

Petunia x *hybrida* is one of the most popular annual, bedding plants worldwide. Thanks to colourful flowers, often with different patterns, over 100 years petunias have decorated gardens and balconies all over the world. The genus *Petunia* belongs to the Solanaceae family that originates from South America and includes 14 species [13]. Petunia was first described by Jussieu in 1803 with plant material originating from Uruguay [13]. *Petunia* x *hybrida* is an interspecies hybridisation of white flowering, moth pollinated *Petunia auxillaris* and purple flowering, bee pollinated *Petunia integrifolia*, but it was also suggested that other petunia species might be the purple parent, like *Petunia interior* [13, 14]. The fact that all native species of petunia and *Petunia* x *hybrida* share the same chromosome number, n=7, allows crossings

between all petunia species [15]. Genomes of *Petunia auxillaris* and *Petunia inflata* have been published in 2016, with the assemblies covering more than 90% of the 1.4 Gb genome [16].

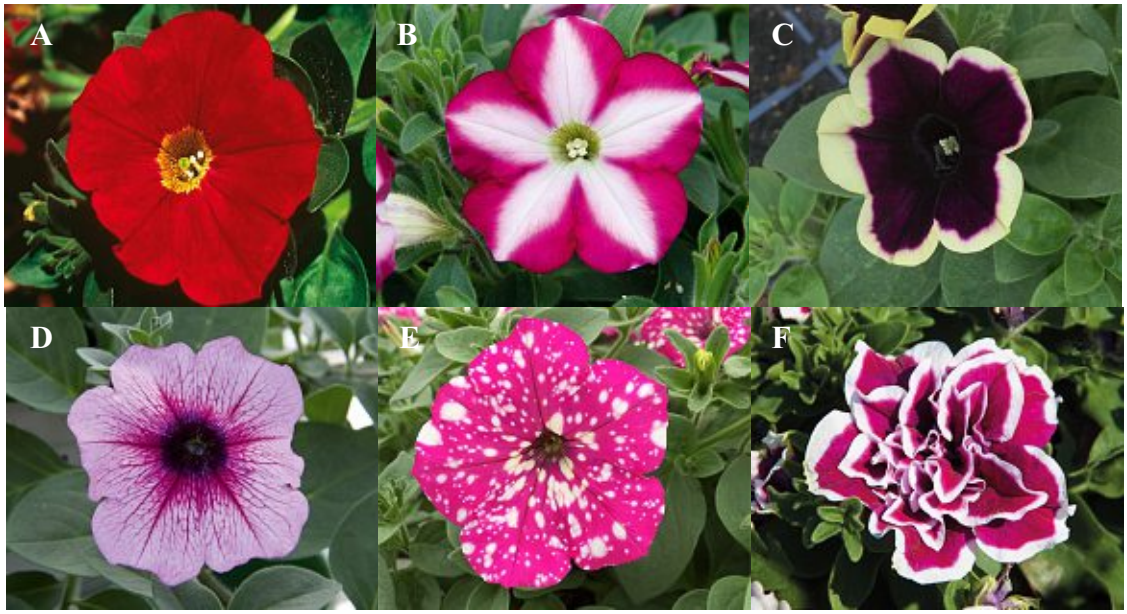


Fig. 4 Examples of petunia cultivars: A: 'AlpeTunia Red', B: 'Bonnie Carmine Star', C: 'Famous Dark Purple Pictee', D: 'AlpeTunia Pink' Vein, E: 'BabyDoll', F: 'SweetSunshine Magenta Picotee'. Picture source: https://www.selecta-one.com/en/assortment/petunia_cultivars/

Nowadays, plenty of petunia cultivars are available. As a result of 200 years of crossing, many flower colour options, patterns and morphology, are available (Fig. 4). Petunias in general are divided into three different groups, depending on flower size: *grandiflora* (big flowers, 10 cm in diameter), *multiflora* (medium size flowers, 5 cm in diameter), *milliflora* (small flowers, 2,5 cm in diameter). Additionally, different patterns on petunia flowers are available, such as star-shape, picotee, dark vein or polka dot. Considering that petunias are not very demanding plants and do not require frequent watering or special soil conditions it makes them perfect for even inexperienced gardeners.

Petunia x *hybrida* is also one of the important model plants in scientific research that allows the study of many topics, such as flower colour, flower development, evolutionary developmental biology, genetics of inflorescence and branch development, response to biotic and abiotic stresses [17]. The most popular research cultivars are 'V26' and 'Mitchell' because of their high transformation efficiency, and cultivar 'W138' that is used for transposon mutagenesis [18]. Further advantages of petunia as a model plant are relatively easy cultivation, short lifecycle (3 - 3.5 months) and easy sexual and asexual propagation [17]. Well established stable and transient transformation protocols are important factors that are reasons for petunias' success as model plants [19, 20].

1.1.3 Flavonoid pathway

Flavonoids belong to the phenylpropanoids, one of the largest secondary metabolite groups in plants. So far, around 8000 compounds that belong to this group have been characterised, and considering the diversity of the plant kingdom, most likely many more are still waiting to be discovered [21]. It is estimated that 20% of the carbon flux in plants goes through the flavonoid pathway [21]. The main classes of flavonoids are: flavans, flavanols, flavandiols, flavanones, flavones, isoflavones, dihydroflavonols, flavonols and anthocyanidins [22]. The flavonoid precursors, malonyl-CoA and *p*-coumaroyl-CoA, are derived from the carbohydrate metabolism; *p*-coumaroyl-CoA specifically is derived from phenyl alanine via the phenylpropanoid pathway [23].

In the first step of the anthocyanin biosynthesis described in Figure 5, chalcone synthase (CHS) catalyses the condensation of one molecule *p*-coumaroyl-CoA with three molecules malonyl-CoA into the first C15-structure, the yellow coloured naringenin chalcone. In the next step, chalcone isomerase (CHI) converts the naringenin chalcone into the flavanone naringenin. Flavanone 3-hydroxylase (FHT) performs hydroxylation in position C-3 to form dihydrokaempferol (DHK). Subsequently, naringenin and DHK can be hydroxylated in position 3' or 3'5' to form eriodyctiol or dihydroquercetin (DHQ) and pentahydroxyflavanone (PHF) or dihydromyricetin (DHM) respectively by flavonoid 3'-hydroxylase (F3'H) or flavonoid 3'5'-hydroxylase (F3'5'). Eriodyctiol and PHF can be converted by FHT to DHQ and DHM. The dihydroflavonols are reduced by dihydroflavonol 4-reductase (DFR) to leucoanthocyanidins, which are converted to the anthocyanidins (pelargonidin, cyanidin or delphinidin) by anthocyanin synthase (ANS). In general, anthocyanidins are unstable at cellular conditions. Glycosylation of the hydroxyl group in position 3 of the C-ring causes much better stability of the compound and is, therefore, regarded as an essential step in the anthocyanidin biosynthesis rather than a modification step [24]. UDP-glucose:flavonoid 3-*O*-glucosyltransferase (FGT) transfers a glucose moiety from UDP-glucose to the hydroxyl group in position 3, which makes them stable in vacuolar conditions. In the next steps anthocyanidins can undergo further modifications by glycosylation and acylation [25].

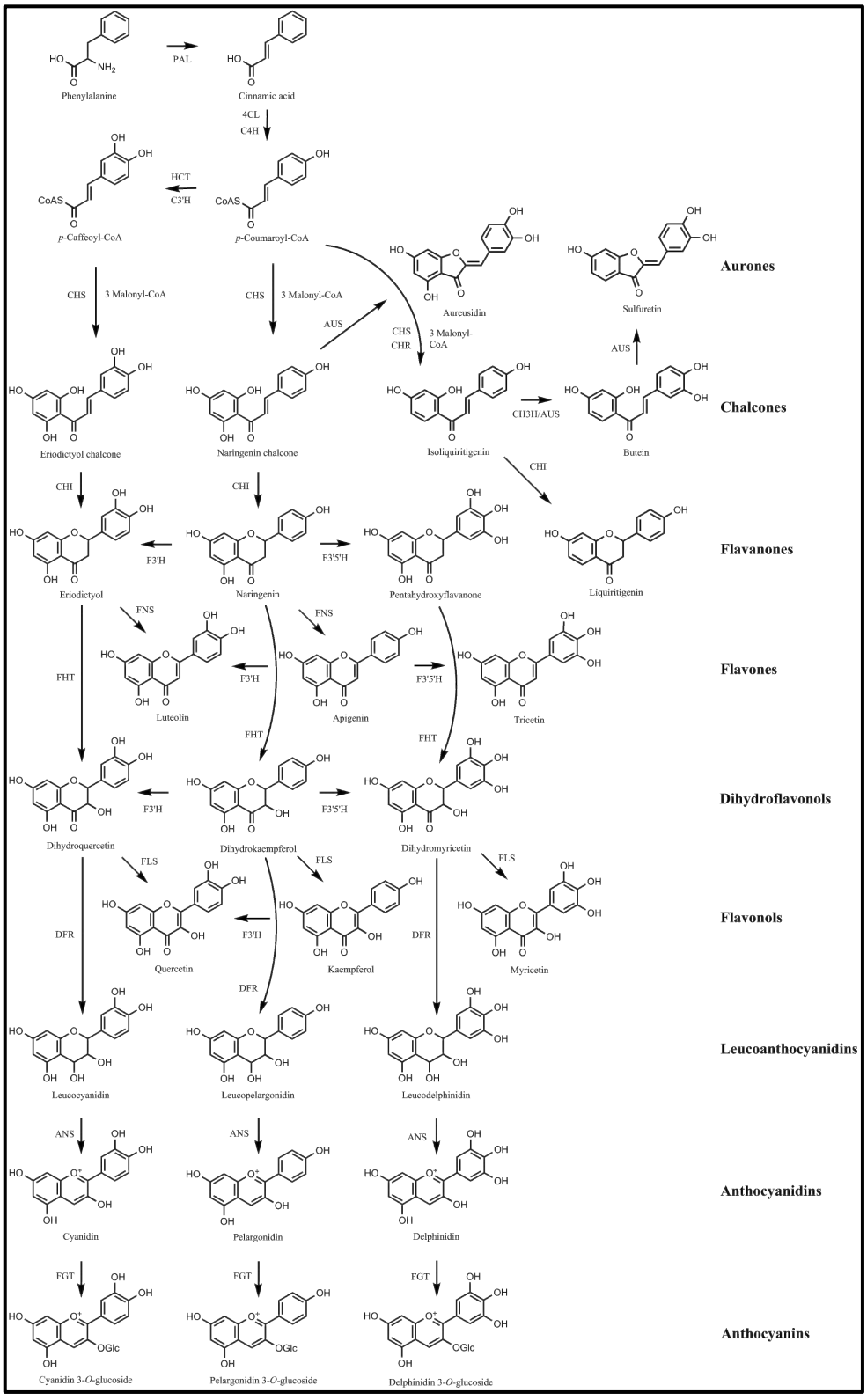


Fig. 5 Flavonoid pathway [26]

The pathway described above results in the formation of anthocyanins, but the flavonoid pathway has many branches that lead to the formation of other flavonoid classes or related structures, for example such metabolites as stilbenes, aurones, 5'-deoxy-(iso)flavonoids,

flavones, flavanols, catechins and pro-anthocyanidins [23]. Flavones are synthesised from naringenin, eriodictyol or pentahydroxyflavanone by flavone synthase (FNS) to apigenin, luteolin or tricetin. Flavones are important for flower colour because they can serve as co-pigments by forming complexes with anthocyanins [27]. Flavonols are synthesised from DHK, DHQ or DHM to kaempferol, quercetin or myricetin, respectively, by flavonol synthase (FLS). Thus, there is competition for common intermediates between the biosynthesis of anthocyanin, flavones and flavonols [28, 29]. It was shown in tobacco and petunia that FLS silencing leads to a higher content of anthocyanins in transgenic flowers [29, 30].

Flavonoids have multiple functions. For example, they protect plants from harmful UV-B light, pathogens and herbivores, aid in surviving abiotic stress, are important for pollen fertility, serve as signal molecules in plant-microbe interaction and attract pollinators by colouring flowers and fruits [31]. This group of compounds also have multiple beneficial health properties for humans, they serve as antioxidants, anticancer agents and are discussed to prevent diverse chronic diseases [32].

1.1.4 F3'H and F3'5'H

Flavonoid 3'-hydroxylase (F3'H) (EC 1.14.13.21) and flavonoid 3'5'-hydroxylase (F3'5'H) (EC 1.14.14.81) belong to the membrane bound cytochrome P450 dependent monooxygenases superfamily [33, 34]. As with all plant cytochrome P450 dependent monooxygenases, they are membrane bound and located at the cytosolic side of the endoplasmic reticulum. They determine the hydroxylation pattern of the B ring of the flavonoids. They perform hydroxylation on the position 3' or 3' and 5', respectively, providing precursors for the synthesis of pink/red and blue/violet-coloured anthocyanins. F3'H converts naringenin to eriodictyol and dihydrokaempferol to dihydroquercetin, whereas F3'5'H converts naringenin to pentahydroxyflavanone (PHF) and DHK to dihydromyricetin (DHM), but can also perform 5' hydroxylation of eriodyctiol and DHQ. They also can catalyse hydroxylation of flavones, flavonols and leucoanthocyanidins [10]. Both genes were isolated for the first time from petunia [35, 36]. In general, they show around 50% identity on the amino acid level to each other [37]. Depside of differences in the primary sequence of P450s the secondary and tertiary structures of the proteins are highly conserved [38]. F3'H belongs to the subfamily CYP75B and F3'5'H to CYP75A, and it is proposed that they diverged before the speciation of higher plants, but as it was showed in Asteraceae that evolution of F3'5'H from F3'H happened independently [37]. It is suggested that F3'5'H evolved from F3'H at least four times in dicotyledonous plants [39]. Gotoh (1992) [40] described six substrate recognition sites (SRS)

that are characteristic for P450s and crucial for substrate contacting and orientation in the catalytic centre. The amino acid sequences in SRS4, SRS5 and SRS6 are more conserved among different P450 groups and SRS1, SRS2 and SRS3 show higher variations [38]. So far it was shown that amino acid at the position 8 of SRS6 is crucial for the enzyme to have 3' or 3' and 5'-hydroxylation activity [38, 39]. It was presented that most F3'H have threonine at this position in contrast to alanine and serine in F3'5'H [38]. In gerbera F3'H, an exchange of just one amino acid at position 8 of SRS6 mediated a slightly elevated F3'5'H activity into F3'H [38]. It was proposed that during the enzymatic reaction in the catalytic centre, the carbon on the 3' position is hydroxylated at first, followed by a slight rotation of the B ring and subsequent hydroxylation on the 5' position. This explains why the presence of the 'correct' amino acid in the catalytic centre is so important for the determination of what kind of hydroxylation the protein can perform [38]. It was suggested that F3'5'H evolved from F3'H in order to attract different groups of pollinators, in particular insects [41].

1.1.5 DFR

Dihydroflavonol 4-reductase (DFR) is another enzyme that has a huge impact on flower colour. DFR (EC 1.1.1.219) is an oxidoreductase that catalyses the NADPH dependent reduction of the keto group in position 4 of dihydroflavonols (DHK, DHQ and DHM), which leads to the production of leucoanthocyanidins (leucopelargonidin, leucocyanidin and leucodelphinidin) and subsequently to anthocyanidins (pelargonidin, cyanidin and delphinidin, respectively). This enzyme has influence on the synthesis of three flavonoid classes: flavonols, flavanols and anthocyanins [42]. The ability to convert dihydroflavonols independently of their hydroxylation pattern is necessary for obtaining three main groups of anthocyanins. Most of the DFRs accept all dihydroflavonols, but there are also some exceptions. Ornamental plants like petunia and *Cymbidium hybrida* do not produce orange/red flowers due to the lack of pelargonidin accumulation, which is not synthesised because the DFR present in this species does not accept DHK as a substrate [43, 44]. The molecular mechanism of DFR substrate specificity is not yet fully understood. Looking on the sequence alignment [45] and by constructing protein chimeras [46], a stretch of 26 amino acid sequence between amino acid 131 and 156 was identified as being putatively responsible for substrate specificity of the protein, which was also confirmed after obtaining the crystal structure [47]. Especially amino acids at the positions that differ in the specific petunia DFR compared to not-specific DFRs, like position 133 (L in petunia, V in others), 134 (D in petunia, N in others), 143 (F in petunia Y in others) and 146 (Q in petunia, E in others) (numbering from petunia) [46]. It was shown

that the amino acid in position 134 is important for substrate specificity of DFR. Exchange of asparagine in this position in unspecific gerbera DFR to leucin created a DFR with a preference for DHK, albeit with low activity [46]. The importance of the amino acid in position 133 (numbering from grape, correspond to position 134 in petunia) for DFR substrate specificity was also confirmed by structural data, but it was also indicated that it's probably not the only amino acid that determines the lowering or inhibition of DHK reduction [47].

1.1.6 Breeding for orange flower colour

Pelargonidin-type anthocyanins are the source of orange to bright red coloured flowers. High concentration of pelargonidin particularly occurs when the activity of FLS, F3'H and F3'5'H is low and a DFR is present that accepts DHK as a substrate. These conditions do not occur naturally in all species, therefore, genetic engineering is a helpful tool in obtaining flowers with orange/bright-red colour.

Petunia is probably the most popular species that lacks orange flowers. This was overcome very early, in the 1980s, by overexpression of the maize *DFR* (the *A1* gene) in the petunia cultivar RL01, which lacks F3'H and F3'5'H activity. This represented the first successful transgenic plant with altered flower colour [48]. Orange flowering petunias were also obtained, when gerbera *DFR* was overexpressed in cultivar RL01 [49]. Orange flowers were obtained when commercial cultivars were used for transformation, but overexpression of rose *DFR* had to be correlated with *F3'H* silencing by RNAi [50]. Pelargonidin accumulation in tobacco flowers was obtained when *FLS* and *F3'H* were supressed by RNAi while gerbera *DFR* was simultaneously overexpressed [51]. In violet flowering torenia, flowers changed their colour to pink, when *F3'H* and *F3'5'H* were downregulated, and pelargonidin was detected in petals. This effect was stronger when *DFR* from rose or pelargonium was additionally overexpressed, which suggests that even in plants in which DFR accepts DHK, better effects can be obtained, if *DFR* with high DHK affinity is overexpressed [52]. In *Osteospermum*, which accumulates mainly delphinidin-type anthocyanins, overexpression of gerbera or strawberry *DFR* did not result in pelargonidin accumulation in flowers but additional silencing of *F3'5'H* was necessary to obtain flowers with altered flower colour [53].

1.1.7 Breeding for blue flower colour

Delphinidin based pigments are most often essential for blue flower colour. Nevertheless, the mechanism of blue colour formation is more complex and other factors than only the presence of delphinidin can be necessary. Other very important factors for blue colour formation are the

pH in the vacuoles or the formation of complexes of anthocyanins with co-pigments and metal ions [54]. Such economically important ornamentals as roses, carnations, chrysanthemums and poinsettia lack delphinidin-type pigments, and as a result lack blue flower colour. Classical breeding approaches in the cases of these species did not result in blue cultivars, therefore, genetic engineering is a promising option for obtaining desired flower colour.

The first blue flowering carnations were obtained by overexpression of petunia *F3'5'H* in a pelargonidin producing carnation cultivar. As a result, 70% of total anthocyanidins were delphinidin derivatives, but that caused only slight colour change towards blue [55]. In the next attempt petunia *F3'5'H* was used again, but together with petunia *DFR* in a DFR deficient carnation cultivar. That resulted in a cultivar that almost exclusively accumulated delphinidin and showed a significant change of its flower colour towards blue. This first transgenic cultivar, cv. 'Moondust', was commercialised by Florigene (Melbourne, Australia) and started the whole 'Moon series' [56]. In another transformation event, pansy *F3'5'H* and petunia *DFR* were used. As a result, plants containing higher amounts of delphinidin and with a more intense violet colour were obtained (cv. 'Moonshadow') [56]. Today, the Florigene Moon series includes many more members in different shades of violet. Plants are sold in North America, Japan, Australia and Russia[2].

A blue flowering cultivar of rose was always desired by breeders and consumers. Obtaining such a cultivar was, however, difficult even when using genetic engineering. Overexpression of viola *F3'5'H* resulted in the presence of delphinidin in petals, but colour change was not observed due to a presence of pelargonidin and cyanidin [57]. The cultivar used for transformation had to show specific traits, such as higher vacuolar pH, low F3'H activity and accumulation of flavonols that can be co-pigments. To obtain plants with more intensive blue colouration, it was necessary to overexpress viola *F3'5'H* together with iris *DFR,* while simultaneously silencing the endogenous rose *DFR*. In all obtained transgenic lines, more than 80% of delphinidin was obtained, and a significant shift of flower colour towards blue was observed [58]. A blue rose cultivar was commercialised by SUNTORY under the commercial name APPLAUS in 2009[3].

There have been a few attempts to obtain blue chrysanthemum. In the first, campanula *F3'5'H* was overexpressed, which resulted in a relative anthocyanidin content of up to 80% delphinidin

[2]https://www.florigene.com/product/

[3]https://www.suntory.com/sic/research/s_bluerose/story/

in transgenic flowers and a shift of flower colour towards violet/blue [59]. In another approach, pansy *F3'5'H* was overexpressed and endogenous *F3'H* was silenced by RNAi. As a result, 80% of accumulated anthocyanidins in the transgenic flowers were delphinidin and a shift of the colour towards violet/blue was observed [60]. In the third attempt, canterbury bells *F3'5'H* was overexpressed together with butterfly pea UDP (uridine diphosphate) glucose:anthocyanin 3'5'-glucosyltransferase (*A3'5'AT*) and silencing of chrysanthemum *F3'H* by RNAi. That resulted in obtaining the first truly blue chrysanthemum. In this case the *A3'5'AT* gene responsible for anthocyanin decoration was the key for obtaining true blue flowers, in comparison with previous attempts, in which the obtained flowers were more violet. By molecular stacking of decorated delphinidin with flavones, the colour of the flower became more blue [61].

Breeding blue flower is often a complex process where a lot of aspects need to be considered. Sasaki (2015) suggested in his review a few bullet points that should be considered during planning a strategy for breeding blue. First, the production of pelargonidin and cyanidin in the modified plant needs to be limited by silencing endogenous *F3'H* and *DFR*. Secondly, delphinidin synthesis might be supported by overexpression of DHM preferring *DFR* (like petunia *DFR*). As a last point, it was suggested to select a cultivar with a suitable background, like a higher vacuolar pH or higher concentrations of co-pigments [62]. Other aspects, like choosing a suitable source of *F3'5'H* and a suitable promoter that ensures strong expression of the transgene, are also important [59]. Overexpression of genes responsible for anthocyanidin decoration seems to be a promising approach in obtaining true blue colour, as modified anthocyanins show more stable colour, especially when they are associated with co-pigments [61].

1.1.8 Genome editing

Genome editing allows introduction of desired changes into organisms in a controlled and efficient fashion. Previously, mutations were introduced by classical breeding approaches like hybridization, radiation or chemical agent treatment, which did not always result in desired phenotypes or demanded a lot of effort [63]. Application of sequence-specific nucleases allows targeted gene modification, by introduction of double strand breaks (DSBs) and in result, introduction of deletions, insertions or substitutions in desired genome locations during repair process. This opens new possibilities for molecular breeding. First genome editing approaches utilised zinc finger nucleases (ZFNs), meganucleases and transcription activation-like effectors

nucleases (TALEN). Unfortunately working with them is very laborious and expensive, thus they were not that widely used [64].

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 (CRISPR associated protein) emerged from an adaptive phage immunity system in archaea and bacteria. Typical CRISPR loci include clustered sets of CRISPR associated genes (Cas) and series of repeated sequences (direct repeats), interspaced by variable sequences (spacers). There are three different classes of Cas system (types I-III) [65]. In type I and III CRISPR, loci contain multiple Cas proteins. In type II, a reduced number of Cas proteins is present and only Cas9 performs targeted DNA cleavage [66]. Other components of type II CRISPR are CRISPR RNA (crRNA) and noncoding trans activating crRNA (tracrRNA) that hybridize with crRNA to facilitate RNA guided targeting of Cas9 [67]. In 2013, first reports were published about the successful application of type II CRISPR for genome editing in mammalian cells [68, 69]. In the same year CRISPR/Cas9 systems were also used for plant genome modifications [70-72].

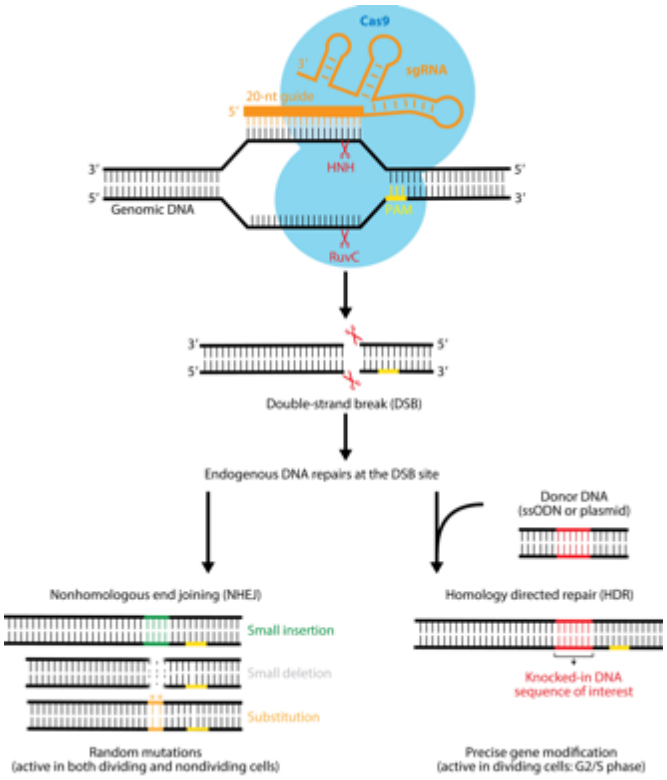


Fig. 6 Schematic representation of CRISPR/Cas9 system [73]

The CRISPR/Cas9 genome editing approach was developed based on the *Streptococcus pyogenes* type II CRISPR/Cas9 system (SpCas9 system) (Fig 6). It is an easy to design, efficient and cheap genome editing system, which very quickly gained popularity. The crRNA and tracrRNA are fused into single-guide RNA (sgRNA), that is complementary to a specific part of the targeted DNA sequence (approximately 20 nucleotides) and contains the sequence

necessary for Cas9 binding. sgRNA guides Cas9, but the protospacer adjacent motif (PAM) is also necessary for recognition and binding of the complex to the targeted sequence. The PAM sequence (*S. pyogenes* system NGG) is located downstream of the targeted side. Cas9 performs DSBs between the 3rd and 4th nucleotides upstream of the PAM motif [74]. After the cut, natural repair mechanisms of the cell repair the break by non-homologous end joining (NHEJ) or homology directed repair (HDR) [75]. During NHEJ, random insertions or deletions are very often introduced into the modified sequence, which commonly leads to pre-mature termination of protein and loss of the protein function [76]. HDR allows introduction of specific insertions and deletions and also nucleotide substitutions, but it requires the additional delivery of a template that allows the introduction of desired changes. In plant genome editing, DSB is most often repaired by the NHEJ, due to difficulties in the delivery of the template sequence. Repair by HDR is much more complicated, but was so far successfully performed in tomato and rice [77, 78]. Interesting alternatives for the introduction of specific mutations are base editors and prime editing. Base editors can be used for precise single nucleotide substitution, successfully used in rice [79, 80]. Prime editing allows the introduction of point mutations, insertions and deletions without DSB and delivery of a donor template. So far this approach was tested in rice and wheat, but unfortunately, editing efficiency was not very high [81, 82].

Genome editing with application of CRISPR/Cas9 was also successfully used in ornamentals. Usually, Cas9 and sgRNA are delivered to the plant by *Agrobacterium*-mediated transformation. The first genome edited ornamental plant was petunia. A phytoene desaturase (Ph*PDS*) gene, necessary for the synthesis of chlorophyll, was targeted and as a result, an albino phenotype was obtained in around 55-87 % of the regenerated shoots [83]. Two other genes were also targeted in petunia, nitrate reductase (Ph*NR*) and 1-aminocyclopropane-1-caroxylate oxidase (Ph*ACO1*), an enzyme of the ethylene biosynthesis, but in this case preassembled purified Cas9-sgRNA ribonucleoprotein was directly delivered into petunia protoplasts [84, 85].

The first modification of the flower colour trait was performed in *Ipomoea nil*. Anthocyanin formation was blocked by targeting *DFR*, resulting transgenic plants with a biallelic mutation that changed flower colour to white [86]. The second modification was performed in the same plant species. In this approach, *carotenoid cleave dioxygenase 4* (*CCD4*) was targeted, resulting in elevated carotenoid levels and flowers that changed their colour to pale yellow [87]. In torenia (*Torenia fournieri*), *FHT* was silenced with CRISPR/Cas9, which resulted in flower colour change from pale blue to white in around 80 % of the transformed plants [88].

Another successful modification was performed in Japanese gentian where three anthocyanin modification genes were knocked out and, as a result, flower colour changed from blue to violet, pink and mauve [89]. In the same species, *glutathione S-transferase* 1 (*GST*1) was targeted, which resulted in the flower colour change from blue to white and confirmed the gene as being responsible for anthocyanin transport to vacuoles [90]. Recently, flower colour in petunia was also modified with CRISPR/Cas9. *FHT* was silenced in the cultivar 'Madness Midnight', which resulted in a flower colour change from blue to pale pink [91].

Genome editing indisputably revolutionized molecular breeding. Its biggest advantage is the possibility of a precise modification of the targeted gene and obtaining the desired phenotype in an easier way than conventional breeding. What is also very important is the possibility to obtain modified, but not-transgenic plants, albeit depending on the legislation. When a desired edition occurred, there is the possibility to cross the T-DNA out. In many countries, those plants are not generally considered as transgenic plants, because they do not contain foreign DNA [86, 92]. This is the great advantage of CRISPR/Cas9 over other transgenic approaches.

Of course, there are also some limitations that should be considered. First of all, for many ornamental plants, genome sequences are currently unavailable, which can cause difficulties in designing the specific sgRNA and makes it impossible to predict off-target activity [93]. In addition, ornamental plants often have a high ploidy level, so the mutations have to occur in all gene copies to obtain the desired phenotype. Another characteristic of ornamentals is that they often have a huge genome size, which also increases the probability of off-target effects [94]. The last important point that needs to be considered is the necessity for an efficient method of delivery of CRISPR/Cas9 components into plant cells. Transformation protocols need to be optimized for each species, and very often for each cultivar, in order to obtain high transformation and edit efficiency [93]. Nevertheless, all those drawbacks are surmountable and genome editing is currently the most promising tool for improvement of ornamental plants available and undoubtedly is the future of modern breeding.

1.2 Goals of the thesis

Traditionally, poinsettia (*Euphorbia pulcherrima*) bracts show a bright red colouration, which is a result of the accumulation of cyanidin-type anthocyanins. Blue colouration, which is mediated by delphinidin, has not been reported so far, although traces of delphinidin appear to be present. Orange-red colouration, which is typically caused by pelargonidin-type anthocyanins, is rare in poinsettia bracts, but can be found in a few cultivars. Currently, commercial poinsettia breeding programmes involve classical crossing to obtain new varieties and radiation mutation to introduce new colour types to existing varieties.

This thesis was embedded in the frame of the Marie Sklodowska Curie Actions ITN FlowerPower 675657, and was part of the biotechnological breeding approach. The main aim was to apply genome editing for the first time in poinsettia to create novel bract colouration. In case that a genome editing approach was not feasible, other biotechnological approaches were to be used. The work was to be structured in three steps:

1. Investigation of the physiological background of pigment formation in the model plant *Petunia* x *hybrida*, of which delphinidin, cyanidin and also a few pelargonidin based varieties are known, to understand the physiological background of colour formation
2. Investigation of the physiological background of pigment formation in red and orange poinsettia to establish the strategy for breeding blue and orange flowering varieties.
3. Applying molecular techniques for breeding novel bract colouration in poinsettia. If possible, genome editing by CRISPR/Cas9 was to be used as a minimally invasive technique, which would, in large parts of the world, not be considered genetic modification, and thus could be exploited without additional registration requirements.

1.3 Methodology

1.3.1 Materials

1.3.1.1 List of chemicals used in this study

Name	*Company*
Acetic acid	VWR
Acetonitrile	VWR
Ammonium nitrate	Roth
Ampicillin	Duchefa
Agar-Agar	Roth
Agarose	VWR
*Bbs*I	New England Biolabs
*Bam*HI	New England Biolabs
6-Benzylaminopurine	Duchefa
Boric acid	Roth
Calcium chloride	Roth
4-chlorophenoxy acetic acid	Duchefa
Cefotaxim	Duchefa
Cobalt chloride hexahydrate	Roth
Copper sulphate pentahydrate	Roth
Cyanidin	Extrasynthese
DC-Cellulose plates (20x20 cm)	Merck
Delphinidin	Extrasynthese
D(+)-Glucose-Monohydrate	Merck
D-Galactose	AppliChem Panreac
Diammonium sulphate	Calbiochem
Dihydrokaempferol	Sigma-Aldrich
Dihydromyricetin	Extrasynthese
Dihydroquercetin	Extrasynthese
D-Sorbitol	Sigma-Aldrich
dNTPs	Thermo Fisher Scientific
DNA Stain Clear G	Serva
*Dra*I	New England Biolabs

The list is continued on the next page

*Eco*RI	New England Biolabs
*Eco*RV	New England Biolabs
Ferrous sulphate hexahydrate	Sigma-Aldrich
Ethyl acetate	Merck
Ethylenediaminetetraacetic acid	Sigma-Aldrich
Ethylenediaminetetraacetic acid ferric sodium	Sigma-Aldrich
Expand High Fidelity PCR System	Roche
Formic acid	VWR
Gentamicin	Duchefa
Glass beads (acid washed)	Sigma-Aldrich
Glycerine 85%	Merck
Glycine	Roth
Go*Taq* DNA-Polymerase	Promega
5 x Green Go*Taq* Reaction Buffer	Promega
Hydrochloric acid	Merck
Isopentenyl adenine	Duchefa
Isopropanol	Merck
Kaempferol	Extrasynthese
Kanamycin sulphate	Duchefa
L-Tryptophan	Sigma-Aldrich
Luna® Universal qPCR Master Mix	New England Biolabs
LR Clonase II Enzyme Mix	Thermo Fisher Scientific
Magnesium sulphate	Roth
Malvidin	Extrasynthese
Methanol	VWR
2-Mercaptoethanol	Sigma-Aldrich
Myo-Inositol	Roth
Myricetin	Extrasynthese
NADPH	Boehringer Mannheim
1-Naphthaleneacetic acid	Duchefa
Naringinase	Sigma-Aldrich
Nicotinic Acid	Roth

The list is continued on the next page

One Shot TOP10- Chemically Competent Cells	Invitrogen
p-Coumaroyl-CoA	TransMIT
Pelargonidin	Extrasynthese
Peonidin	Extrasynthese
Petunidin	Extrasynthese
Pepton	Merck
PEG 4,000	Sigma-Aldrich
Polyclar AT	Serva
Potassium chloride	Roth
Monopotassium phosphate	Roth
Dipotassium phosphate	Merck
Potassium hydroxide	Roth
Potassium iodide	Roth
Potassium nitrate	Roth
*Pvu*II	New England Biolabs
Pyridoxine	Duchefa
Q5 Hot Start High-Fidelity DNA Polymerase	New England Biolabs
Quartz sand	Sigma-Aldrich
Quercetin	Extrasynthese
RevertAid H Minus RT	Thermo Scientific
Rifampicin	Duchefa
SDS	Sigma-Aldrich
Sephadex G25	GE Healthcare
Sodium ascorbate	Sigma-Aldrich
Sodium chloride	Roth
Tri-sodium citrate dehydrate	Roth
Sodium hypochlorite	Roth
Sodium hydroxide	Roth
Sodium molybdate dihydrate	Duchefa
Spectinomycin	Duchefa
StarGate Competent Cells E. coli TOP10	Iba
Sucrose	Roth

The list is continued on the next page

*Stu*I	New England Biolabs
T4 DNA Ligase	New England Biolabs
T4 DNA Ligase 10 x Buffer	New England Biolabs
Thiamine	Sigma-Aldrich
Timentin	Duchefa
Tris(hydroxymethyl)aminomethane	Merck
Tryptone	Fluka Analytical
Tween 20	Roth
Yeast Extract	Roth
Yeast Nitrogen Base without amino acids	Sigma-Aldrich
Zinc sulphate heptahydrate	Roth

1.3.1.2 List of equipment

Device	***Company***
Allegra™21 R Centrifuge	Beckmann
Autoclave Systec V-55	Systec
Avanti® J-26xP Centrifuge	Beckmann Coulter™
Balance PJ 3000	Mettler
Balance Research R 160 P	Sartorius Research
Berthold LB 2842 Linear Analyzer	Berthold
Certomat® BS-1	Braun Biotech Int.
Centrifuge Sigma 1-14	Sigma GmbH
Centrifuge Sigma 2-16 K	Sigma GmbH
DU 800 UV/Vis Spectrophotometer	Beckman Coulter
Electrophoresis Power Supply (EPS600)	Pharmacia
Electrophoresis System MSMINIDUO	Sigma-Aldrich
Exsikkator	Glaswerk Wertheim
Heating block (QBT2)	Grant
HPLC UltiMate 3000	Thermo Fisher Scientific
Incubator Function Line T12	Heraeus
Incubator Heratherm IGS60	Thermo Scientific
Incubator Certomat BS-1	Sartorius AG
Laminar TL2448	Holten

The list is continued on the next page

Laminar Heraguard ECO	Thermo Scientific
MicroPulser Electroporator	Bio-Rad
Microprocessor pH/ION Meter pMX 3000	WTW
PCR Mastercycler ep-gradient	Eppendorf
Spectrostar Nano	BMG Labtech
StepOne™ Real-Time PCR System	Thermo Fisher Scientific
Thermomixer Comfort	Eppendorf
Typhoon 5 Biomolecular Imager	Amersham
UV-working bench Reprostar II	Camag
Vacuum pump Laboport	KNF Neuberger, Inc.
Vortex-Genie 2	Scientific Industries
Water bath MP-PA/KÜ	Julabo

1.3.1.3 List of kits used in this study

Name	**Company**
DNeasy plant mini kit	Qiagen
GenElute Plasmid Mini Prep Kit	Sigma-Aldrich
GenomeWalker Universal Kit	Clontech Takara
InVisorb Plant mini kit	Stratec
µMACS mRNA isolation Kit	Miltenyi Biotec
mirPremier Kit	Sigma-Aldrich
NEBNext Multiplex Oligos for Illumina (Index Primer Set 1)	New England Biolabs
NEBNext Ultra II DNA Library Prep kit for Illumina	New England Biolabs
Q5 Site-Directed Mutagenesis Kit	New England Biolabs
SMARTer RACE 5′/3′ Kit	Clontech Takara
Sc. EasyComp Transformation Kit	Invitrogen
StarGate Combi Entry Reagent Set	Iba
StarGate Transfer Reagent Set	Iba
Wizard SV Gel and PCR Clean-up System	Promega

1.3.1.4 List of vectors

Vector	Company
pCR2.1-TOPO	Invitrogen
pCR™8 TOPO. TA	Invitrogen
pDe-Sa_Cas9	Steinert et al. 2015 [97]
pEN-Sa_Chimera	Steinert et al. 2015 [97]
pENTRY-IBA	Iba
pGEX-6P-1	GE Healthcare
p9N-35s	DNA Cloning Service Hamburg
pYES2.1/V5-His-TOPO	Invitrogen
pYSG IBA103	Iba

1.3.1.5 Media

1.3.1.5.1 LB Media

For the cultivation of *E. coli*, an LB medium was prepared, consisting of 10 g/L Peptone, 10 g/L NaCl, 5 g/L Yeast extract, with pH adjusted to 7.0 with 10 N NaOH. For use as solid medium 15 g/L of Agar were added, and for expression use 5 ml of 50 % Glycerol and 1 ml of 1 M $MgSO_4$ were added. The LB medium was autoclaved at 121 °C for 20 minutes and subsequently allowed to cool. Antibiotics were added once a temperature of approximately 65 °C was reached. Antibiotics used include 100 mg/L ampicillin, 100 mg/L spectinomycin, or 50 mg/L kanamycin. The medium was stored at 4 °C until use.

1.3.1.5.2 SOB Media

For the cultivation of *Agrobacterium* strain GV3101, an SOB medium was prepared, consisting of Tryptone 20 g/L, Yeast extract 5 g/L, NaCl 0.5 g/l, KCl 0.186 g/L, $MgCl_2$ 0.952 g/L, $MgSO_4$ 1.2 g/L, with pH adjusted to 7.0 with 10 N NaOH. For use as solid medium 15 g/L of Agar were added. The SOB medium was autoclaved at 121 °C for 20 minutes and subsequently allowed to cool. Antibiotics were added once a temperature of approximately 65 °C was reached. Antibiotics used include spectinomycin 100 mg/L rifampicin 50 mg/L, 30 mg/L gentamicin. The medium was stored at 4 °C until use.

1.3.1.5.3 SOC Media

The SOC medium was used during *E. coli* TOP10 and BL21 Lemo transformation and was provided with the competent cells.

1.3.1.5.4 YEP Media

For the cultivation of *Agrobacterium* strain GV3101, a YEP medium was prepared, consisting of 10 g/L peptone, 5 g/L NaCl, 10 g/L yeast extract, with pH adjusted to 7.0 with 10 N NaOH. For use as solid medium 15 g/L of Agar were added. The YEP medium was autoclaved at 121 °C for 20 minutes and subsequently allowed to cool. Antibiotics were added once a temperature of approximately 65 °C was reached. Antibiotics used include spectinomycin 100 mg/L rifampicin 50 mg/L, 30 mg/L gentamicin. The medium was stored at 4 °C until use.

1.3.1.5.5 SGI Media

For the cultivation of yeast strain INVSc1, an SGI medium was prepared, consisting of Bacto Casamino Acid 1,00 g/L, Yeast Nitrogen Base w/o amino acids 6.70 g/L, L-Tryptophan 0.02 g/L, α -D-Glucose 20.00 g/L (autoclaved separately). For use as solid medium 15 g/L of Agar were added. The SGI medium was autoclaved at 121 °C for 20 minutes and subsequently allowed to cool. The medium was stored at 4 °C until use.

1.3.1.5.6 YPGE Media

For the protein expression in yeast strain INVSc1, a YPGE medium was prepared, consisting of Yeast Extract 10 g/L, Peptone 10 g/L, α -D-Glucose 5 g/L (autoclaved separately), 3 % (v/v) Ethanol (added after autoclaving). For use as solid medium 15 g/L of Agar were added. The YPGE medium was autoclaved at 121 °C for 20 minutes and subsequently allowed to cool. The medium was stored at 4 °C until use.

1.3.1.5.7 Minimal A Media

For the cultivation of *Agrobacterium* strain GV3101, for plant transformation a Minimal A Medium was prepared, consisting of K_2HPO_4 10.5 g/L, KH_2PO_4 4.5 g/L, $(NH_4)_2SO_4$ 1 g/L, $Na_3C_6H_5O_7$ x 2 H_2O 0.52 g/L, glucose 0.1 % (sterile filtrated), MgSO4 x 7 H2O 0. 005 % (autoclave separately), thiamine 0.00025 % (added after autoclaving). The Minimal A Medium was autoclaved at 121 °C for 20 minutes and subsequently allowed to cool. The medium was stored at 4 °C until use.

1.3.1.5.8 MS Media

Media used in the plant tissue culture. Recipe according to Murashige and Skoog (1962) [95]. Media were supplemented with sucrose 30 g/L. pH was adjusted with KOH to 5.7-5.8. For the solid media 7.5 g/L of agar was added. Media were autoclaved at 121 °C for 20 minutes, store at 4 °C. For poinsettia regeneration following MS media modifications were used:

Callus induction media (CIM): MS media supplemented with 0.2 mg/L 4-chlorophenoxy acetic acid (CPA) and 0.2 mg/L and 6-Benzylaminopurine (BAP). Antibiotics: cefotaxim 250 mg/L, timentin 150 mg/L.

Somatic embryo induction media (SEIM) – MS media supplemented with 0.2 mg/L 1-Naphthaleneacetic acid (NAA) and 0.1 mg/L isopentenyl adenine (2ip). Antibiotics: cefotaxim 250 mg/L, timentin 150 mg/L and kanamycin 2.5 mg/L.

Somatic embryo maturation medium (SEMM) – MS medium supplemented with 0.05 mg/L BAP. Antibiotics: cefotaxim 250 mg/L, timentin 150 mg/L and kanamycin 200 or 50 mg/L.

1.3.1.6 Buffers and solutions

1.3.1.6.1 Buffers used during protein expression in yeast

TEK buffer: 50 mM Tris, 1m M EDTA, 100m M KCl

pH was adjusted with 10 N KOH to 7.4.

TES-B* buffer: 50 mM Tris, 1 mM EDTA, 600 mM Sorbitol, 2 mM DTT

pH was adjusted with 10 N KOH to 7.4.

TEG* buffer: 50 mM Tris, 1 mM EDTA, 2m M DTT, 20% Vol. Glycerine

pH was adjusted with 10 N KOH to 7.4.

The buffer was prepared fresh every day, stored at 4 °C until used.

1.3.1.6.2 TAE buffer

TAE buffer was used during gel electrophoresis.

Composition 1 x TAE: 40 mM Tris, 1 mM EDTA, 20 mM acetic acid

Store at room temperature.

1.3.1.6.3 Monopotassium phosphate buffer

Buffer used during enzyme assays.

Composition: 0.1M KH_2PO_4, 0.4% Na-ascorbate

pH was adjusted with 10 N KOH to the desired value (DFR assay pH 6.5, F3'H assay 7.5). The buffer was prepared fresh every day.

1.3.1.6.4 McIlvaine buffer

The McIlvaine buffer was used during enzymatic hydrolysis of HPLC samples.

Composition: 0.1 M Na_2HPO_4, 0.4 % Na-ascorbate

Set up the pH to 4 with citric acid. The buffer was prepared fresh every day.

1.3.1.6.5 Antibiotics stock solutions (x1000)

Ampicillin 100 mg/mL, Kanamycin 100 mg/mL Spectinomycin 100 mg/mL, Gentamycin 100 mg/mL: 1 g of antibiotic was diluted in 10 mL of distilled water, filter sterilised, aliquoted in 2 mL Eppendorf tubes, stored at -20 °C.

Timentin 150 mg/mL: 1.5 g of antibiotic was diluted in 10 mL of distilled water, filter sterilised, aliquoted in 2 mL Eppendorf tubes, stored at -20 °C.

Cefotaxim 250 mg/mL: 2.5 g of antibiotic was diluted in 10 mL of distilled water, filter sterilised, aliquoted in 2 mL Eppendorf tubes, stored at -20 °C.

Rifampicin 50 mg/mL: 0.5 g of antibiotic was diluted in 10 mL of DMSO, aliquoted in 2 mL Eppendorf tubes, stored in -20 °C.

1.3.1.6.6 Plant hormone stock solutions

For all hormones (BAP, CPA, NAA, 2ip), 0.1 mg/mL stock solutions were prepared, by dilutions of 10 mg of hormone in 1-2 mL of 1 M KOH and adding distilled water to the volume 100 mL. All stock solutions were stored at 4 °C.

1.3.1.7 Poinsettia plant material

The analysis was carried out with bracts of the commercially available *Euphorbia pulcherrima* cv. 'Premium Red' (Dümmen Orange GmbH, Germany), cv. 'Christmas Feelings', cv. 'Christmas Beauty', cv. 'Christmas Eve' (Klemm + Sohn GmbH & Co. KG, Germany), and cv. 'Harvest Orange' (Ecke Ranch, USA). The plant material was collected and frozen in liquid nitrogen, and stored at -80 °C until analysis.

Poinsettia *Euphorbia pulcherrima* cv. 'Christmas Eve' (Klemm + Sohn GmbH & Co. KG, Germany) and cv. 'Premium Red' (Dümmen Orange GmbH, Germany) were used for transformation. Plants were cultivated in the greenhouse under long day conditions (16 h day/8 h night). For *Agrobacterium*-mediated transformation, internode stem explants were used. Around 2 - 3 cm internode stem segments were harvested. Surface sterilization was performed by washing excised stems for 10 minutes in 1,5 % solution of sodium hypochlorite with one drop of Tween 20 followed by washing two times for 10 minutes in sterile water. In the next step, internode stems were cut (around 1 mm thick discs) and placed on callus induction media (CIM – no antibiotics). Explant discs were then used for transformation.

1.3.1.8 Petunia plant material

Analysis was carried out on the flowers of the commercially available petunia cv. 'Salmon Ray' (Danziger, Israel), cv. 'Viva Orange' (Florensis, The Netherlands), cv. 'Electric Orange'

(Klemm + Sohn GmbH & Co. KG, Germany), cv. 'BabyDoll' (Klemm + Sohn GmbH & Co. KG, Germany), cvs. 'Corso Rot', 'Corso Blau' and 'Blackberry' which were purchased from Austrosaat (Vienna, Austria). Furthermore 154 individual crossings of a commercial breeding program were used. Flowers or leaves were shock frozen with liquid nitrogen and kept at -80°C until analysis.

1.3.2. Methods

1.3.2.1. Nucleic acid extraction

Genomic DNA was extracted with InVisorb Plant mini kit (Stratec, Germany) or with DNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's protocols.
Total RNA was extracted by mirPremier Kit (Sigma Aldrich, Austria) according to the manufacturer's protocol. mRNA was extracted from poinsettia bracts with the µMACS mRNA isolation Kit (Miltenyi Biotec, Germany). cDNA was synthesized using the SuperScript II Reverse Transcriptase (Invitrogen, USA) or RevertAid H Minus Reverse Transcriptase (Thermo Scientific, US) and the primer oligo-dT SMART (AAGCAGTGGTATCAACGCAGAGTAC$(T)_{23}$VN).

1.3.2.2 Polymerase Chain Reaction (PCR)

For standard PCR, such as colony PCR, transgenic plants screening or other qualitative PCR, Go*Taq* polymerase (Promega, Germany) was used. The reaction mix contained in a total volume of 20 µL: 4 µL of 5 x Go*Taq* Green Reaction Buffer, 0.4 µL of dNTPs (10 mM), 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 1 µL of template, and 0.2 µL of Go*Taq* polymerase. Reaction conditions: 2 minutes 94 °C initial denaturation, 35 or 40 cycles (94 °C, 30 seconds denaturation; X °C, 30 seconds primer annealing; 72 °C, 1 minute/1 kb extension), 10 minutes, 72 °C final extension. Annealing temperatures depended on the primers used for the reaction. PCR products were separated on 1 % or 2 % agarose gel.
When PCR product was used for cloning or sequence analysis, proof reading Q5 High Fidelity DNA Polymerase (New England Biolabs, Austria) was used. The reaction mix contained in a total volume of 20 µL: 4 µL of 5 x Q5 Reaction Buffer, 0.4 µL of dNTPs (10 mM), 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 1 µL of template, 0.2 µL of Q5 polymerase. PCR program: 98 °C 30 seconds for initial denaturation, (98 °C, 30 seconds denaturation, X °C, 30 seconds primer annealing, 72 °C 30 seconds/1 kb extension) x 30 or 40 cycles, 2 minutes 72 °C final extension. Annealing temperatures depended on the primer used

for the reaction. PCR products were separated on 1 % or 2 % agarose and extracted using Wizard SV Gel and PCR Clean-up System (Promega, Germany).

1.3.2.3 RACE PCR

In order to obtain the full gene sequence RACE (rapid amplification of cDNA ends) PCR was performed. SMARTer RACE 5'/3' kit (Clontech Takara, US) was used according to the manufacturer's protocol.

1.3.2.4 Cloning into pYes2.1/V5-His-TOPO

The pYes2.1/V5-His-TOPO was used for overexpression of protein in yeast. Cloning into the pYes2.1/V5-His-TOPO vector (Invitrogen, US) was performed according to the manufacturer's protocol.

1.3.2.5 Cloning into pCR2.1-TOPO

The pCR2.1-TOPO was used for cloning and sequencing PCR products. Cloning into the pCR2.1-TOPO vector (Invitrogen, US) was performed according to the manufacturer's protocol.

1.3.2.6 Cloning into pYSG-IBA103

The pYSG-IBA103 was used for protein overexpression in yeast. Cloning into the pYSG-IBA103 vector (IBA GmbH, Germany) was performed according to the manufacturer's protocol, with introduction of the gene of interest in the first step in the pENTRY-IBA51 vector (IBA GmbH, Germany).

1.3.2.7 Cloning into pGEX-6P-1

The pGEX-6P-1 vector was used for overexpression of protein in *E. coli.* Cloning into pGEX-6P-1 was performed with application of sticky end PCR [96]. Two PCR products were obtained by performing two reactions with two different primer combination: forward long + reverse short and forward short + reverse long. The two PCR products were analysed on agarose gel, eluted and purified. PCR products from both PCR reactions were mixed in equimolar amounts, denatured and reannealed, resulting in ¼ double stranded product with sticky *Bam*HI (GATC) and *Eco*RI (AATT) sequences at the ends. The PCR product was directly ligated into the linearized pGEX-6P-1 with T4 DNA ligase (New England Biolabs, Austria).

1.3.2.8 sgRNA design and cloning into pDe-Sa_Cas9

Binary vector pDe-Sa_Cas9 [97] was used for poinsettia transformation. sgRNA sequence was designed, with application of online tool CHOPCHOP [98, 99]. A 20 nucleotide long sgRNA sequence, without PAM (TCGGGT), was cloned first into a pEN-Sa_Chimera vector [97]. Primers were annealed by incubation for one hour at 37 °C then heating up the reaction mix to 95 °C and cooling down to 25 °C (cooling rate 0.1 °C per second). The reaction mix contained in 10 µL: 1 µL forward primer (100 µM), 1 µL reverse primer (100 µM), 1 µL 10 x T4 ligase buffer, 0.5 µL T4 Ligase. Subsequently, annealed primers were ligated into the entry vector by incubation overnight at 4 °C. The ligation mix contained in 10 µL: 50 ng of *Bbs*I digested pEN-Sa_Chimera vector, 2 µL of oligo duplex (diluted 1:20), 0.5 µL ATP (10 mM), 0.7 µL 10 x T4 DNA Ligase buffer, 1 µL T4 DNA ligase. After incubation the ligation mix was extracted with chloroform and used for *E. coli* TOP10 bacteria transformation. In the second step a sgRNA expression cassette was transferred to the destination vector (pDe-Sa_Cas9) by Gateway LR reaction (Thermo Fisher Scientific, Germany). The reaction mix contained: 100 ng of the entry clone and 150 ng of the destination vector. The volume was filled up to 8 µL with TE buffer pH 8 then 2 µL of LR Clonase II enzyme mix (Thermo Fisher Scientific, Germany) was added. The reaction was incubated at 25 °C for one hour, stopped with 1 µL of Proteinase K solution and incubation for 10 minutes at 37 °C. Thereafter, 2 µL of the mixture was used for *E. coli* TOP10 bacteria transformation. Cloning success was confirmed by commercial sequencing (Microsynth, Austria).

1.3.2.9 Cloning into p9N-35s

The vector p9n35s was used for overexpression of *F3'5'H* in poinsettia. First, the gene of interest (GOI) was cloned into the entry vector pCR™8 TOPO® TA (Invitrogen, US) according to the manufacturer's protocol. In the next step the GOI was transferred to the destination vector p9N-35s by Gateway LR reaction (Thermo Fisher Scientific). LR reaction was modified due to the fact that entry and acceptor vectors have the same antibiotic resistance gene (spectinomycin). Instead of using the entry vector for the reaction, the gene of interest, flanked by attL1 and attL2, was amplified with M13 primers and then 150 ng of PCR product was used in the LR reaction (Thermo Fisher Scientific, Germany). The LR reaction was performed as described above.

1.3.2.10 Bacteria transformation

E. coli One Shot TOP10 (Invitrogen, US) chemically competent cells were transformed according to the manufacturer's protocol and plated on LB plates with appropriate antibiotics and incubated overnight at 37 °C.

E. coli BL21 Lemo (New England Biolabs, Austria) chemically competent cells were transformed by mixing 100 ng of plasmid with 5 µl of competent cells, incubation for 30 minutes on ice followed by 30 seconds of heat shock at 42 °C and adding 250 µL of SOC medium. After one hour of incubation at 37 °C with shaking, bacteria were plated on the LB media with the appropriate antibiotic and incubated overnight at 37 °C.

Agrobacterium tumefaciens strain GV3101 electrocompetent cells were transformed by mixing 40 µL of competent cells with 100 ng of plasmid. After electroporation (2.5 kV, 25 µF, 400 ohm) 2 mL of YEP medium was added and then transformation mix was incubated for two to four hours at 28 °C with shaking. Next bacteria were plated on the YEP plates with the appropriate antibiotic and incubated for two to four days at 28 °C.

1.3.2.11 Plasmid preparation

The plasmid was extracted from 5 ml of *E. coli* overnight culture with application of GenElute Plasmid Mini Prep Kit (Sigma-Aldrich, Germany) according to the manufacturer's protocol.

1.3.2.12 Yeast transformation

For transformation into yeast cells, the *Sc*. EasyComp Transformation Kit (Invitrogen, USA) was used. 5 µL of competent yeast, strain INVSc1 was mixed with 0.25-0.5 µg of plasmid DNA in 80 µL of Solution III. All components were mixed for one minute and incubated at 30 °C for 1 hour (with mixing every for 15 seconds every15 minutes). Transformation mix was plated on the SGI media and incubated at 30 °C for three to four days.

1.3.2.13 Protein expression in *E. coli*

5 mL of LB medium with the appropriate antibiotic was inoculated with *E. coli* BL21 Lemo caring desired plasmid. This overnight culture was used to inoculate 100 mL of LB media with the appropriate antibiotic that was incubated at 37 °C with shaking. When the culture reached an OD of 0.6, protein production was induced by adding 100 µL of 1mM IPTG. After three hours incubation at 28 °C, cells were harvested by centrifugation at 10,000 x g for 5 minutes. Afterwards, pelleted cells were frozen in liquid nitrogen and stored at -80 °C.

1.3.2.14 Protein expression in yeast

In order to express protein in yeast, 5 mL of SGI media were inoculated with the yeast strain INVSc1 carrying the desired plasmid. After 2-3 days of incubation at 28 °C with shaking (200 rpm), this culture was used for inoculation of 50 mL of SGI medium. After 24 hours incubation at 28 °C with shaking (200 rpm) the OD of the culture was measured and an appropriate volume of culture was used to inoculate 250 ml of YPGE media (OD 2.0 – 25 ml, OD 1.7 – 30 ml, OD 1.4 – 35 ml, OD 1.25 – 40 ml, OD 1.1 – 45 ml, OD ≤ 1 – 50 ml of yeast suspension). The culture was induced with 27 ml of sterile filtrated 20 % D-galactose when the OD was between 0.8 and 1.2. Protein was harvested 12 to 15 hours after induction. Yeast suspension was transferred on ice and centrifuged at 5,000 x g for 10 minutes at 4 °C. The pellet was then resuspended in 27 mL of TEK buffer and incubated for 5 minutes at room temperature. Subsequently it was centrifuged at 4,000 rpm and 4 °C for 5 minutes. Pellets were resuspended in 2.5 mL of TES-B* buffer and 7.5 g of glass beads (acid washed) were added. The cells were lysed by shaking the tube for 20 minutes (30 seconds shaking, 30 seconds cooling on ice). The suspension was washed three times with 5 mL of TES-B* buffer, each time the supernatant was collected. In the next step the supernatant was centrifuged at 16,000 rpm for 10 minutes. In the next step, the supernatant was collected and the volume was adjusted to 25 ml with TES-B* buffer. 0.94 ml of 4 M NaCl and 2.5 g of PEG 4,000 were added to the solution, dissolved and incubated on ice for 15 minutes. Next the solution was centrifuged for 10 minutes at 10,500 rpm and the pellet was washed with 2 mL of TES-B*, following resuspension in 1.5 mL of TEG* buffer. The suspension was homogenised in a glass tissue homogenizer, aliquoted in 100 µL portions, frozen in liquid nitrogen and stored at -80 °C

1.3.2.15 Mutagenesis

Mutagenesis was performed with Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Austria) according to the manufacturer's protocol. Mutagenesis primers were designed with the NEBaseChanger v 1.25 web app provided at http://nebasechanger.neb.com. Success of the mutations were confirmed by sequencing (Microsynth, Austria).

1.3.2.16 Gene expression studies

Gene expression was evaluated by qPCR using the StepOnePlus system (Applied Biosystems, Germany) and the Luna® Universal qPCR Master Mix (New England Biolabs, Austria) according to the manufacturer's protocol. The analysis was performed in three independent replicates and the results were normalized to the reference genes (*actin*, *glyceraldehyde 3-*

phosphate dehydrogenase (GAPDH) or translation elongation factor 1-alpha (*EF1A*) [100] for poinsettia and *actin* or *SAND* [101] for petunia). The relative expression ratio was calculated according to Pfaffl (2004) [102]. Product specificity was confirmed by analysis of melting curves. Primer efficiency was calculated according to Pfaffl (2004) [102].

1.3.2.17 Enzyme preparation

Crude protein extracts from poinsettia bracts or petunia flowers were obtained by using protocol 1, described in Halbwirth (2009) [103]. 1 g of poinsettia bracts or petunia flowers were homogenized with 0.5 g quartz sand and 0.5 g Polyclar AT in 6 mL of 0.1 M monopotassium phosphate buffer (pH 6.5, containing 0.4 % Na ascorbate). Low molecular compounds were removed by passing the crude protein extract preparation through a gel chromatography column (Sephadex G25, GE Healthcare, Germany).

1.3.2.18 Enzyme assay

DFR assays were performed using DHK, DHQ and DHM as substrates. The reaction contained in the final volume of 50 µL: 1–5 µL enzyme preparation, 0.048 nmol (^{14}C)-dihydroflavonol, 0.25 nmol NADPH, and 40–44 µL 0.1 M KH_2PO_4 buffer (pH 6.5 for DHK; 6.25 for DHQ; 5.75 for DHM). The amount of added enzyme was adjusted to ensure the maximum conversion rate of the best substrate at around 50% (linear range of reaction). The reaction mixtures with DHK or DHQ as substrates were incubated at 40 °C for 20 minutes, stopped, and extracted with 70 µL of ethyl acetate. The organic phases were applied to pre-coated thin-layer cellulose plates without fluorescence indication (Merck, Germany) and developed in chloroform/acetic acid/water (10:9:1, v:v:v). Assays with DHM as substrate were incubated at 40 °C for 20 minutes and stopped with 10 µL of 100% acetic acid and 30 µL of methanol. The mixture was chromatographed on 20 cm × 1 cm stripes of paper (Schleicher Schuell, 2041 b, Germany) in chloroform/acetic acid/water (10:9:1, v:v:v). Results were evaluated on a Berthold LB 2842 Linear Analyzer (Berthold, Germany) by integration of the peak areas.

For F3′H assays, the reaction contained in the final volume of 100 µL: 20 µL enzyme preparation (1 µg/µL enzyme), 0.048 nmol (^{14}C)-naringenin or DHK, 0.05 nmol NADPH, and 75 µL 0.1 M KH_2PO_4 buffer pH 7.5. The reaction mixture was incubated at 30 °C for 30 minutes and stopped with 10 µL 100% acetic acid and extracted with 70 µL ethyl acetate. The organic phases were chromatographed on the pre-coated thin-layer cellulose plates without fluorescence indication (Merck, Germany) and developed in chloroform/acetic acid/water

(10:9:1, v:v:v). Results were evaluated on a Berthold LB 2842 Linear Analyzer (Berthold, Germany) by integration of the peak areas.

1.3.2.19 HPLC Analysis

For HPLC analysis, 0.5 g to 1 g plant material was extracted with 1 mL or 1.5 mL 2 M hydrochloric acid in methanol. For anthocyanin composition analysis, 40 µL of the extract was incubated with 160 µL 4 N HCl for 60 minutes at 95 °C. For analysis of other flavonoid content, 20 µL of the extract was digested by 10 U Naringinase (Sigma-Aldrich, Vienna, Austria) and then hydrolysed for 20 minutes at 40 °C in 0.1 M McIlvaine buffer at a pH of 4. After acid or enzymatic hydrolysis, solid compounds were removed by centrifugation and the supernatant was passed through 0.2 µm syringe filters. 4 µL of the filtrated supernatants were used for injection into the HPLC system.

Analysis was performed on a Thermo Scientific Dionex UltiMate® 3000 RSLC System with DAD-3000RS Photodiode Array Detector (Thermo Scientific, Germany) using an Acclaim™ column RSLC 120 C18, 2.2 µm, 120Å, 2.1 × 150 mm (Dionex Bonded Silica Products: No. 071399) operated at 25 °C. For anthocyanidin analysis, elution solvents were (A) 10 % formic acid and (B) 10 % formic acid/22.5 % acetonitrile/22.5 % methanol in water (v/v) using a slightly modified method from Thermo Scientific Application note 281 (gradient: −10 to 0 minute 9 % B, 0–30 minute 9–90 % B, 30–40 minute 90 % B; flow rate 0.2 mL/minute). For other flavonoids, elution solvents were (A) 0.1 % formic acid and (B) 0.1 % formic acid in acetonitrile (gradient: −3 to 0 minute 20 % B, 0–15 minute 20–53 % B, 15–20 minute 53–95 % B; 20–30 minute 95 % B, 31–35 minute 20 % B; flow rate 0.2 mL/minute). Anthocyanidins were detected at 520 nm and other flavonoids at 290 nm. Identification of the compounds was carried out by analysis of their retention times and comparison of UV-VIS spectra from 190 to 800 nm. Peak areas and the standard curves obtained for the standards were used for calculation of the concentration of identified compounds.

1.3.2.20 Phylogenetic analysis of F3′Hs

MUSCLE [104] was used to prepare the alignment of amino acid sequences. The obtained alignment was used for reconstruction of phylogenetic relationships on the JTT matrix-based model [105]. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model. Evolutionary analyses were performed in MEGA7 [106]. Amino acid sequences used for this analysis were *Ep*CB_F3′H (KY273439), *Ep*CF_F3′H (KY273440), *Ep*PR_F3′H

(KY489667), *Ep*HO_F3′H (KY273441), *Arabidopsis thaliana* F3′H (AF271651), *Callistephus chinensis* F3′H (AF313488), *Gentiana triflora* F3′H (AB193313), *Gerbera hybrida* F3′H (ABA64468), *Glycine max* F3′H (AF499731), *Hieracium pilosella* F3′H (DQ319866), *Ipomoea nil* F3′H (AB113264), *Lobelia erinus* F3′H (BAF49324), *Matthiola incana* F3′H (AF313491), *Osteospermum hybrida* F3′H (ABB29899), *Pelargonium hortorum* F3′H (AF315465) *Perilla frutescens* F3′H (AB045593), *Petunia hybrida* F3′H (AF155332), *Torenia hybrida* F3′H AB057673, *Prunus avium* F3′H (ADZ54783), *Jatropha curcas* F3′H (XP_012080364), *Ricinus communis* F3′H (XP002514665), *Vitis vinifera* F3′H (ALP48438), *Camelina sativa* F3′H (XP_010491421), *Vaccinium ashei* F3′H (BAO58432). Flavone synthase (FNSII) sequences: *Glycine max* FNSII (ACV65037), *Medicago truncatula* FNSII (ABC86159), *Dahlia pinnata* FNSII (AGA17938).

1.3.2.21 Transformation and regeneration of poinsettia

The *Agrobacterium* strain GV3101 carrying the pDe-Sa_Cas9 or p9n35s vector were cultivated in SOB media supplemented with 50 mg/L rifampicin, 100 mg/L spectinomycin and 30 mg/L gentamicin for 24 hours with shaking (200 rpm) at 28 °C. Then 5 mL of this culture was used to inoculate 50 mL of Minimal A medium, and bacteria were further cultivated in identical conditions until OD reached around 0.5. The bacteria were used for transformation. Explants were incubated directly with 10 mL of *Agrobacterium* inoculum with addition of three drops of Tween 20 for 30 minutes with gentle shaking and dried on sterile paper, then placed on the CIM media for two days of co-cultivation. Next, discs were washed in sterile water containing antibiotics 250 mg/L cefotaxim and 150 mg/L timentin for 30 minutes and dried on sterile paper, then placed on the CIM supplemented with 250 mg/L cefotaxim and 150 mg/L timentin for callus induction. After 21 days on CIM, explants that produced calli were transferred to somatic embryo induction media (SEIM) supplemented with 250 mg/L cefotaxim, 150 mg/L timentin and 2.5 mg/L kanamycin. After 3 to 6 weeks, when somatic embryos started to appear, explants were transferred to somatic embryo maturation medium (SEMM) supplemented with 250 mg/L cefotaxim, 150 mg/L timentina and 200 or 50 mg/L kanamycin for further cultivation and selection of transgenic plants. Fully regenerated plants were transferred on MS media without antibiotics for further cultivation and propagation. In the next step transgenic plants were transferred to the greenhouse and cultivated under short-day conditions (11 h day/13 h night) for the development of bract colour.

1.3.2.22 Sequence analysis by NGS

For sequence analysis with application of Next Generation Sequencing (NGS), genomic DNA from poinsettia leaves was extracted using DNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's protocol. Amplicons were obtained by amplification of *F3'H* fragments with EpF3'HpYes-F and EpF3'H-crispr-R primers and Q5 High Fidelity DNA Polymerase (New England Biolabs, Austria). PCR program: 30 seconds 98 °C for initial denaturation, (98 °C 30 seconds denaturation, 64 °C 30 seconds primer annealing, 72 °C 30 seconds extension) x 30 cycles, 2 minutes 72 °C final extension. PCR products were separated on 1 % agarose and extracted using Wizard SV Gel and PCR Clean-up System (Promega, Germany). Construction of Sub-libraries was performed with application of NEBnext Ultra II DNA Library Prep kit for Illumina (New England Biolabs, Austria) according to manufacturer's protocol. NEBnext Multiplex Oligos for Illumina (Index Primer Set 1) (New England Biolabs, Austria) was used for indexing. Concentration of sub-libraries was measured with Qubit (Invitrogen, USA). Sub-libraries were pooled in order to obtain a bulked library. Concentration of the bulked library was controlled with Qubit and Fragment Analyser (Agilent, USA), followed by library denaturation and dilution to 8 pM according to 'MiSeq System Denature and Dilute Libraries Guide' available at https://support.illumina.com. As a control, PhiX (Illumina, USA) was spiked into the bulked library in a concentration of 1.3 %. Sequencing was performed on Illumina MiSeq system, with the application of the MiSeq v2 Reagent Kit 300 cycles (Illumina, USA) (2 x 150 reads). Sequencing results were analysed with CRISPResso2 [107].

1.3.2.23 Genome walking

To obtain junctions between T-DNA and petunia DNA in transgenic petunia cv. 'Viva Orange', the Genome Walker Universal Kit (Clontech Takara, USA) was used according to the manufacturer's protocol. Four GenomeWalker DNA libraries were constructed using restriction enzymes: *Dra*I, *Eco*RV, *Pvu*II and *Stu*I.

1.4 Brief summaries of publications and own contribution

Publication 1

C. Haselmair-Gosch, S. Miosic, **D. Nitarska**, B.L Roth, B. Walliser, R. Paltram, R.C Lucaciu, L. Eidenberger, T. Rattei, K. Olbricht, K. Stich, and H. Halbwirth (2018) Great cause—small effect: undeclared genetically engineered Orange Petunias Harbor an inefficient Dihydroflavonol 4-Reductase. *Frontiers in plant science*, *9*, p.149. doi.org/10.3389/fpls.2018.00149

Summary

In this publication, we investigated orange petunia cultivars, which just had turned out to be genetically modified and had unrecognized contaminated the global petunia market. The source of the transgene in the three independent orange petunia cultivars was identified as a construct that was used by Meyer in 1980s for the creation of a genetically modified petunia for scientific purpose. It was shown that similar to common red and blue petunia cultivars and in contrast to expectations, enzyme preparations obtained from GM orange petunias, were not able to convert DHK. In addition, expression of the exogenous *DFR* was much lower comparing to petunia *DFR*. The molecular background of orange colour formation in petunia was elucidated. It was shown that a special genetic background is essential for the establishment of pelargonidin formation in GM petunias. When the activity of FLS and B ring hydroxylating enzymes is not present or significantly reduced, even DFR with low DHK specificity is sufficient to obtain pelargonidin accumulation.

Own contribution

C. Haselmair-Gosch, K. Stich and H. Halbwirth designed the study. I analysed qPCR data (B.L Roth and B. Walliser), transcriptome data (R.C. Lucaciu and T. Rattei), enzyme activities (S. Miosic), data from HPLC analysis (R. Paltram), and derived from the sum of these data the rare genetic background required for the establishment of orange colour in petunia. I performed sequence analysis together with C. Haselmair-Gosch and supported C. Haselmair-Gosch and H. Halbwirth in manuscript preparation.

Publication 2

D. Nitarska, C. Stefanini, C. Haselmair-Gosch, S. Miosic, B. Walliser, M. Mikulic-Petkovsek, I. Regos, A. Slatnar, T. Debener, D. Terefe-Ayana, V. Vilperte, J. Hadersdorfer, K. Stich and H. Halbwirth (2018) The rare orange-red colored *Euphorbia pulcherrima* cultivar 'Harvest Orange'shows a nonsense mutation in a flavonoid 3'-hydroxylase allele expressed in the bracts. *BMC plant biology*, *18*(1), pp.1-12. doi.org/10.1186/s12870-018-1424-0

Summary

In this publication, the molecular background of colour formation in red and orange-red cultivars of poinsettia was investigated. It was shown that red cultivars prevalently accumulate cyanidin-type anthocyanins, whereas orange-red cultivars accumulate large amounts of pelargonidin-type anthocyanins. The *F3'H* and *DFR* genes were isolated from poinsettia and the substrate specificity of the encoded enzymes was investigated. It was shown that poinsettia DFR prefers DHQ and DHM over DHK, which explains why red cultivars are most common among poinsettias. During this work it was demonstrated that a mutation in the *F3'H* sequence in the orange-red cultivar 'Harvest Orange' causes a shift in the open reading frame, which results in premature protein termination and non-functional protein. These results showed that in the absence of F3'H activity, poinsettia can accumulate pelargonidin-type anthocyanins and defined F3'H silencing as promising strategy for orange poinsettia breeding.

Own contribution

H. Halbwirth, K. Stich and T. Debener designed the study. I cloned genes responsible for anthocyanin formation in poinsettia and performed enzyme assays (with support from C. Haselmair-Gosch, S. Miosic and B. Walliser), and wrote the manuscript together with H. Halbwirth. C. Stefanini, M. Mikulic-Petkovsek, I. Regos and A. Slatnar provided HPLC data. D. Terefe-Ayana and V. Vilperte, under the supervision of T. Debener, performed the bioinformatic analysis.

Publication 3

D. Nitarska, R. Boehm, T. Debener, R.C. Lucaciu and H. Halbwirth (2021) First genome edited poinsettias: Breeding for orange bract colour by application of CRISPR/Cas9. *Plant Cell, Tissue and Organ Culture (PCTOC),* submitted

Summary

In this work, the poinsettia *F3'H* gene was silenced with application of CRISPR/Cas9 to obtain a cultivar that accumulates prevalently pelargonidin-type anthocyanins. A construct carrying *Cas9* and sgRNA was introduced into poinsettia by *Agrobacterium*-mediated transformation. Three transgenic lines were obtained. Plants propagated from one of the lines showed a change of bract colouration from vivid red to vivid reddish-orange. The cyanidin level in the genome edited plants was significantly lower in comparison to the parent cultivar. NGS analysis revealed that orange flowering plants contained mutated *F3'H* variant, but the original, unchanged *F3'H* was also present. Cloning and heterologous expression of the three *F3'H* variants expressed in genome-edited line revealed that the mutated *F3'Hs* encoded non-functional proteins. This work is a proof-of-concept for the hypothesis that silencing of *F3'H*

is a good strategy for breeding orange poinsettia and also demonstrated that even if F3'H activity was not eliminated, completely significant change of colour towards orange can be observed. Here was presented first successful application of genome editing in poinsettia breeding.

Own contribution

I designed the study together with R. Boehm, T. Debener and H. Halbwirth. I performed plant transformation and regenerated plant analysis, and wrote the manuscript with support from H. Halbwirth. R.C Lucaciu performed the bioinformatic analysis.

Publication 4

D. Nitarska, R. Boehm, T. Debener, H. Halbwirth (2021) Molecular breeding for blue poinsettia. Draft.

Summary

In this work, the possibility of breeding blue poinsettias with application of genome editing approach was evaluated. First it was tested if an exchange of amino acids in the *F3'H* could introduce the F3'5'H activity, but obtained mutated proteins did not show expected F3'5'H activity. This showed that currently *F3'H* modification is not a suitable strategy for breeding blue poinsettia by genome editing approach. Therefore, *Cyclamen persicum F3'5'H* was introduced into poinsettia by *Agrobacterium*-mediated transformation. Only one transgenic line with significantly higher delphinidin amount was obtained. However, because of the high level of cyanidin and pelargonidin-type anthocyanins there was no change in the colour of the poinsettia bracts. That was first successful attempt to introduce exogenous F3'5'H into poinsettia. Nevertheless, further modifications are necessary to obtain expected colour change towards blue.

Own contribution

I designed the study together with R. Boehm, T. Debener and H. Halbwirth. I performed mutations, enzyme assays, plant transformations and regenerated plants analysis, and wrote the manuscript with support from H. Halbwirth.

Publication 5

C. Haselmair-Gosch*, D. Nitarska*, B. Walliser, H. Flachowsky, S. Marinovic and H. Halbwirth (2020) Event-specific qualitative polymerase chain reaction analysis for two T-DNA copies in genetically modified orange Petunia. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *142*(2), pp.415-424. * shared co-first authorship
doi.org/10.1007/s11240-020-01871-w

Summary

In this publication, we investigated the escaped orange petunias as follow-up study to publication 1. It was shown that most likely two T-DNA copies are present in genetically modified orange petunia cultivars. The junctions between T-DNA and petunia genome were identified by performing genome walking. For the T-DNA1, the 3' junction was isolated whereas the 5' junction sequence could not be obtained. For the T-DNA2, only the 5' junction could be identified. Event specific PCR primers were designed and tested with 126 GM offspring from commercial breeding program. All investigated plants carried both T-DNAs copies, which underpins that most likely a single transgenic line was the source of all escaped genetically modified orange petunias.

Own contribution

C. Haselmair-Gosch, H. Flachowsky and H. Halbwirth designed the study. I performed genome walking experiments and sequence analysis together with C. Haselmair-Gosch and B. Walliser, and established the event-specific PCR assay together with C. Haselmair-Gosch. I performed screening of 126 petunia cultivars and analyzed the data, and supported C. Haselmair-Gosch, H. Flachowsky and H. Halbwirth in manuscript preparation.

1.5 Scientific contribution of the PhD thesis

Poinsettia (*Euphorbia pulcherrima*) is one of the most important elements of Christmas all over the world. Breeding companies each year try to deliver novel cultivars that get the consumers attention and allow them to gain a competitive edge. Bract colour is one of the most important and obvious traits of poinsettia. The most popular are red flowering cultivars, but plants with white, pink or yellow bracts, different patterns or bract shapes have also been gaining in popularity in recent years [12]. Novel cultivars of poinsettia are obtained by classical breeding approaches like crossing or radiation mutation, however, these methods usually take a lot of time and effort before a plant with the desired phenotype is achieved. This can be a fatal drawback in a rapidly developing market. Some phenotypes are unattainable by classical breeding due to a lack of the necessary genetic background. Molecular breeding, especially genome editing might be a solution for the fast and efficient development of plants with desired traits, and can streamline the delivery of new interesting cultivars to customers.

Flavonoids, especially anthocyanins, are among the most important compounds that are responsible for the colouration of flowers and fruit [22]. The hydroxylation pattern of the B-ring of the anthocyanin structure determines the colour of the compound. Orange to orange-red colours are produced by pelargonidin with one hydroxyl group in position 4'. Red to pink cyanidin has two hydroxyl groups in positions 3' and 4'. Violet to blue delphinidin has three hydroxyl groups in positions 3', 4' and 5'. Flavonoid 3' -hydroxylase (F3'H) and flavonoid 3'5'-hydroxylase (F3'5'H) are cytochrome P450 dependent monooxygenases that are responsible for the establishment of the hydroxylation pattern of anthocyanins [10]. The presence of F3'H and F3'5'H activities is necessary for the formation of cyanidin-type and delphinidin-type anthocyanins, respectively, and thus, to obtain red/pink and violet/blue flowers. Consequently, their absence is a prerequisite for the accumulation of pelargonidin-type anthocyanins, which results in orange-red petal colour [22]. Another important enzyme for flower colour formation is dihydroflavonol 4-reductase (DFR), and its ability to convert dihydrokaempferol (DHK), dihydroquercetin (DHQ) and dihydromyricetin (DHM) to the corresponding leucoanthocyanidin, which are further converted to pelargonidin, cyanidin and delphinidin, respectively [47]. The genes that code these three enzymes, F3'H, F3'5'H and DFR, are the main targets for breeding orange and blue poinsettia.

The first part of the thesis was dedicated to a better understanding of the physiological background of colour formation in poinsettia and focused on anthocyanin formation in

poinsettia bracts, as well as in *Petunia* x *hybrida* flowers. The aim was to establish the strategy for the subsequent breeding experiments, which subsumes the second part.

1.5.1 Physiological background of colour formation in petunia flowers

Petunia x *hybrida* has a very well characterised flavonoid pathway and has been used for decades as a genetic model system [17]. Thus, the whole-genome sequences of *P. axillaris* and *P. inflata*, who are the wild parents of the garden petunia, are available [16]. The long-lasting research on formation and regulation of flower colour in this species has resulted in several breakthroughs, including the involvement of RNAi in flavonoid gene suppression or the substrate specificity of DFR [46].

Garden petunia shows a high diversity of colours with cyanidin-type and delphinidin-type anthocyanins being prevalent. For a long time, cultivars accumulating pelargonidin-type anthocyanins were not available, and this was explained with an exceptional substrate specificity of petunia DFR, which does not convert DHK, and therefore, does not produce the precursors for the formation of pelargonidin [43, 46]. Thus, petunia DFR has become the role model in the flavonoid community for substrate specific DFRs [44]. Only in the last decade, a few orange petunia cultivars started to appear on the market which accumulate pelargonidins. It could be assumed that the ability to produce pelargonidin pigments is caused by a change of the DFR's specificity [108], however enzyme preparations from orange petunia flowers did not support this hypothesis [109]. Independently of the flower colour, enzyme preparations from petunia petals always showed the similar, well known substrate specificity and an inability to convert DHK. The orange, pelargonidin-accumulating petunias, turned out to be escaped genetically modified plants, harbouring a maize DFR, but independently from this, the question remained, how pelargonidin-type pigments can accumulate in petunia flowers despite the high substrate specificity of DFR. This was especially interesting in the case of orange and blue poinsettia breeding, where DFR specificity could be one of the key factors in successful blue and orange breeding.

To investigate this, the FlowerPower project team, in which this thesis is embedded, decided to perform transcriptome analysis of three pelargonidin accumulating petunia cultivars (cv. 'Electric Orange', cv. 'Viva Orange', cv. 'Salmon Ray') and compare them to the transcriptomes of a cyanidin-accumulating (cv. 'Corso Rot') and a delphinidin-accumulating (cv. 'Blackberry') cultivar. Transcriptome analysis in combination with qPCR studies of selected flavonoid genes, enzyme analysis and substrate specificity studies finally established

for the first time that pelargonidin accumulation can occur, despite of a very low substrate specificity of DFR for DHK. This can happen very slowly over a long time in a rare constellation where sufficient leucopelargonidin precursor for pelargonidin-type flowers will be synthesized because (i) of an absent or low F3′H and F3′5′H activity so that only 4' -hydroxylated dihydroflavonol precursor are present, (ii) and the DHK pool is not redirected by a highly active FLS toward flavonols [109]. In the same way, cyanidin-based red flowering petunia varieties occur naturally, despite the fact that the petunia DFR shows stringent specificity for DHM as substrate [109]. This was a central finding of my thesis and was included in the peer reviewed publication of Haselmair-Gosch et al. (2018) [109]. For the poinsettia breeding approach, it was important to understand, that in the first instance it was possible to concentrate on the F3′H and F3′5′H activities, with DFR substrate specificity being a minor issue. In addition, the choice of a suitable poinsettia cultivar for the biotechnological breeding approach would be important. The ideal candidate would be characterized by an anthocyanin pathway with modest side branches leading to the accumulation of other flavonoid classes.

1.5.2 Physiological background of colour formation in poinsettia bracts

In this part of the thesis, I analysed the genes and enzymes of F3'H as a key target for the breeding approach, and also DFR as a minor target. The investigation started with pigment analysis of red and rare orange-red poinsettia. It was shown by HPLC analysis that main pigments in red poinsettia bracts are of the cyanidin-type, while we could find only a few orange-red cultivars, in which pelargonidins were more prevalent [110]. Poinsettia in general does not produce delphinidin-type pigments in significant amounts, although unexpected traces can be detected in the bracts [111]. The next step was the cloning of poinsettia *F3'H* and *DFR* from red and orange-red poinsettias to study the gene expression and the biochemical characteristics and substrate specificity of the encoded enzymes. Recombinant F3'Hs obtained from heterologous expression of *F3'H*s from red poinsettias in yeast, were functionally active, accepting naringenin and DHK as substrate. The *F3'H* sequence obtained from cv. 'Harvest Orange', however, carries a point mutation, which results in a shift in the open reading frame and premature protein termination after 44 amino acids, and thus a non-functional enzyme [110]. The mutated allele was, however, highly expressed. *F3'H* in cv. 'Harvest Orange' turned out to be heterozygous, because an allele encoding a functionally active *F3'H* was present in addition to the mutated *F3'H* [110].

The studies included a second cultivar accumulating pelargonidin-type anthocyanins. In comparison to cv. 'Harvest Orange', cv. 'Premium Red' had a more vivid red colour but also a pelargonidin prevalence of 85 % percent [110]. The physiological background for this remained unclear, as the *F3'H* was highly expressed and encoded a functionally active enzyme. Thus, it was obvious that there are several mechanisms for the establishment of orange-red bract colouration in poinsettia, but as demonstrated for cv. 'Harvest Orange', suppression of F3'H could be sufficient. It was also shown for petunia, tobacco and torenia that the suppression of F3'H activity is a very important aspect in obtaining orange flowers [50-52].

In this thesis, DFR of poinsettia was also studied. *DFR*s from all cultivars did not show much diversity and after heterologous expression in *E. coli*, the resulting recombinant enzymes were able to accept all three dihydroflavonol types (DHK, DHQ and DHM), although in most cases DHQ and DHM were preferred [110]. To change the poinsettia DFR specificity, exchange of the valine in position 132 (amino acid numbering from poinsettia *DFR*) to leucine was performed. The poinsettia VDV motif was changed to LDV, which is characteristic for petunia DFR with a high affinity for DHM [46]. The mutated DFR that was obtained was able to convert only DHM, although the activity seemed to be strongly reduced [110].

Red and orange-red poinsettias were both able to convert DHK, but for recombinant enzymes of orange-red cultivars the conversion rate was much higher than for red ones [110]. Overall, my studies of poinsettia DFR underpinned the assumption resulting from the petunia studies that it is possible to breed orange-red and blue poinsettia without DFR manipulation. Although, the outcome would probably be better if poinsettia DFR were to show higher specificity, for DHK in the case of breeding orange poinsettia, and for DHM for the breeding blue approach, concentration on F3'H suppression and introduction of F3'5'H activity, respectively, would be sufficient for a first proof-of-concept.

Breeding for blue bract colouration in poinsettia first aimed at applying a genome editing approach, in which the modification of the poinsettia F3'H sequence would result in an activity change and the induction of F3'5'H activity, which is an important prerequisite for obtaining blue flower colour [54]. The idea was to apply a strategy that was first suggested by Seitz (2007) [38], who introduced partial F3'5'H activity into gerbera F3'H by mutation of a single amino acid in position 8 of substrate recognition site 6 (SRS6), or by creation of chimeric proteins, where the C-terminus of gerbera F3'H was exchanged for the F3'5'H of *Osteospermum hybrida*. This approach seemed to be very promising, as genome editing would be a perfect tool for such small sequence changes. As a proof of concept, the strategy was first

tested by mutating *F3'H*s of poinsettia, apple and *Arabidopsis* by site directed mutagenesis and testing the resulting activities of the recombinant enzymes. After the exchange of the threonine in position 8 of SRS6 of poinsettia F3'H to alanine or serine, which are highly conserved in F3'5'Hs, mutated proteins were functionally active but still showed their original F3'H activity [112]. An exchange of the C-terminus of poinsettia F3'H to the C-terminus of *Osteospermum* F3'5'H resulted in functional inactivation rather than the desired introduction of F3'5'H activity in poinsettia F3'H [112]. At this stage, it became obvious that genome editing of F3'H would not be feasible. Thus, the strategy was changed towards a common transgenic approach in which the *F3'5'H* cDNA clone of a blue flowering ornamental plant should be transformed into poinsettia cells with *Agrobacterium tumefaciens*.

Recently it was shown that F3'Hs from rice and *Medicago*, which are classified as CYP75B class can perform 3' and 5' hydroxylation [113, 114]. These enzymes carry a glycine in position 8 of SRS6 instead of the conserved threonine of F3'Hs [114]. This supports the hypothesis that obtaining F3'5'H activity in the F3'H is in general possible and position 8 in SRS6 is crucial, but in case of poinsettia further investigation of possible additional amino acids that should be targeted is necessary.

1.5.3 Breeding for orange-red colour in poinsettia bracts

The study of red and orange-red poinsettia cultivars [110], suggested that breeding for orange cultivars could be achieved by a genome editing approach with CRISPR/Cas9, in which *F3'H* would be targeted with the aim of F3'H inactivation. Genome editing by application of CRISPR/Cas9 is regarded as a very promising tool in modern plant breeding. So far there are several records of a successful application of this method in the breeding of ornamentals, such as the modification of flower colour in Japanese Morning Glory [86, 87], torenia [88], gentian [89, 90] and petunia [91]. The potential for obtaining GM free plants after crossing out T-DNA is a big advantage [86, 92].

Agrobacterium-mediated transformation in poinsettia is a challenging and arduous process. To date it was performed by Clarke (2008), Islam (2013) and Sagvaag (2015) [115-117]. Transformation efficiency is usually quite low [115], and when the plant material for transformation comes from the greenhouse there are problems with efficient surface sterilization. For the transformation, cultivar 'Christmas Eve' was selected as the one with the best regeneration rates. The process of transformation led to three transgenic lines that showed the presence of *nptII*, *cas9* and gRNA on the gDNA and RNA level. Among those three, just

one line (B2) showed a change in bract colour from vivid red (RHS 45B) to vivid reddish-orange (RHS 33A) [118]. HPLC analysis showed that plants propagated from this line prevalently accumulate pelargonidin-type anthocyanins. Gene expression studies demonstrated that *F3'H* expression levels in the transgenic lines are not different than in the WT, which concurs with what was previously observed in rice [119]. Also, expression of *cas9* was detected in all transgenic plants, with the highest expression level in line B2. It was presented in Jang et al (2016) and Mikami et al (2015) [119, 120] that a higher expression level of *cas9* correlates with higher editing efficiency. Next generation sequencing (NGS) revealed that in line B2 editing in the *F3'H* sequence took place. However, original WT reads were also present in higher amounts than the modified ones. Modified reads showed just one type of mutation, insertion of one thymidine three nucleotides before the PAM sequence [118], which is a typical cutting point for Cas9 [73]. Two other transgenic lines showed only WT type reads. During cloning of expressed versions of poinsettia *F3'H*, however, three different cDNA clones were obtained. One sequence was identical to the WT, whereas the second showed the insertion of one T in position 170 (like in NGS results) and the third *F3'Hs* version was 126 nucleotides shorter than that of the WT [118]. The recombinant proteins resulting from the mutated *F3'Hs* were, however, not functionally active, indicating that the genome editing suppressed F3'H activity as desired. Despite the fact that line B2 expressed the unmutated, functionally active *F3'H*, the cyanidin level in this line decreased significantly [118]. This is similar to the case of cultivar 'Harvest Orange', where we could also detect two alleles of *F3'H*, and the allele encoding a functioning enzyme was expressed at a lower rate than that which encodes a non-functioning enzyme [110]. This would explain why we can still detect cyanidin during HPLC analysis, albeit at a much lower content than in WT plants.

These results are a proof that silencing of *F3'H* is a good strategy for breeding orange flowering poinsettia. Even when *F3'H* was not silenced completely, we could observe a significant shift in bract colour toward orange [118]. Given that orange-red cultivars are very rare, it would probably be difficult to obtain this trait by classical breeding. Those results prove that genome editing with application of CRSISPR/Cas9 is possible in poinsettia, and is more suitable for breeding orange than classical breeding techniques. In order to obtain plants that exclusively accumulate pelargonidin, additional modification of poinsettia *DFR* to encode an enzyme that accepts only DHK could be performed. As was shown previously [46], modification of a single amino acid in the putative substrate recognition site of DFR can lead to DHK specificity. Genome editing gives possibilities to perform such mutations, which, together with *F3'H*

silencing, would lead to a poinsettia plant that accumulates only pelargonidin, which would result in more vibrant orange colours in poinsettia bracts.

1.5.4 Breeding for blue colour in poinsettia bracts

For breeding blue poinsettia, *Cyclamen persicum F3'5'H* or *Sollya heterophylla F3'5'H* were overexpressed in the poinsettia cultivar 'Premium Red'. This cultivar was selected because of its good regeneration efficiency and lower cyanidin content, which is a favourable trait in breeding approaches for blue tissue colour [62]. However, no transgenic plant was obtained when the construct carrying *Sollya F3'5'H* was used. During *Agrobacterium*-mediated transformation of the poinsettia cultivar 'Premium Red' with a construct carrying cyclamen *F3'5'H*, we obtained just one transgenic line (E360). In the transgenic line E360 the delphinidin level was significantly higher than in the WT, however, the ratio to cyanidin and pelargonidin did not shift sufficiently to produce a discernible colour change [112].

In chrysanthemum, around 30 % of delphinidin was necessary to obtain a visible colour change [60]. It seems that for poinsettia, an additional elimination of F3'H activity would be important to decrease cyanidin formation. This would reduce the competition of exogenous F3'5'H and endogenous F3'H for the same substrates and thus, increase delphinidin formation. As a result, higher delphinidin levels and also a higher delphinidin ratio in comparison to other anthocyanidins could be expected. This could be achieved by different strategies, e.g. transformation of a cultivar lacking F3'H activity or silencing of endogenous *F3'H*, as was done previously in chrysanthemum [61], but with application of genome editing. Another possible improvement in breeding blue poinsettia would be the use of DFR that accepts only DHM, as was performed in roses [58]. This could be achieved, either by using exogenous DFR or changing the specificity of endogenous DFR by genome editing. It has previously been shown that the exchange of the amino acid in position 132 (V to L, numbering from poinsettia) produced DFR that accepts only DHM, so this could be a promising approach. It was also proposed that to obtain truly blue colours, overexpression of the genes responsible for anthocyanin decoration might be beneficial. Glycosylated or acylated anthocyanins are more stable and often present more intense bluish colours and are more easily associated with co-pigments [25]. Overexpression of *F3'5'H* together with *UDP (uridine diphosphate) glucose:anthocyanin 3'5'-glucosyltransferase* (*A3'5'AT*) and silencing of *F3'H* by RNAi in chrysanthemum resulted in the creation of first truly blue plants [61]. A similar approach could also be used in poinsettia in order to obtain bracts that have truly blue colour. But to start with, a simpler approach addressing a lower number of targets would probably be more appropriate.

Anyhow, a novel poinsettia cultivar that accumulates prevalently delphinidin and shows only violet colour like blue roses and carnations [56, 58] would be a very important milestone. At this point, probably the best approach to obtain blue poinsettia would be overexpression of exogenous *F3'5'H* and modification of poinsettia *F3'H* and *DFR* by a genome editing approach. During work on this thesis the first step in breeding blue poinsettia was made and this could be a starting point for further modifications that leads to the first blue flowering poinsettia. Unfortunately, I obtained only one transgenic line expressing the transgene, and an extension of this work would have been out of any timeframe available for this thesis.

1.5.5 The escaped genetically modified orange petunias

Petunia hybrida is a popular bedding plant with a long commercial history and several hundred cultivars available. As mentioned before, however, petunia cultivars, which accumulate prevalently pelargonidin-type anthocyanins appeared only very recently on the market, in Austria it was in 2013. The availability of cultivars with colours made from the three main anthocyanin types (pelargonidin, cyanidin, delphinidin), was exploited for our transcriptome studies as a model system in order to understand the key factors in colour establishment. In 2017, it turned out that all orange petunia cultivars are unauthorised genetically modified (GM) plants, harbouring maize DFR and had to be therefore withdrawn from the market. This gave rise to an unexpected additional scientific focus of my thesis, which was dedicated to the escaped genetically modified petunias.

The unauthorized GM-petunias caused significant economic losses for many ornamental breeding companies worldwide [121]. To make sure that all relevant cultivars were withdrawn, petunia breeders had to investigate their assortment, breeding lines and progenies of previous breeding programmes for the presence of transgenes. It turned out that the phenotype alone does not indicate a genetic modification. In fact, many other, not orange flowering cultivars can undetectably carry the transgene, if they resulted from a breeding process in which a GM-cultivar had been involved at some stage during the past years of petunia breeding. As a matter of fact, there was a strong interest in a better understanding where and how the GM-petunia had entered the commercial breeding programmes.

The GM-petunias carry a vector that contains a maize *DFR* (*A1*) [108]. This pointed at two possible sources of the transgene: one was a petunia transformation performed by Meyer (1987) [48] in the 1980s and the second a transformation performed by Elomaa (1995) [49] in the 1990s. In both cases, the *A1* gene was used but its orientation to *nptII* gene was different; sense

in case of the Meyer (1987) construct [48], and antisense in the Elomaa (1995) construct [49], which allowed to easily distinguish them [109]. Screening all tested cultivars showed that they carry the same sequence used in the Meyer (1987) construct [48] and that all elements characteristic (p*35S*, *A1*, *Cin4-1*, t*35S*, p*NOS*, *nptII*, t*OCS*) for the Meyer construct are present [108, 109]. This pointed at a single source of the unauthorised GM orange petunias.

As a result of the GM-petunia escape, petunia growers now have to provide certificates that confirm that their plants are not genetically modified. This is typically done by certified commercial laboratories which test for amplification of fragments of *35S* or *nptII*. Because of the frequent use of the *35S* promoter and *nptII* as selection marker gene in biotechnological breeding, this is of course useful for the broad detection of GM events, however, it is does not allow a specific detection. I therefore decided to take a closer look at the orange petunias and to identify the locus where the transgene is integrated to establish an event specific PCR method for the GM-petunias carrying the Meyer (1987) construct [48]. The best way to establish event specific methods for transgene detection is the development of a primer specifically designed at the junction region between genomic DNA and T-DNA.

The genome walking experiments indicated that most likely there are two copies of the transgene incorporated in the petunia genome [122]. This was also confirmed by the Southern blot data [122]. The identification of the locus, where the transgenes were integrated turned out to be trickier than expected. This was owed to the fact that the petunia genomes where available only from the two parent species [16], and not from *Petunia hybrida*, and that they were apparently integrated in a highly repetitive sequence.

For the T-DNA2, the 5' junction sequence was detected, whereas on the 3' end only the T-DNA sequence was obtained. For T-DNA1, the 3' junction sequence was obtained, whereas the 5' junction sequence was not isolated [122]. We assume that the T-DNA1 could have been truncated somewhere in the *A1* region, and that therefore, primers used for genome walking could not bind. Those data are supported by Southern blot results, where just one copy of the gene was detected when a probe specific to *A1* was used, and two to three copies when a probe specific to *nptII* was applied [122]. It also explains why just one copy of the transgene was detected by Fraiture (2019) and Voorhuijzen (2020) [123, 124], since the primers they used could not bind to truncated T-DNA.

After isolation of the junctions' sequences for the two T-DNAs, two pairs of primers (specific to the T-DNA1 3' and T-DNA2 5' junctions) and one pair of element specific primers (specific

to 3' end of T-DNA2) were designed. 126 GM offspring of a commercial breeding program and three orange petunia cultivars from breeders of different countries were screened. All gave positive signals for both T-DNA junctions and the element specific PCR. This confirmed the assumption that most likely all GM petunia originated from a single transgenic line [122].

It remains, however, unclear which transgenic line that was and how it entered commercial breeding programs. Orange petunias obtained by Meyer (1987) [48] were used for a field trial in Germany in 1990 and later for breeding purposes [121, 125]. Recently, it was excluded that line RL01-17, which was used in the field trials, could be the source of the escaped orange GM-petunias [124]. However, several lines were made available to breeders, but of course not for commercial breeding. Currently, the most plausible explanation is that during fusions of companies, the GM-background of orange petunia was forgotten and they were used simply by accident in the breeding process [121]. The fact that the orange phenotype occurs only in the rare physiological background, while remaining hidden in cultivars showing high F3'H, F3'5'H and FLS activities, has certainly facilitated the spread of the transgene in many breeding programs worldwide.

Recently, another transgenic element was detected in GM pink petunias [124]. The vector was carrying F3'5'H, and as a source of the transgene two previous scientific petunia, transformation events are possible [50, 126]. The escaped orange petunia case and the second example of escaped GM-petunias demonstrate that despite many regulations, unintended escapes of GM-plants are possible, and might be difficult to detect immediately. The only way of preventing this is to test the breeding material for the presence of common transgenic elements and identification of GM sources by event specific PCR [124].

Apart from the catchy story of the petunia escape, which contrary to my expectations went all but unnoticed by the public, the orange petunias are also interesting model plants, e.g., for studying the physiological relevance of DFR substrate specificity for flower colour establishment. In this thesis, the impact of the *A1* gene on pigment formation in orange petunia was also investigated. As mentioned before, enzyme preparations from the orange petunias were not able to convert DHK, despite carrying the *A1* gene [109]. Actually, *in vitro* the DFR substrate specificity did not differ between differently coloured cultivars, which is on the one hand a result of the very low expression of *A1* in comparison to petunia *DFR* in all flower developmental stages. Moreover, recombinant A1, similar to recombinant petunia DFR did not show DHK conversion. However, it showed a much higher DHQ conversion than the petunia DFR [109]. As outlined before, pelargonidin in petunia flowers is synthesized only very slowly

over a long time, if no other dihydroflavonol precursor is present and the accumulating DHK is not consumed by other enzymes. Thus, the question remains, if the maize DFR or the petunia DFR is responsible, particularly considering the low expression ratio of *A1* compared to the endogenous *DFR* in petunia. This could be investigated by silencing petunia *DFR* and *A1* in orange GM petunias for example with application of genome editing approaches like CRISPR/Cas9, which has been used in petunia with good results [83-85, 91]. A few trials with the Virus-induced gene silencing (VIGS) system were not successful, and within the time frame of the thesis this question could not be answered. Interestingly, there is still a chance that orange petunia can appear naturally when a mutation in the substrate binding side of petunia DFR occurs. Previously it was shown [46] that even a point mutation in this region can increase DHK specificity of DFR, albeit in this case the activity was strongly reduced. If such a mutation would occur in a petunia plant with a favourable genetic background, this could result in petunias with orange flower colour that are not genetically modified.

1.5.6 Outlook

In summary, this thesis has made a first step in breeding novel flower colour in poinsettias via the CRISPR/Cas9 method. There is no doubt that genome editing, and CRISPR/Cas9 in particular, will be of increasing importance, both for plant research and plant breeding. However, my work has also recalled the fact that even procedures that are well established in microorganisms or animal model systems can frequently create problems in plants. Many of these problems occur as a result of the larger genomes which are associated with a high level of non-coding DNA and repetitive sequences, commonly occurring polyploidy and long generation times.

In the European Union, the level of acceptance of transgenic plants is still very low. In 2001 EU introduced very strict regulations concerning cultivation of GM plants and in 2018 the European Court of Justice decided that genome edited plants should be legally treated as genetically modified. Although genome edited plants, after crossing out T-DNA do not possess foreign DNA elements in their genome, they are regarded as being GM. The European Federation of Academies of Sciences and Humanities (ALLEA), together with 120 researches from different European countries, are appealing to the EU to review these restrictions[1]. Genome editing is the future of plant breeding, and hopefully use of this technique will soon be allowed in plant breeding programs in Europe. This would result in crops with improved quality that will be beneficial for producers, consumers and the environment.

[1]https://sciencebusiness. net/news/fresh-call-eu-allow-gene-edited-crops

1.6 References

1. *Floriculture Crops 2019 Summary.* United States Department of Agriculture, 2019: p. https://www.nass.usda.gov/Publications/Todays_Reports/reports/floran20.pdf.
2. Taylor, J.M., et al., *The poinsettia: History and transformation.* Chronica Horticulturae, 2011. **51**(3): p. 23-28.
3. Gillespie, L.J. and W.S. Armbruster, *A contribution to the Guianan flora: Dalechampia, Haematostemon, Omphalea, Pera, Plukenetia, and Tragia (Euphorbiaceae) with notes on subfamily Acalyphoideae.* Smithsonian Contributions to Botany, 1997.
4. *The plant list: Euphorbiaceae.* Royal Botanic Gardens Edinburgh and Missouri Botanic Gardens: p. http://www.theplantlist.org/1.1/browse/A/Euphorbiaceae/.
5. Webster, G.L., *Synopsis of the genera and suprageneric taxa of Euphorbiaceae.* Annals of the Missouri Botanical Garden, 1994: p. 33-144.
6. BEMPONG, M. and K. SINK, *The karyotype of Poinsettia.* Journal of Heredity, 1968. **59**(4): p. 259-260.
7. Trejo, L., et al., *Poinsettia's wild ancestor in the Mexican dry tropics: Historical, genetic, and environmental evidence.* American Journal of Botany, 2012. **99**(7): p. 1146-1157.
8. Lee, I.-M., *Phytoplasma casts a magic spell that turns the fair poinsettia into a Christmas showpiece.* Plant Health Progress, 2000. **1**(1): p. 16.
9. Mol, J., E. Grotewold, and R. Koes, *How genes paint flowers and seeds.* Trends in Plant Science, 1998. **3**(6): p. 212-217.
10. Halbwirth, H., *The creation and physiological relevance of divergent hydroxylation patterns in the flavonoid pathway.* International journal of molecular sciences, 2010. **11**(2): p. 595-621.
11. Horn, J.W., et al., *Phylogenetics and the evolution of major structural characters in the giant genus Euphorbia L.(Euphorbiaceae).* Molecular Phylogenetics and Evolution, 2012. **63**(2): p. 305-326.
12. Barrett, J., *Consumer Poinsettia Preference.* Lawn and Garden Retailer, 2005. **4**(2): p. 1-4.
13. Stehmann, J.R., et al., *The genus petunia*, in *Petunia*. 2009, Springer. p. 1-28.
14. Segatto, A.L.A., et al., *Molecular insights into the purple-flowered ancestor of garden petunias.* American Journal of Botany, 2014. **101**(1): p. 119-127.
15. Watanabe, H., et al., *Cross compatibility of Petunia cultivars and P. axillaris with native taxa of Petunia in relation to their chromosome number.* Journal of the Japanese Society for Horticultural Science, 1996. **65**(3): p. 625-634.
16. Bombarely, A., et al., *Insight into the evolution of the Solanaceae from the parental genomes of Petunia hybrida.* Nature plants, 2016. **2**(6): p. 1-9.
17. Vandenbussche, M., et al., *Petunia, your next supermodel?* Frontiers in plant science, 2016. **7**: p. 72.
18. Koes, R., et al., *Targeted gene inactivation in petunia by PCR-based selection of transposon insertion mutants.* Proceedings of the National Academy of Sciences, 1995. **92**(18): p. 8149-8153.
19. van der Meer, I.M., *Agrobacterium-mediated transformation of Petunia leaf disks.* Plant cell culture protocols, 1999: p. 327-334.
20. Conner, A.J., N.W. Albert, and S.C. Deroles, *Transformation and regeneration of Petunia*, in *Petunia*. 2009, Springer. p. 395-409.

21. Tohge, T., L.P. de Souza, and A.R. Fernie, *Current understanding of the pathways of flavonoid biosynthesis in model and crop plants.* Journal of Experimental Botany, 2017. **68**(15): p. 4013-4028.
22. Winkel-Shirley, B., *Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology.* Plant physiology, 2001. **126**(2): p. 485-493.
23. Schijlen, E.G., et al., *Modification of flavonoid biosynthesis in crop plants.* Phytochemistry, 2004. **65**(19): p. 2631-2648.
24. Mazza, G. and R. Brouillard, *Recent developments in the stabilization of anthocyanins in food products.* Food Chemistry, 1987. **25**(3): p. 207-225.
25. Zhang, Y., E. Butelli, and C. Martin, *Engineering anthocyanin biosynthesis in plants.* Current opinion in plant biology, 2014. **19**: p. 81-90.
26. Eidenberger, L., *Reinigung und Charakterisierung einer Dehydrogenase des Phloridzinbiosynthesewegs.* TU Wien, 2018.
27. Martens, S. and A. Mithöfer, *Flavones and flavone synthases.* Phytochemistry, 2005. **66**(20): p. 2399-2407.
28. Thill, J., et al., *'Le Rouge et le Noir': A decline in flavone formation correlates with the rare color of black dahlia (Dahlia variabilis hort.) flowers.* BMC plant biology, 2012. **12**(1): p. 1-14.
29. Luo, P., et al., *Disequilibrium of flavonol synthase and dihydroflavonol-4-reductase expression associated tightly to white vs. red color flower formation in plants.* Frontiers in plant science, 2016. **6**: p. 1257.
30. Holton, T.A., F. Brugliera, and Y. Tanaka, *Cloning and expression of flavonol synthase from Petunia hybrida.* The Plant Journal, 1993. **4**(6): p. 1003-1010.
31. Samanta, A., G. Das, and S.K. Das, *Roles of flavonoids in plants.* Carbon, 2011. **100**(6): p. 12-35.
32. Halliwell, B., J. Rafter, and A. Jenner, *Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not?* The American journal of clinical nutrition, 2005. **81**(1): p. 268S-276S.
33. Chapple, C., *Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases.* Annual review of plant biology, 1998. **49**(1): p. 311-343.
34. Tanaka, Y., *Flower colour and cytochromes P450.* Phytochemistry Reviews, 2006. **5**(2-3): p. 283-291.
35. Holton, T.A., et al., *Cloning and expression of cytochrome P450 genes controlling flower colour.* Nature, 1993. **366**(6452): p. 276-279.
36. Brugliera, F., et al., *Isolation and characterization of a flavonoid 3'-hydroxylase cDNA clone corresponding to the Ht1 locus of Petunia hybrida.* The Plant Journal, 1999. **19**(4): p. 441-451.
37. Seitz, C., et al., *Cloning, functional identification and sequence analysis of flavonoid 3'-hydroxylase and flavonoid 3', 5'-hydroxylase cDNAs reveals independent evolution of flavonoid 3', 5'-hydroxylase in the Asteraceae family.* Plant molecular biology, 2006. **61**(3): p. 365-381.
38. Seitz, C., S. Ameres, and G. Forkmann, *Identification of the molecular basis for the functional difference between flavonoid 3'-hydroxylase and flavonoid 3', 5'-hydroxylase.* FEBS letters, 2007. **581**(18): p. 3429-3434.
39. Seitz, C., et al., *Multiple evolution of flavonoid 3', 5'-hydroxylase.* Planta, 2015. **242**(3): p. 561-573.
40. Gotoh, O., *Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences.* Journal of Biological Chemistry, 1992. **267**(1): p. 83-90.

41. Harborne, J.B., *Introduction to ecological biochemistry*. 1993: Gulf Professional Publishing.
42. Halbwirth, H., et al., *Synthesis of (14C)-labeled 5-deoxyflavonoids and their application in the study of dihydroflavonol/leucoanthocyanidin interconversion by dihydroflavonol 4-reductase.* Plant Science, 2006. **170**(3): p. 587-595.
43. Forkmann, G. and B. Ruhnau, *Distinct substrate specificity of dihydroflavonol 4-reductase from flowers of Petunia hybrida.* Zeitschrift für Naturforschung C, 1987. **42**(9-10): p. 1146-1148.
44. Johnson, E.T., et al., *Cymbidium hybrida dihydroflavonol 4-reductase does not efficiently reduce dihydrokaempferol to produce orange pelargonidin-type anthocyanins.* The Plant Journal, 1999. **19**(1): p. 81-85.
45. Beld, M., et al., *Flavonoid synthesis in Petunia hybrida: partial characterization of dihydroflavonol-4-reductase genes.* Plant molecular biology, 1989. **13**(5): p. 491-502.
46. Johnson, E.T., et al., *Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase.* The Plant Journal, 2001. **25**(3): p. 325-333.
47. Petit, P., et al., *Crystal structure of grape dihydroflavonol 4-reductase, a key enzyme in flavonoid biosynthesis.* Journal of molecular biology, 2007. **368**(5): p. 1345-1357.
48. Meyer, P., et al., *A new petunia flower colour generated by transformation of a mutant with a maize gene.* Nature, 1987. **330**(6149): p. 677-678.
49. Elomaa, P., et al., *Transgene inactivation in Petunia hybrida is influenced by the properties of the foreign gene.* Molecular and General Genetics MGG, 1995. **248**(6): p. 649-656.
50. Tsuda, S., et al., *Flower color modification of Petunia hybrida commercial varieties by metabolic engineering.* Plant Biotechnology, 2004. **21**(5): p. 377-386.
51. Nakatsuka, T., et al., *Production of red-flowered plants by genetic engineering of multiple flavonoid biosynthetic genes.* Plant cell reports, 2007. **26**(11): p. 1951-1959.
52. Nakamura, N., et al., *Generation of pink flower varieties from blue Torenia hybrida by redirecting the flavonoid biosynthetic pathway from delphinidin to pelargonidin.* Plant biotechnology, 2010. **27**(5): p. 375-383.
53. Seitz, C., et al., *Redirection of anthocyanin synthesis in Osteospermum hybrida by a two-enzyme manipulation strategy.* Phytochemistry, 2007. **68**(6): p. 824-833.
54. Yoshida, K., M. Mori, and T. Kondo, *Blue flower color development by anthocyanins: from chemical structure to cell physiology.* Natural product reports, 2009. **26**(7): p. 884-915.
55. Holton, T.A., *Transgenic plants exhibiting altered flower colour and methods for producing same*. 1996, Google Patents.
56. Tanaka, Y., F. Brugliera, and S. Chandler, *Recent progress of flower colour modification by biotechnology.* International journal of molecular sciences, 2009. **10**(12): p. 5350-5369.
57. Brugliera, F., Y. Tanaka, and J. Mason, *Flavonoid 3', 5' hydroxylase gene sequences and uses therefor*. 2013, Google Patents.
58. Katsumoto, Y., et al., *Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin.* Plant and Cell Physiology, 2007. **48**(11): p. 1589-1600.
59. Noda, N., et al., *Genetic engineering of novel bluer-colored chrysanthemums produced by accumulation of delphinidin-based anthocyanins.* Plant and cell physiology, 2013. **54**(10): p. 1684-1695.
60. Brugliera, F., et al., *Violet/blue chrysanthemums—metabolic engineering of the anthocyanin biosynthetic pathway results in novel petal colors.* Plant and Cell Physiology, 2013. **54**(10): p. 1696-1710.

61. Noda, N., et al., *Generation of blue chrysanthemums by anthocyanin B-ring hydroxylation and glucosylation and its coloration mechanism.* Science advances, 2017. **3**(7): p. e1602785.
62. Sasaki, N. and T. Nakayama, *Achievements and perspectives in biochemistry concerning anthocyanin modification for blue flower coloration.* Plant and Cell Physiology, 2015. **56**(1): p. 28-40.
63. Kishi-Kaboshi, M., R. Aida, and K. Sasaki, *Genome engineering in ornamental plants: current status and future prospects.* Plant Physiology and Biochemistry, 2018. **131**: p. 47-52.
64. Sprink, T., J. Metje, and F. Hartung, *Plant genome editing by novel tools: TALEN and other sequence specific nucleases.* Current opinion in biotechnology, 2015. **32**: p. 47-53.
65. Makarova, K.S., et al., *Evolution and classification of the CRISPR–Cas systems.* Nature Reviews Microbiology, 2011. **9**(6): p. 467-477.
66. Garneau, J.E., et al., *The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA.* Nature, 2010. **468**(7320): p. 67-71.
67. Deltcheva, E., et al., *CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III.* Nature, 2011. **471**(7340): p. 602-607.
68. Cong, L., et al., *Multiplex genome engineering using CRISPR/Cas systems.* Science, 2013. **339**(6121): p. 819-823.
69. Mali, P., et al., *RNA-guided human genome engineering via Cas9.* Science, 2013. **339**(6121): p. 823-826.
70. Shan, Q., et al., *Targeted genome modification of crop plants using a CRISPR-Cas system.* Nature biotechnology, 2013. **31**(8): p. 686-688.
71. Nekrasov, V., et al., *Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease.* Nature biotechnology, 2013. **31**(8): p. 691-693.
72. Li, J.-F., et al., *Multiplex and homologous recombination–mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9.* Nature biotechnology, 2013. **31**(8): p. 688-691.
73. Jiang, F. and J.A. Doudna, *CRISPR–Cas9 structures and mechanisms.* Annual review of biophysics, 2017. **46**: p. 505-529.
74. Jinek, M., et al., *A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity.* science, 2012. **337**(6096): p. 816-821.
75. Puchta, H., *The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution.* Journal of experimental botany, 2005. **56**(409): p. 1-14.
76. Chiruvella, K.K., Z. Liang, and T.E. Wilson, *Repair of double-strand breaks by end joining.* Cold Spring Harbor perspectives in biology, 2013. **5**(5): p. a012757.
77. Čermák, T., et al., *High-frequency, precise modification of the tomato genome.* Genome biology, 2015. **16**(1): p. 232.
78. Sun, Y., et al., *Engineering herbicide-resistant rice plants through CRISPR/Cas9-mediated homologous recombination of acetolactate synthase.* Molecular plant, 2016. **9**(4): p. 628-631.
79. Li, J., et al., *Generation of targeted point mutations in rice by a modified CRISPR/Cas9 system.* Molecular plant, 2017. **10**(3): p. 526-529.
80. Ren, B., et al., *Improved base editor for efficiently inducing genetic variations in rice with CRISPR/Cas9-guided hyperactive hAID mutant.* Molecular plant, 2018. **11**(4): p. 623-626.
81. Li, H., et al., *Precise modifications of both exogenous and endogenous genes in rice by prime editing.* Molecular plant, 2020. **13**(5): p. 671-674.

82. Lin, Q., et al., *Prime genome editing in rice and wheat.* Nature biotechnology, 2020. **38**(5): p. 582-585.
83. Zhang, B., et al., *Exploiting the CRISPR/Cas9 system for targeted genome mutagenesis in petunia.* Scientific reports, 2016. **6**: p. 20315.
84. Subburaj, S., et al., *Site-directed mutagenesis in Petunia× hybrida protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins.* Plant cell reports, 2016. **35**(7): p. 1535-1544.
85. Xu, J., et al., *CRISPR/Cas9-mediated editing of 1-aminocyclopropane-1-carboxylate oxidase1 enhances Petunia flower longevity.* Plant biotechnology journal, 2020. **18**(1): p. 287-297.
86. Watanabe, K., et al., *CRISPR/Cas9-mediated mutagenesis of the dihydroflavonol-4-reductase-B (DFR-B) locus in the Japanese morning glory Ipomoea (Pharbitis) nil.* Scientific reports, 2017. **7**(1): p. 1-9.
87. Watanabe, K., et al., *Alteration of flower colour in Ipomoea nil through CRISPR/Cas9-mediated mutagenesis of carotenoid cleavage dioxygenase 4.* Transgenic research, 2018. **27**(1): p. 25-38.
88. Nishihara, M., et al., *Application of the CRISPR/Cas9 system for modification of flower color in Torenia fournieri.* BMC plant biology, 2018. **18**(1): p. 1-9.
89. Tasaki, K., et al., *Effects of knocking out three anthocyanin modification genes on the blue pigmentation of gentian flowers.* Scientific reports, 2019. **9**(1): p. 1-10.
90. Tasaki, K., et al., *Molecular characterization of an anthocyanin-related glutathione S-transferase gene in Japanese gentian with the CRISPR/Cas9 system.* BMC plant biology, 2020. **20**(1): p. 1-14.
91. Yu, J., et al., *Simultaneous targeting of duplicated genes in Petunia protoplasts for flower color modification via CRISPR-Cas9 ribonucleoproteins.* Plant Cell Reports, 2020: p. 1-9.
92. Nekrasov, V., et al., *Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion.* Scientific reports, 2017. **7**(1): p. 1-6.
93. Erpen-Dalla Corte, L., et al., *Development of Improved Fruit, Vegetable, and Ornamental Crops Using the CRISPR/Cas9 Genome Editing Technique.* Plants (Basel, Switzerland), 2019. **8**(12).
94. Zhao, H. and J.D. Wolt, *Risk associated with off-target plant genome editing and methods for its limitation.* Emerging Topics in Life Sciences, 2017. **1**(2): p. 231-240.
95. Murashige, T. and F. Skoog, *A revised medium for rapid growth and bio assays with tobacco tissue cultures.* Physiologia plantarum, 1962. **15**(3): p. 473-497.
96. Zeng, G., *Sticky-end PCR: new method for subcloning.* Biotechniques, 1998. **25**(2): p. 206-208.
97. Steinert, J., et al., *Highly efficient heritable plant genome engineering using Cas9 orthologues from Streptococcus thermophilus and Staphylococcus aureus.* The Plant Journal, 2015. **84**(6): p. 1295-1305.
98. Labun, K., et al., *CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering.* Nucleic acids research, 2016. **44**(W1): p. W272-W276.
99. Montague, T.G., et al., *CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing.* Nucleic acids research, 2014. **42**(W1): p. W401-W407.
100. Zhang, L., et al., *Selection of reliable reference genes for gene expression studies in the biofuel plant Jatropha curcas using real-time quantitative PCR.* International journal of molecular sciences, 2013. **14**(12): p. 24338-24354.
101. Mallona, I., et al., *Validation of reference genes for quantitative real-time PCR during leaf and flower development in Petunia hybrida.* BMC plant biology, 2010. **10**(1): p. 1-11.

102. Pfaffl, M.W., *Quantification strategies in real-time PCR.* AZ of quantitative PCR, 2004. **1**: p. 89-113.
103. Halbwirth, H., et al., *Measuring flavonoid enzyme activities in tissues of fruit species.* Journal of agricultural and food chemistry, 2009. **57**(11): p. 4983-4987.
104. Edgar, R.C., *MUSCLE: multiple sequence alignment with high accuracy and high throughput.* Nucleic acids research, 2004. **32**(5): p. 1792-1797.
105. Jones, D.T., W.R. Taylor, and J.M. Thornton, *The rapid generation of mutation data matrices from protein sequences.* Computer applications in the biosciences: CABIOS, 1992. **8**(3): p. 275-282.
106. Kumar, S., G. Stecher, and K. Tamura, *MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets.* Molecular biology and evolution, 2016: p. msw054.
107. Clement, K., et al., *CRISPResso2 provides accurate and rapid genome editing sequence analysis.* Nature Biotechnology, 2019. **37**(3): p. 224-226.
108. Bashandy, H. and T.H. Teeri, *Genetically engineered orange petunias on the market.* Planta, 2017. **246**(2): p. 277-280.
109. Haselmair-Gosch, C., et al., *Great cause—small effect: undeclared genetically engineered Orange Petunias Harbor an inefficient Dihydroflavonol 4-Reductase.* Frontiers in plant science, 2018. **9**: p. 149.
110. Nitarska, D., et al., *The rare orange-red colored Euphorbia pulcherrima cultivar 'Harvest Orange'shows a nonsense mutation in a flavonoid 3'-hydroxylase allele expressed in the bracts.* BMC plant biology, 2018. **18**(1): p. 216.
111. Slatnar, A., et al., *Anthocyanin and chlorophyll content during poinsettia bract development.* Scientia Horticulturae, 2013. **150**: p. 142-145.
112. Nitarska, D., et al., *Molecular breeding for blue poinsettia.* 2021.
113. Lam, P.Y., et al., *Recruitment of specific flavonoid B-ring hydroxylases for two independent biosynthesis pathways of flavone-derived metabolites in grasses.* New Phytologist, 2019. **223**(1): p. 204-219.
114. Lui, A.C., et al., *Convergent recruitment of 5'-hydroxylase activities by CYP75B flavonoid B-ring hydroxylases for tricin biosynthesis in Medicago legumes.* New Phytologist, 2020. **228**(1): p. 269-284.
115. Clarke, J.L., et al., *Agrobacterium tumefaciens-mediated transformation of poinsettia, Euphorbia pulcherrima, with virus-derived hairpin RNA constructs confers resistance to Poinsettia mosaic virus.* Plant Cell Reports, 2008. **27**(6): p. 1027-1038.
116. Islam, M.A., et al., *Overexpression of the AtSHI gene in poinsettia, Euphorbia pulcherrima, results in compact plants.* PloS one, 2013. **8**(1).
117. Sagvaag, A.K., *Genetic transformation of Poinsettia (Euphorbia pulcherrima)*. 2015, Norwegian University of Life Sciences, Ås.
118. Nitarska, D., et al., *First genome edited poinsettias: Breeding for orange bract colour by application of CRISPR/Cas9.* Plant Cell, Tissue and Organ Culture (PCTOC), submitted 2021.
119. Jang, G., et al., *Genetic chimerism of CRISPR/Cas9-mediated rice mutants.* Plant biotechnology reports, 2016. **10**(6): p. 425-435.
120. Mikami, M., S. Toki, and M. Endo, *Parameters affecting frequency of CRISPR/Cas9 mediated targeted mutagenesis in rice.* Plant cell reports, 2015. **34**(10): p. 1807-1815.
121. Servick, K., *The strange case of the orange petunias*. 2017, American Association for the Advancement of Science.
122. Haselmair-Gosch, C., et al., *Event-specific qualitative polymerase chain reaction analysis for two T-DNA copies in genetically modified orange Petunia.* Plant Cell, Tissue and Organ Culture (PCTOC), 2020. **142**(2): p. 415-424.

123. Fraiture, M.-A., et al., *MinION sequencing technology to characterize unauthorized GM petunia plants circulating on the European Union market.* Scientific reports, 2019. **9**(1): p. 1-8.
124. Voorhuijzen, M.M., et al., *Molecular Characterization and Event-Specific Real-Time PCR Detection of Two Dissimilar Groups of Genetically Modified Petunia (Petunia x hybrida) Sold on the Market.* Frontiers in plant science, 2020. **11**: p. 1047.
125. Oud, J.S., et al., *Breeding of transgenic orange Petunia hybrida varieties.* Euphytica, 1995. **84**(3): p. 175-181.
126. Shimada, Y., et al., *Expression of chimeric P450 genes encoding flavonoid-3', 5'-hydroxylase in transgenic tobacco and petunia plants 1.* Febs Letters, 1999. **461**(3): p. 241-245.

2 Publications

2.1 Publication 1

Great cause—small effect: undeclared genetically engineered Orange Petunias Harbor an inefficient Dihydroflavonol 4-Reductase

C. Haselmair-Gosch, S. Miosic, **D. Nitarska**, B.L Roth, B. Walliser, R. Paltram, R.C Lucaciu, L. Eidenberger, T. Rattei, K. Olbricht, K. Stich, and H. Halbwirth

Frontiers in plant science, *9*, p.149. (2018), doi.org/10.3389/fpls.2018.00149

frontiers in Plant Science

ORIGINAL RESEARCH
published: 28 February 2018
doi: 10.3389/fpls.2018.00149

Great Cause—Small Effect: Undeclared Genetically Engineered Orange Petunias Harbor an Inefficient Dihydroflavonol 4-Reductase

*Christian Haselmair-Gosch[1], Silvija Miosic[1], Daria Nitarska[1], Barbara L. Roth[1], Benjamin Walliser[1], Renate Paltram[1], Rares C. Lucaciu[2], Lukas Eidenberger[1], Thomas Rattei[2], Klaus Olbricht[3], Karl Stich[1] and Heidi Halbwirth[1]**

[1] Institute of Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, Vienna, Austria, [2] Department of Microbiology and Ecosystem Science, University of Vienna, Vienna, Austria, [3] Thaer-Institute of Agricultural and Horticultural Sciences Humboldt University Berlin, Berlin, Germany

OPEN ACCESS

Edited by:
Mariana Mondragón-Palomino, University of Regensburg, Germany

Reviewed by:
Teemu Heikki Teeri, University of Helsinki, Finland
Xiang Gao, Key Laboratory of Molecular Epigenetics of MOE, Northeast Normal University, Changchun, China
Michal Oren-Shamir, Agricultural Research Organization, Israel

***Correspondence:**
Heidi Halbwirth
heidrun.halbwirth@tuwien.ac.at

Specialty section:
This article was submitted to Plant Biotechnology, a section of the journal Frontiers in Plant Science

Received: *11 September 2017*
Accepted: *29 January 2018*
Published: *28 February 2018*

Citation:
Haselmair-Gosch C, Miosic S, Nitarska D, Roth BL, Walliser B, Paltram R, Lucaciu RC, Eidenberger L, Rattei T, Olbricht K, Stich K and Halbwirth H (2018) Great Cause—Small Effect: Undeclared Genetically Engineered Orange Petunias Harbor an Inefficient Dihydroflavonol 4-Reductase. Front. Plant Sci. 9:149. doi: 10.3389/fpls.2018.00149

A recall campaign for commercial, orange flowering petunia varieties in spring 2017 caused economic losses worldwide. The orange varieties were identified as undeclared genetically engineered (GE)-plants, harboring a maize dihydroflavonol 4-reductase (*DFR*, A_1), which was used in former scientific transgenic breeding attempts to enable formation of orange pelargonidin derivatives from the precursor dihydrokaempferol (DHK) in petunia. How and when the A_1 cDNA entered the commercial breeding process is unclear. We provide an in-depth analysis of three orange petunia varieties, released by breeders from three countries, with respect to their transgenic construct, transcriptomes, anthocyanin composition, and flavonoid metabolism at the level of selected enzymes and genes. The two possible sources of the A_1 cDNA in the undeclared GE-petunia can be discriminated by PCR. A special version of the A_1 gene, the A_1 type 2 allele, is present, which includes, at the 3′-end, an additional 144 bp segment from the non-viral transposable *Cin4-1* sequence, which does not add any functional advantage with respect to DFR activity. This unequivocally points at the first scientific GE-petunia from the 1980s as the A_1 source, which is further underpinned e.g., by the presence of specific restriction sites, parts of the untranslated sequences, and the same arrangement of the building blocks of the transformation plasmid used. Surprisingly, however, the GE-petunia cannot be distinguished from native red and blue varieties by their ability to convert DHK in common *in vitro* enzyme assays, as DHK is an inadequate substrate for both the petunia and maize DFR. Recombinant maize DFR underpins the low DHK acceptance, and, thus, the strikingly limited suitability of the A_1 protein for a transgenic approach for breeding pelargonidin-based flower color. The effect of single amino acid mutations on the substrate specificity of DFRs is demonstrated. Expression of the A_1 gene is generally lower than the petunia *DFR* expression despite being under the control

of the strong, constitutive p35S promoter. We show that a rare constellation in flavonoid metabolism—absence or strongly reduced activity of both flavonol synthase and B-ring hydroxylating enzymes—allows pelargonidin formation in the presence of DFRs with poor DHK acceptance.

Keywords: *Petunia* × *hybrida*, *Zea mays*, dihydroflavonol 4-reductase, A_1 type 2 allele, anthocyanin, pelargonidin, orange flower color, transgenic plant

INTRODUCTION

The color of anthocyanin pigments is determined by their B-ring hydroxylation pattern (**Figure 1**), ranging from orange to bright red (one hydroxy group), dark red to magenta (two hydroxy groups), and violet to blue (three hydroxy groups; Halbwirth, 2010). This basically depends on two factors, which have both been exploited by biotechnological methods to influence flower color (Meyer et al., 1986; Tanaka et al., 2009, 2010): the presence of enzymes introducing hydroxy groups vicinal to that in position 4′ [flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′5′-hydroxylase (F3′5′H)], and the substrate specificity of DFR (Winkel-Shirley, 2001).

Important ornamental plants such as petunia, African violet and cyclamen do not naturally produce orange/bright-red flowers (Johnson et al., 1999) because they lack the ability to synthesize pelargonidin-type anthocyanin pigments. This is based on the presence of a substrate specific dihydroflavonol 4-reductase (DFR) enzyme, which does not accept the essential precursor, dihydrokaempferol (DHK), as a substrate.

DFR is an oxidoreductase (EC 1.1.1.219) that catalyzes the NADPH dependent stereospecific reduction of the keto group of (+)-(*2R,3R*)-dihydroflavonols in position 4 to the respective (*2R,3S,4S*)-flavan-2,3-*trans*-3,4-*cis*-diols (leucoanthocyanidins), as well as the reverse reaction in the presence of $NADP^+$ (Halbwirth et al., 2006; Petit et al., 2007). DFR is the first of the so-called "late" enzymes of the flavonoid pathway which shows a major impact on the formation of anthocyanin pigments, flavan 3-ols and flavonols. DFR provides the immediate precursors for the formation of anthocyanidins and flavan 3-ols, the building blocks of condensed tannins. On the formation of flavonols, DFR has an indirect effect. DFR competes with flavonol synthase (FLS), which opens a side branch of the anthocyanin pathway, for common substrates (Winkel-Shirley, 2001; **Figure 1**). Several DFRs can convert dihydroflavonols irrespective of their hydroxylation pattern, but petunia possesses a DFR that does not convert DHK into leucopelargonidin. In the 1980s and 1990s genetically engineered (GE)-petunias with orange flowers were created by introducing either a maize DFR encoded by the A_1 gene (Meyer et al., 1986; Elomaa et al., 1995) or a gerbera DFR (Elomaa et al., 1995).

The petunia belongs to the predominant balcony and bedding plants worldwide. A few years ago petunia varieties, showing a novel orange flower color, started to appear on the market and were swiftly adopted in private and public flower arrangements, in Europe and the US. Recently, the vast majority of them turned out to be genetically modified, after PCR-screening for the 35S-promoter and the A_1 gene (Bashandy and Teeri, 2017; David, 2017; Servick, 2017).

We selected three varieties, released by breeders from three countries, for an-in-depth investigation of the presence and nature of a transgenic construct and its impact on the flavonoid metabolism. We show that they all carry the same construct, and that this can be traced back to the first GE-petunia (Meyer et al., 1986) with near absolute certainty. But surprisingly, the orange petunias were not characterized by a drastically changed DFR substrate specificity compared to common red and blue petunia flowers, as would have been expected. We aimed on elucidating this paradox and demonstrate that the orange petunia owe their color primarily to a rare biochemical background. We underpin this by flavonoid analyses together with enzyme assays and expression and transcriptome studies.

MATERIALS AND METHODS

Material

Flowers (stage 1: buds of 0.6–3 cm length, stage 2: buds of 3–5 cm length, stage 3: open flowers) of cv. Salmon Ray (Danziger, Moshav Mishmar Hashiva, Israel), cv. Viva Orange (Florensis, Ambacht, The Netherlands), and cv. Electric Orange (Selecta One, Stuttgart, Germany) were harvested in the summers 2015–2017. Non-transgenic control plants of *Petunia* × *hybrida* cv. BabyDoll were obtained from Selecta One, cvs. Corso Rot, Corso Blau and Blackberry were purchased from Austrosaat (Vienna, Austria). The plant material was harvested from balcony pots or garden beddings, shock-frozen and kept at −80°C until analysis. Images of the petunia varieties are found in **Figures 1**, **2** and Supplementary Figure S3.

Reference compounds (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin, dihydromyricetin, dihydroquercetin, kaempferol, myricetin, and quercetin) were purchased from Extrasynthese (Genay, France), dihydrokaempferol from Sigma Aldrich (Vienna, Austria). Radiolabeled substrates were synthesized as previously described (Halbwirth et al., 2006).

HPLC Analysis

For analyzing the flavonoid class/anthocyanidin type composition in the petals, sugar moieties were removed by acidic or enzymatic hydrolysis. 1 g plant material was extracted with 1 ml 2 M hydrochloric acid in methanol. For anthocyanin analysis, 40 µl of the supernatant after centrifugation were incubated with 160 µl 4 N HCl for 60 min at 95°C. For analysis of other flavonoids, 20 µl of the supernatant were subjected to enzymatic hydrolysis by 10 U Naringinase (Sigma-Aldrich,

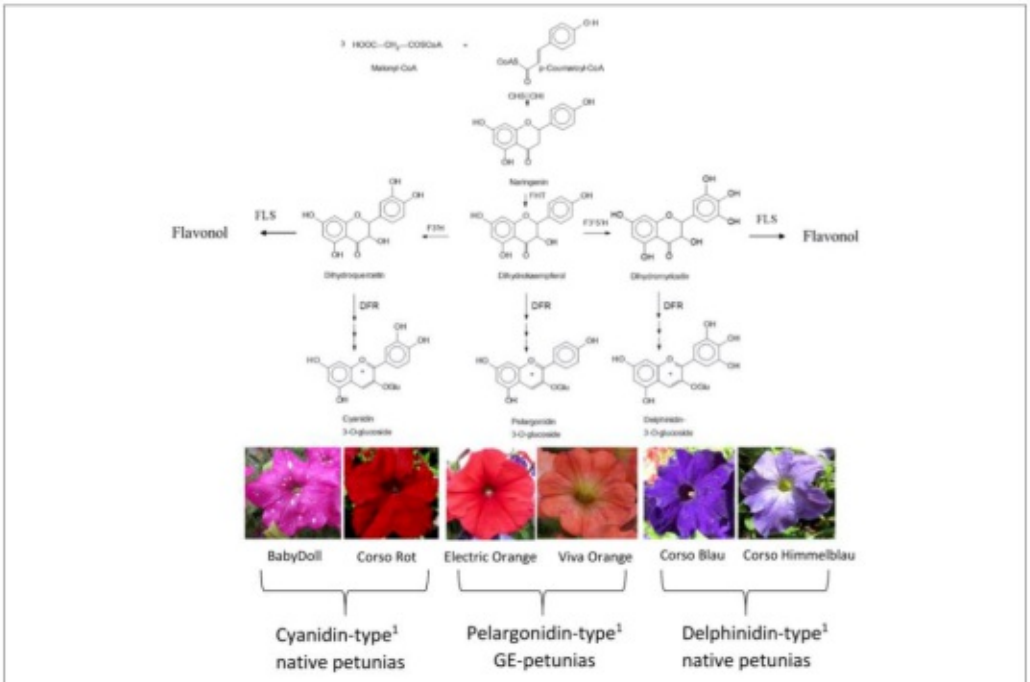


FIGURE 1 | Simplified flavonoid pathway demonstrating the influence of the B-ring hydroxylation pattern on the establishment of petunia flower color. [1]Petals contain prevalently the respective anthocyanidin type. CHS, Chalcone synthase; CHI, chalcone isomerase; DFR, dihydroflavonol 4-reductase; FHT, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; FLS, flavonol synthase.

Vienna, Austria) and hydrolysed for 20 min at 40°C in 0.1 M McIlvaine buffer pH 4. After hydrolysis, solid compounds were removed by centrifugation, and 4 µl of the supernatants were injected after filtration by 0.2 µm syringe filters.

HPLC analysis was performed on a Thermo Scientific Dionex UltiMate® 3000 RSLC System with DAD-3000RS Photodiode Array Detector (Thermo Scientific, Germany) using an Acclaim™ column RSLC 120 C18, 2.2 µm, 120Å, 2.1 × 150 mm (Dionex Bonded Silica Products: No. 071399) operated at 25°C. For analysis of anthocyanidins, elution solvents were (A) 10% formic acid and (B) 10% formic acid/22.5% acetonitrile/22.5% methanol in water (v/v) using a slightly modified method from Thermo Scientific Application note 281 (gradient: −10 to 0 min 9% B, 0–30 min 9–90% B, 30–40 min 90% B; flow rate 0.2 ml/min). For analysis of other flavonoids, elution solvents were (A) 0.1% formic acid and (B) 0.1% formic acid in acetonitrile (gradient: −3 to 0 min 20% B, 0–15 min 20–53% B, 15–20 min 53–95% B; 20–30 min 95% B, 31–35 min 20% B; flow rate 0.2 ml/min). Anthocyanidins were detected at 520 nm, other flavonoids at 290 nm. All compounds were identified by retention times and comparison of their UV-VIS spectra from 190 to 800 nm. The concentrations were calculated from the peak areas of samples and standard lines obtained with the respective reference compounds. Methylated anthocyanidins are not listed separately (**Table 1**), but were included according to their number of hydroxy groups in the delphinidin type (petunidin, malvidin) or cyandin type (peonidin) anthocyanidins. Relative contents of anthocyanidin types (% Pg/Cy/Dp based pigments was calculated from the [µg/g] values in **Table 1**. Flavonoid class distribution (% anthocyandins/flavonols/dihydroflavonols) were calculated from the [µg/g] values in **Table 1** in relation to a mathematical total amount of flavonoids resulting from the three types.

PCR, qPCR

Genomic DNA was obtained according to Lipp et al. (1999). mRNA was extracted with the µMACS mRNA isolation Kit (Miltenyi Biotec, Germany) and cDNA was synthesized as described (Thill et al., 2012). PCR and qPCR primers are listed in Supplementary Table S1. PCR was performed with the GoTaq DNA polymerase (Promega, Germany). Quantitative gene expression (at least in biological triplicates with three technical replications each) of *DFR*, *A*$_1$, *F3'H*, and *FLS* in comparison to the *actin* reference gene (Mallona et al., 2010)

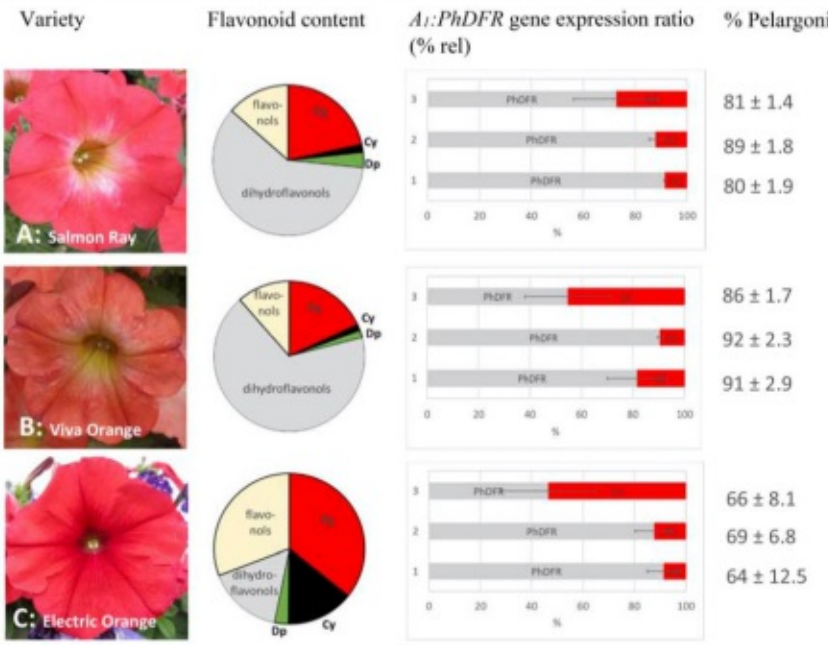


FIGURE 2 | Flavonoid content, A_1:*PhDFR* gene expression ratio and relative pelargonidin content of flowers from *Petunia* × *hybrida* cv. Salmon Ray **(A)**, cv. Viva Orange **(B)**, and cv. Electric Orange **(C)**. Pie chart (bright red: pelargonidin (Pg), black: cyanidin (Cy), green: delphinidin (Dp), gray: dihydroflavonols, yellow: flavonols) of flavonoid contents (for absolute values see **Table 1**). Bar chart with the ratio of *A1*:*PhDFR* gene expression in % rel for each developmental stage (1: buds of 0.6–3 cm length, 2: buds of 3–5 cm length, 3: open flowers). Standard deviation (error bars) in A_1 and *PhDFR* gene expression ratios for each developmental stage are shown. Portions of pelargonidin based pigments of the anthocyanidins in total are given in % pelargonidin in the three stages of each cultivar.

were analyzed with a StepOnePlus system (Applied Biosystems, CA, USA) and the Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, UK). The relative expression ratio was calculated according to Pfaffl (2001). The efficiency of the PCR-reaction was determined on the basis of standard curves which were obtained by applying different DNA concentrations and calculated from the given slopes in the StepOne software according to equation $E = 10^{(-1/\text{slope})}$ (Pfaffl, 2001). All qPCR primers had an efficiency between 90 and 110% (for amplification efficiencies see Supplementary Table S1). The product specificity was confirmed by melting curve analysis and gel electrophoresis. Sequencing of PCR products was done by a commercial supplier (Microsynth, Vienna, Austria).

Transcriptome Analysis

Plant material was harvested in summer 2016 and shock-frozen with liquid nitrogen. Preparation of rRNA, depleted RNA, random-primed cDNA and Illumina PE sequencing (50 million 150 bp, paired-end reads) was performed by a commercial supplier (vertis AG, Freising, Germany) on an Illumina NextSeq 500 system using 2 × 150 bp read length.

The random tagged prime RNA-seq data was first analyzed with the common NGS (next generation sequencing) analysis tools: First, the entire rRNA database provided by the tool was analyzed with sortmerna (v 2.1; Kopylova et al., 2012). From the remaining reads, the low-quality reads (below 20 quality score) were trimmed using trimmomatic (v 0.36) (Bolger et al., 2014) and the parameters TRAILING:20 AVGQUAL:20 SLIDINGWINDOW:5:20 MINLEN:75. Reads were mapped against the available *Petunia axillaris* genome (Bombarely et al., 2016), in which we incorporated the sequence of A_1 (NCBI CAA28734) to allow a quantification of the transgene expression. FPKM (Fragments Per Kilobase of transcript per Million mapped reads) were obtained using the method for quantification of RNA expression RSEM (v 1.3.0) (Li and Dewey, 2011).

Recombinant DFR

The DFR cDNA clone of *P. hybrida* was synthesized by GeneCust Europe (Dudelange, Luxembourg) based on the sequence available in the database (NCBI X15537). The A_1 cDNA clone (NCBI CAA28734) from maize was provided by Udo Wienand

TABLE 1 | Flavonoid composition (µg/g FW and % rel) of methanolic extracts of three orange flowering petunias after acidic and enzymatic hydrolysis in comparison to the non-GE cultivars Corso Rot (red), Corso Blau (blue) and BabyDoll (pink with white dots).

Pigment composition after acidic or enzymatic hydrolysis*		Salmon ray	Viva Orange	Electric Orange	Corso Rot	Corso Blau	BabyDoll
Total anthocyanidins**	[µg/g FW]	684.6 ± 299.6	522.7 ± 2.1	684.6 ± 299.6	552.0 ± 44.7	737.5 ± 194.4	959.3 ± 148.4
Pelargonidin type	[µg/g FW]	555.5 ± 193.4	448.5 ± 93.0	474.6 ± 314.2	n.d.	n.d.	n.d.
Cyanidin type	[µg/g FW]	43.0 ± 32.0	32.7 ± 7.3	191.7 ± 74.2	521.8 ± 45.0	n.d.	853.7 ± 123.8
Delphinidin type	[µg/g FW]	86.0 ± 15.7	41.5 ± 3.8	36.9 ± 43.9	30.3 ± 14.4	737.5 ± 194.4	105.6 ± 24.6
Total dihydroflavonols	[µg/g FW]	1,538.9 ± 1,040.6	1,680.2 ± 88.8	212.4 ± 47.8	455.0 ± 66.9	n.d.	188.2 ± 32.3
Dihydrokaempferol (DHK)	[µg/g FW]	1,502.4 ± 1,040.6	1,652.5 ± 88.1	179.9 ± 34.0	n.d.	n.d.	n.d.
Dihydroquercetin (DHQ)	[µg/g FW]	36.5 ± 29.5	27.8 ± 10.6	32.5 ± 26.2	455.0 ± 66.9	n.d.	188.2 ± 32.3
Dihydromyricetin (DHM)	[µg/g FW]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total flavonols	[µg/g FW]	348.8 ± 276.2	284.1 ± 130.5	408.53 ± 190.6	857.2 ± 189.6	1,458.3 ± 42.4	1,942.1 ± 232.4
Kaempferol	[µg/g FW]	298.3 ± 205.3	284.1 ± 130.5	180.3 ± 27.7	n.d.	n.d.	168.9 ± 30.5
Quercetin	[µg/g FW]	50.5 ± 71.4	n.d.	228.1 ± 164.2	857.2 ± 189.6	1,407.1 ± 38.5	1,773.0 ± 199.9
Myricetin	[µg/g FW]	n.d.	n.d.	n.d.	n.d.	51.2. ± 3.9	n.d.
Dihydroflavonols	%	57 ± 4.1	68 ± 4.1	17 ± .1.5	29 ± .6.5	n.d.	6 ± .1.3
Flavonols	%	12 ± 2.7	11 ± 4.9	41 ± 2.1	33 ± 4.8	66 ± 5.1	63 ± .5.1
Anthocyanidins**	%	31 ± 6.4	21 ± 1.2	42 ± 3.7	32 ± 7.9	34 ± 5.1	31 ± .6.4
Pelargonidin type	%	81 ± 1.4	86 ± 1.7	66 ± 8.1	n.d.	n.d.	n.d.
Cyanidin type	%	6 ± .2.2	6 ± .1.2	30 ± .8.3	95 ± .2.7	n.d.	11 ± .2.9
Delphinidin type	%	13 ± .2.2	8 ± .0.8	4 ± .3.1	5 ± .2.7	100 ± .2.7	89 ± .1.7

Pictures of the cultivars are incorporated in ***Figure 1****.*

**Average values and standard deviations were calculated from at least three biological replications collected at different sites. Large standard deviations of absolute values [µg/g fresh weight (FW)] partially result from the strong variation of flavonoid contents with external factors such as lighting conditions. But even from the same plants, flowers with divergent color intensity could be collected (details not shown). Despite this, the relative distribution [%] between the flavonoid classes (anthocyanins, dihydroflavonols, and flavonols) and within anthocyanidins (pelargonidin-, cyanidin-, delphinidin based pigments) was quite stable.*

***Methylated anthocyanidins are not shown separately, but were included according to their number of hydroxy groups in the delphinidin type (petunidin, malvidin) or cyandin type (peonidin) anthocyanidins.*

n.d. not detected.

(University of Hamburg, Germany). The cDNA clones were used for subcloning into the bacterial expression vector pGEX-6P-1 (GE Healthcare, Munich, Germany) for overexpression of the *DFRs* as GST-fusion proteins, as described previously (Gosch et al., 2014), using primers A_1DFR-FL, A_1DFR-FS, A_1DFR-RL, A_1DFR-RS, and PhDFR-FL, PhDFR-FS, PhDFR-RL, PhDFR-RS, respectively (Supplementary Table S2).

Enzyme Assays

DFR assays with recombinant enzyme or preparations from flowers were performed as described previously (Gosch et al., 2014).

Site Directed Mutagenesis

Mutants were generated by use of the Q5® Site-Directed Mutagenesis Kit (NewEngland Biolabs, Vienna, Austria). Primers were designed using the NEBase Changer™ provided at http://nebasechanger.neb.com. The sequences are given in Supplementary Table S2. The integrity of the constructs was confirmed by commercial sequencing (Microsynth, Vienna, Austria).

Statistical Analysis

The statistical analysis of the qPCR data was performed using RStudio v 1.0.136 and R v 3.3.3 with the package "agricolae" (Ihaka and Gentleman, 1996; De Mendiburu, 2017). Shapiro-Wilk test was used for testing on normality (Razali and Wah, 2011). A Wilcoxon rank sum test was used for not normal distributed data and a paired *t*-test was used for normal distributed data, respectively. The correlation between pelargonidin content and the expression ratio of *PhDFR* was calculated using the Pearson correlation coefficient (Duncan, 1955). Group-wise comparison of gene expression between different developmental stages and varieties was calculated utilizing Duncan's new multiple range test (MRT) (Duncan, 1955). For all statistical significance tests, the significance level was set to 0.05 (5%).

RESULTS

Pigment Composition

We analyzed the pigment composition of three commercially available orange varieties. In all three, pelargonidin based pigments were the prevalent anthocyanins (pie chart in **Figure 2**, **Table 1**). Whereas cv. Salmon Ray and cv. Viva Orange contained more than 80% pelargonidin based pigments and only small amounts of cyanidin and delphinidin derivatives, cv. Electric Orange showed a relatively high content of cyanidin based pigments (30%), traces of delphinidin based pigments and 66%

pelargonidin based pigments. The anthocyanin pattern was relatively stable during flower development and varied only to a minor extent between buds of different size and fully developed flowers (**Figure 2** right). Common red, pink, or blue petunia varieties, which were analyzed as controls, accumulated cyanidin or delphinidin based pigments, depending on the color, but no pelargonidin based pigments could be detected (**Table 1**).

Besides anthocyanins, dihydroflavonols and flavonols were present in the petals (**Figure 2**, **Table 1**). In the orange cultivars Salmon Ray and Viva Orange, dihydroflavonols were the prevalent flavonoid class, with more than 55% of the total flavonoids, whereas relatively small amounts of flavonols (below 15%) could be found (**Table 1**, pie charts in **Figure 2**). As with the anthocyanins, cv. Electric Orange showed a somewhat different composition, with 41% flavonols and 17% dihydroflavonols. In the red cv. Corso Rot, concentrations of anthocyanins, dihydroflavonols and flavonols were almost equal, whereas the blue and pink cultivars contained more than 60% flavonols and no (cv. Corso Blau) or only traces of (cv. BabyDoll) dihydroflavonols (**Table 1**).

Evidence for a Genetic Modification Event and Identification of the A_1 Source

We screened the varieties by PCR for sequences that would indicate the presence of a biotechnological construct. With specific primers for A_1, *nptII* and the *35S* promoter, PCR fragments could be amplified from genomic DNA of all three varieties (**Figure 3**), thereby confirming a genetic modification event. To illuminate the origin of the A_1 source, we analyzed the transgene present in the three orange varieties. Two A_1 constructs previously used to create orange GE-lines (Meyer et al., 1986; Elomaa et al., 1995) for scientific purposes are the most probable sources. Discrimination by PCR between these is possible (Table in **Figure 4**), based on the direction of the A_1 gene, which was either sense (Meyer et al., 1986) or antisense (Elomaa et al., 1995) to the *nptII* gene. All three orange petunia varieties showed an approximately 2.3 kb amplicon when PCR with genomic DNA as template and A_1 specific forward and *nptII* specific reverse primers was performed (**Figure 4**), as expected only for the construct of Meyer et al. (1986). We furthermore sequenced a 3.3 kb PCR product obtained from genomic DNA of the three

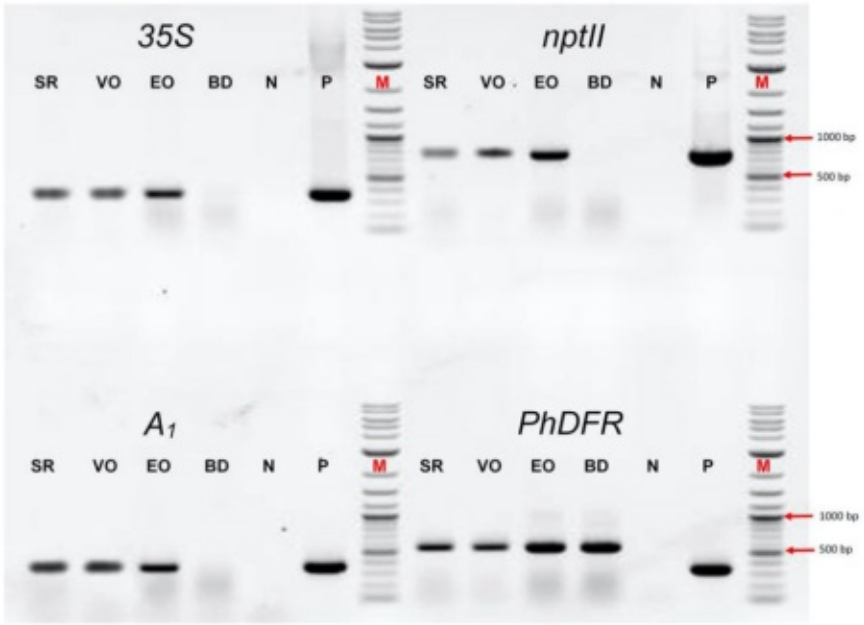


FIGURE 3 | PCR evaluation of genomic DNA of the three orange cultivars Salmon Ray (SR), Viva Orange (VO), and Electric Orange (EO) and the pink-white cultivar BabyDoll (BD) for the presence of specific DNA sequences. Water was used as negative control (N), and plasmids harboring the genes of interest as positive control (P) instead of genomic DNA. M: DNA marker 2-Log DNA Ladder; 1,000 and 500 bp fragments are marked with red arrows. *35S*: partial promoter sequence of the *35S* Cauliflower mosaic virus gene; *nptII*: partial coding sequence of the *nptII* selectable marker gene; A_1: partial coding sequence of the A_1 gene; *PhDFR*: partial coding sequence of the DFR of *Petunia* × *hybrida*. Primer sequences are provided in Supplementary Table S1. For genomic DNA, fragments of 365 bp (*35S*), 779 bp (*nptII*), 346 bp (A_1), and 565 bp (*PhDFR*) are expected. For *35S*, *nptII* and A_1, expected fragment sizes are identical for control plasmid. For *PhDFR*, a fragment of 337 bp for control plasmid DNA (cDNA clone) instead of 565 bp (genomic DNA) is expected.

	Primer 1	Primer 2	Amplicon expected for construct of		Amplicon obtained with cDNA from three orange petunia varieties
			Meyer	Elomaa	
A	DFR-A1a-F2	nptII-R	yes (2357 bp)	no	yes
B	DFR-A1a-R	nptII-R	no	yes	no

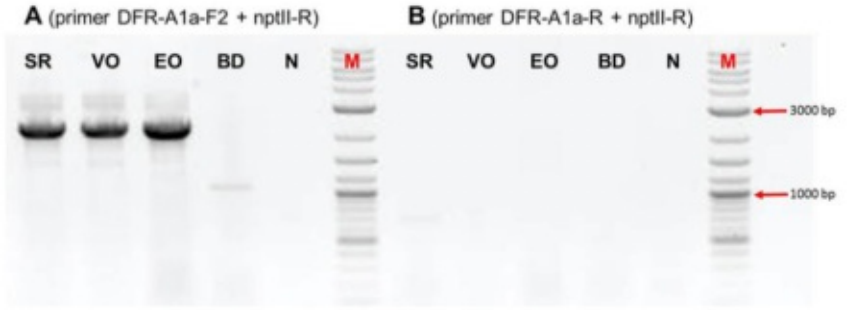


FIGURE 4 | PCR discrimination between the transformation plasmids of Meyer et al. (*A1* and *nptII* cloned in the sense orientation) and Elomaa et al. (*A1* and *nptII* cloned in the antisense direction). PCRs were performed with primer combinations **(A)** (primer DFR-*A1*a-F2 + *nptII*-R) and **(B)** (primer DFR-*A1*a-R + *nptII*-R) with genomic DNA of cvs. Salmon Ray (SR), Viva Orange (VO), Electric Orange (EO), and BabyDoll (BD). N, negative control in which genomic DNA is replaced by water; M, DNA marker 2-Log DNA Ladder; 3,000 and 1,000 bp fragments are marked with red arrows.

orange lines as templates using a *35S* promoter specific forward and an *OCS terminator* specific reverse primer.

All three amplicons showed identical sequences (Supplementary Figure S1) at the nucleotide level (NCBI MF521566). This included a partial sequence of the *35S* promoter (217 bp), followed by the restriction sites of *Xba*I and *Eco*RI, a 5′ untranslated region (UTR) of the A_1 cDNA clone of maize, the 1,074 bp full size A_1 cDNA clone, and an adjacent 3′ UTR with a 144 bp insertion consisting of the partial *Cin4-1* transposable element with a polyA stretch as present in the type 2 allele of A_1 described by Schwarz-Sommer et al. (1987a,b). After the polyA stretch, we identified an *Eco*RI and *Xba*I restriction site, a 226 bp t*35S* terminator flanked by an *Eco*RI restriction site, and a selection gene cassette including the p*NOS* promoter, *nptII* cDNA clone and t*OCS* terminator (1,402 bp fragment with only partial t*OCS*).

DFR Substrate Specificity

Protein preparations obtained from the three orange petunia varieties were, surprisingly, not able to convert DHK under *in vitro* assay conditions, although they showed DFR activity with dihydromyricetin (DHM) as a substrate (**Table 2**). DHK conversion with enzyme preparation of the orange petunia petals could be observed neither in buds nor in petals. DHQ was not accepted either, with exception of the enzyme preparations from cv. Electric Orange, which converted DHQ to some extent, although at dramatically lower level than DHM (**Table 2**). Common blue and red petunias, which were used as controls, showed dihydroflavonol conversion levels that were almost comparable to those of the GE-petunia. High conversion rates were obtained with DHM as substrate, whereas DHK was not converted (**Table 2**). Conversion of DHQ was much lower than with DHM and lower than those observed with preparations from cv. Electric Orange. To exclude a false negative result, the integrity of DHK was confirmed with enzyme preparations from strawberry (**Table 2**) which possess a DFR showing high DHK substrate specificity (Miosic et al., 2014). Thus, the orange petunias showed the same lack of DHK acceptance under common DFR assay conditions as the blue and red flowering non-GE controls.

To shed further light on the substrate specificity of the maize DFR encoded by A_1, we heterologously expressed an A_1 cDNA clone as GST-fusion protein in *E. coli*. After removal of the GST-tag, the purified recombinant A_1 protein showed high substrate specificity, converting DHQ and DHM to a comparable extent, but no conversion of DHK could be observed during time spans sufficient to exhaust DHQ and DHM (**Table 2**). Even when incubation times with DHK were extended over night or up to 24 h at 4°C, no conversion of DHK to leucopelargonidin could be observed *in vitro*. Kinetic data indicate a comparable substrate specificity for DHM (K_m 3.1 µM, V_{max} 1.0 × 10^{-3} µM/s; k_{cat} 0.87 s^{-1}; k_{cat}/K_m 0.28 $s^{-1}µM^{-1}$) and DHQ (K_m

TABLE 2 | Specific DFR activity with DHK, DHQ, and DHM as substrate using enzyme preparations from petals of petunia cultivars and with recombinant DFR from maize and petunia.

Enzyme source	A_1 present	Specific activity [nmol/g protein] measured with substrate DHK	DHQ	DHM
Salmon Ray buds/open flowers	Yes	–/–*	–/–	1.96/1.94
Electric Orange buds/open flowers	Yes	–/–*	0.13/0.12	1.96/1.96
Viva Orange buds/open flowers	Yes	–/–*	–/–	1.96/1.96
BabyDoll	No	–/–*	–/–	1.96/1.93
Corso Rot	No	–/–*	0.01/–	1.96/1.94
Corso Blau	No	–/–*	0.03/–	1.96/1.96
Control reaction (strawberry)	No	1.96	0.25	0
recombinant A_1 protein		–	0.33	0.55
Recombinant wild type *Ph*DFR (137L, 138D)**		–	–	1.47
Recombinant *Ph*DFR mutant (137V, 138D)**		–	–	1.52
Recombinant *Ph*DFR mutant (137V, 138N)**			1.41	1.14
Recombinant *Ph*DFR mutant (137L, 138N)**		–	1.48	1.48

Enzyme preparations from strawberry fruits were used as control to demonstrate integrity of the DHK and DHQ substrates.
**DHK conversion was not observed even if incubation time was extended to 24 h at 4°C; **Numbering according to the deduced amino acid sequence of A_1 as shown in Supplementary Figure S2; –: below detection level.*

2.9 µM, V_{max} 1.25 × 10^{-3} µM/s; k_{cat} 0.70 s^{-1}; k_{cat}/K_m 0.24 $s^{-1}µM^{-1}$).

To compare the substrate specificities of the DFRs of maize and *Petunia* × *hybrida*, studies were also performed with recombinant *Petunia* × *hybrida* DFR (*Ph*DFR). Purified recombinant, *Ph*DFR showed no DHQ or DHK conversion and thus, a clear specificity for DHM (**Table 2**), which was also confirmed by the kinetic data (K_m 1.3 µM; V_{max} 0.5 × 10^{-3} µM/s; k_{cat}/K_m 2.14 s^{-1}; k_{cat}/K_m 1.6 s^{-1} $µM^{-1}$). Amino acid sequence identity between *Ph*DFR and A_1 is only 54%. Particularly the region presumably determining substrate specificity shows a difference in positions 132–134 (amino acid numbering according *Ph*DFR). To investigate whether the striking difference in the DHQ acceptance between the two recombinant DFRs is based on this region as suggested (Johnson et al., 2001), we created mutants with altered amino acids (**Table 2**). Whereas an exchange of the leucine with a valine in position 137 did not result in increased DHQ conversion, the exchange of aspartic acid with asparagine in position 138 raised DHQ conversion from zero to levels equaling those of DHM.

Gene Expression in the Orange Petunia Varieties

The transcriptomes of orange (cv. Salmon Ray), red (cv. Corso Rot) and black (cv. Blackberry) petunias were analyzed for differences in their gene expression pattern with respect to the phenotype. We particularly focused on the genes involved in color formation (Bombarely et al., 2016). The three varieties did not provide a uniform picture (Supplementary Figure S3). Whereas cv. Salmon Ray showed lower gene expressions in many of the structural genes of the flavonoid pathway, but not of the phenylpropanoid pathway, most of the structural genes in the early and late flavonoid pathway seemed to be up-regulated in the two other varieties. However, an increased *DFR:FLS* expression ratio was observed in the red and in all three orange cultivars compared to the black. This was further examined by quantitative real-time PCR.

Quantitative real-time PCR performed with primers discriminating between the DFRs from maize (A_1) and petunia (*PhDFR*) (Supplementary Table S1), showed that *PhDFR* expression strongly dominated over A_1expression (**Figure 2**, bar charts) in developing buds of the orange varieties, despite being under the control of the strong, constitutive p35*S* promoter. A_1/*PhDFR* expression ratios of approximately 1 could only be observed in open flowers. The qPCR studies also confirmed that the three orange petunia varieties had a low expression of *F3'H* and *FLS* and thus a very high *DFR:FLS* expression ratio during the flower life cycle (**Figure 6**). There was, however, no statistically significant correlation between the A_1/*PhDFR* expression ratios and the pelargonidin-type anthocyanidin concentration in the petals (**Figure 2**).

DISCUSSION

The DFR of petunia has ever been the role model for studies on DFR substrate specificity, and the resulting lack of orange flower color in petunia has always been the best example for the complex mechanisms of color establishment in flowers. The creation of an orange petunia by a transgenic approach in the 1980s at the Max Planck Institute for Plant Breeding Research in Cologne, Germany was a further landmark in the field of flower color research and the subsequent field trial attracted attention far beyond the horticultural community. Thus, the fact that petunia does not naturally possess orange flower color, and the underlying biochemical reason, has been established knowledge for a few decades (Meyer et al., 1986, 1992; Johnson et al., 1999, 2001). Therefore, the appearance of orange petunia varieties on the European market attracted the interest of scientists familiar with anthocyanin flower color. As they were not declared as genetically modified plants, which would have been compulsory if a transgenic breeding method had been used, they were apparently a result of classical breeding. Recent research demonstrated, however, that the vast majority of the commercially available orange petunia varieties, but not all, are genetically engineered and harbor the A_1 cDNA clone from *Zea mays* (Bashandy and Teeri, 2017). Despite the general consensus that the transgenic construct most probably derived from the first scientific GE-petunia (Meyer et al., 1986), it always remained unmentioned that there was a second scientific petunia (Elomaa et al., 1995), which was constructed with the same GE-elements, which could have been a possible source of the putatively unintentionally escaped A_1 cDNA clone. Our data unequivocally demonstrate, however, that of these two possible

known sources, an unintentional release of the construct of the Elomaa et al. (1995) can indeed be excluded.

In an independent approach we analyzed three varieties released by breeders from three countries, The Netherlands, Israel and Germany. All three amplicons showed identical sequences (Supplementary Figure S1) at the nucleotide level (NCBI MF521566), demonstrating that a single A_1 source had entered breeding programmes worldwide. The sequence we obtained was in line with NCBI KY964325 (Bashandy and Teeri, 2017). There is, however, an overlap, as our primers started 699 bp downstream at the 5′-end, but provided an additional 375 bp stretch at the 3′-end.

Our sequencing results identified with near absolute certainty the transformation construct of Meyer et al. (1986) as the A_1 source, based on the following characteristics (**Figure 5**, Supplementary Figure S1): the same arrangement of *35S* promoter, A_1, *35S* terminator, *nopaline synthase* (*NOS*) promoter, *nptII*, *octopine synthase (OCS)* terminator, and the restriction sites used for the p35A1 plasmid construction, as described for the Meyer et al. (1986) construct, was identified (**Figure 5**). Pre-eminently, the transgene found in the three orange petunia varieties contains the A_1 type 2 allele previously used for plasmid p35A1 construction (Meyer et al., 1986), which includes, at the 3′-end, an additional segment from the non-viral transposable *Cin4-1* sequence (Schwarz-Sommer et al., 1987a). In addition, in the transition zone between the *35S* promoter and the A_1 gene, parts of the untranslated sequences of the 5′ flanking region, described previously (Schwarz-Sommer et al., 1987b), are present. This rules out other potential, as yet unidentified, sources, as it is unlikely that a putative third, yet unknown construct would harbor this special A_1 allele, particularly as the transposable element does not add any functional advantage with respect to color formation.

Unexpectedly, the orange petunias showed the same lack of DHK acceptance under common DFR assay conditions as the blue and red flowering non-GE controls. Concordantly, the orange petals accumulate large amounts of DHK derivatives (**Table 1**) that have not been ultimately converted to pelargonidin-based pigments. Such elevated dihydroflavonol levels were not found in common petunia varieties with a high F3′5′H activity, as this favors creation of delphinidin based pigments. Likewise, in the absence of F3′5′H activity, accumulated DHK and DHQ can be converted to flavonols, if FLS is active in the petals, as is the case in the red variety (**Table 1**). The low substrate specificity for DHK was surprising, given the fact that A_1 had been introduced to explicitly enable conversion of DHK and thus, formation of pelargonidin based pigments. Much better color effects had been achieved by transformation with an unspecific gerbera *DFR* (Elomaa et al., 1995), thereby already pointing to a low substrate specificity of the maize DFR for DHK, although observed effects were rather attributed to the instability of monocotyledonous cDNA in the dicotyledonous petunia (Elomaa et al., 1995). The low substrate specificity of the protein encoded by the A_1 gene was confirmed with recombinant maize DFR obtained by heterologous expression in *E. coli*.

Only low expression rates were observed for the A_1 gene, which is in accordance with findings of epigenetic downregulation effects in GE-petunia (Linn et al., 1990; Meyer and Heidmann, 1994; Meyer, 1998). A_1 gene expression generally remained below the rates measured for the *PhDFR*. Highest expression was found in fully developed flowers, where an A_1/*PhDFR* ratio of up to 1 could be measured. Despite this, the content of pelargonidin-based pigments remained surprisingly unchanged during flower development (**Figure 2**). There was, however, no statistically relevant correlation between the pelargonidin-based pigment concentration and the A_1 gene expression. Moreover, we never observed DHK conversion with enzyme preparations of the orange petunia petals independently of a high (open flowers) or low A_1/*PhDFR* (buds) expression ratio, raising the question, if, besides the maize DFR, the petunia DFR could also contribute to pelargonidin-precursor production.

To determine how the orange coloration may occur at all, if a poorly expressed non-petunia *DFR*, with an additionally low substrate specificity for DHK, was present in GE-petunia petals, we compared the transcriptomes of the three orange varieties (cvs. Salmon Ray, Viva Orange, Electric Orange), red (cv. Corso Rot) and black (cv. Blackberry) petunias (Supplementary Figure S3) and analyzed the genes particularly involved in color formation (Bombarely et al., 2016). An increased *DFR:FLS* expression ratio in the red and orange cultivars was observed, compared to the black, which was confirmed by qPCR (**Figure 6**) and by the relatively small amounts of flavonols found in the petals of orange varieties (**Table 1**).

Apparently, the establishment of orange petunia flower color can occur only in the absence of interfering F3′H and FLS activities (**Figure 6**). Considering the low substrate specificity of both *Ph*DFR and A_1 protein for DHK, sufficient

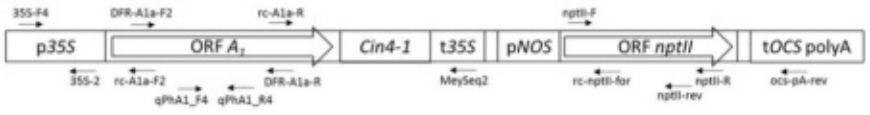


FIGURE 5 | Schematic representation of the transgenic insert found in the three GE-petunia varieties cvs. Salmon Ray, Viva Orange and Electric Orange (NCBI MF521566) p35S, promoter sequence of the *35S* Cauliflower mosaic virus gene; A_1, coding sequence of the A_1 gene; *Cin4-1*, partial *Cin4-1* transposable element present in type 2 allele of A_1 according to Schwarz-Sommer et al. (1987a,b); t35S, terminator sequence of the *35S* Cauliflower mosaic virus gene; pNOS, promoter sequence of the nopaline synthase gene; *nptII*, coding sequence of the neomycine phosphotransferase II selectable marker gene; tOCS, terminator sequence of the octopine synthase gene. Positions of primers are marked by arrows. Primer sequences are provided in Supplementary Table S1.

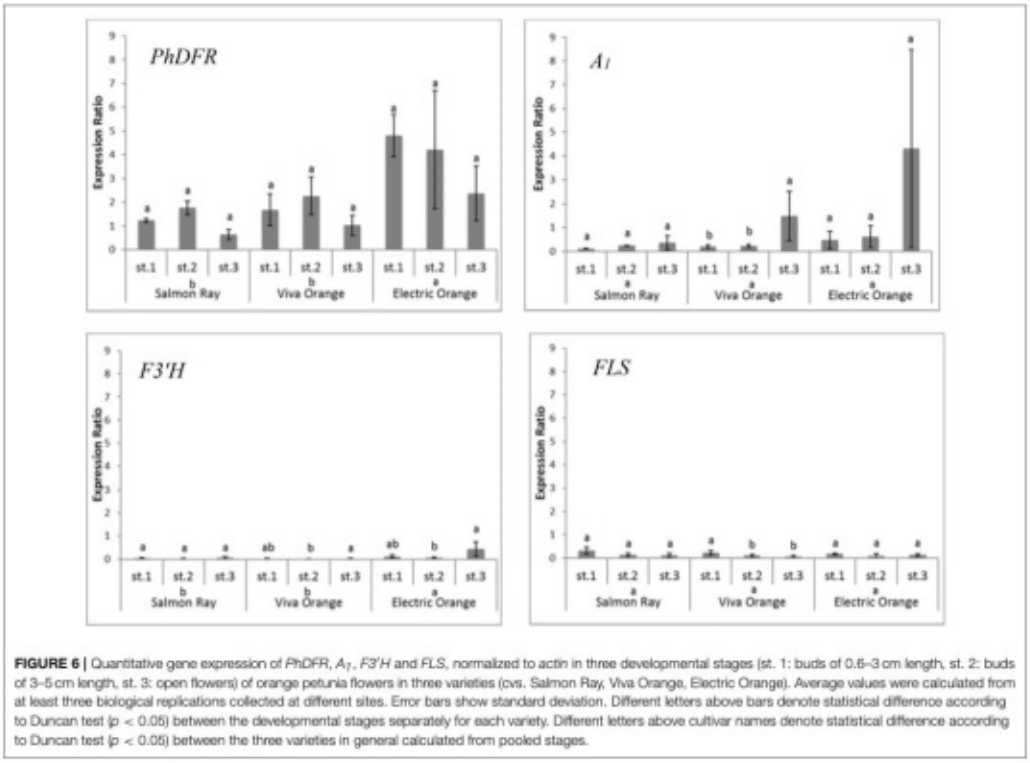


FIGURE 6 | Quantitative gene expression of *PhDFR*, A_1, *F3'H* and *FLS*, normalized to actin in three developmental stages (st. 1: buds of 0.6–3 cm length, st. 2: buds of 3–5 cm length, st. 3: open flowers) of orange petunia flowers in three varieties (cvs. Salmon Ray, Viva Orange, Electric Orange). Average values were calculated from at least three biological replications collected at different sites. Error bars show standard deviation. Different letters above bars denote statistical difference according to Duncan test ($p < 0.05$) between the developmental stages separately for each variety. Different letters above cultivar names denote statistical difference according to Duncan test ($p < 0.05$) between the three varieties in general calculated from pooled stages.

leucopelargonidin precursor for pelargonidin-type flowers will be synthesized (i) very slowly over a long time, (ii) only if no other dihydroflavonol precursor is present (absent or low F3′H and F3′5′H activity), and (iii) if the accumulating DHK is not redirected by a highly active FLS toward flavonols. In the same way, cyanidin-based red flowering petunia varieties occur naturally, despite the fact that *Ph*DFR shows stringent specificity for DHM as substrate (**Table 2**). The red cultivar used as a control accumulates a substantial amount of dihydroflavonols, however, thereby confirming the low *FLS* expression and elevated *DFR:FLS* expression ratio in the red cultivars already indicated by the transcriptome analysis. Considering the DFR substrate specificity of petunia for DHM, increased flavonol formation, at the expense of anthocyanin accumulation, and thus, only a pale color, would be expected in the case of a highly active petunia FLS. The elaborate creation of transgenic petunia and the current global commotion surrounding the escaped A_1 gene seem to be a great cause, in comparison to the relatively small color effects attained by the use of the inefficient maize DFR.

American and European authorities unambiguously stated that the GE-petunia is not harmful to consumers and environment. It is still unknown, how plants harboring the A_1 construct of Meyer et al. (1986) entered classical breeding programmes. Nefarious use of GE-plants is unlikely, due to foreseeable troubles when plants inevitably attract attention. There are, however, several scenarios how the GE-petunia could have escaped. After its creation at the Max Planck Institute for Plant Breeding Research in Cologne (Meyer et al., 1986), and the contentious field trial in Germany in 1990, the plants were kept in several institutions, and were also used for breeding purposes (Oud et al., 1995; Servick, 2017), followed by field trials in the US. Currently, the most favored explanation (Servick, 2017) seems to be that during a chain of company fusions the GE-background of orange petunias was forgotten, and the lines could therefore enter new breeding programmes. Our results demonstrate why the presence of the A_1 does not result in orange phenotypes in a common biochemical petunia background, which facilitates undetected dispersion. Thus, the original GE-petunia (Meyer et al., 1986) or progenies thereof, created by classical breeding (Oud et al., 1995) or—less likely—by escaped pollen, in the field or in the greenhouse, could have infiltrated classical breeding chains, unless it re-emerged as orange petunia in a rare event of a proper genetic background. The large spectrum of undeclared GE-petunia varieties can be explained by the use of early orange varieties as parent plants in classical breeding attempts for further orange varieties by other companies and by the use of non-orange

breeding material harboring an unrecognized A_1. The fact that a single construct was found so far in the orange petunia varieties, as opposed to plural different constructs, points at a single event in the breeding chain rather than at multiple parallel events.

In the current debate, it was iterated that real orange petunia flower colors cannot occur naturally (David, 2017). Some few petunia varieties, however, show a pattern of red and yellow pigments that, at a glance, might be mistaken for orange (Bashandy and Teeri, 2017). But even minor mutations in the active site of the DFR can result in higher DHK specificity, as demonstrated by the existing patent for a DHK specific DFR (Johnson et al., 2001). A spontaneous mutation occurring in a suitable background, although unlikely, could indeed provide a naturally orange-flowering petunia. This could also be achieved by cutting-edge genome editing methods causing a targeted mutation, which can currently not be distinguished from mutations induced by well accepted methods such as mutation via chemicals or radiation. It remains to be seen how the escaped GE-petunias will influence the current debate about the classification of genome editing as a genetic engineering method, and on biotech patents in general, which was provoked by the recent barley patents obtained by large brewing companies.

AVAILABILITY OF DATA AND MATERIALS

All data supporting the findings is contained in the manuscript and its supplementary files. Transcriptome data are available at the Short Read Archive of the International Nucleotide Sequence Database Collaboration: SAMN07988829 (*Petunia* × *hybrida* cv. Blackberry), SAMN07988830 (*Petunia* × *hybrida* cv. Corso Rot), SAMN07988831 (*Petunia* × *hybrida* cv. Viva Orange), SAMN07988832 (*Petunia* × *hybrida* cv. Electric Orange), SAMN07988833 (*Petunia* × *hybrida* cv. Salmon Ray).

AUTHOR CONTRIBUTIONS

HH, CH-G, and KS: Conceived the research and wrote the manuscript; SM, DN, BR, BW, RP, RL, and LE: conducted the experiments; TR and KO: analyzed the data. All authors approved the manuscript.

FUNDING

We acknowledge funding by the Austrian Science Fund (FWF) P 28134-B25 and from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 675657.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00149/full#supplementary-material

REFERENCES

Bashandy, H., and Teeri, T. H. (2017). Genetically engineered orange petunias on the market. *Planta* 246, 277–280. doi: 10.1007/s00425-017-2722-8

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Bombarely, A., Moser, M., Amrad, A., Bao, M., Bapaume, L., Barry, C. S., et al. (2016). Insight into the evolution of the Solanaceae from the parental genomes of *Petunia hybrida*. *Nat. Plants* 2:16074. doi: 10.1038/nplants.2016.74

David, M. (2017). U.S. flower sellers rush to destroy illegal GE petunias. *Sci. Post. Plants Anim. Sci. Policy*. doi: 10.1126/science.aal1200

De Mendiburu, F. (2017). *Agricolae: Statistical Procedures for Agricultural Research*. R package version 1.2-8. Available online at: https://CRAN.R-project.org/package=agricolae.

Duncan, D. B. (1955). Multiple range and multiple F tests. *Biometrics* 11, 1–42. doi: 10.2307/3001478

Elomaa, P., Helariutta, Y., Kotilainen, M., Teeri, T. H., Griesbach, R. J., and Seppänen, P. (1995). Transgene inactivation in *Petunia hybrida* is influenced by the properties of the foreign gene. *Mol. Gen. Genet.* 248, 649–656. doi: 10.1007/BF02191704

Gosch, C., Nagesh, K. M., Thill, J., Miosic, S., Plaschil, S., Milosevic, M., et al. (2014). Isolation of Dihydroflavonol 4-Reductase cDNA Clones from Angelonia × angustifolia and Heterologous Expression as GST Fusion Protein in *Escherichia coli*. *PLoS ONE* 9:e107755. doi: 10.1371/journal.pone.0107755

Halbwirth, H. (2010). The creation and physiological relevance of divergent hydroxylation patterns in the flavonoid pathway. *Int. J. Mol. Sci.* 11, 595–621. doi: 10.3390/ijms11020595

Halbwirth, H., Kahl, S., Jager, W., Reznicek, G., Forkmann, G., and Stich, K. (2006). Synthesis of (^{14}C)-labeled 5-deoxyflavonoids and their application in the study of dihydroflavonol/leucoanthocyanidin interconversion by dihydroflavonol 4-reductase. *Plant Sci.* 170, 587–595. doi: 10.1016/j.plantsci.2005.10.013

Ihaka, R., and Gentleman, R. (1996). R: a language for data analysis and graphics. *J. Comput. Graph. Stat.* 5, 299–314.

Johnson, E. T., Ryu, S., Yi, H., Shin, B., Cheong, H., and Choi, G. (2001). Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. *Plant J.* 25, 325–333. doi: 10.1046/j.1365-313x.2001.00962.x

Johnson, E. T., Yi, H., Shin, B., Oh, B. J., Cheong, H., and Choi, G. (1999). Cymbidium hybrida dihydroflavonol 4-reductase does not efficiently reduce dihydrokaempferol to produce orange pelargonidin-type anthocyanins. *Plant J.* 19, 81–85. doi: 10.1046/j.1365-313X.1999.00502.x

Kopylova, E., Noé, L., and Touzet, H. (2012). SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* 28, 3211–3217. doi: 10.1093/bioinformatics/bts611

Li, B., and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12:323. doi: 10.1186/1471-2105-12-323

Linn, F., Heidmann, I., Saedler, H., and Meyer, P. (1990). Epigenetic changes in the expression of the maize A1 gene in *Petunia hybrida*: role of numbers of integrated gene copies and state of methylation. *Mol. Gen. Genet.* 222, 329–336. doi: 10.1007/BF00633837

Lipp, M., Brodmann, P., Pietsch, K., Pauwels, J., Anklam, E., Borchers, T., et al. (1999). IUPAC collaborative trial study of a method to detect genetically modified soy beans and maize in dried powder. *J. AOAC Int.* 82, 923–928.

Mallona, I., Lischewski, S., Weiss, J., Hause, B., and Egea-Cortines, M. (2010). Validation of reference genes for quantitative real-time PCR during leaf and flower development in *Petunia hybrida*. *BMC Plant Biol.* 10:4. doi: 10.1186/1471-2229-10-4

Meyer, P. (1998). Stabilities and instabilities in transgene expression. *Trans. Plant Res.* 263–275.

Meyer, P., and Heidmann, I. (1994). Epigenetic variants of a transgenic petunia line show hypermethylation in transgene DNA: an indication for specific recognition of foreign DNA in transgenic plants. *Mol. Gen. Genet.* 243, 390–399.

Meyer, P., Heidmann, I., Forkmann, G., and Saedler, H. (1986). A new petunia flower colour generated by transformation of a mutant with a maize gene. *Nature* 330, 677–678. doi: 10.1038/330677a0

Meyer, P., Linn, F., Heidmann, I., Meyer, H., Niedenhof, I., and Saedler, H. (1992). Endogenous and environmental factors influence 35S promoter methylation of a maize A1 gene construct in transgenic petunia and its colour phenotype. *Mol. Gen. Genet.* 231, 345–352. doi: 10.1007/BF00292701

Miosic, S., Thill, J., Milosevic, M., Gosch, C., Pober, S., Molitor, C., et al. (2014). Dihydroflavonol 4-reductase genes encode enzymes with contrasting substrate specificity and show divergent gene expression profiles in Fragaria Species. *PLoS ONE* 9:e112707. doi: 10.1371/journal.pone.0112707

Oud, J. S., Schneiders, H., Kool, A. J., and Van Grinsven, M. Q. (1995). Breeding of transgenic orange *Petunia hybrida* varieties. *Euphytica* 84, 175–181. doi: 10.1007/BF01681809

Petit, P., Granier, T., d'estaintot, B. L., Manigand, C., Bathany, K., Schmitter, J. M., et al. (2007). Crystal structure of grape Dihydroflavonol 4-Reductase, a Key Enzyme in Flavonoid Biosynthesis+. *J. Mol. Biol.* 368, 1345–1357. doi: 10.1016/j.jmb.2007.02.088

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.* 29:e45. doi: 10.1093/nar/29.9.e45

Razali, N. M., and Wah, Y. B. (2011). Power comparisons of shapiro-wilk, kolmogorov-smirnov, lilliefors and Anderson-darling tests. *J. Stat. Model. Anal.* 2, 21–33.

Schwarz-Sommer, Z., Leclercq, L., Göbel, E., and Saedler, H. (1987a). Cin4, an insert altering the structure of the A1 gene in Zea mays, exhibits properties of nonviral retrotransposons. *EMBO J.* 6:3873.

Schwarz-Sommer, Z., Shepherd, N., Tacke, E., Gierl, A., Rohde, W., Leclercq, L., et al. (1987b). Influence of transposable elements on the structure and function of the A1 gene of Zea mays. *EMBO J.* 6:287.

Servick, K. (2017). The strange case of the orange petunias. *Science* 356:792. doi: 10.1126/science.356.6340.792

Tanaka, Y., Brugliera, F., and Chandler, S. (2009). Recent progress of flower colour modification by biotechnology. *Int. J. Mol. Sci.* 10, 5350–5369. doi: 10.3390/ijms10125350

Tanaka, Y., Brugliera, F., Kalc, G., Senior, M., Dyson, B., Nakamura, N., et al. (2010). Flower color modification by engineering of the flavonoid biosynthetic pathway: practical perspectives. *Biosci. Biotechnol. Biochem.* 74, 1760–1769. doi: 10.1271/bbb.100358

Thill, J., Miosic, S., Ahmed, R., Schlangen, K., Muster, G., Stich, K., et al. (2012). 'Le Rouge et le Noir': a decline in flavone formation correlates with the rare color of black dahlia (*Dahlia variabilis* hort.) flowers. *BMC Plant Biol.* 12:225. doi: 10.1186/1471-2229-12-225

Winkel-Shirley, B. (2001). Flavonoid biosynthesis. a colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol.* 126:485. doi: 10.1104/pp.126.2.485

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Table S1: Primers used for molecular evaluation of the GE-petunias.
If applicable, their position in the construct is shown in Fig. 5.

Gene	Primer:	sequence (5'-3')	Annealing T (°C)	Purpose	Amplicon size (bp) gDNA	cDNA
p35S	35S-F4:	CATGGTGGAGCACGACACTC	60	transgene detection, sequencing	365	-
	35S-2	GATAGTGGGATTGTGCGTCA				
nptII	nptII-rev:	ATCGGGAGCGGCGATACCGTA	-	sequencing	-	-
	rc-nptII-for	CAGTCATAGCCGAATAGCCTC	-	sequencing	-	-
	nptII-F:	CAAGATGGATTGCACGCAGG	61	transgene detection	779	779
	nptII-R:	AACTCGTCAAGAAGGCGATAG				
A_f	DFR-A1a-F2:	GGGCATGCAAGGAGGCCGG	65	transgene detection	346	346
	DFR-A1a-R:	GAGGTCGTCGAGGTGGATGAGC				
	qPhA1_F4	CGACTTCTGCCGTCGCG	72	quantitative PCR*	-	118
	qPhA1_R4	GATGATGGTGACCAGGTCCAG				
	rc-A1a-R	GCTCATCCACCTCGACGACCTC	-	sequencing	-	-
	rc-A1a-F2	CCGGCCTCCTTGCATGCCC	-	sequencing	-	-
t35S	MeySeq2:	AACACATGAGCGAAACCCTATA	-	sequencing	-	-
PhDFR	Pet-DFR-F1:	TCACTTCATCTGCTGGAACTCTCGATG	65	transgene detection	565	337
	Pet-DFR-R:	GCCTCACAAAGATCATCCAAATGCACATAT				
	qPhDFR_F:	CCTTCCCGGAGCAACGG	72	quantitative PCR*	-	94
	qPhD_R:	TGGCGACGTGGAAGACG				
tOCS	ocs-pA-rev:	GTAACGGGTGATATATTCATTAGAATG	-	sequencing	-	-
Actin	PetAct11_qF:	TGCACTCCCACATGCTATCCT	72	quantitative PCR*	-	114
	PetAct11_qR:	TCAGCCGAAGTGGTGAAAGAG				
FLS	PetFLSq_F:	GATGAGGCTATCGCGGATTTAC	72	quantitative PCR*	-	102
	PetFLSq_R:	GTCGTTTGATCCTGGAGTCTTG				
F3'H	PHT1F3H_qF	CTCATTTCTTCTACAATTCATTCTTAGATC	72	quantitative PCR*	-	114
	PHT1F3H_qF	GGTCCAAGATGGACTAGGTTTC				

*amplification efficiencies: *A_f* (2.00), *Actin* (2.04), *PhDFR* (2.05), *FLS* (2.02), *F3'H* (2.00)

Supplementary Table S2: List of primers used for subcloning *DFR*s into the bacterial expression vector pGEX-6P-1 and site-directed mutagenesis

Primer Name	Sequences (5´>3´- direction)	T_m (°C)
A_1DFR-FL	GATCCATGGAGAGAGGTGCCGGT	58.0
A_1DFR-FS	CATGGAGAGAGGTGCCGGT	58.0
A_1DFR-RL	AATTCTTAAGCGCCAATCGTCG	56.0
A_1DFR-RS	CTTAAGCGCCAATCGTCG	56.0
PhDFR-FL	GATCCATGCCCCTTCACCTCCG	58.0
PhDFR-FS	CATGCCCCTTCACCTCCG	58.0
PhDFR-RL	AATTCCTAGACTTCAACATTGCTTAACATTTCTG	61.0
PhDFR-RS	CCTAGACTTCAACATTGCTTAACATTTCTG	61.0
Ph_DFR_135V_f	TGCTGGAACTGTCGATGTGCA	65.0
Ph_DFR_135V_r	GATGAAGTGAAAACCAGCC	60.0
Ph_DFR_136N_f	TGGAACTCTCAATGTGCAAGAG	59.0
Ph_DFR_136N_r	GCAGATGAAGTGAAAACC	57.0
Ph_DFR_135NV_f	TGTGCAAGAGCAACAAAAACTTTTC	57.0
Ph_DFR_135NV_r	TTGACAGTTCCAGCAGATGAAGTG	61.0

Supplementary Figure S1: Sequence alignment of the 3.3 kb transgene obtained from three orange petunia cultivars (1: Salmon Ray; 2: Viva Orange, 3: Electric Orange). p*35S*, partial promoter sequence of the *35S* Cauliflower mosaic virus gene; 5'UTR, 5' untranslated region according to Schwarz-Sommer et al. (1987a,b), A_1, coding sequence of the A_1 gene; 3'UTR, 3' untranslated region according to Schwarz-Sommer et al. (1987a,b), ISE, insertion site of elements identified in mutable A_1 alleles (Schwarz-Sommer et al., 1987a,b), *Cin4-1*, partial *Cin4-1* transposable element present in type 2 allele of A_1 (Schwarz-Sommer et al. 1987a,b); t*35S*, terminator sequence of the *35S* Cauliflower mosaic virus gene; p*NOS*, promoter sequence of the nopaline synthase gene; *nptII*, coding sequence of the neomycine phosphotransferase II selectable marker gene; t*OCS*, partial terminator sequence of the octopine synthase gene. For better visualization, every second functional DNA segment (p*35S*, A_1, *ISE*, t*35S*, p*NOS*, t*OCS*) is highlighted with grey background. Restriction enzyme sites are boxed.

partial p*35S*

```
    1                                                        50
1                                       AGAT GGACCCCCAC CCACGAGGAG
2   GATGCCTCTG CCGACAGTGG TCCCAAAGAT GGACCCCCAC CCACGAGGAG
3   GATGCCTCTG CCGACAGTGG TCCCAAAGAT GGACCCCCAC CCACGAGGAG
```

Partial p*35S*

```
    51                                                      100
1   CATCGTGGAA AAAGAAGACG TTCCAACCAC GTCTTCAAAG CAAGTGGATT
2   CATCGTGGAA AAAGAAGACG TTCCAACCAC GTCTTCAAAG CAAGTGGATT
3   CATCGTGGAA AAAGAAGACG TTCCAACCAC GTCTTCAAAG CAAGTGGATT
```

Partial p*35S*

```
    101                                                     150
1   GATGTGATAT CTCCACTGAC GTAAGGGATG ACGCACAATC CCACTATCCT
2   GATGTGATAT CTCCACTGAC GTAAGGGATG ACGCACAATC CCACTATCCT
3   GATGTGATAT CTCCACTGAC GTAAGGGATG ACGCACAATC CCACTATCCT
```

partial p*35S*

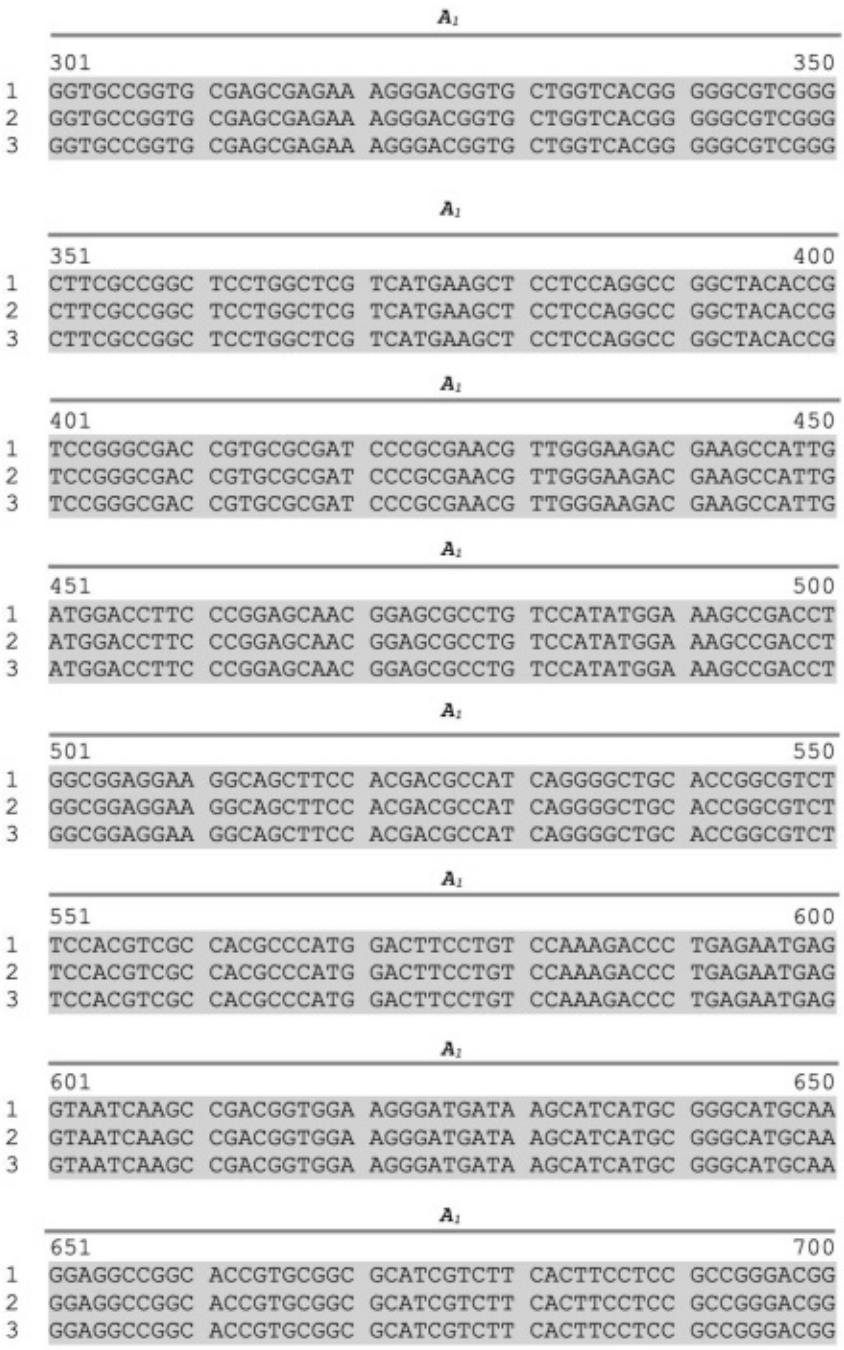
```
    151                                                     200
1   TCGCAAGACC CTTCCTCTAT ATAAGGAAGT TCATTTCATT TGGAGAGGAC
2   TCGCAAGACC CTTCCTCTAT ATAAGGAAGT TCATTTCATT TGGAGAGGAC
3   TCGCAAGACC CTTCCTCTAT ATAAGGAAGT TCATTTCATT TGGAGAGGAC
```

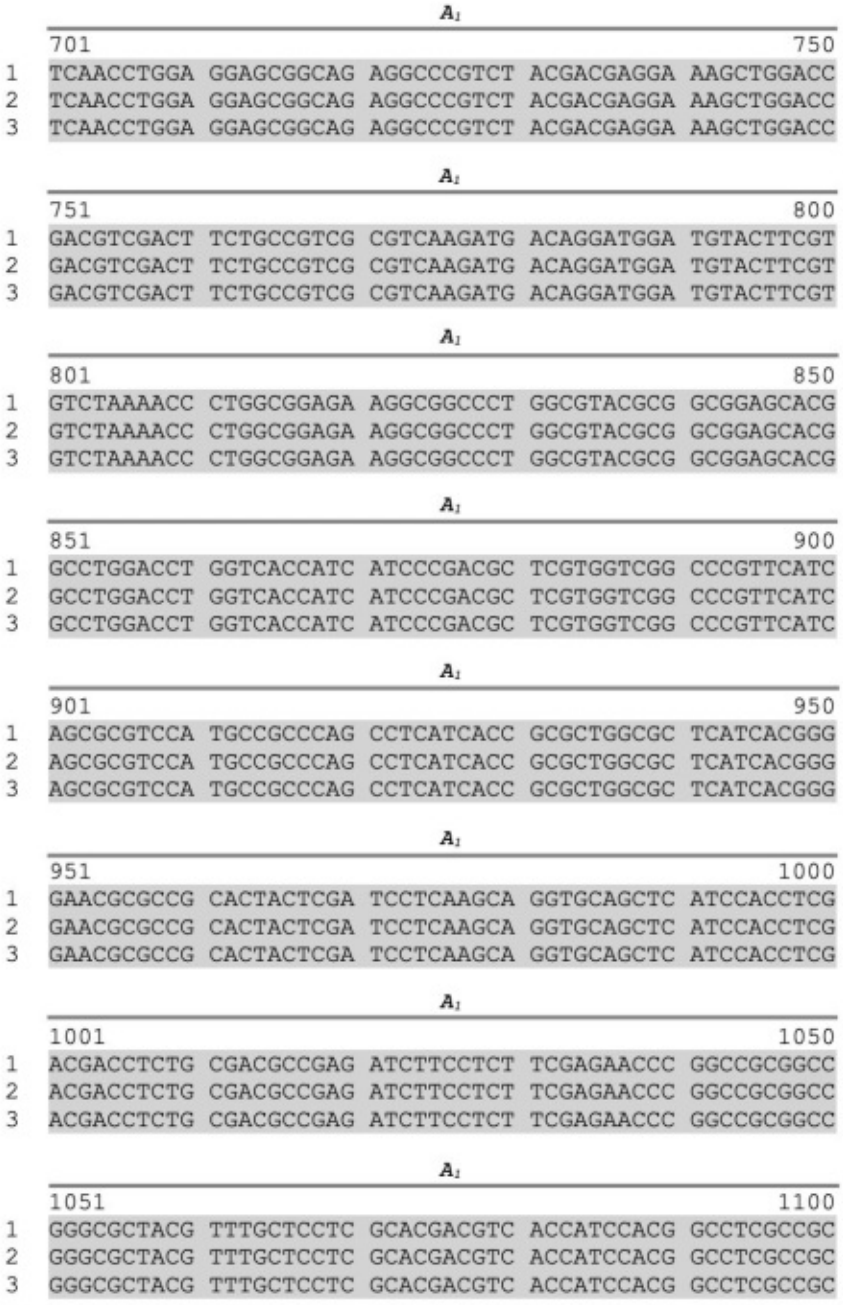
partial p*35S* *Xba*I *Eco*RI 5'UTR

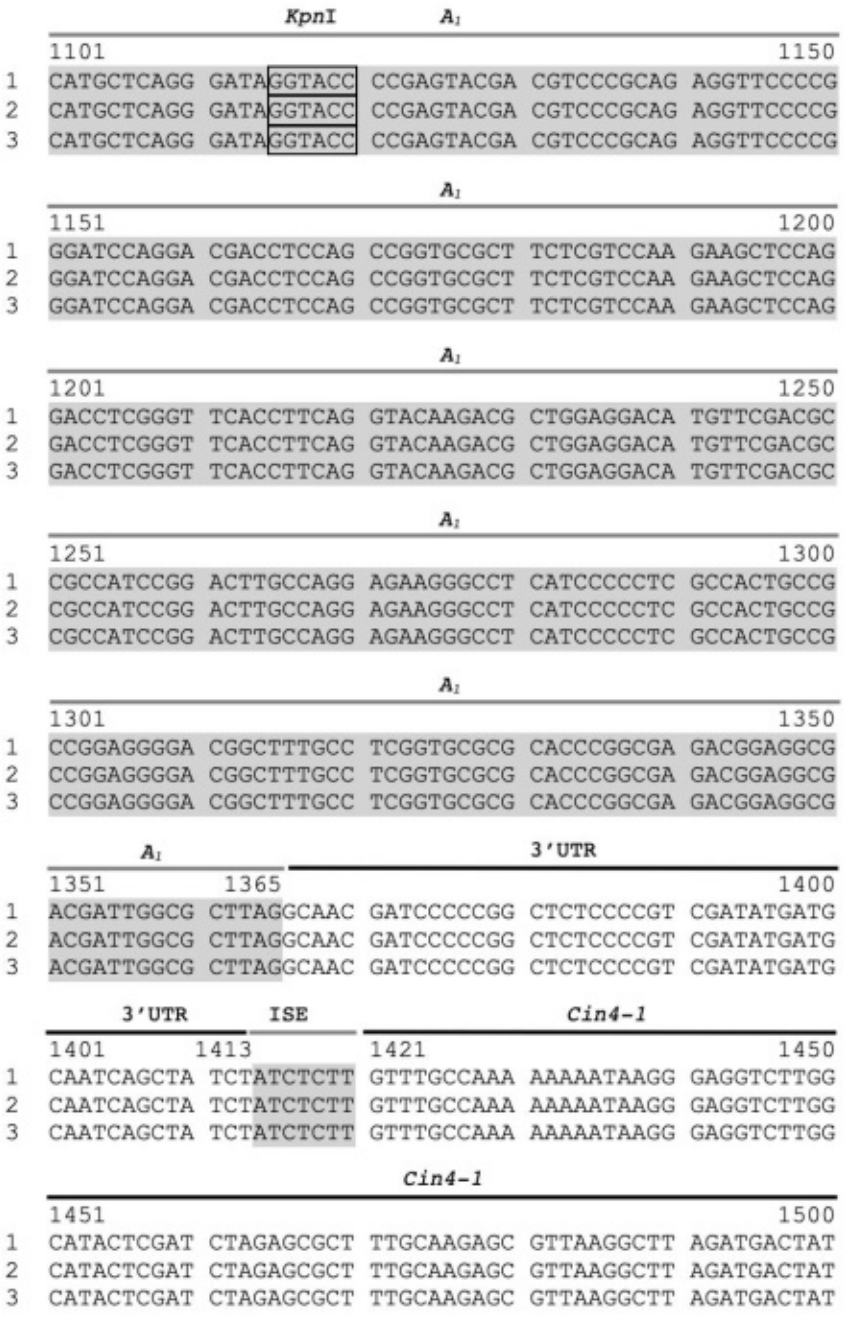
```
    201             217                 233             250
1   AGGGTACCCG GGGATCCTCT AGAGAATTCC AGCTGCTCAC TCAGTCCTGC
2   AGGGTACCCG GGGATCCTCT AGAGAATTCC AGCTGCTCAC TCAGTCCTGC
3   AGGGTACCCG GGGATCCTCT AGAGAATTCC AGCTGCTCAC TCAGTCCTGC
```

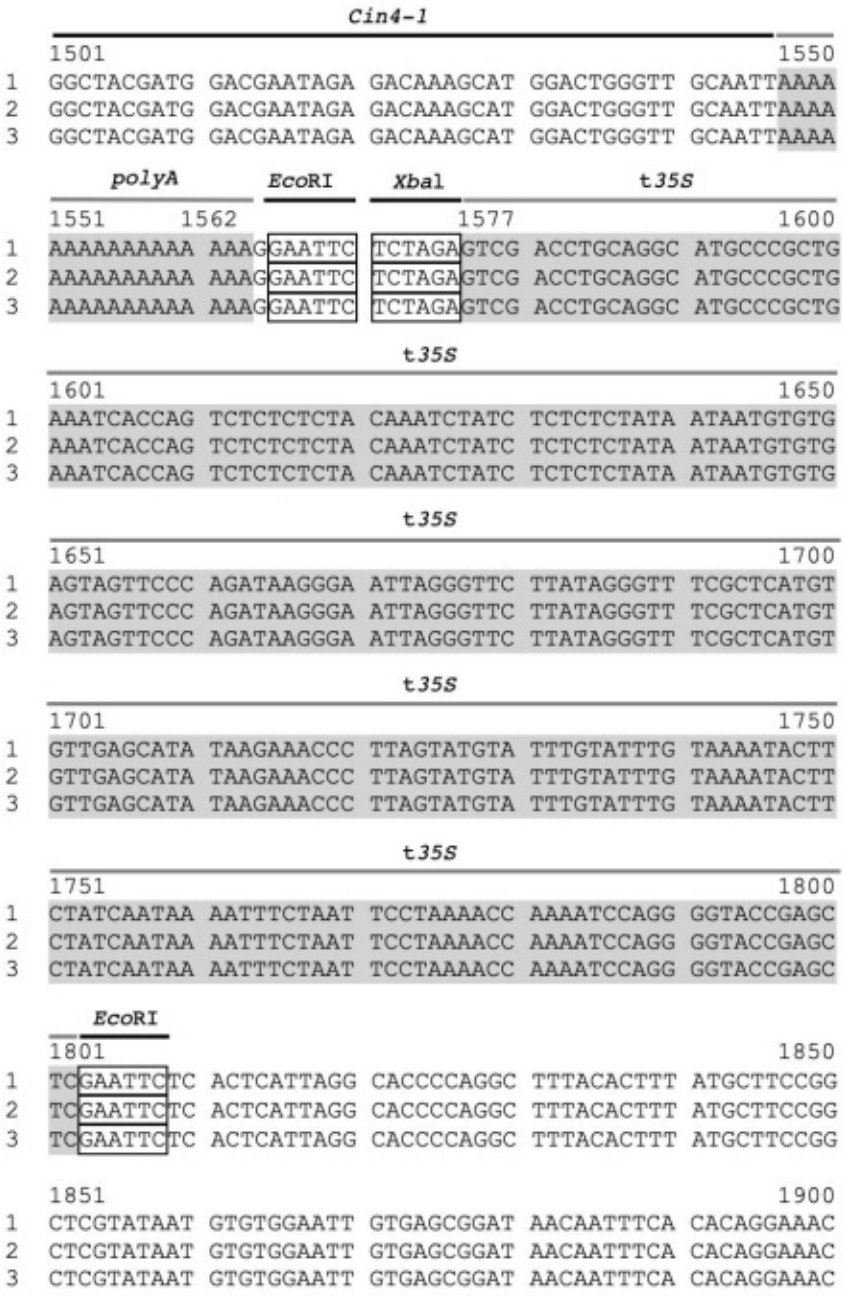
5'UTR A_1

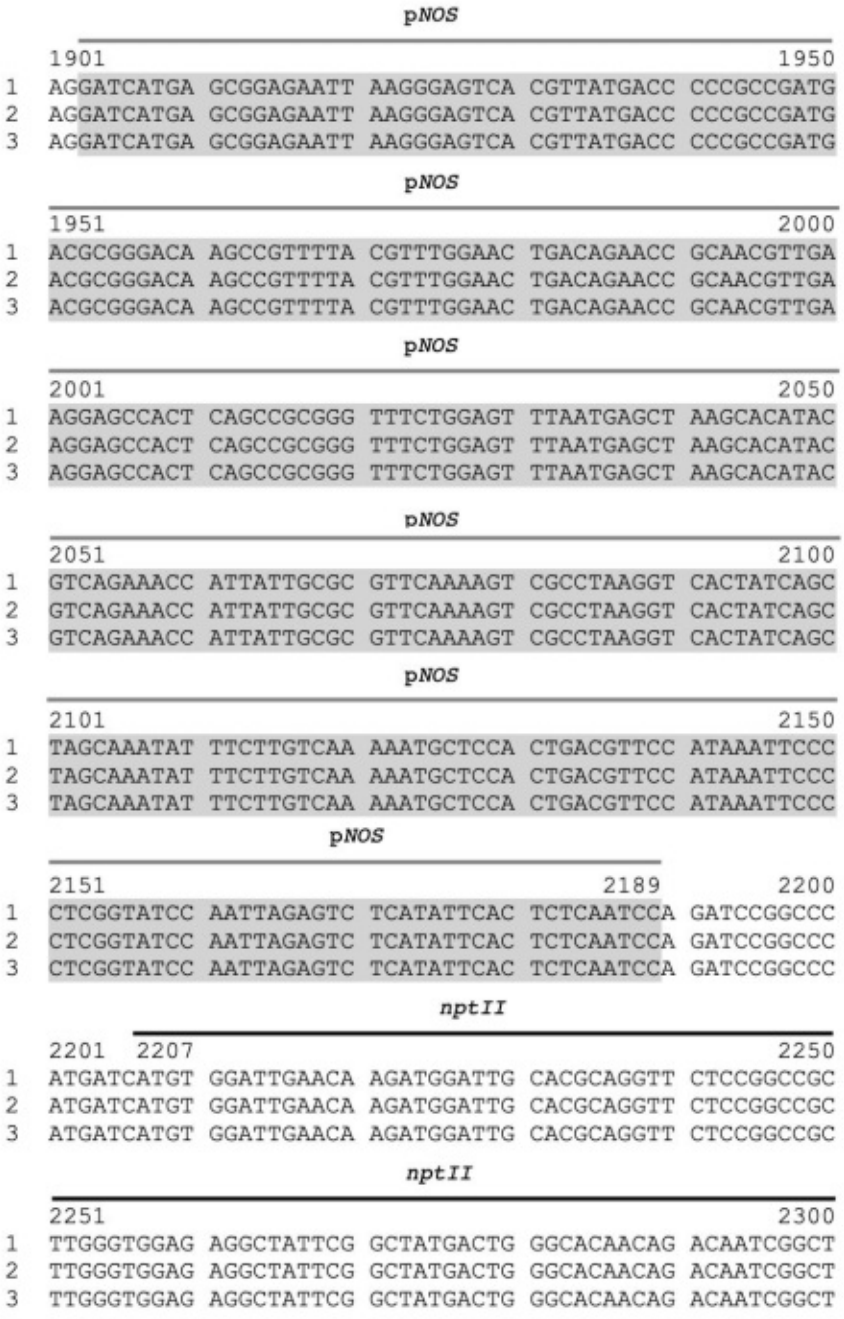
```
    251                                             292  300
1   GCAAGAGCTC GCTCTCGGAG AAAAAAACGC GGGAGGCGAT AATGGAGGGA
2   GCAAGAGCTC GCTCTCGGAG AAAAAAACGC GGGAGGCGAT AATGGAGGGA
3   GCAAGAGCTC GCTCTCGGAG AAAAAAACGC GGGAGGCGAT AATGGAGGGA
```

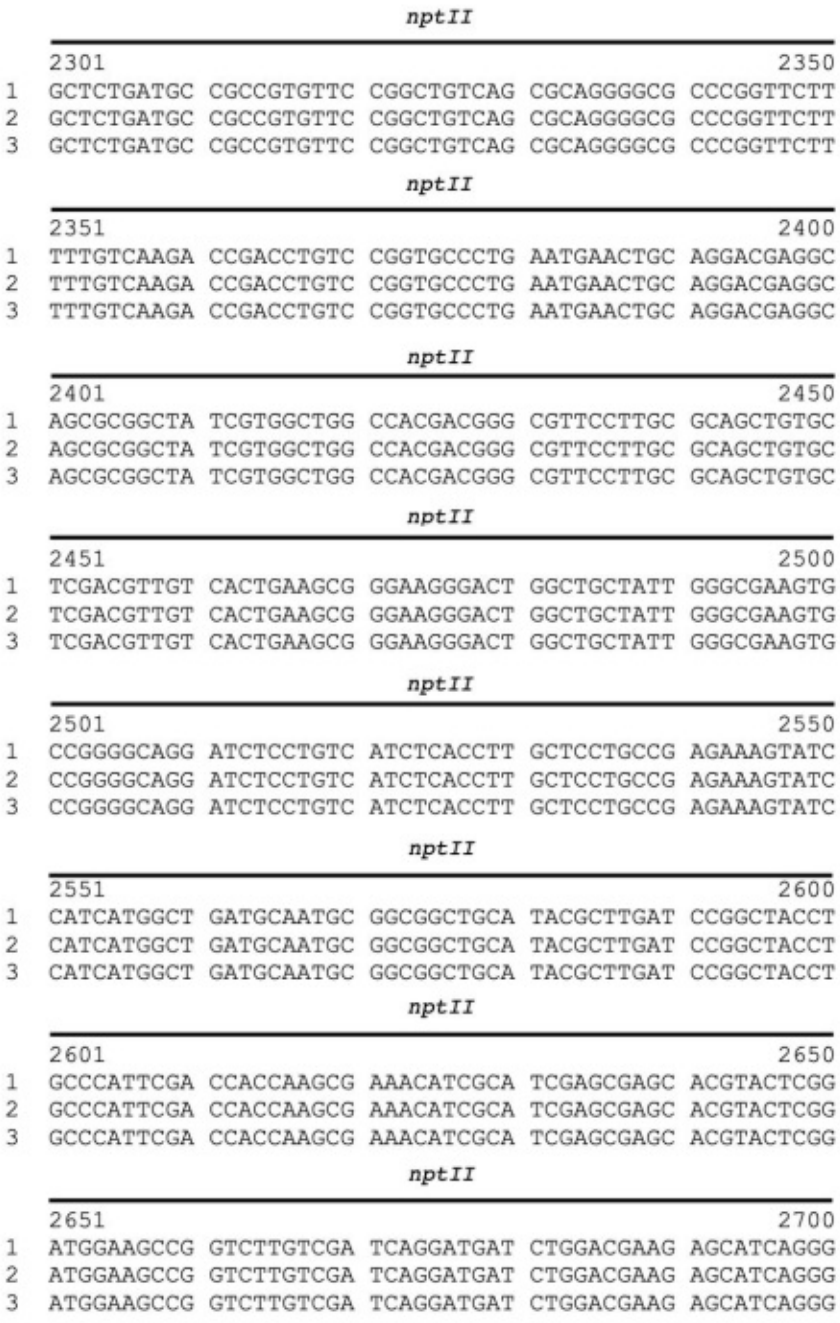


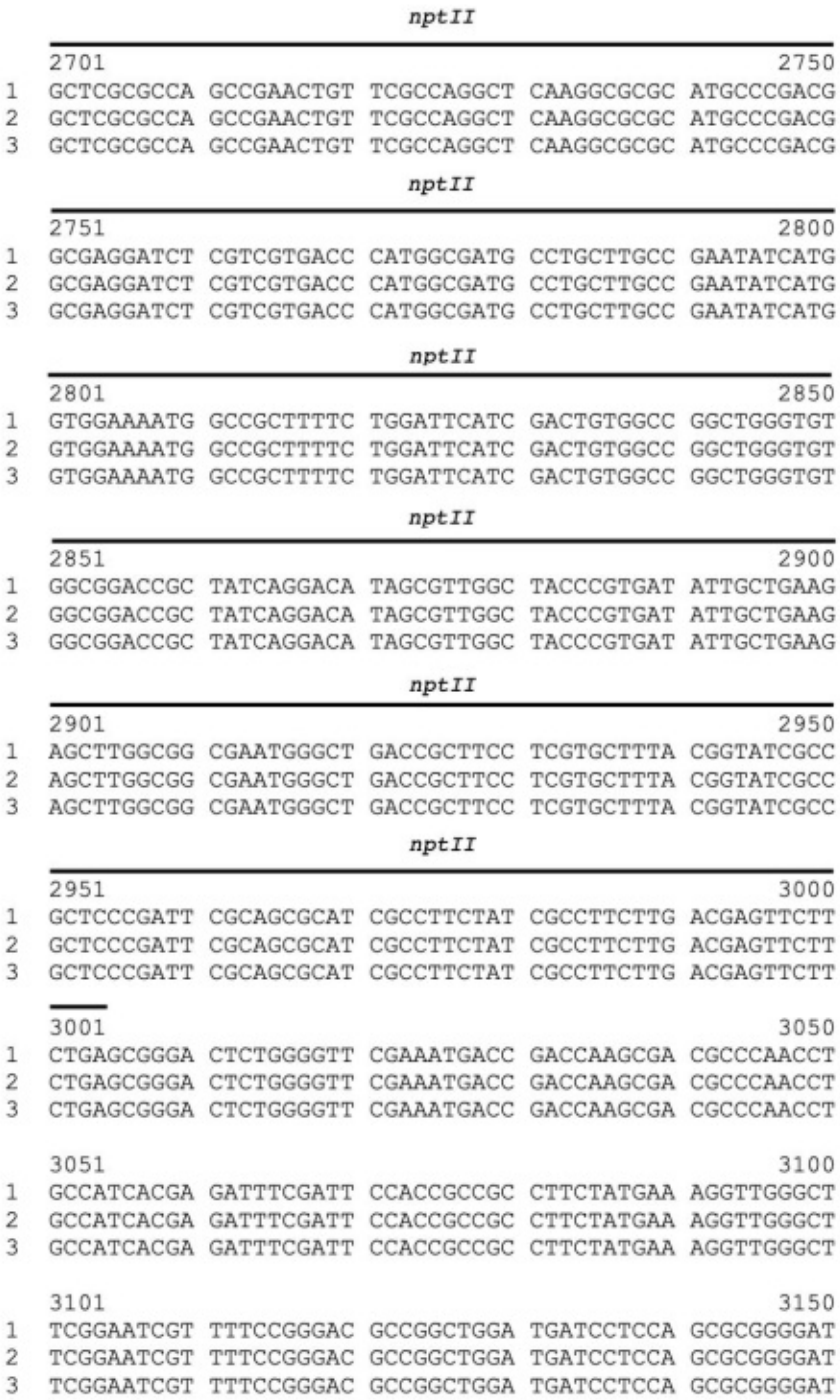


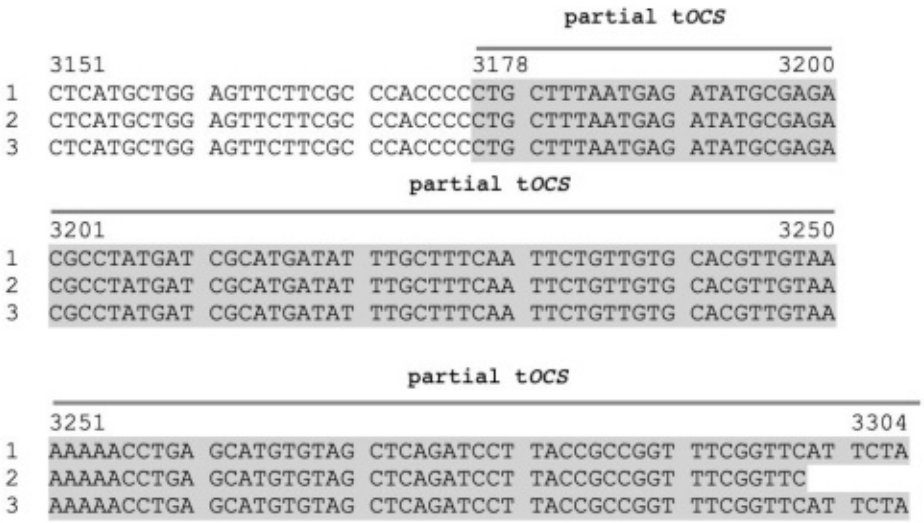












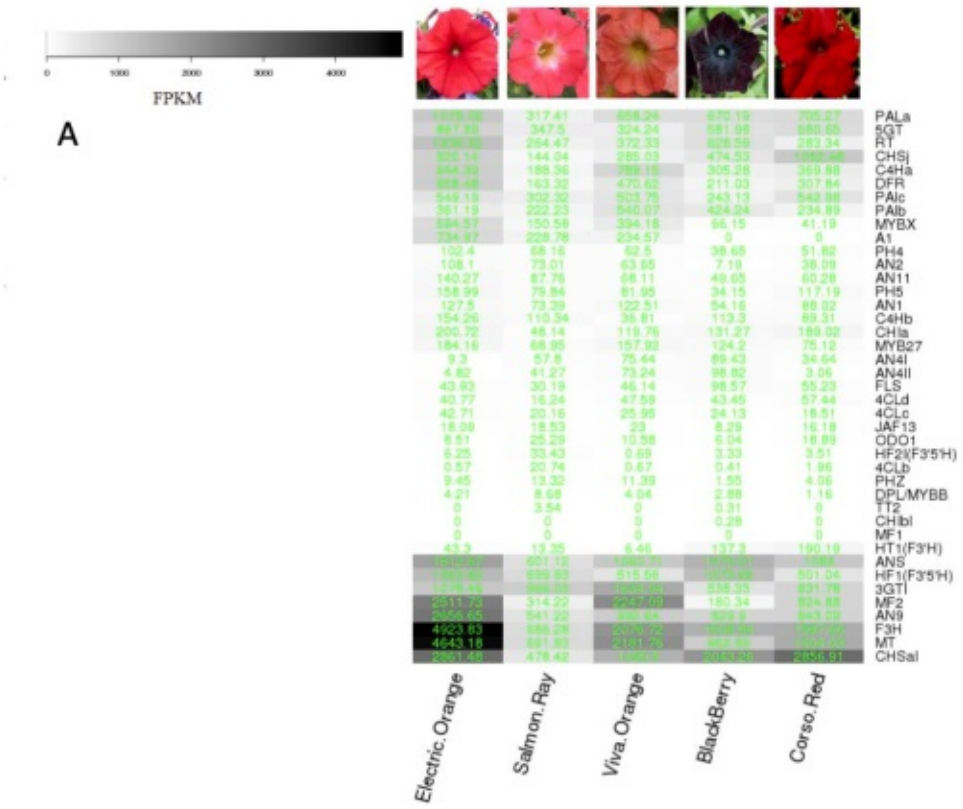
Supplementary Figure S2: Pairwise alignment of the deduced amino acid sequences of *DFR* cDNA clones of *Petunia* × *hybrida* (NCBI X15537) and *Zea mays* (A_1, NCBI CAA28734).

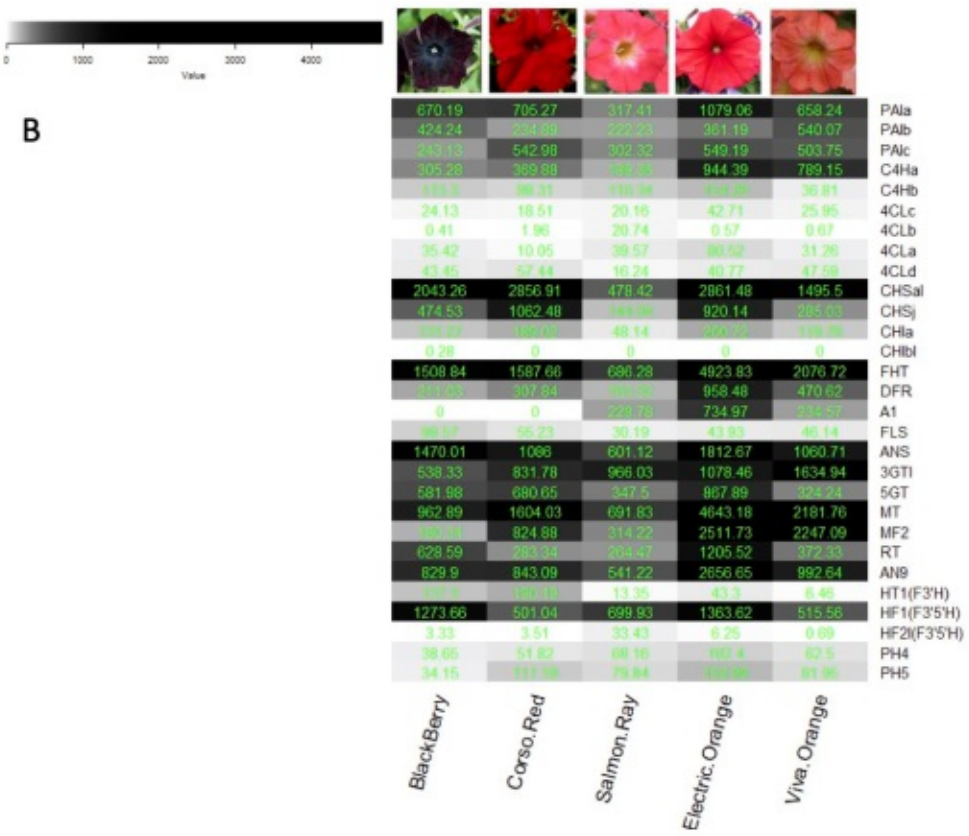
The underlined amino acids represent the 26 amino acid stretch highlighted by Johnson et al. (2001) as putative substrate specificity determining region. The amino acids exchanged by site-directed mutagenesis in this work are highlighted by grey shades.

```
                1                                                   50
          A1    MEGGAGASEK GTVLVTGASG FAGSWLVMKL LQAGYTVRAT VRDPANVGKT
PetuniaX15537   ..MPLHLRCS ATVCVTGAAG FIGSWLVMRL LERGYNVHAT VRDPENKKKV

               51                                                  100
          A1    KPLMDLPGAT ERLSIWKADL AEEGSFHDAI RGCTGVFHVA TPMDFLSKDP
PetuniaX15537   KHLLELPKAD TNLTLLKADL TVEGSFDEAI QGCQGVFHVA TPMDFESKDP

              101                                                  150
          A1    ENEVIKPTVE GMISIMRACK EAGTVRRIVF TSSAGTVNLE ERQRPVYDEE
PetuniaX15537   ENEVIKPTVR GMLSIIESCA KANTVKRLVF TSSAGTLDVQ EQQKLFYDQT

              151                                                  200
          A1    SWTDVDFCRR VKMTGWMYFV SKTLAEKAAL AYAAEHGLDL VTIIPTLVVG
PetuniaX15537   SWSDLDFIYA KKMTGWMYFA SKILAEKAAM EEAKKKNIDF ISIIPPLVVG

              201                                                  250
          A1    PFISASMPPS LITALALITG NAPHYSILKQ VQLIHLDDLC DAEIFLFENP
PetuniaX15537   PFITPTFPPS LITALSLITG NEAHYCIIKQ GQYVHLDDLC EAHIFLYEHP

              251                                                  300
          A1    AAAGRYVCSS HDVTIHGLAA MLRDRYPEYD VPQRFPGIQD DLQPVRFSSK
PetuniaX15537   KADGRFICSS HHAIIYDVAK MVREKWPEYY VPTEFKGIDK DLPVVSFSSK

              301                                                  350
          A1    KLQDLGFTFR YKTLEDMFDA AIRTCQEKGL IPLATAAGGD GFASVRAPGE
PetuniaX15537   KLTDMGFQFK Y.TLEDMYKG AIDTCRQKQL LPFSTRSAED NGHNREAIAI

              351                    376
          A1    TEATIGA... .......... ......
PetuniaX15537   SAQNYASGKE NAPVANHTEM LSNVEV
```

Supplementary Figure S3: RNA expression values of genes related to colour formation according to Bombarly et al. (2016) in *Petunia* × *hybrida* for cv. Electric Orange, cv. Salmon Ray, cv. Viva Orange, cv. Blackberry and cv. Corso Rot using the *Petunia axillaris* genome as reference.

The green numbers represent the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values of the reads after normalization. A: full gene set; B: selection of structural genes ordered according to the phenylpropanoid/flavonoid pathway for better overview (next page).





Evaluation of the housekeeping gene for qPCR studies:

For our results, qPCR data are particularly important to confirm indications of the transcriptome data and to investigate gene expression of selected flavonoid genes relative to each other in the three developmental stages of the three varieties. In this case, the influence of the reference gene is almost negligible as it is eliminated in e.g. *A_1:PhDFR* gene expression ratios. We nevertheless made strong efforts to choose a reliable reference gene and screened primers for reference genes used in literature for *Petunia* sp. (Mallona, 2010). The majority showed insufficient specificity based on the melt curves we obtained and were therefore sorted out. We evaluated two candidates of reference genes, *actin* (Suppl. Table S1) and *SAND* (Mallona, 2010) with respect to expression stability and quality of signals. The two reference genes showed somewhat differing absolute values in the expression during flower development but both confirmed the very low gene expression of *F3'H* and *FLS* in comparison to *PhDFR* in the tested samples. Based on this results, we used *actin*, as reference gene, because it showed best melting curves and lower C_t-values than *SAND*.

2.2 Publication 2

The rare orange-red colored *Euphorbia pulcherrima* cultivar 'Harvest Orange'shows a nonsense mutation in a flavonoid 3'-hydroxylase allele expressed in the bracts

D. Nitarska, C. Stefanini, C. Haselmair-Gosch, S. Miosic, B. Walliser, M. Mikulic-Petkovsek, I. Regos, A. Slatnar, T. Debener, D. Terefe-Ayana, V. Vilperte, J. Hadersdorfer, K. Stich and H. Halbwirth

BMC plant biology, *18*(1), pp.1-12. (2018), doi.org/10.1186/s12870-018-1424-0

Nitarska et al. BMC Plant Biology (2018) 18:216
https://doi.org/10.1186/s12870-018-1424-0

BMC Plant Biology

RESEARCH ARTICLE Open Access

The rare orange-red colored *Euphorbia pulcherrima* cultivar 'Harvest Orange' shows a nonsense mutation in a flavonoid 3'-hydroxylase allele expressed in the bracts

Daria Nitarska[1], Carmen Stefanini[2], Christian Haselmair-Gosch[1], Silvija Miosic[1], Benjamin Walliser[1], Maja Mikulic-Petkovsek[3], Ionela Regos[2], Ana Slatnar[3], Thomas Debener[4], Diro Terefe-Ayana[4], Vinicius Vilperte[4], Johannes Hadersdorfer[2], Karl Stich[1] and Heidi Halbwirth[1*]

Abstract

Background: Commercially available poinsettia (*Euphorbia pulcherrima*) varieties prevalently accumulate cyanidin derivatives and show intense red coloration. Orange-red bract color is less common. We investigated four cultivars displaying four different red hues with respect to selected enzymes and genes of the anthocyanin pathway, putatively determining the color hue.

Results: Red hues correlated with anthocyanin composition and concentration and showed common dark red coloration in cultivars 'Christmas Beauty' and 'Christmas Feeling' where cyanidin derivatives were prevalent. In contrast, orange-red bract color is based on the prevalent presence of pelargonidin derivatives that comprised 85% of the total anthocyanin content in cv. 'Premium Red' and 96% in cv. 'Harvest Orange' (synonym: 'Orange Spice'). cDNA clones of flavonoid 3'-hydroxylase (*F3'H*) and dihydroflavonol 4-reductase (*DFR*) were isolated from the four varieties, and functional activity and substrate specificity of the corresponding recombinant enzymes were studied. Kinetic studies demonstrated that poinsettia DFRs prefer dihydromyricetin and dihydroquercetin over dihydrokaempferol, and thus, favor the formation of cyanidin over pelargonidin. Whereas the *F3'H* cDNA clones of cultivars 'Christmas Beauty', 'Christmas Feeling', and 'Premium Red' encoded functionally active enzymes, the *F3'H* cDNA clone of cv. 'Harvest Orange' contained an insertion of 28 bases, which is partly a duplication of 20 bases found close to the insertion site. This causes a frameshift mutation with a premature stop codon after nucleotide 132 and, therefore, a non-functional enzyme. Heterozygosity of the *F3'H* was demonstrated in this cultivar, but only the mutated allele was expressed in the bracts. No correlation between *F3'H*-expression and the color hue could be observed in the four species.

Conclusions: Rare orange-red poinsettia hues caused by pelargonidin based anthocyanins can be achieved by different mechanisms. F3'H is a critical step in the establishment of orange red poinsettia color. Although poinsettia DFR shows a low substrate specificity for dihydrokaempferol, sufficient precursor for pelargonidin formation is available *in planta*, in the absence of F3'H activity.

Keywords: Poinsettia (*Euphorbia pulcherrima*), Bract coloration, Flavonoid 3'-hydroxylase (F3'H), Dihydroflavonol 4-reductase (DFR), Substrate specificity, Anthocyanin, Pelargonidin, Cyanidin

* Correspondence: heidrun.halbwirth@tuwien.ac.at
[1]Institute of Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, 1060 Vienna, Austria
Full list of author information is available at the end of the article

Background

Poinsettia (*Euphorbia pulcherrima*) is a prominent ornamental plant of particular seasonal interest. The deep red coloration of their bracts induced by short days is typically associated with Christmas time in North America, Europe and Asia [1]. The bracts escort the relatively small and unimpressive reproductive structures and - as flowers - serve the function of attracting pollinators. Phylogenetically, they are leaves changing their function from photosynthesis providing assimilates for growth towards pollinator attraction [2–4]. Because of increasing competition and growing price pressure, more and more varieties are released. Breeding of poinsettia focuses on plant shape, shipping tolerability, robustness in cultivation, disease resistance, as well as on flowering time and showy color. Although the majority of cultivars show intense red bract coloration, other colors have become more popular in recent years such as pink, white, cinnamon and yellow or even bicolored, scattered or marbled types [1].

Red colors of poinsettia bracts are caused by anthocyanins [5], which are widely distributed plant pigments in flowers, fruits and other plant tissues. Anthocyanins can impart the full spectrum of red hues to poinsettia bracts, from orange, red, rosy and, pink to crimson. In the most common red poinsettias, cyanidin type anthocyanins (two hydroxy groups in B-ring) are prevalent, but pelargonidin type anthocyanins (one hydroxy group in the B-ring) are also present to some extent [6] (Fig. 1a). Even traces of the delphinidin type pigments (three hydroxy groups in B-ring), have been previously found in poinsettia [6]. The hydroxylation pattern of the B-ring of the dihydroflavonol precursors ultimately determines the anthocyanin type that is accumulated. Dihydroflavonols with one hydroxy group (dihydrokaempferol, DHK) are the precursors for orange-red pigments (pelargonidin type), with two hydroxy groups (dihydroquercetin, DHQ) for red and pink pigments (cyanidin type) and with three hydroxy groups (dihydromyricetin, DHM) purple to blue pigments (delphinidin type).

The hydroxylation pattern of flavonoids and anthocyanins is determined by different enzymes (Fig. 1a). Flavonoid 3′ -hydroxylase (F3′H) and flavonoid 3′5′-hydroxylase (F3′5′H) are essential for the introduction of a second and third hydroxy group in the B-ring of flavonoids [7]. The F3′H (EC 1.14.13.21) belongs to the subfamily CYP75B of cytochrome P450-dependent monooxygenases (P450). This enzyme class is remarkably diverse and their members are present in all types of organisms [8, 9]. Plant P450s are usually membrane-bound enzymes associated with the endoplasmic reticulum [10]. The F3′H can accept flavanones and dihydroflavonols as well as leucoanthocyanidins (flavan 3,4-diols) as substrates, and can, therefore, influence the B-ring hydroxylation pattern at all precursor levels of anthocyanidin formation [11].

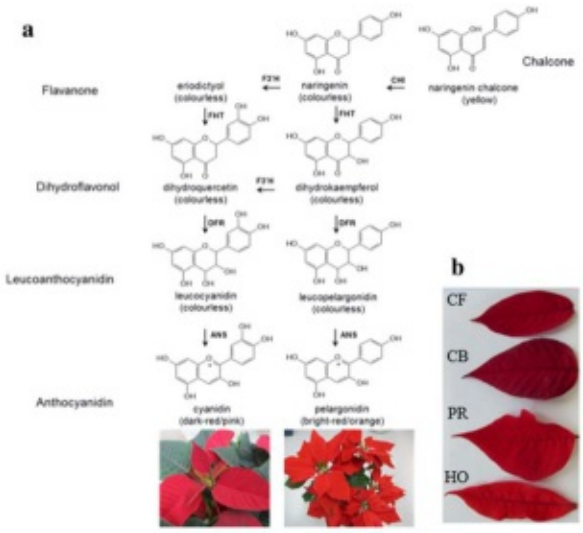


Fig. 1 a Simplified overview of the anthocyanin pathway. Abbrev: ANS: anthocyanidin synthase, CHI: chalcone isomerase, CHS: chalcone synthase, DFR: dihydroflavonol 4-reductase, FHT: flavanone 3-hydroxylase, F3′H: flavonoid 3′-hydroxylase, F3′5′H: flavonoid 3′,5′-hydroxylase. **b** *Euphorbia pulcherrima* cv. 'Christmas Feeling' (CF), cv. 'Christmas Beauty' (CB), cv. 'Premium Red' (PR), cv. 'Harvest Orange' (HO)

Another enzyme with impact on flower color is the dihydroflavonol 4-reductase (DFR, EC 1.1.1.219). The oxidoreductase catalyzes in the presence of NADPH the stereospecific reduction of the keto group in position 4 of dihydroflavonols, producing leucoanthocyanidins as precursors for anthocyanidin formation [12, 13]. The enzyme can show substrate specificity with respect to the B-ring hydroxylation pattern of the dihydroflavonol substrate and can, therefore, have an influence on the type of anthocyanin formed [14]. Flowers that accumulate pelargonidin type anthocyanins are usually characterized by low or absent F3′H and F3′5′H activities and possess a DFR that converts DHK (one hydroxy group in the B-ring) to a sufficient extent [15].

We studied the anthocyanin formation of two commonly dark-red cultivars (cvs. 'Christmas Feelings' and 'Christmas Beauty'), and two orange-red cultivars (cvs. 'Premium Red' and 'Harvest Orange'), (Fig. 1b). We show that the orange-red coloration of cv. 'Harvest Orange' is based on the almost exclusive accumulation of pelargonidin type pigments and that this correlates with a nonsense mutation in the *F3′H* gene, whereas in cv. 'Premium Red', which prevalently accumulates pelargonidin type anthocyanins, a functionally active F3′H is present. Our study establishes the base for designing strategies for breeding orange-red poinsettias accumulating prevalently pelargonidin type anthocyanin pigments.

Methods

Chemicals

(2-^{14}C)-Malonyl-coenzyme A (55 mCi/mmol) was purchased from New England Nuclear Corp. GmbH (Vienna, Austria). (^{14}C)-Labeled flavonoids naringenin, DHK, DHQ, and DHM were synthesized as previously described [16, 17] using recombinant F3′5′H from *Sollya heterophylla* and recombinant F3′H from *Arabidopsis thaliana*.

Pelargonidin-3-*O*-glucoside chloride, pelargonidin-3-*O*-rutinoside chloride, and, cyanidin-3-*O*-galactoside chloride available from Carbosynth (Berkshire, UK), cyanidin-3-*O*-glucoside was purchased from Extrasynthese (Genay, France) and cyanidin-3-*O*-rutinoside was obtained from Roth (Karlsruhe, Germany).

Plant material

The analysis was carried out with young bracts of commercially available *Euphorbia pulcherrima* cv. 'Premium Red' (PR) (Dümmen Orange GmbH, Rheinsberg, Germany), cv. 'Christmas Feelings' (CF) and cv. 'Christmas Beauty' (CB) (Klemm + Sohn GmbH & Co. KG, Stuttgart, Germany), and cv. 'Harvest Orange' (HO) (Ecke Ranch, Encinitas, USA). After the takeover of Ecke Ranch by Dümmen Orange, cv. 'Harvest Orange' was sold as cv. 'Orange Spice'. The plant material was collected in December 2015 and December 2016, frozen in liquid nitrogen and stored at − 80 °C. For HPLC analysis, samples were freeze-dried and ground in a ball mill.

Analysis of anthocyanins

Anthocyanin extraction was performed by adding 500 μL of 5% acetic acid in methanol containing 3-methoxyflavone (0.02 mg/ml) as internal standard to 100 mg of powder for a period of 45 min in an ultrasonic water bath at 5 °C. After centrifugation (10,000 x g, 10 min, 4 °C), the clear supernatant was transferred to an Eppendorf tube. A 10 μL sample of the extract was injected for HPLC analysis. The anthocyanins were separated with an RP-HPLC system consisting of two pumps (model 422, Kontron Instruments, Germany), an automatic sample injector (model 231, Gilson Abimed Systems, Germany) and a diode array detector (Kontron 540, Kontron Instruments). Chromatography was performed on a Nucleosil column (250 × 4 mm, Macherey-Nagel, Germany) with a mobile phase consisting of water containing 5% formic acid (solvent A) and methanol (solvent B) with gradient elution (Additional file 1: Table S1). Anthocyanins were monitored and analyzed on their maximum UV-Vis absorption at 540 nm. Cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, pelargonidin-3-*O*-glucoside and pelargonidin-3-*O*-rutinoside were available as authentic reference compounds. Quantification was performed using an internal standard method and calculating response factors for the standards at each concentration point on the calibration curve within the linear range. Linearity was measured at 5 concentrations. Calibration curves were constructed by plotting peak area versus concentration at 5 concentrations between 0.1–1 mg/mL for all reference compounds. Linearity was described by a regression equation and by the determination of the correlation coefficient. The identity of the anthocyanins was additionally confirmed by LC-MS analysis of cv. 'Premium Red' (Additional file 2: Table S2). LC-MS analysis was performed as previously described [6] using a mass spectrometer (LCQ Deca XP MAX, Thermo Scientific) with electrospray ionization (ESI) operating in positive ion mode using MS^2 scanning mode from *m/z* 115 to 900.

Enzyme preparation

Crude protein extracts from poinsettia bracts were obtained using protocol 1 as described earlier [18]. Briefly, 1 g bracts were homogenized with 0.5 g quartz sand and 0.5 g Polyclar AT with 6 ml 0.1 M KH_2PO_4/K_2PO_4 buffer (pH 6.5, containing 0.4% Na ascorbate). Low molecular compounds were removed by passing the crude protein extract preparation through a gel chromatography column (Sephadex G25, GE Healthcare, Freiburg, Germany). For *Euphorbia pulcherrima* DFR (*Ep*_DFR)

enzyme characterization, enzyme preparation from commercially available red poinsettia was used.

Enzyme assays

DFR assays with enzyme preparations from poinsettia bracts were performed using DHK, DHQ and DHM as substrates. The reaction contained in the final volume of 50 µL: 1–5 µL enzyme preparation, 0.048 nmol (^{14}C)-dihydroflavonol, 0.25 nmol NADPH, and 40–44 µL 0.1 M KH_2PO_4/K_2PO_4 buffer (pH 6.5 for DHK; 6.25 for DHQ; 5.75 for DHM) containing 0.4% Na ascorbate. The amount of enzyme was set up to provide that the maximum conversion rate of the best substrate was around 50% (linear range of reaction). The reaction mixture with DHK and DHQ as a substrate was incubated at 40 °C for 20 min, and stopped and extracted with 70 µL of ethyl acetate. The organic phases were transferred to pre-coated thin-layer cellulose plates without fluorescence indication (Merck, Germany) and developed in chloroform/acetic acid/water (10:9:1, v:v:v). Assays with DHM as substrate were incubated at 40 °C for 20 min and stopped with 10 µL of 100% acetic acid and 30 µL of methanol. The mixture was chromatographed on 20 cm × 1 cm stripes of paper (Schleicher Schuell, 2041 b, Germany) in chloroform/acetic acid/water (10:9:1, v:v:v). Results were evaluated on a Berthold LB 2842 Linear Analyzer (Berthold, Germany) by integration of the peak areas.

For F3′H assays with crude protein preparations from bracts or recombinant enzymes obtained from yeast, the reaction contained in the final volume of 100 µl: 40 µL enzyme preparation (1 µg/µL enzyme), 0.048 nmol (^{14}C)-naringenin or DHK, 0.05 nmol NADPH, and 55 µL 0.1 M KH_2PO_4/K_2PO_4 buffer pH 7.5 containing 0.4% Na ascorbate. The reaction mixture was incubated at 30 °C for 30 min and stopped with 10 µL 100% acetic acid. Substrate and product of the reaction were extracted with 70 µL ethyl acetate. The organic phases were transferred to pre-coated thin-layer cellulose plates without fluorescence indication (Merck, Germany) and developed in chloroform/acetic acid/water (10:9:1, v:v:v). Results were evaluated on a Berthold LB 2842 Linear Analyzer (Berthold, Germany) by integration of the peak areas.

Assays with enzyme preparations for chalcone synthase/chalcone isomerase (CHS/CHI), flavanone 3-hydroxylase (FHT) and flavonol synthase (FLS) were performed as described [18]. Separate detection of CHS and CHI is not possible because of the immediate chemical conversion of naringenin chalcone by CHI to naringenin without any cofactor requirements.

Transcriptome analysis

De novo transcriptome assembly was performed using the bioinformatic tool Trinity v2.2.0 [19]. Homology searches and functional annotation were performed using Blast2GO v4.0 and the non-redundant protein sequence database of NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db).

Cloning of *F3'Hs*

mRNA was extracted from poinsettia bracts with the µMACS mRNA isolation Kit (Miltenyi Biotec, Germany). cDNA was synthesized using the SuperScript II Reverse Transcriptase (Invitrogen, USA) and the primer oligo-dT SMART (AAGCAGTGGTATCAACGCA GAGTAC(T_{23})VN). Based on specific sequence information of *F3′H* fragments from an *E. pulcherrima* transcriptome study (Debener, unpublished), 5′-partial *F3′H* cDNA clones were isolated from the four poinsettia cultivars. The start codon was identified by alignment with the F3′H of the closely related species *Jatropha curcas* (Accession number XM_012224974). The 3′ end was identified by application of the 3′-RACE technique, using the SMARTer RACE 5′/3′ Kit (Clontech, Takara Bio Europe, France). Full size cDNA was amplified with the primer pair Ep_F3′H_full (Additional file 3: Table S3) using the *Taq/Pwo* Expand High Fidelity PCR System (Roche, Germany).

Cloning of *DFRs*

Based on *DFR* sequences available in the NCBI database, the degenerated primer pair Ep-DFR1(deg) was designed (Additional file 3: Table S3). After amplification, *DFR* cDNA fragments were isolated, ligated in the vector pCR2.1-TOPO (Invitrogen, USA) and transformed into the *E. coli* strain TOP10. The obtained sequence information was used to design specific 3′- and 5′-RACE primers. Amplification of DFR 5′- and 3′-ends was performed using the SMARTer RACE 5′/3′ Kit (Clontech, Takara Bio Europe, France). The full size primer pair EpDFRfull was designed (Additional file 3: Table S3) and used for amplification of four full size *DFRs* from cv. 'Christmas Beauty', cv. 'Christmas Feelings', cv. 'Premium Red' and cv. 'Harvest Orange'.

Heterologous expression of *DFR* in *E. coli*

An established standard procedure for the production of soluble enzymes in *E. coli* was used for the heterologous expression of the DFR cDNA clone [20]. For each variety two PCR reactions with different primers were performed with *Pfu* DNA polymerase (Fermentas, Germany) (PCR1: *Ep_DFR*_LF and *Ep_DFR*_SR; PCR 2: *Ep_DFR*_SF and *Ep_DFR*_LR) (Additional file 3: Table S3). The PCR products were analyzed on agarose gel, eluted and purified. PCR products from both PCRs were mixed in an equimolar amount, denatured and reannealed, resulting in 1/4 double stranded DFR with sticky *Bam*HI (GATC) and *Eco*RI (AATT) recognition sequences at the ends for direct ligation into the linearized

plasmid pGEX-6P-1 with T4 DNA ligase (Promega, Germany). After transformation into *E. coli* TOP10, plasmids were isolated and the presence of the insert confirmed by sequencing (Microsynth Austria AG, Austria). DFR sequences obtained during the present study were deposited in the NCBI database with the following accession numbers: KY273436 (*Ep*CB_DFR), KY273437 (*Ep*CF_DFR), KY499617 (*Ep*PR_DFR), KY273438 (*Ep*HO_DFR).

Heterologous expression of *F3'H* in yeast

Heterologous expression of the *F3'H* cDNA clones, which encode membrane bound enzymes, was performed in the yeast *Saccharomyces cerevisiae* according to established procedures [21]. *F3'H* cDNA clones were amplified with the *Taq/Pwo* Expand High Fidelity PCR System (Roche, Germany), and ligated into the vector pYES2.1/V5-His-TOPO (Invitrogen, USA). Plasmids were isolated and the presence and sense orientation of the insert was confirmed by sequencing (Microsynth Austria AG, Austria). The vectors containing the *F3'H* cDNAs of the four cultivars were transformed into the yeast strain INVSc1 using the *Sc*. EasyComp Transformation Kit (Invitrogen, USA). Heterologous expression and preparation of protein fractions were carried out as described previously [21]. Protein fractions were shock frozen in liquid nitrogen and stored at − 80 °C.

Phylogenetic analysis of F3'Hs

F3'H sequences obtained during the present study were deposited in the NCBI database with the following accession numbers: KY273439 (*Ep*CB_F3'H), KY273440 (*Ep*CF_F3'H), KY489667 (*EpPR*_F3'H) and KY273441 (*Ep*HO_F3'H). Multiple alignments were carried out with the software MultAlin [22]. Amino acid sequences were aligned using MUSCLE [23]. The alignment was used for reconstruction of phylogenetic relationships on the JTT matrix-based model [24]. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model. Evolutionary analyses were performed in MEGA7 [25]. Amino acid sequences used for this analysis were *Ep*CB_F3'H (KY273439), *Ep*CF_F3'H (KY273440), *Ep*PR_F3'H (KY489667), *Ep*HO_F3'H (KY273441), *Arabidopsis thaliana* F3'H (AF271651), *Callistephus chinensis* F3'H (AF313488), *Gentiana triflora* F3'H (AB193313), *Gerbera hybrida* F3'H (ABA64468), *Glycine max* F3'H (AF499731), *Hieracium pilosella* F3'H (DQ319866), *Ipomoea nil* F3'H (AB113264), *Lobelia erinus* F3'H (BAF49324), *Matthiola incana* F3'H (AF313491), *Osteospermum hybrida* F3'H (ABB29899), *Pelargonium hortorum* F3'H (AF315465) *Perilla frutescens* F3'H (AB045593), *Petunia hybrida* F3'H (AF155332), *Torenia hybrida* F3'H AB057673, *Prunus avium* F3'H (ADZ54783), *Jatropha curcas* F3'H (XP_012080364), *Ricinus communis* F3'H (XP002514665), *Vitis vinifera* F3'H (ALP48438), *Camelina sativa* F3'H (XP_010491421), *Vaccinium ashei* F3'H (BAO58432). Flavone synthase (FNSII) sequences: *Glycine max* FNSII (ACV65037), *Medicago truncatula* FNSII (ABC86159), *Dahlia pinnata* FNSII (AGA17938).

qPCR studies

The *F3'H* gene expression was evaluated by qPCR using the StepOnePlus system (Applied Biosystems, Germany) and the SybrGreenPCR Master Mix (Applied Biosystems, Austria) according to the manufacturer's protocol. The analysis was performed in three independent replicates and the results were normalized to the two control genes, *actin* and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). The relative expression ratio was calculated according to MW Pfaffl [26]. During the qPCR analysis primer pairs were used according to (Additional file 3: Table S3), to quantify the relative expression of *F3'H* (q*EpF3'H*) in comparison to the housekeeping genes *actin* (q*EpAct*) and *GAPDH* (q*EpGAPDH*). Product specificity was confirmed by analysis of melting curves and gel electrophoresis.

Site-directed mutagenesis

Mutagenesis was performed by use of the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Austria) and the pGEX-6P-1 vector containing EpCF_DFR. Primers Ep_DFR_132L were designed with the NEBase Changer™ v 1.25 provided at http://nebasechanger.neb.com. The sequences of the primers are presented in (Additional file 3: Table S3). Success of mutation was confirmed by sequencing.

Zygosity status of F3'H

The primer pair *Ep*F3'H_fra flanking the variable region at the N-terminal end of F3'H for all three varieties were designed (Additional file 3: Table S3). Gene fragments were amplified from genomic DNA, which was obtained according to Lipp et al. [27] using the *Taq/Pwo* Expend High Fidelity system (Roche, Germany). The expected band sizes were 107 (*Ep*CB_*F3'H*, and *Ep*PR_*F3'H*), 110 (*Ep*CF_*F3'H*), and 137 bp (*Ep*HO_*F3'H*) respectively. The PCR products were analyzed by electrophoresis in a 3% agarose gel and extracted with Wizard SV Gel and PCR Clean-up System (Promega, USA). After extraction from the gel, the PCR products were ligated into the vector pCR2.1-TOPO (Invitrogen, USA) and sequenced.

Results

Identification of anthocyanins

The anthocyanin contents and concentrations showed significant differences between cultivars exhibiting dark red bracts and the cultivars with orange-red bracts.

Highest anthocyanin concentrations were found in cv. 'Christmas Beauty' (Table 1). The two dark red cultivars showed higher anthocyanin concentrations than the orange-red cultivars. The dark red cultivars contained cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-galactoside, pelargonidin-3-*O*-glucoside and pelargonidin-3-*O*-rutinoside (Table 1, Additional file 2: Table S2, Additional file 4: Figure S1) as reported earlier by Asen et al. [28], with cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside as prevalent pigments. The orange-red cv. 'Harvest Orange' in contrast, produced only the two pelargonidin glycosides and in a few, but not all, biological replications, traces of cyanidin 3-*O*-glucoside (Table 1, Additional file 4: Figure S1). The orange-red cv. 'Premium Red' contained 82% pelargonidin glycosides and 18% cyanidin glycosides (Table 1). Pelargonidin-3-*O*-glucoside was the prevalent pigment in the orange-red cultivars.

Enzyme activities of the anthocyanin pathway

In the enzyme preparations obtained from intense red and orange-red poinsettia bracts, the activity of CHS/CHI, FHT, DFR, and F3′H, the key enzymes for anthocyanin formation, were measured (Additional file 5: Table S4.). The activity of the membrane F3′H could not be observed, maybe because of a loss of activity during destruction of the cell membranes [10].

Enzyme preparations from bracts converted all three types of dihydroflavonol substrates (Additional file 6: Table S4), DHK, DHQ and DHM. To study the substrate specificity of DFR, kinetic studies were performed with enzyme preparations obtained from bracts of cv. 'Christmas Feelings'. DFR reactions were optimized for each substrate. Reaction time and protein concentration was chosen in a way that the maximum conversion rate for the best substrate did not reach more than 50%. The kinetic data demonstrated the substrate specificity of DFR for DHM and DHQ in comparison to DHK. The k_{cat}/K_m values (Table 2) indicate that the best substrate for DFR is DHM, and that there is only low substrate specificity for DHK.

Cloning and characterization of *F3'Hs* from *Euphorbia*

Exploiting the preliminary data from a *Euphorbia* transcriptome study (Debener, unpublished) and the homology of the closely related species *Jatropha curcas*, four putative *F3'H* cDNA clones from poinsettias cvs. 'Harvest Orange', 'Premium Red', 'Christmas Beauty', and 'Christmas Feelings' were obtained (Accession numbers: KY273441, KY489667, KY273439, KY273440). The four cDNA clones showed 98.8% to 99.8% nucleotide sequence identities to each other (Additional file 6: Figure S2) and 67% to 76% to *F3′H* sequences from other species. The *F3′H* cDNA clones had open reading frames of 510 (*Ep*CB_*F3′H*, *Ep*PR_*F3′H*), 511 amino acids (*Ep*CF_*F3′H*), and 44 amino acids (*Ep*HO_F3′H), respectively (Fig. 2). The deduced *Ep*CF_*F3′H* amino acid sequence showed an additional phenylalanine in position 17 (numbering according to cv. 'Christmas Feelings') in the region responsible for anchoring the enzyme in the membrane [29].

The nucleotide sequence of the *Ep*HO_*F3′H* cDNA clone was 31 bp longer compared to *Ep*CB_*F3′H*, *Ep*PR_*F3′H* and 28 bp longer compared to *Ep*CF_*F3′H* (Additional file 6: Figure S2). The *Ep*HO_*F3′H* nucleotide sequence carried an insertion of 28 bp in positions 42 to 69 (numbering according to cv. 'Harvest Orange'). This included a stretch of 20 bp in positions 50–69, which is a repetition of the sequence 22ACCATTTTT TCTGCAATTTT41 (Fig. 3), and most importantly, results in a frameshift leading to an only 44 amino acids truncated F3′H fragment (Fig. 2).

Table 1 The anthocyanins in bracts of poinsettia cultivars and their respective concentration as determined by HPLC and LC-MS analysis of extracts

Anthocyanin composition [mg/g FW]	'Christmas Beauty'	'Christmas Feeling'	'Harvest Orange'	'Premium Red'
Total anthocyanins	29.7 ± 1.7	29.0 ± 0.5	15.4 ± 0.7	9.3 ± 3.9
Pelargonidin 3-*O*-glucoside	6.4 ± 0.4	5.2 ± 0.9	9.9 ± 0.1	3.8 ± 0.6
Pelargonidin 3-*O*-rutinoside	3.0 ± 0.2	2.9 ± 0.6	5.0 ± 0.6	2.0 ± 0.1
Other pelargonidin derivatives[a]	≥ 0.02	≥0.01	≥0.02	≥0.02
Total pelargonidin based anthocyanins	9.4 ± 0.6	8.2 ± 0.1	14.9 ± 0.2	7.9 ± 3.6
Cyanidin-3-*O*-glucoside	5.3 ± 0.2	5.0 ± 0.9	0.06 ± 0.1	0.4 ± 0.1
Cyanidin-3-*O*-rutinoside	9.9 ± 0.4	10.4 ± 0.2	n.d.	0.7 ± 0.1
Cyanidin-3-*O*-galactoside	5.0 ± 0.7	5.4 ± 0.7	n.d.	0.2 ± 0.03
Other cyanidin derivatives[b]	≥0.06	≥ 0.05	n.d.	≥0.01
Total cyanidin based anthocyanins	20.3 ± 1.4	20.9 ± 0.3	0.06 ± 0.1	1.3 ± 0.2
Total delphinidin based anthocyanins	≥0.01	≥0.01	n.d.	≥0.01

[a]Pelargonidin 3-*O*-(6″malonylglucoside), Pelargonidin 3-*O*-(6″malonyldihexoside) [b]Cyanidin-3-*O*-xyloside

Table 2 Characterisation of DFR from enzyme preparations of cv. 'Christmas Feeling' bracts

Substrate	DHK	DHQ	DHM
pH optimum	6.50	6.25	5.75
Temperature optimum	40 °C	40 °C	40 °C
Time linearity [min]	20	20	20
Protein linearity [μg in assay]	2.6	0.2	0.2
Apparent k_{cat} [μmol/kg*s]	97	78	3105
Apparent K_m [μM]	14	0.9	29
k_{cat}/K_m [l/s*kg]	7	86	107

The phylogenetic relationship of the poinsettia F3′Hs in comparison to F3′Hs from a further 23 species was analyzed using FNSII as outgroup. The deduced poinsettia F3′H amino acid sequences clustered together and showed closest relationship to putative F3′H sequences of *Ricinus communis* and *Jatropha curcas* (Additional file 7: Figure S3), which also belong to the same family Euphorbiaceae.

The cDNA clones were transferred into the pYES2.1/V5-His-TOPO vector and heterologously expressed in yeast. The recombinant enzymes *Ep*CB_F3′H, *Ep*CF_F3′H, and *Ep*PR_F3′H were functionally active and catalysed the NADPH dependent conversion of both naringenin and DHK to eriodictyol and DHQ, respectively. Both substrates were accepted to a comparable extent (Table 3). As expected, no activity of *Ep*HO_F3′H was observed (Table 3).

qPCR studies

The expression profile of *F3′H* was evaluated in the four poinsettia varieties using two sets of plants of different age and kept at different conditions. The quantitative real-time PCR data for *F3′H* were normalized against *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*, Fig. 4) and *actin* (data not shown). Results obtained from both housekeeping genes were comparable. Generally higher expression ratios were observed for the 3 year old plants kept in the greenhouse which could be owing to the better light conditions. In both cases, however, there was no correlation between *F3′H* expression and cyanidin formation. Highest expression rates were found in the orange-red cv. 'Harvest Orange' whereas the dark red cv. 'Christmas Beauty' showed the lowest *F3′H* expression (Fig. 4).

Zygosity status

During the isolation of the *F3′H* cDNA clones of the four cultivars, electropherograms provided by the sequencing company did not indicate that more than one version of *F3′H* is present. To confirm this and particularly to check if *Ep*HO_*F3′H* possesses another allele with a correct open reading frame, we designed primers (Additional file 3: Table S3) flanking the inserted region and performed PCR with genomic DNA and cDNA as template. Based on the isolated cDNA clones, we expected band sizes of 107 for *F3′Hs* from cvs. 'Christmas Beauty' and 'Premium Red' and of 110 for the *F3′Hs* from 'Christmas Feelings', respectively. For 'Harvest Orange', a size of 137 bp was expected in the case of a fragment carrying the insertion, whereas the presence of an allele without the insertion mutation would be indicated by a 109 bp amplicon. After the separation of the obtained amplicons on a 3% agarose gel, in the cultivars 'Christmas Beauty', 'Christmas Feeling' and 'Premium Red', only one band was present and the sizes corresponded to the expected fragment sizes (Fig. 5). For cv. 'Harvest Orange', the situation was different. When genomic DNA was used as a template, two bands of slightly different size were observed, of which, however, only one was expressed in the bracts. With cDNA as a template only the larger band, carrying the insert resulting in a frameshift, was obtained, whereas the smaller band was almost not visible (Fig. 5). Sequencing of the two fragments confirmed that in cv. 'Harvest Orange' two alleles of the *F3′H* gene are present of which one carries the insert mutation provoking the premature stop codon.

Cloning and characterization of *DFRs* from poinsettia

Putative *DFR* cDNA clones were isolated from the bracts of cvs. 'Christmas Beauty', 'Christmas Feelings', 'Premium Red' and 'Harvest Orange'. All four consisted of 1056 nucleotides, with open reading frames of 352 deduced amino acids (Additional file 8: Figure S4). The *DFR* cDNA clones showed a very high sequence identity between 98.3 and 99.4% on the amino acid level, even across the four independent varieties. During the isolation and characterization, a second allelic variant has not been isolated.

The *DFR* cDNA clones from cvs. 'Harvest Orange' (KY273438), 'Premium Red' (KY499617), 'Christmas Feeling' (KY273437), and 'Christmas Beauty' (KY273436) were cloned into the pGEX-6P-1 vector and heterologously expressed in *E. coli*. All four recombinant proteins were active, catalyzing the NADPH dependent conversion of dihydroflavonols to leucoanthocyanidins. They accepted DHQ and DHM as a substrate to a comparable extent (Table 3). DHK was accepted by recombinant *Ep*CF_DFR, *Ep*HO_DFR and *Ep*PR_DFR, whereas recombinant *Ep*CB_DFR showed only a low conversion rate of DHK (Table 3).

Substitution of the valine in position 132 (numbering from *Ep*CF_DFR) of *Ep*CF_DFR to leucine was performed, to change the VDV motif (Additional file 8: Figure S4) of the poinsettia DFR into the LDV motif commonly found in e.g. the petunia DFR (AAF60298)

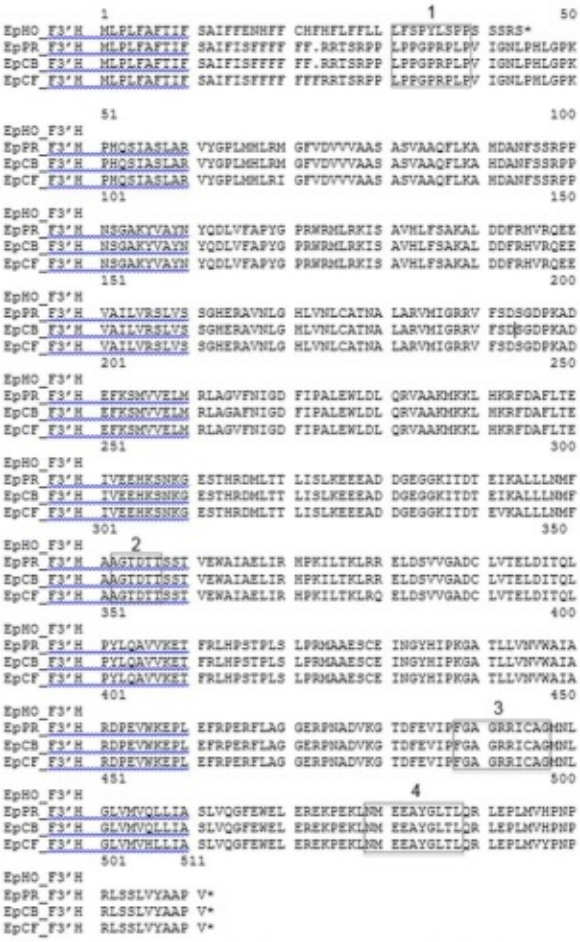


Fig. 2 Multiple alignment of deduced amino acid sequences of *F3'H* cDNA clones of *Euphorbia pulcherrima* cvs. 'Harvest Orange' (*EpHO_F3'H*, KY273441), 'Premium Red' (*EpPR_F3'H*, KY489667), 'Christmas Beauty' (*EpCB_F3'H*, KY273439), and 'Christmas Feeling' (*EpCF_F3'H*, KY273440). Grey frames highlight characteristic sequences of the P450 protein family. 1. Proline-rich region [40]; 2. Oxygen binding pocket [41]; 3. Heme binding motif (Prosite pattern PS00086, [42]); 4. Substrate recognition site (SRS) 6 according to Seitz et al. [43]

[14]. This resulted in an increase of DHM specificity but also in a major loss of enzyme activity (Table 3).

Discussion

Anthocyanins are most frequently found in flowers and fruits, where they serve as colorful signals to pollinators and seed dispersers [30]. However, other tissues such as, leaves, roots, stems and shoots can accumulate anthocyanins as well. In the latter case, the function of anthocyanins is less well understood, but has repeatedly been shown to be involved in the protection against biotic and abiotic stress [31]. Anthocyanins in leaves have been suggested to fulfil a range of functions including screening against sun and UV-B radiation, antioxidative protection, osmoregulation and herbivory and pathogen defence [32].

Cyanidin, which carries 2 hydroxy groups in the B-ring, is regarded to be the ancestral pigment.

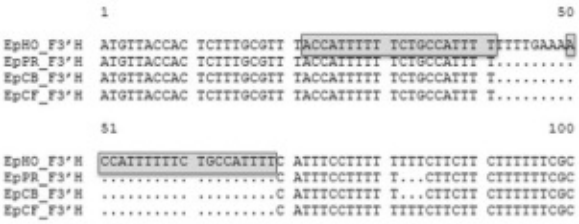


Fig. 3 Multiple alignment of a selected part of the nucleotide sequences at the 5'-terminus of *F3'H* cDNA clones of *Euphorbia pulcherrima* cvs. 'Harvest Orange' (*EpHO_F3'H*, KY273441), 'Premium Red' (*EpPR_F3'H*, KY489667), 'Christmas Beauty' (*EpCB_F3'H*, KY273439), and 'Christmas Feeling' (*EpCF_F3'H*, KY273440). The grey-shaded frame highlights the repetition of ACCATTTTTTCTGCCATTTT from position 22 to 41 in position 50 to 69 (numbering from *EpHO_F3'H*)

Formation of pelargonidin and delphinidin, which carry 1 and 3 hydroxy groups, respectively, evolved in flowers by loss-of function mutations and gain of function mutations, respectively, as an adaptation to the colour sense of specific pollinators. Thus, cyanidin based anthocyanins are predominant in less advanced tissues such as leaves [33]. As bracts are specialized leaves associated with reproductive structures, it does not seem to be surprising that an intense red coloration prevalently based on cyanidin derivatives seems to be the standard within the huge spectrum of available commercial varieties of red poinsettia [5, 6, 28, 34]. Orange-red hues seem to be a rare occurrence in poinsettia and not to be simply the result of a specific selection of breeders for intense, dark-red colour hues. In this study, we analysed the anthocyanin content and the correlating enzyme activities and gene expressions of four poinsettia cultivars to identify possible mechanisms leading to orange-red bract coloration.

Recently DFR was suggested to take a key role in the conversion of green leaves into red bracts in poinsettia [35]. In addition, the formation of cyanidin type anthocyanins strongly depends on the presence of F3´H hydroxylating enzymes, but can also be influenced by DFR substrate specificity [16]. Our studies therefore concentrated on these two enzymes.

Table 3 Functional activity test with recombinant enzymes from *Euphorbia pulcherrima*

	DFR (DHK/DHQ/DHM)	F3'H (NAR/DHK)
Cultivar	nmol/s*kg	nmol/s*kg
'Harvest Orange'	804/1260/800	0/0
'Premium Red'	1560/2043/1960	424/345
'Christmas Beauty'	200/2040/1987	420/370
'Christmas Feeling'	1300/1870/1630	430/345
	0/0/187[a]	

[a]EpCF_DFR132L mutant

The orange-red cvs. 'Harvest Orange' and 'Premium Red' were characterized by a generally lower anthocyanin concentration and a prevalent presence of pelargonidin-type anthocyanins. The lower amounts of total anthocyanins present in the orange-red bracts correlated well with the observed low specificity of DFR for DHK, which could result in a lower total conversion rate of dihydroflavonols, if only DHK is present. The bright orange-red coloration of the cvs. 'Harvest Orange' and 'Premium Red' demonstrate however, that sufficient precursors for pelargonidin formation can be provided by poinsettia DFR despite its low substrate specificity for DHK. Similar observations were reported for carnations where both pelargonidin and cyanidin based phenotypes can be formed, despite a strong preference of DFR for DHQ and DHM in comparison to DHK [36]. A comparable situation was recently reported for petunia DFR [37]. Substrate specificity of DFR was reported to be determined in a highly variable region of 26 amino acids in the N-terminal part of the enzyme, apparently with particular relevance of amino acid 133 [14]. The DFRs of the four varieties showed high homology in this area and there was no indication of the presence of an allelic variant of the DFR in contrast to F3´H. All cDNA clones identified showed high activity and concordant substrate specificity. The preference for DHQ over DHK, if both are simultaneously present, could well explain the prevalence of cyanidin and also indicates that F3´H is the key enzyme in the formation of orange-red color in poinsettia as described earlier for other species [38].

For *F3´H*, in contrast, we were able to show the presence of two allelic variants, of which only one was expressed in the petals. The isolated full-size *F3´H* cDNA clones of cvs. 'Christmas Beauty', 'Christmas Feeling' and 'Premium Red' encoded functionally active enzymes with very few differences in their deduced amino acid sequences. The cDNA clone obtained from cv. 'Harvest Orange' had an insertion of 28 bases, which causes a frame shift and an early termination of the

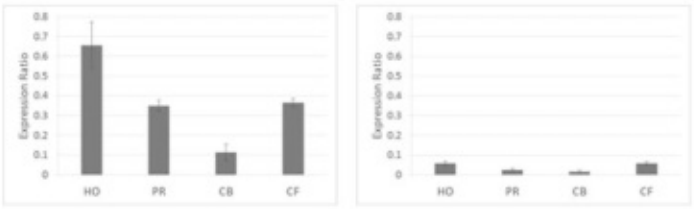


Fig. 4 Quantitative expression of *F3′H* normalized to glycerine aldehyde 3-phosphate dehydrogenase (GAPDH) in *Euphorbia pulcherrima* cvs. 'Harvest Orange' (HO), 'Premium Red' (PR), 'Christmas Beauty' (CB), and 'Christmas Feeling' (CF). Left: Three year old plants kept in the greenhouse. Right: Plants in their first year cultivated in house under standard conditions. Data were calculated from three biological replicates with at least two technical replicates and with error bars representing standard deviation

translation at amino acid 44, and, consequently, a non-functional F3′H, as demonstrated by heterologous expression in yeast. The insertion is, however, only present in the allele, which is actually expressed in the bracts. Expression of the other allele, which encodes presumably a functionally active F3′H without the insert mutation, was almost negligible. This provides a sufficient explanation for the almost exclusive presence of pelargonidin-type anthocyanins and the orange-red coloration in cv. 'Harvest Orange'. The 20 nucleotide repetition in the insertion indicated that the frameshift mutation could have been caused by a transposition event [39]. It is possible that, as a result of transposition, a part of the sequence was repeated and one additional nucleotide remained after retransposition.

Quantification of the *F3′H* gene expression by real-time PCR in the four cultivars did not indicate any correlation with the color type. Lowest *F3′H* expression was measured for the prevalently cyanidin type anthocyanins containing cv. 'Christmas Beauty'. The relatively high *F3′H* expression in the orange-red cv. 'Premium Red' was surprising because *Ep*PR_*F3′H* cDNA encoded a functionally active enzyme. At this stage it remains open if a post-transcriptional or a post-translational event or a simple competition between enzymes is responsible for the prevalence of pelargonidin derivatives formed in this cultivar.

Conclusion

In bracts, anthocyanins serve the same purpose as in flowers, i.e. attraction of pollinators and their biosynthesis follows similar mechanisms as numerously reported for flowers [2]. Our studies have shown that the red hues of poinsettias are primarily influenced by the anthocyanin composition and that attractive orange-red color of poinsettia bracts essentially depends on the

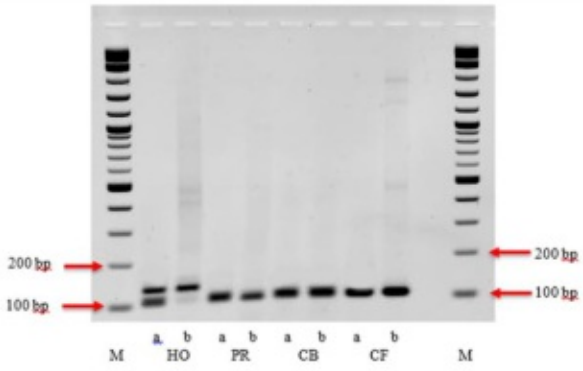


Fig. 5 Amplification of *F3′H* with the primer pair *EpF3′H_fraF* and *EpF3′H_fraR* (Additional file 5: Table S4) flanking the variable region at the N-terminal end using genomic DNA (a) and cDNA (b) from the four poinsettia cultivars 'Harvest Orange' (HO), 'Premium Red' (PR), Christmas (CB) and 'Christmas Feeling' (CF). For cv. 'Harvest Orange', amplification from gDNA delivered two fragments of the expected size (calculated values: 109 and 138 bp), whereas only the larger fragment was obtained with cDNA. With gDNA and cDNA from the other cultivars only a single fragment of the smaller size was obtained. Size marker (M) was the 2-Log DNA Ladder (New England Biolabs, UK) with digested DNA fragments ranging from 100 bp to 10 kbp (100 bp steps between 100 and 1000); 100 and 200 bp fragments are highlighted on the gel with red arrows

absence of cyanidin formation, which can be obtained by different mechanisms. An F3′H knock-out via a non-sense mutation could therefore be a promising approach for breeding orange-red poinsettia bracts by molecular breeding techniques. Future work will concentrate on application of these findings in molecular breeding approaches.

Additional files

Additional file 1: Table S1. Gradient elution time-table program in the RP-HPLC system using mobile phase A (water with 5% HCO_2H) and mobile phase B (MeOH). (DOCX 17 kb)

Additional file 2: Table S2. Identification of anthocyanins in poinsettia flowers by using their HPLC–DAD, LC–MS and LC–MS/MS data in the positive ion mode. (DOCX 16 kb)

Additional file 3: Table S3. List of primers used. (DOCX 16 kb)

Additional file 4: Figure S1. High performance liquid chromatographic profile of anthocyanins in (a) cv. Christmas Beauty and (b) cv. Harvest Orange. The anthocyanins in order of increasing retention time were cyanidin-3-*O*-galactoside (A1), cyanidin-3-*O*-glucoside (A2), pelargonidin-3-*O*-glucoside (A3), cyanidin-3-*O*-rutinoside (A4) and pelargonidin-3-*O*-rutinoside (A5). (DOCX 109 kb)

Additional file 5: Table S4. Activities of 3 key enzymes of the anthocyanin pathway in *Euphorbia pulcherrima*. *(DOCX 16 kb)*

Additional file 6: Figure S2. Multiple alignment of the open reading frames of the F3′H cDNA clones of *Euphorbia pulcherrima* cvs. Harvest Orange (EpHO_F3′H, KY273441), Premium Red (EpPR_F3′H, KY489667), Christmas Beauty (EpCB_F3′H, KY273439), and Christmas Feelings (EpCF_F3′H, KY273440). (DOCX 23 kb)

Additional file 7: Figure S3. Phylogenetic analysis of F3′Hs from the three poinsettia cvs. Christmas Feelings (KY273440), Christmas Beauty (KY273439) and Premium Red (KY489667) by application of the Maximum Likehood method based on the deduced amino acid sequences of isolated poinsettia F3′H cDNA clones and deduced F3′H amino acid sequences of other species available in the NCBI database. Sequences of FNSII were used as outgroup. The JJT matrix-based model was used as a substitution model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. (DOCX 20 kb)

Additional file 8: Figure S4. Multiple Alignment of the deduced amino acid sequences of the DFRs of *Euphorbia pulcherrima* cvs. Harvest Orange (EpHO_DFR, KY273438), Premium Red (EpPR_DFR, KY499617), Christmas Beauty (EpCB_DFR, KY273436), and Christmas Feelings (EpCF_DFR, KY273437). Grey frames highlight the VDV region in position 132 to 134. Grey shades highlight differences in the amino acid sequence. (DOCX 21 kb)

Abbreviations

CB: 'Christmas Beauty'; CF: 'Christmas Feeling'; CHS/CHI: Chalcone synthase/chalcone isomerase; cv(s): Cultivar(s); DFR: Dihydroflavonol 4-reductase; DHK: Dihydrokaempferol; DHM: Dihydromyricetin; DHQ: Dihydroquercetin; F3′5′H: Flavonoid 3′,5′-hydroxylase; F3′H: Flavonoid 3′-hydroxylase; FHT: Flavanone 3-hydroxylase; FNS: Flavone synthase; gDNA: Genomic DNA; HO: 'Harvest Orange'; PR: 'Premium Red'

Acknowledgments

We would like to dedicate this work to Dieter Treutter who was one of the key researchers of FlowerPower and sadly passed away on May 7, 2016. We would also like to thank Christopher Schlosser for critical reading of the manuscript.

Funding

We gratefully acknowledge funding by the Austrian Science Fund (FWF) P 28134-B25, Slovenian Research Agency (programme Horticulture No. P4–0013-0481) and from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 675657 FlowerPower.

Availability of data and materials

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DN, CS, SM, BW, MMP, IR, AS, DT-A, VV conducted the experiments and generated data. TD, JH, KS and HH conceived the research and designed the experiments. DN, CHG and HH wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All plant material used in this study is commercially available from the sources listed under methods subsection Plant Material.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

[1]Institute of Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, 1060 Vienna, Austria. [2]Fruit Science, Technical University of Munich, 85354 Freising, Germany. [3]Agronomy Department, Fruit, Wine and Vegetable Growing, Biotechnical Faculty, University of Ljubljana, 1000 Ljubljana, Slovenia. [4]Institute of Plant Genetics, Leibniz Universität Hannover, 30419 Hannover, Germany.

Received: 25 October 2017 Accepted: 13 September 2018
Published online: 03 October 2018

References

1. Ecke P. The Ecke poinsettia manual. Batavia. In: Ill.: Ball Pub; 2004.
2. Mol J, Grotewold E, Koes R. How genes paint flowers and seeds. Trends Plant Sci. 1998;3(6):212–7.
3. Halbwirth H. The creation and physiological relevance of divergent hydroxylation patterns in the flavonoid pathway. Int J Mol Sci. 2010;11(2):595–621.
4. Horn JW, van Ee BW, Morawetz JJ, Riina R, Steinmann VW, Berry PE, Wurdack KJ. Phylogenetics and the evolution of major structural characters in the giant genus Euphorbia L.(Euphorbiaceae). Mol Phylogenet Evol. 2012;63(2):305–26.
5. Stewart R, Asen S, Massie D, Norris K. The identification of poinsettia cultivars by HPLC analysis of their anthocyanin content. Biochem Syst Ecol. 1979;7(4):281–7.
6. Slatnar A, Mikulic-Petkovsek M, Veberic R, Stampar F, Schmitzer V. Anthocyanin and chlorophyll content during poinsettia bract development. Sci Hortic. 2013;150:142–5.
7. Tanaka Y. Flower colour and cytochromes P450. Phytochem Rev. 2006;5(2–3):283–91.
8. Schuler MA, Werck-Reichhart D. Functional genomics of P450s. Annu Rev Plant Biol. 2003;54:629–67.
9. Nelson D, Werck-Reichhart D. A P450-centric view of plant evolution. Plant J. 2011;66(1):194–211.
10. Chapple C. Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. Annu Rev Plant Biol. 1998;49(1):311–43.
11. Schwinn K, Miosic S, Davies K, Thill J, Gotame TP, Stich K, Halbwirth H. The B-ring hydroxylation pattern of anthocyanins can be determined through activity of the flavonoid 3′-hydroxylase on leucoanthocyanidins. Planta. 2014;240(5):1003–10.
12. Petit P, Granier T, d'Estaintot BL, Manigand C, Bathany K, Schmitter J-M, Lauvergeat V, Hamdi S, Gallois B. Crystal structure of grape dihydroflavonol 4-reductase, a key enzyme in flavonoid biosynthesis. J Mol Biol. 2007;368(5):1345–57.

13. Heller W, Forkmann G, Britsch L, Grisebach H. Enzymatic reduction of (+)-dihydroflavonols to flavan-3, 4-cis-diols with flower extracts from Matthiola incana and its role in anthocyanin biosynthesis. Planta. 1985; 165(2):284–7.
14. Johnson ET, Ryu S, Yi H, Shin B, Cheong H, Choi G. Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. Plant J. 2001;25(3):325–33.
15. Johnson ET, Yi H, Shin B, Oh BJ, Cheong H, Choi G. Cymbidium hybrida dihydroflavonol 4-reductase does not efficiently reduce dihydrokaempferol to produce orange pelargonidin-type anthocyanins. Plant J. 1999;19(1):81–5.
16. Miosic S, Thill J, Milosevic M, Gosch C, Pober S, Molitor C, Ejaz S, Rompel A, Stich K, Halbwirth H. Dihydroflavonol 4-reductase genes encode enzymes with contrasting substrate specificity and show divergent gene expression profiles in Fragaria Species. PloS one. 2014;9(11):e112707.
17. Fischer TC, Halbwirth H, Meisel B, Stich K, Forkmann G. Molecular cloning, substrate specificity of the functionally expressed dihydroflavonol 4-reductases from Malus domestica and Pyrus communis cultivars and the consequences for flavonoid metabolism. Arch Biochem Biophys. 2003;412(2):223–30.
18. Halbwirth H, Waldner I, Miosic S, Ibanez M, Costa G, Stich K. Measuring flavonoid enzyme activities in tissues of fruit species. J Agric Food Chem. 2009;57(11):4983–7.
19. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011;29(7):644–52.
20. Gosch C, Nagesh KM, Thill J, Miosic S, Plaschil S, Milosevic M, Olbricht K, Ejaz S, Rompel A, Stich K. Isolation of Dihydroflavonol 4-Reductase cDNA Clones from Angelonia x angustifolia and Heterologous Expression as GST Fusion Protein in *Escherichia coli*. PloS one. 2014;9(9):e107755.
21. Seitz C, Ameres S, Schlangen K, Forkmann G, Halbwirth H. Multiple evolution of flavonoid 3', 5'-hydroxylase. Planta. 2015;242(3):561–73.
22. Corpet F. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 1988;16(22):10881–90.
23. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32(5):1792–7.
24. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. Computer applications in the biosciences: CABIOS. 1992;8(3):275–82.
25. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33(7):1870–4. https://doi.org/10.1093/molbev/msw054.
26. Pfaffl MW. Quantification strategies in real-time PCR. AZ of quantitative PCR. 2004;1:89–113.
27. Lipp M, Brodmann P, Pietsch K, Pauwels J, Anklam E, Borchers T, Braunschweiger G, Busch U, Eklund E, Eriksen F. IUPAC collaborative trial study of a method to detect genetically modified soy beans and maize in dried powder. Journal-AOAC International. 1999;82:923–8.
28. Asen S, Stewart R, Norris K. Co-pigmentation of anthocyanins in plant tissues and its effect on color. Phytochemistry. 1972;11(3):1139–44.
29. Kemper B. Structural basis for the role in protein folding of conserved proline-rich regions in cytochromes P450. Toxicol Appl Pharmacol. 2004; 199(3):305–15.
30. Durst F, Nelson DR. Diversity and evolution of plant P450 and P450-reductases. Drug Metabol Drug Interact. 1995;12:189–206.
31. De Castro E, Sigrist CJ, Gattiker A, Bulliard V, Langendijk-Genevaux PS, Gasteiger E, Bairoch A, Hulo N. ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. Nucleic Acids Res 2006;34:suppl 2:W362-W365.
32. Seitz C, Ameres S, Forkmann G. Identification of the molecular basis for the functional difference between flavonoid 3'-hydroxylase and flavonoid 3', 5'-hydroxylase. FEBS Lett. 2007;581(18):3429–34.
33. Schlangen K, Miosic S, Thill J, Halbwirth H. Cloning, functional expression, and characterization of a chalcone 3-hydroxylase from Cosmos sulphureus. J Exp Bot. 2010;61(12):3451–9.
34. Harborne JB. Introduction to ecological biochemistry: academic press; 1993.
35. Feild TS, Lee DW, Holbrook NM. Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. Plant Physiol. 2001; 127(2):566–74.
36. Manetas Y. Why some leaves are anthocyanic and why most anthocyanic leaves are red? Flora-Morphology, Distribution, Functional Ecology of Plants. 2006;201(3):163–77.
37. Gould KS. Nature's Swiss army knife: the diverse protective roles of anthocyanins in leaves. Biomed Res Int. 2004;2004(5):314–20.
38. Behe BK, Redman PB, Dole JM. Consumers prefer red poinsettia cultivars. HortTechnology. 1997;7(4):438–41.
39. Gu Z, Chen H, Yang R, Ran M. Identification of DFR as a promoter of anthocyanin accumulation in poinsettia (Euphorbia pulcherrima, willd. Ex Klotzsch) bracts under short-day conditions. Sci Hortic. 2018;236:158–65.
40. Stich K, Eidenberger T, Wurst F, Forkmann G. Enzymatic conversion of dihydroflavonols to flavan-3, 4-diols using flower extracts of Dianthus caryophyllus L.(carnation). Planta. 1992;187(1):103–8.
41. Haselmair-Gosch C, Miosic S, Nitarska D, Roth BL, Walliser B, Paltram R, Lucaciu RC, Eidenberger L, Rattei T, Olbricht K, et al. Great cause—small effect: undeclared genetically engineered Orange Petunias Harbor an inefficient Dihydroflavonol 4-Reductase. Front Plant Sci. 2018;9:149.
42. Tsuda S, Fukui Y, Nakamura N, Katsumoto Y, Yonekura-Sakakibara K, Fukuchi-Mizutani M, Ohira K, Ueyama Y, Ohkawa H, Holton TA. Flower color modification of Petunia hybrida commercial varieties by metabolic engineering. Plant Biotechnology. 2004;21(5):377–86.
43. Flavell AJ, Pearce SR, Kumar A. Plant transposable elements and the genome. Curr Opin Genet Dev. 1994;4(6):838–44.

Table S1: Gradient elution time-table program in the RP-HPLC system using mobile phase A (water with 5 % HCO_2H) and mobile phase B (MeOH)

Time (min)	Flow (ml/min)	%A	%B
0-5	0.5	95	5
5-10	0.5	95-90	5-10
10-15	0.5	90	10
15-35	0.5	90-85	10-15
35-55	0.5	85	15
55-70	0.5	85-80	15-20
70-80	0.5	80	20
80-95	0.5	80-75	20-25
95-125	0.5	75-70	25-30
125-145	0.5	70-60	30-40
145-160	0.5	60-50	40-50
160-175	0.5	50-10	50-90
175-195	0.5	10	90

Table S2: Identification of anthocyanins in poinsettia flowers by using their HPLC–DAD, LC–MS and LC–MS/MS data in the positive ion mode.

Anthocyanin	$[M^+]$ (*m/z*)	MS^2 $[M^+]$ (*m/z*)
Cyanidin 3-*O*-galactoside	449	287
Cyanidin 3-*O*-glucoside	449	287
Cyanidin 3-*O*-rutinoside	595	449/287
Pelargonidin 3-*O*-glucoside	433	271
Pelargonidin 3-*O*-rutinoside	579	433/271
Cyanidin 3-*O*-xyloside	419	287
Pelargonidin 3-*O*-(6"malonylglucoside)	519	433/271
Cyanidin 3-*O*-(6"malonylglucoside)-5-*O*-glucoside	697	535/449/287
Delphinidin 3-*O*-(2G-xylosylrutinoside)	743	597/435/303

Table S3: List of primers used

Primer	Forward 5'-3' (F)	Reverse 5'-3' (R)
Ep_F3'H_full	GCCAACTACATCTAATCTAACCCAACATG	GACAAACAGTACTGGAGTAATAAGCAACG
Ep_DFR1(deg)	GG(AGCT)TT(CT)AT(ACT)GG(GCT)TC(AT)TG GCT(CT)(AG)TCATGA	TC(AGT)A(CT)(AGCT)GC(ACT)CC(ACT)(AGC T)(CT)(AG)(AT)ACAT(AG)TCCTC
Ep_DFR_full	TGGGGAAGTTNTCTTGTTTTCA	TCCCATTATTTTTATTGTCTCCCTAGGTA
Ep_DFR_L	GATCCATGGGTGAAGTGCCTGAGATTGTG	AATTCTCCCATTATTTTTATTGTCTCCCTAG GTA
Ep_DFR_S	CATGGGTGAAGTGCCTGAGATTGTG	CTCCCATTATTTTTATTGTCTCCCTAGGTA
Ep_DFR_132L	GGCTGGCACACTTGATGTTGAG	GAGGATGTGAATATGATCC
qEpGAPDH	GTCAAGCAGGCTTCTCACTT	GCCAACCGGCTTGACAT
qEpAct	GCTCAGTCCAAGAGAGGTATTT	CCATGTCATCCCAATTGCTTAC
qEpF3'H	ACAGGAAGAAGTGGCAATCC	TCTTCTGCCAATCATTACGC
EpF3'H_fra	GCCAACTACATCTAATCTAACCCAACATG	GCGAGAGGTACGGCGAA

Figure S1: High performance liquid chromatographic profile of anthocyanins in (a) cv. Christmas Beauty and (b) cv. Harvest Orange. The anthocyanins in order of increasing retention time were cyanidin-3-*O*-galactoside (A1), cyanidin-3-*O*-glucoside (A2), pelargonidin-3-*O*-glucoside (A3), cyanidin-3-*O*-rutinoside (A4) and pelargonidin-3-*O*-rutinoside (A5)

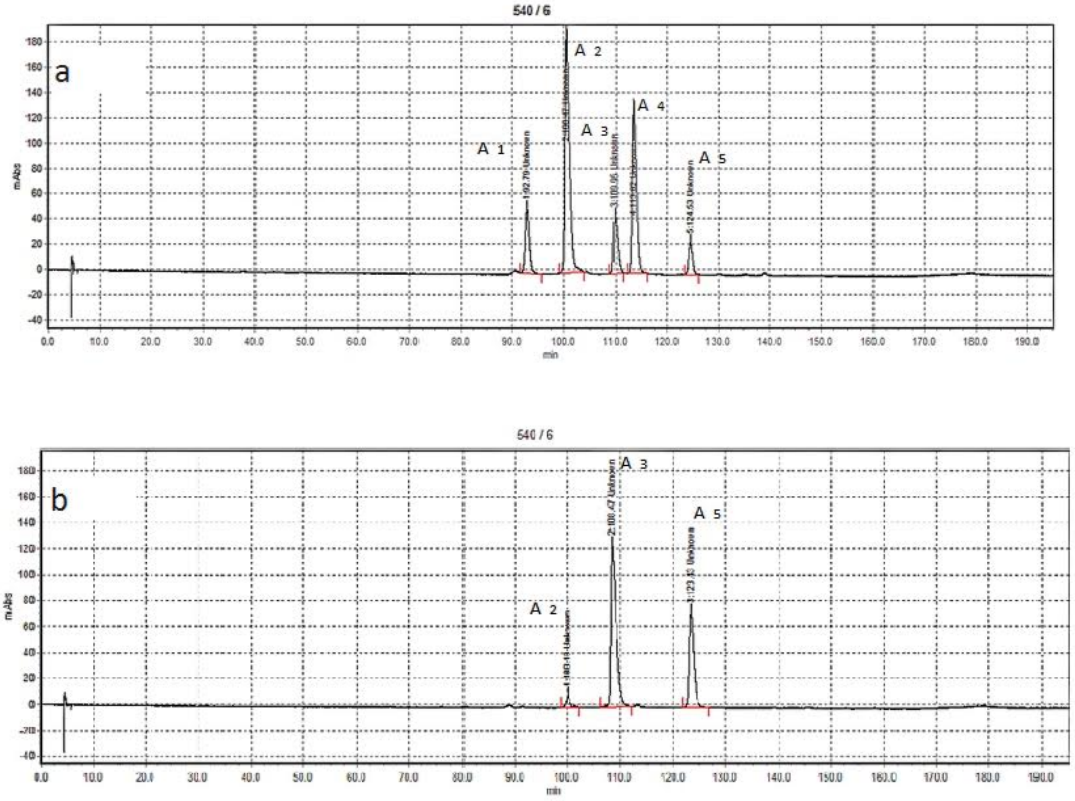


Table S4: Activities of 3 key enzymes of the anthocyanin pathway in *Euphorbia pulcherrima*

	CHS/CHI	**FHT**	**DFR** (DHK/DHQ/DHM)
Cultivar	nmol/s*g	nmol/s*g	nmol/s*g
Harvest Orange	0.9	1.2	4.3/4.4/4.4
Premium Red	0.3	0.3	1.9/4.5/4.4
Christmas Feelings	0.6	1.0	4.2/4.3/4.5
Christmas Beauty	0.6	1.1	4.4/4.4/4.6

Figure S2: Multiple alignment of the open reading frames of the *F3'H* cDNA clones of *Euphorbia pulcherrima* cvs. Harvest Orange (*Ep*HO_*F3'H*, KY273441), Premium Red (*Ep*PR_*F3'H*, KY489667), Christmas Beauty (*Ep*CB_*F3'H*, KY273439), and Christmas Feelings (*Ep*CF_*F3'H*, KY273440).

```
           1                                                   50
EpHO_F3'H  ATGTTACCAC TCTTTGCGTT TACCATTTTT TCTGCCATTT TTTTTGAAAA
EpPR_F3'H  ATGTTACCAC TCTTTGCGTT TACCATTTTT TCTGCCATTT T.........
EpCB_F3'H  ATGTTACCAC TCTTTGCGTT TACCATTTTT TCTGCCATTT T.........
EpCF_F3'H  ATGTTACCAC TCTTTGCGTT TACCATTTTT TCTGCCATTT T.........

           51                                                  100
EpHO_F3'H  CCATTTTTTC TGCCATTTTC ATTTCCTTTT TTTTCTTCTT CTTTTTTCGC
EpPR_F3'H  .......... .........C ATTTCCTTTT T...CTTCTT CTTTTTTCGC
EpCB_F3'H  .......... .........C ATTTCCTTTT T...CTTCTT CTTTTTTCGC
EpCF_F3'H  .......... .........C ATTTCCTTTT TTTTCTTCTT CTTTTTTCGC

           101                                                 150
EpHO_F3'H  CGTACCTCTC GCCCCCCTCT TCCTCCCGGT CCTAGACCAC TGCCTGTAAT
EpPR_F3'H  CGTACCTCTC GCCCCCCTCT TCCTCCCGGT CCTAGACCAC TGCCTGTAAT
EpCB_F3'H  CGTACCTCTC GCCCCCCTCT TCCTCCCGGT CCTAGACCAC TGCCTGTAAT
EpCF_F3'H  CGTACCTCTC GCCCCCCTCT TCCTCCCGGT CCTAGACCAC TGCCTGTAAT

           151                                                 200
EpHO_F3'H  TGGAAACCTG CCTCATTTAG GCCCCAAACC CCACCAGTCA ATAGCCTCCT
EpPR_F3'H  TGGAAACCTG CCTCATTTAG GCCCCAAACC CCACCAGTCA ATAGCCTCCT
EpCB_F3'H  TGGAAACCTG CCTCATTTAG GCCCCAAACC CCACCAGTCA ATAGCCTCCT
EpCF_F3'H  TGGAAACCTG CCTCATTTAG GCCCCAAACC CCACCAGTCA ATAGCCTCCT

           201                                                 250
EpHO_F3'H  TGGCTCGGGT TTATGGCCCC CTTATGCACC TCCGTATAGG CTTTGTCGAC
EpPR_F3'H  TGGCTCGGGT TTATGGCCCC CTTATGCACC TCCGTATGGG CTTTGTCGAC
EpCB_F3'H  TGGCTCGGGT TTATGGCCCC CTTATGCACC TCCGTATGGG CTTTGTCGAC
EpCF_F3'H  TGGCTCGGGT TTATGGCCCC CTTATGCACC TCCGTATAGG CTTTGTCGAC

           251                                                 300
EpHO_F3'H  GTCGTTGTGG CGGCGTCGGC GTCCGTTGCT GCCCAGTTCT TGAAAGCTCA
EpPR_F3'H  GTCGTTGTGG CGGCGTCGGC GTCCGTTGCT GCCCAGTTCT TGAAAGCTCA
EpCB_F3'H  GTCGTTGTGG CGGCGTCGGC GTCCGTTGCT GCCCAGTTCT TGAAAGCTCA
EpCF_F3'H  GTCGTTGTGG CGGCGTCGGC GTCCGTTGCT GCCCAGTTCT TGAAAGCTCA

           301                                                 350
EpHO_F3'H  TGACGCTAAT TTCTCGAGCC GGCCGCCTAA TTCGGGTGCT AAGTATGTTG
EpPR_F3'H  TGACGCTAAT TTCTCGAGCC GGCCGCCTAA TTCGGGTGCT AAGTATGTTG
EpCB_F3'H  TGACGCTAAT TTCTCGAGCC GGCCGCCTAA TTCGGGTGCT AAGTATGTTG
EpCF_F3'H  TGACGCTAAT TTCTCGAGCC GGCCGCCTAA TTCGGGTGCT AAGTATGTTG

           351                                                 400
EpHO_F3'H  CTTATAATTA CCAAGATCTT GTTTTTGCCC CGTACGGACC TCGCTGGCGC
EpPR_F3'H  CTTATAATTA CCAAGATCTT GTTTTTGCCC CGTACGGACC TCGCTGGCGC
EpCB_F3'H  CTTATAATTA CCAAGATCTT GTTTTTGCCC CGTACGGACC TCGCTGGCGC
EpCF_F3'H  CTTATAATTA CCAAGATCTT GTTTTTGCCC CGTACGGACC TCGCTGGCGC

           401                                                 450
EpHO_F3'H  ATGCTCAGGA AAATCAGTGC CGTGCATCTC TTCTCGGCTA AGGCCTTGGA
EpPR_F3'H  ATGCTCAGGA AAATCAGTGC CGTGCATCTC TTCTCGGCTA AGGCCTTGGA
EpCB_F3'H  ATGCTCAGGA AAATCAGTGC CGTGCATCTC TTCTCGGCTA AGGCCTTGGA
EpCF_F3'H  ATGCTCAGGA AAATCAGTGC CGTGCATCTC TTCTCGGCTA AGGCCTTGGA

           451                                                 500
EpHO_F3'H  TGATTTCCGC CATGTTAGAC AGGAAGAAGT GGCAATCCTT GTACGGTCTC
EpPR_F3'H  TGATTTCCGC CATGTTAGAC AGGAAGAAGT GGCAATCCTT GTACGGTCTC
EpCB_F3'H  TGATTTCCGC CATGTTAGAC AGGAAGAAGT GGCAATCCTT GTACGGTCTC
EpCF_F3'H  TGATTTCCGC CATGTTAGAC AGGAAGAAGT GGCAATCCTT GTACGGTCTC
```

```
            501                                                 550
EpHO_F3'H   TAGTAAGTTC CGGGCACGAA AGAGCGGTCA ATTTAGGGCA TCTGGTGAAC
EpPR_F3'H   TAGTAAGTTC CGGGCACGAA AGAGCGGTCA ATTTAGGGCA TCTGGTGAAC
EpCB_F3'H   TAGTAAGTTC CGGGCACGAA AGAGCGGTCA ATTTAGGGCA TCTGGTGAAC
EpCF_F3'H   TAGTAAGTTC CGGGCACGAA AGAGCGGTCA ATTTAGGGCA TCTGGTGAAC

            551                                                 600
EpHO_F3'H   CTGTGCGCCA CAAATGCACT GGCACGCGTA ATGATTGGCA GAAGAGTATT
EpPR_F3'H   CTGTGCGCCA CAAATGCACT GGCACGCGTA ATGATTGGCA GAAGAGTATT
EpCB_F3'H   CTGTGCGCCA CAAATGCACT GGCACGCGTA ATGATTGGCA GAAGAGTATT
EpCF_F3'H   CTGTGCGCCA CAAATGCACT GGCACGCGTA ATGATTGGCA GAAGAGTATT

            601                                                 650
EpHO_F3'H   CAGCGACAGC GGTGATCCGA AGGCCGACGA GTTCAAGTCA ATGGTGGTGG
EpPR_F3'H   CAGCGACAGC GGTGATCCGA AGGCCGACGA GTTCAAGTCA ATGGTGGTGG
EpCB_F3'H   CAGCGACAGC GGTGATCCGA AGGCCGACGA GTTCAAGTCA ATGGTGGTGG
EpCF_F3'H   CAGCGACAGC GGTGATCCGA AGGCCGACGA GTTCAAGTCA ATGGTGGTGG

            651                                                 700
EpHO_F3'H   AACTGATGAG ACCTGCCGGA GTATTCAATA TAGGGGATTT TATTCCGGCA
EpPR_F3'H   AACTGATGAG ACTTGCCGGA GTATTCAATA TAGGGGATTT TATTCCGGCA
EpCB_F3'H   AACTGATGAG ACTTGCCGGA GCATTCAATA TAGGGGATTT TATTCCGGCA
EpCF_F3'H   AACTGATGAG ACTTGCCGGA GTATTCAATA TAGGGGATTT TATTCCGGCA

            701                                                 750
EpHO_F3'H   CTGGAGTGGC TGGATTTACA GCGAGTAGCA GCTAAAATGA AGAAACTCCA
EpPR_F3'H   CTGGAGTGGC TGGATTTACA GCGAGTAGCA GCTAAAATGA AGAAACTCCA
EpCB_F3'H   CTGGAGTGGC TGGATTTACA GCGAGTAGCA GCTAAAATGA AGAAACTCCA
EpCF_F3'H   CTGGAGTGGC TGGATTTACA GCGAGTAGCA GCTAAAATGA AGAAACTCCA

            751                                                 800
EpHO_F3'H   TAAGAGATTC GATGCGTTTT TGACTGAAAT CGTCGAGGAA CACAAGAGTA
EpPR_F3'H   TAAGAGATTC GATGCGTTTT TGACTGAAAT CGTCGAGGAA CACAAGAGTA
EpCB_F3'H   TAAGAGATTC GATGCGTTTT TGACTGAAAT CGTCGAGGAA CACAAGAGTA
EpCF_F3'H   TAAGAGATTC GATGCGTTTT TGACTGAAAT CGTCGAGGAA CACAAGAGTA

            801                                                 850
EpHO_F3'H   ACAAAGGAGA GTCAACTCAC AGAGACATGT TGACTACTTT AATCTCGTTA
EpPR_F3'H   ACAAAGGAGA GTCAACTCAC AGAGACATGT TGACTACTTT AATCTCGTTA
EpCB_F3'H   ACAAAGGAGA GTCAACTCAC AGAGACATGT TGACTACTTT AATCTCGTTA
EpCF_F3'H   ACAAAGGAGA GTCAACTCAC AGAGACATGT TGACTACTTT AATCTCGTTA

            851                                                 900
EpHO_F3'H   AAGGAGGAAG AAGCTGATGA CGGTGAGGGA GGGAAAATCA CTGACACCGA
EpPR_F3'H   AAGGAGGAAG AAGCTGATGA CGGTGAGGGA GGGAAAATCA CTGACACCGA
EpCB_F3'H   AAGGAGGAAG AAGCTGATGA CGGTGAGGGA GGGAAAATCA CTGACACCGA
EpCF_F3'H   AAGGAGGAAG AAGCTGATGA CGGTGAGGGA GGGAAAATCA CTGACACCGA

            901                                                 950
EpHO_F3'H   AATTAAAGCC CTGCTTCTGA ACATGTTTGC AGCAGGCACC GACACTACAT
EpPR_F3'H   AATTAAAGCC CTGCTTCTGA ACATGTTTGC AGCAGGCACC GACACTACAT
EpCB_F3'H   AATTAAAGCC CTGCTTCTGA ACATGTTTGC AGCAGGCACC GACACTACAT
EpCF_F3'H   AGTTAAAGCC CTGCTTCTGA ACATGTTTGC AGCAGGCACC GACACTACAT

            951                                                1000
EpHO_F3'H   CAAGCACGGT TGAGTGGGCC ATTGCTGAGC TCATCAGGCA CCCCAAAATA
EpPR_F3'H   CCAGCACAGT TGAGTGGGCC ATTGCTGAGC TCATCAGGCA CCCCAAAATA
EpCB_F3'H   CCAGCACAGT TGAGTGGGCC ATTGCTGAGC TCATCAGGCA CCCCAAAATA
EpCF_F3'H   CAAGCACGGT TGAGTGGGCC ATTGCTGAGC TCATCAGGCA CCCCAAAATA

            1001                                               1050
EpHO_F3'H   CTAACCAAAC TCCGGCAAGA ACTCGACTCC GTCGTCGGCG CCGATTGTCT
EpPR_F3'H   CTAACCAAAC TCCGGCGAGA ACTCGACTCC GTCGTCGGCG CCGATTGTCT
EpCB_F3'H   CTAACCAAAC TCCGGCGAGA ACTCGACTCC GTCGTCGGCG CCGATTGTCT
EpCF_F3'H   CTAACCAAAC TCCGGCAAGA ACTCGACTCC GTCGTCGGCG CCGATTGTCT
```

```
            1051                                                1100
EpHO_F3'H   CGTAACCGAG CTAGACATCA CTCAACTCCC CTACCTCCAA GCCGTCGTCA
EpPR_F3'H   CGTAACCGAG CTAGACATCA CTCAACTCCC CTACCTCCAA GCCGTCGTCA
EpCB_F3'H   CGTAACCGAG CTAGACATCA CTCAACTCCC CTACCTCCAA GCCGTCGTCA
EpCF_F3'H   CGTAACCGAG CTAGACATCA CTCAACTCCC CTACCTCCAA GCCGTCGTCA

            1101                                                1150
EpHO_F3'H   AAGAAACCTT CCGCCTCCAC CCATCAACTC CCCTCTCTCT CCCTCGAATG
EpPR_F3'H   AAGAAACCTT CCGCCTCCAC CCATCAACTC CCCTCTCTCT CCCTCGAATG
EpCB_F3'H   AAGAAACCTT CCGCCTCCAC CCATCAACTC CCCTCTCTCT CCCTCGAATG
EpCF_F3'H   AAGAAACCTT CCGCCTCCAC CCATCAACTC CCCTCTCTCT CCCTCGAATG

            1151                                                1200
EpHO_F3'H   GCGGCCGAAA GCTGCGAAAT CAACGGCTAC CACATCCCAA AAGGCGCCAC
EpPR_F3'H   GCGGCCGAAA GCTGCGAAAT CAACGGCTAC CACATCCCAA AAGGCGCCAC
EpCB_F3'H   GCGGCCGAAA GCTGCGAAAT CAACGGCTAC CACATCCCAA AAGGCGCCAC
EpCF_F3'H   GCGGCCGAAA GCTGCGAAAT CAACGGCTAC CACATCCCAA AAGGCGCCAC

            1201                                                1250
EpHO_F3'H   GCTTCTGGTC AACGTGTGGG CAATAACTCG CGATCCAGAA GTATGGAAAG
EpPR_F3'H   GCTTCTGGTC AACGTGTGGG CAATAGCTCG CGATCCAGAA GTATGGAAAG
EpCB_F3'H   GCTTCTGGTC AACGTGTGGG CAATAGCTCG CGATCCAGAA GTATGGAAAG
EpCF_F3'H   GCTTCTGGTC AACGTGTGGG CAATAGCTCG CGATCCAGAA GTATGGAAAG

            1251                                                1300
EpHO_F3'H   AGCCGCTGGA GTTTCGACCG GAGAGGTTTC TCGCCGGCGG AGAAAGGCCG
EpPR_F3'H   AGCCGCTGGA GTTTCGACCG GAGAGGTTTC TCGCCGGCGG AGAAAGGCCG
EpCB_F3'H   AGCCGCTGGA GTTTCGACCG GAGAGGTTTC TCGCCGGCGG AGAAAGGCCG
EpCF_F3'H   AGCCGCTGGA GTTTCGACCG GAGAGGTTTC TCGCCGGCGG AGAAAGGCCG

            1301                                                1350
EpHO_F3'H   AACGCCGACG TGAAAGGGAC GGATTTTGAG GTGATTCCGT TTGGGGCAGG
EpPR_F3'H   AACGCCGACG TGAAAGGGAC GGATTTTGAG GTGATTCCGT TTGGGGCAGG
EpCB_F3'H   AACGCCGACG TGAAAGGGAC GGATTTTGAG GTGATTCCGT TTGGGGCAGG
EpCF_F3'H   AACGCCGACG TGAAAGGGAC GGATTTTGAG GTGATTCCGT TTGGGGCAGG

            1351                                                1400
EpHO_F3'H   GCGGAGAATT TGCGCAGGGA TGAATTTAGG GTTAGTAATG GTTCATCTGC
EpPR_F3'H   GCGGAGAATT TGCGCAGGGA TGAATTTAGG GTTAGTAATG GTTCAGCTGC
EpCB_F3'H   GCGGAGAATT TGCGCAGGGA TGAATTTAGG GTTAGTAATG GTTCAGCTGC
EpCF_F3'H   GCGGAGAATT TGCGCAGGGA TGAATTTAGG GTTAGTAATG GTTCATCTGC

            1401                                                1450
EpHO_F3'H   TTATTGCGAG TTTAGTACAA GGATTTGAAT GGGAATTGGA AAGGGAAAAA
EpPR_F3'H   TTATTGCGAG TTTAGTACAA GGATTTGAAT GGGAATTGGA AAGGGAAAAA
EpCB_F3'H   TTATTGCGAG TTTAGTACAA GGATTTGAAT GGGAATTGGA AAGGGAAAAA
EpCF_F3'H   TTATTGCGAG TTTAGTACAA GGATTTGAAT GGGAATTGGA AAGGGAAAAA


            1451                                                1500
EpHO_F3'H   CCAGAGAAAT TGAACATGGA GGAAGCTTAT GGGCTGACCT TACAACGACT
EpPR_F3'H   CCAGAGAAAT TGAACATGGA GGAAGCTTAT GGGCTGACCT TACAACGACT
EpCB_F3'H   CCAGAGAAAT TGAACATGGA GGAAGCTTAT GGGCTGACCT TACAACGACT
EpCF_F3'H   CCAGAGAAAT TGAACATGGA GGAAGCTTAT GGGCTGACCT TACAACGACT

            1501                                                1550
EpHO_F3'H   TGAGCCATTG ATGGTGTACC CAAACCCTAG GTTGTCGTCT CTAGTCTATG
EpPR_F3'H   TGAGCCATTG ATGGTGCACC CAAACCCTAG GTTGTCGTCT CTAGTCTATG
EpCB_F3'H   TGAGCCATTG ATGGTGCACC CAAACCCTAG GTTGTCGTCT CTAGTCTATG
EpCF_F3'H   TGAGCCATTG ATGGTGTACC CAAACCCTAG GTTGTCGTCT CTAGTCTATG

            1551       1564
EpHO_F3'H   CTGCTCCTAT TTGA
EpPR_F3'H   CTGCGCCTGT TTGA
EpCB_F3'H   CTGCGCCTGT TTGA
EpCF_F3'H   CTGCTCCTGT TTGA
```

Figure S3: Phylogenetic analysis of F3'Hs from the three poinsettia cvs. Christmas Feelings (KY273440), Christmas Beauty (KY273439) and Premium Red (KY489667) by application of the Maximum Likehood method based on the deduced amino acid sequences of isolated poinsettia F3'H cDNA clones and deduced F3'H amino acid sequences of other species available in the NCBI database. Sequences of FNSII were used as outgroup. The JJT matrix-based model was used as a substitution model. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

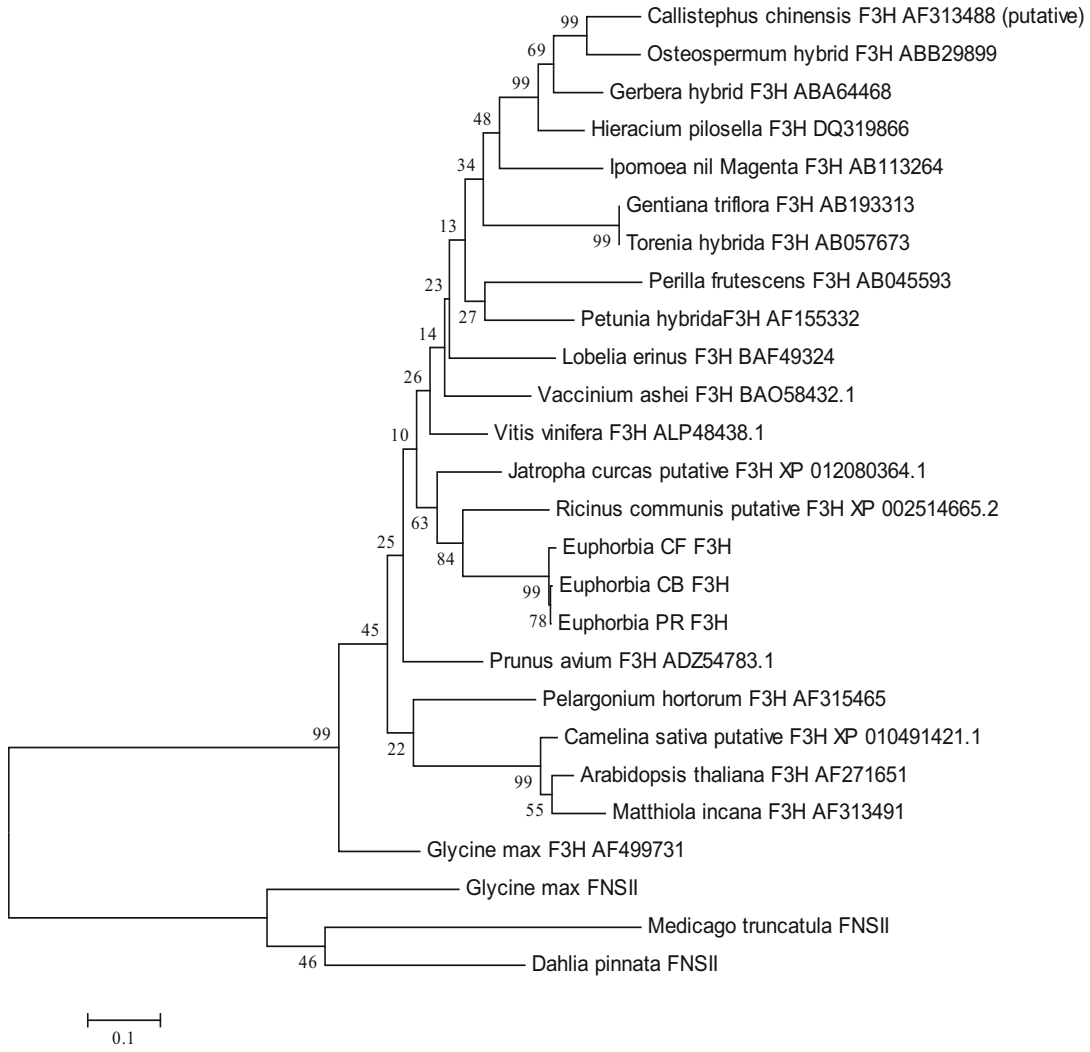


Figure S4: Multiple Alignment of the deduced amino acid sequences of the DFRs of *Euphorbia pulcherrima* cvs. Harvest Orange (*Ep*HO_DFR, KY273438), Premium Red (*Ep*PR_DFR, KY499617), Christmas Beauty (*Ep*CB_DFR, KY273436), and Christmas Feelings (*Ep*CF_DFR, KY273437). Grey frames highlight the VDV region in position 132 to 134. Grey shades highlight differences in the amino acid sequence.

```
           1                                                  50
EpHO_DFR   MGEVPEIVCV TGASGFIGSW LIMRLLERGY RVRATVRDPG NISKVQHLIE
EpPR_DFR   MGEVPEIVCV TGASGFIGSW LIMRLLERGY RVRATVRDPG NISKVQHLIE
EpCB_DFR   MGEVPEIVCV TGASGFIGSW LIMRLLERGY RVRATVRDPG NISKVQHLIE
EpCF_DFR   MGEVPEIVCV TGASGFIGSW LIMRLLERGY RVRATVRDPG NISKVQHLIE

           51                                                 100
EpHO_DFR   LPNAMTNLSL WKADLSVEGS FDEAIKGCSG VFHVATPMDF DSKDPENEVI
EpPR_DFR   LPNAKTNLSL WKADLSVEGS FDEAIKGCSG VFHVATPMDF DSKDPENEVI
EpCB_DFR   LPNAMTNLSL WKADLSVEGS FDEAIKGCSG VFHVATPMDF DSKDPENEVI
EpCF_DFR   LPNAKTNLSL WKADLSVEGS FDEAIKGCSG VFHVATPMDF DSKDPENEVI

           101                                                150
EpHO_DFR   KPTVSGVLDI MKACSKAKTV RRIIFTSSAG TVDVEQHKKP LYDESCWSDL
EpPR_DFR   KPTVSGVLDI MKACSKAKTV RRIIFTSSAG TVDVEQHKKP LYDESCWSDL
EpCB_DFR   KPTVSGVLDI MKACSKAKTV RRIIFTSSAG TVDVEQHKKP LYDESCWSDL
EpCF_DFR   KPTVRGVLDI MKACSKAKTV RRIIFTSSAG TVDVEQHKKP LYDENCWSDL

           151                                                200
EpHO_DFR   DFILSTKMTG WMYFVSKTMA EKAAWKYAEE NNIDLISIIP TLVVGPFIMP
EpPR_DFR   DFILATKMTG WMYFVSKTMA EKAAWKFAEE NNIDLISIIP TLVVGPFIMP
EpCB_DFR   DFILATKMTG WIYFVSKTMA EKAAWKFAEE NNIDLISIIP TLVVGPFIMP
EpCF_DFR   DFILATKMTG WMYFVSKTMA EKAAWKFAEE NNIDLISIIP TLVVGPFIMP

           201                                                250
EpHO_DFR   SMPPSLITAL SPITGNEAHY SIIKQGHYIH LDDLCNAHIY LFEHSKAKGR
EpPR_DFR   SMPPSLITAL SPITGNEAHY SIIKQGHYIH LDDLCNAHIY LFEHSKAKGR
EpCB_DFR   SMPPSLITAL SPITGNEAHY SIIKQGHYIH LDDLCNAHIY LFEHSKAKGR
EpCF_DFR   SMPPSLITAL SPITGNEAHY SIIKQGHYIH LDDLCNAHIY LFEHSKAKGR

           251                                                300
EpHO_DFR   YFCSSHDATI HEIARLLRQK YPQFNIPAKI KGVEENVKNL IFSSKKLEEA
EpPR_DFR   YFCSSHDATI HEIARLLRQK YPQFNIPTKI KGVEENVKNL IFSSKKLEEA
EpCB_DFR   YFCSSHDATI HEIARLLRQK YPQFNIPTKI KGVEENVKNL IFSSKKLEEA
EpCF_DFR   YFCSSHDATI HEIARLLRQK YPQFNIPTKI KGVEENVKNL IFSSKKLEEA

           301                                                  352
EpHO_DFR   GFEFKYSLED MFEGAVETCL AKGLLHAADE KQEPNKVETN DVITSTAEVSCH
EpPR_DFR   GFEFKYSLED MFEGAVETCL AKGLLHAADE KQEPNKVETN DVITSTAEVSCG
EpCB_DFR   GFEFKYSLED MFEGAVETCL AKGLLHAADE KQEPNKVETN DVITSTAEVSCG
EpCF_DFR   GFEFKYSLED MFEGAVETCL AKGLLHAADE KQEPNKVETN DVITSTAEVSCG
```

2.3 Publication 3

First genome edited poinsettias: Breeding for orange bract colour by application of CRISPR/Cas9

D. Nitarska, R. Boehm, T. Debener, R.C. Lucaciu and H. Halbwirth

Plant Cell, Tissue and Organ Culture (PCTOC), submitted

First genome edited poinsettias: Breeding for orange bract colour by application of CRISPR/Cas9

Daria Nitarska[1], Robert Boehm[2], Thomas Debener[3], Rares Calin Lucaciu[1] and Heidi Halbwirth[1*]

[1]Institute of Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, 1060, Vienna, Austria

[2]Klemm + Sohn GmbH & Co., 70379, Stuttgart, Germany

[3]Institute of Plant Genetics, Leibniz Universität Hannover, 30419, Hannover, Germany

[*]corresponding author, Tel. (43) 1 58801166559, Fax. (43) 1 5880117399, heidrun.halbwirth@tuwien.ac.at

Key words (4-6)

Poinsettia (*Euphorbia pulcherrima*), genome editing, CRISPR/Cas9, orange bracts colour, flavonoid 3'-hydroxylase

Abstract (150-250 words)

The CRISPR/Cas9 system is a remarkably promising tool for targeted gene mutagenesis, and becoming ever more popular for modification of ornamental plants. In this study we performed the knockout of *flavonoid 3'-hydroxylase* (*F3'H*) with application of CRISPR/Cas9 in the red flowering poinsettia (*Euphorbia pulcherrima)* cultivar 'Christmas Eve', in order to obtain plants with orange bract colour. F3'H is an enzyme that is necessary for formation of cyanidin-type anthocyanins, which are responsible for the red colour of poinsettia bracts. Even though *F3'H* was not completely silenced, the bract colour of transgenic plants changed from vivid red (RHS 45B) to vivid reddish-orange (RHS 33A), and cyanidin levels decreased significantly compared with the wild type. In the genetically modified plants, an increased ratio of pelargonidin to cyanidin was observed. By cloning and expression of mutated proteins, the lack of F3'H activity was showed. This confirms that a loss of function mutation in the poinsettia *F3'H* gene is sufficient for obtaining poinsettia with orange bract colour. This is the first report of successful use of CRISPR/Cas9 for genome editing in poinsettia.

Introduction

The winter-flowering *Euphorbia pulcherrima* (poinsettia or Christmas Star) belongs to the most economically important potted ornamental plants, especially during the Christmas season (Ecke 2004). Traditionally, the mass market prefers intense scarlet or dark-red colouration of the bracts (Taylor et al. 2011), the latter being leaves that change their colour from green to red to support the plain cymes in pollinator attraction. However, during the last two decades, a huge diversity of red hues and novelty colours and styles has arisen in the poinsettia assortment, because a considerable number of consumers is willing to pay higher prices for unusual varieties (Barrett 2005). Among these, orange-red bract colour seems to attract European and North-American consumers, and has the potential to extend the market e.g. as a Halloween speciality (Barrett 2005).

The pigments responsible for poinsettia bract colouration, at least for the red hues, are the anthocyanins, a well-known group of secondary metabolites (Stewart et al. 1979; Slatnar et al. 2013). Two main types of anthocyanins can be distinguished in poinsettia, based on the number of hydroxyl groups in the B-ring, the pelargonidin-type (one hydroxyl group) and the cyanidin-type (two hydroxyl groups). Recently, we have shown that the rare orange-red bract colouration of poinsettia (*Euphorbia pulcherrima*) is associated with a prevalence of pelargonidin-type anthocyanins and a somewhat decreased anthocyanin concentration in general. We also reported that accumulation of pelargonidin-based pigments in poinsettia is caused by different mechanisms, of which one is a strong reduction of flavonoid 3'-hydroxylase (F3′H) activity in the bracts (Nitarska et al. 2018). F3′H (EC 1.14.13.21) is a membrane bound enzyme associated with the cytosolic site of the endoplasmic reticulum (Schuler and Werck-Reichhart 2003) and belongs to the subfamily CYP75B of cytochrome P450-dependent monooxygenase (P450) (Tanaka 2006; Chapple 1998). F3'H is responsible for the introduction of a second hydroxyl group in the B-ring thereby leading to the formation of cyanidin-type anthocyanins. F3'H, however, does not act on anthocyanins themselves, but on one of the intermediates upstream of the anthocyanin forming step (Schwinn et al. 2014). Particularly, the number of hydroxyl groups in the B-ring of the dihydroflavonol precursors determines the colour of each group of anthocyanins. Dihydrokaempferol (DHK), with one hydroxyl group, is converted to orange-red pelargonidin-type anthocyanins, and dihydroquercetin (DHQ), with two hydroxyl groups, to red-pink cyanidin-type (Halbwirth 2010) (Fig. 1). As has been described (Nakatsuka et al.

2007), silencing of *F3'H* is an important factor for obtaining plants that accumulate prevalently pelargonidin-type anthocyanins in tobacco.

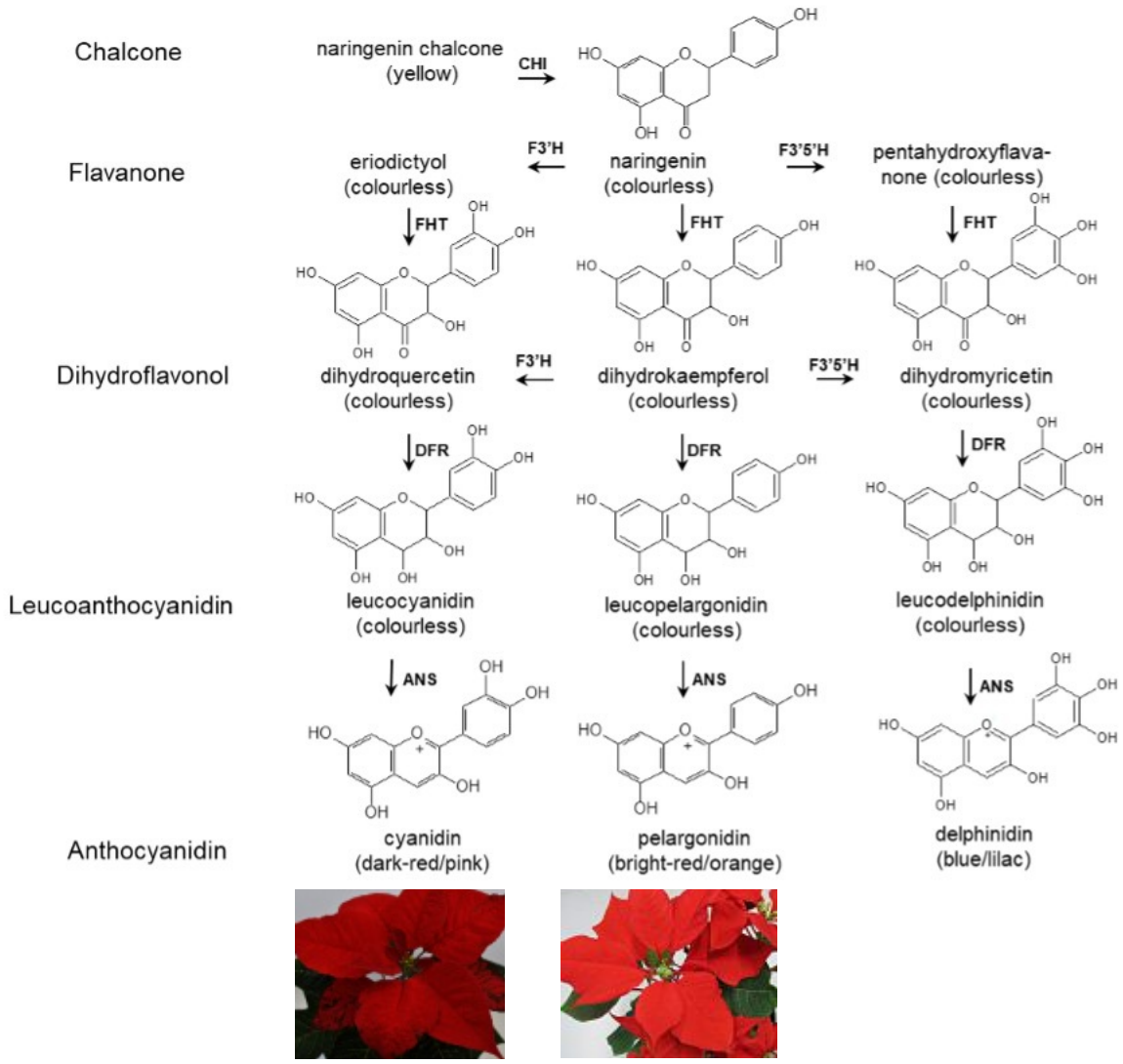


Fig. 1 Simplified overview of the anthocyanin pathway. Abbrev: ANS: anthocyanidin synthase, CHI: chalcone isomerase, DFR: dihydroflavonol 4-reductase, FHT: flavanone 3-hydroxylase, F3′H: flavonoid 3′-hydroxylase, F3′5′H: flavonoid 3′,5′-hydroxylase

Recently, the CRISPR (Clustered Regularly-Interspaced Short Palindromic Repeats)/Cas9 system was reported as a promising tool for targeted genome modification in plants (Erpen-Dalla Corte et al. 2019) and it has quickly developed into the most widely used genome editing method (Kishi-Kaboshi et al. 2018). In this system, double-strand breaks (DSBs) are introduced by Cas9 nuclease, guided by a 20 nucleotide sequence, single guide RNA (sgRNA) (Jinek et al. 2012). There are two mechanisms of DSB repair, homology recombination (HDR) and non -homologous end joining (NHEJ) (Puchta 2005). The second mechanism usually leads to the introduction of a mutation, insertion or deletion, in the gene sequence and consequently to a loss of function in the protein (Chiruvella et al. 2013). This approach is now commonly used

for specific gene knockout in many plant species (Ma et al. 2016). For the first time, the CRISPR/Cas9 system was used in ornamentals for silencing *phytoene desaturase* (*PDS)* in petunia (Zhang et al. 2016). The first successful alteration of flower traits was reported in Japanese morning glory (*Ipomoea nil*), where *dihydroflavonol 4-reductase* (*DFR*) was targeted (Watanabe et al. 2017). Currently this approach is becoming more and more popular for the modification of ornamentals. Up to now it was used for targeting multiple secondary metabolism genes in orchid (Tong et al. 2020), chrysanthemum (Kishi-Kaboshi et al. 2017), lily (Yan et al. 2019), torenia (Nishihara et al. 2018) and gentian (Tasaki et al. 2019).

This study is a proof of concept for the conclusion from our previous work (Nitarska et al. 2018), that a mutation in the *F3'H* gene is sufficient to obtain pelargonidin accumulating poinsettia. We attempted to knockout the poinsettia *F3'H* gene by applying the CRISPR/Cas9 method to obtain poinsettias prevalently accumulating pelargonidin-type anthocyanins in their bracts. To our knowledge, this is the first report of a genome-edited poinsettia.

Materials and methods

Chemicals

(2-^{14}C)-Malonyl-coenzyme A (55 mCi/mmol) was purchased from New England Nuclear Corp. GmbH (Vienna, Austria). Synthesis of radiolabeled substrates was performed as described in (Halbwirth et al. 2006). Reference substances for HPLC analysis (cyanidin, pelargonidin, delphinidin, malvidin, petunidin,) were purchased from Extrasynthese (Genay, France)

Plant material

Poinsettia *Euphorbia pulcherrima* cultivar 'Christmas Eve' (Klemm + Sohn GmbH & Co. KG, Germany) was used for transformation. Plants were grown in the greenhouse under long day conditions (16 h day/8 h night). For *Agrobacterium*-mediated transformation internode stem explants were used. Excised stems were surface sterilized by washing for 10 minutes in 1.5 % solutions of NaOCl with 1 drop of Tween 20 and then washed twice for 10 minutes in sterile water. In the next step, internode stems were cut (around 1 mm thick) and placed on callus induction media (CIM – MS media (Murashige and Skoog 1962) supplemented with 0.2 mg/L 4-chlorophenoxy acetic acid (CPA) and 0.2 mg/L 6-Benzylaminopurine (BAP)) for 4 to 5 days and then explant discs were used for transformation.

sgRNA design and cloning

For transformation, the binary vector pDe-Sa_Cas9 (Steinert et al. 2015), carrying a kanamycin resistance gene (*nptII*) to facilitate selection was used. A sgRNA sequence was designed based on the sequences available in the Gene Bank (KY273440.1), with application of the online tool CHOPCHOP (Labun et al. 2016; Montague et al. 2014). A 20 nucleotide long sgRNA sequence (CAGTCAATAGCCTCCTTGGC), without PAM sequence (TCGGGT), was cloned into a pEN-Sa_Chimera vector (primers for cloning: pEN-Sa_Chim_EpF3HgRNA_F ATTGCAGTCAATAGCCTCCTTGGC and pEN-Sa_Chim_EpF3HgRNA_R AAACGCCAAGGAGGCTATTGACTG)(Steinert et al. 2015). In the second step, a sgRNA expression cassette was transferred to the destination vector (pDe-Sa_Cas9) by single-site Gateway LR reaction (Thermo Fisher Scientific, US) according to manufacturer's protocol. Cloning success was confirmed by sequencing by a commercial supplier (LGC, Germany).

Transformation and regeneration

Agrobacterium tumefaciens strain GV3101, carrying the pDe-Sa_Cas9 vector with cloned sgRNA was cultivated in SOB media (2 % w/v tryptone, 0.5 % w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) supplemented with 50 mg/L rifampicin, 100 mg/L spectinomycin and 30 mg/L gentamicin for 24 hours with shaking (200 rpm) at 28 °C. Then 5 mL of this culture was used to inoculate 50 mL of Minimal A medium (10.5 g/L K_2HPO_4, 4.5 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 0.52 g/L $Na_3C_6H_5O_7 \times 2\ H_2O$, 0.1 % w/v glucose, 0. 005 % $MgSO_4 \times 7\ H_20$, 0.00025 % thiamine) and was further cultivated at identical conditions till OD reached 0.5. Then bacteria were used for transformation. Explants were incubated with 10 mL of *Agrobacterium* inoculum, with addition of three drops of Tween 20, for 30 minutes with gentle shaking and dried on sterile paper, then placed on the CIM for 2 days of co-cultivation. As control explants were incubated with 10 mL of Minimal A medium. Subsequently, discs were washed in sterile water containing the antibiotics 250 mg/L cefotaxim and 150 mg/L timentin for 30 minutes and dried on sterile paper, then placed on the CIM supplemented with 250 mg/L cefotaxim and 150 mg/L timentin for callus induction. After 21 days on CIM, explants were transferred to somatic embryo induction media (SEIM – MS medium with 0.2 mg/L 1-Naphthaleneacetic acid (NAA) and 0.1 mg/L isopentenyl adenine (2ip)) supplemented with 250 mg/L cefotaxim, 150 mg/L timentin and 2.5 mg/L kanamycin, for induction of somatic embryogenesis. After 3 to 6 weeks, when somatic embryos were visible, explants were transferred to somatic embryo maturation medium (SEMM – MS medium with 0.05 mg/L BAP) supplemented with 250 mg/L cefotaxim, 150 mg/L timentin and

200 or 50 mg/L kanamycin for further cultivation and selection. Fully regenerated plants were placed on MS media without antibiotics, propagated and transferred to the greenhouse.

Screening of regenerated plants

Genomic DNA was extracted from leaves of regenerated poinsettia with InVisorb Plant mini kit (Stratec, Germany). Transgenic plants were selected by PCR, where presence of *nptII*, *cas9* and sgRNA sequences was detected (primers' sequences are provided in Suppl. Table S1). For screening, Go*Taq* polymerase (Promega, Germany) was used. The reaction contained a final volume of 20 µL: 4 µL 5X Go*Taq* Green buffer, 0.4 µL dNTPs, 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 2 µL DNA, 0.2 µL *Taq*; reaction conditions: 2 minutes 94 °C initial denaturation, 40 cycles (98 °C, 30 seconds denaturation, 60/62 °C, 30 seconds primer annealing, 72 °C, 45 seconds extension), 10 minutes 72 °C final extension. From positive plants, the total RNA was extracted by mirPremier Kit (Sigma Aldrich, Austria) according to the provided protocol. cDNA was synthesized with RevertAid H Minus Reverse Transcriptase (Thermo Scientific, US) according to the manufacturer's protocol. Primers and PCR conditions were the same as described above. Integrity of DNA as cDNA was confirmed by amplification of poinsettia endogenous genes (*actin*, *GAPDH* or Ep*F3'H* fragment).

Sequence analysis

Genomic DNA from genome-edited plants' leaves was extracted using DNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's protocol. Gene fragments were amplified with EpF3'HpYes-F and EpF3'H-crispr-R primers (Suppl. Table S1) with Q5 High Fidelity DNA Polymerase (New England Biolabs, Austria). PCR conditions: 30 seconds 98 °C for initial denaturation, 30 cycles (98 °C 30 seconds denaturation, 62 °C 30 seconds primer annealing, 72 °C 30 seconds extension), 2 minutes 72 °C final extension. PCR products were separated on 1 % agarose and extracted using Wizard SV Gel and PCR Clean-up System (Promega, Germany). Sub-libraries were constructed with application of NEBNext Ultra II DNA Library Prep kit for Illumina (New England Biolabs, Austria) according to the manufacturer's protocol. For indexing, NEBNext Multiplex Oligos for Illumina (Index Primer Set 1) (New England Biolabs, Austria) were used. Concentration of each sub-library was measured with Qubit (Invitrogen, USA). The bulked library was obtained by mixing equal amounts of each sub-library. The concentration of the bulked library was measured with Qubit and checked on Fragment Analyzer (Agilent, USA), then the bulked library was denatured, and diluted to 8 pM. As a control PhiX (Illumina, USA) was spiked into bulked library in a concentration of 1.3 %. Sequencing runs were performed on Illumina MiSeq system, with

application of MiSeq v2 Reagent Kit 300 cycles (Illumina, USA) (2 x 150 reads). Sequencing results were analysed with CRISPResso2 software (Clement et al. 2019).

HPLC analysis

Poinsettia bract pigment analysis was performed by HPLC as previously described (Haselmair-Gosch et al. 2018). For the extraction, 0.5 g of shock frozen poinsettia bracts were used in 1.5 mL of 2M HCl in methanol.

Gene expression studies

The *F3'H* and *Cas9* gene expression was evaluated by qPCR using the StepOnePlus system (Applied Biosystems, Germany) and the Luna® Universal qPCR Master Mix (New England Biolabs, Austria) according to the manufacturers' protocols. The analysis was performed in three independent replicates and the results were normalized to the two control genes, *actin* and *translation elongation factor 1-alpha* (*EF1 A*)(Zhang et al. 2013). Primer efficiency (Suppl. Table S1) and the relative expression ratio were calculated according to Pfaffl (Pfaffl 2004). Product specificity was confirmed by analysis of melting curves.

Poinsettia F3'H cloning and heterologous expression in yeast

F3'H gene from transgenic plants and WT were cloned into pYes2.1/V5-His-TOPO vector (Invitrogen, US) using EpF3'HpYes-F and EpF3'HpYes-R primers (Suppl. Table S1). Heterologous expression in yeast and the F3'H enzyme assay was performed as previously described (Nitarska et al. 2018).

Statistical analysis

The statistical analysis was performed in GraphPad Prims version 8.4.3 for Mac. Normality of data was checked with the Shapiro-Wilk test. Statistical significance was calculated using a double-tailed, unpaired *t*-test when the variances were equal or unpaired *t*-test with Welch's correction when variances were different. The Mann-Whitney test was used for not normally distributed data.

Results

Transformation and regeneration

In order to obtain poinsettia plants with inactive *F3'H*, a CRISPR/Cas9 approach was used. Poinsettia stem segments were transformed via *Agrobacterium*-mediated transformation with the pDe-Sa_Cas9 vector carrying a sgRNA sequence specific to poinsettia *F3'H*. For transformation, cultivar 'Christmas Eve' was selected as the best for regeneration among four

other tested cultivars (data not shown). In total, seven experiments were performed, which resulted in the regeneration of 105 putatively transgenic plants (Table 1). Due to the fact that the stem segments originated from the greenhouse and the surface sterilization process was not always sufficient, a huge number of explants was lost during the first days after transformation, due to fungal and bacterial infections. Although the callus formation process was very efficient, the embryo formation could be observed only on a few explants. In experiments A to D, 200 mg/L of kanamycin was used for selection, which resulted in the regeneration of only 7 plants, because most of the embryos and plantlets did not survive the selection step. In order to obtain more regenerated plants in experiments E to G we lowered the kanamycin content to 50 mg/L as was suggested in literature (Clarke et al. 2008). At these conditions, we obtained an additional 98 putatively transformed plants, which would suggest that the previous selection conditions could have been too harsh for the cultivar 'Christmas Eve'.

Table 1. Overview of the transformation of the poinsettia cultivar 'Christmas Eve'

Transformation event	*Kanamycin concentration [mg/L]*	*Explants*	*Lost explants*	*Embryogenic explants*	*Regenerated plants*
A	200	46	7	36	3
B	200	349	235	49	4
C	200	66	66	0	0
D	200	446	337	0	0
E	50	389	335	18	20
F	50	275	241	31	25
G	50	504	216	113	53
Total	-	1975	1437	247	105

Screening of regenerated plants

Screening of regenerated plants was performed in two steps. In the first step, genomic DNA from leaves of regenerated plants was extracted and the presence of the transgene was investigated by PCR (targets: *nptII*, *cas9*). In step II, the total RNA was extracted from plants harbouring the transgene as identified in step I. Thereafter, cDNA was synthesized and used as a template for PCR (targets: *nptII*, *cas9*, gRNA) to check the expression of the transgene. In the first screening step, a total of 14 positive plants were found (Table 2, Fig. 2). The high number of negative plants (91) can be attributed to the decrease of the kanamycin concentration in the SEM media, which caused a bigger number of escapes. The second step of screening revealed that expression of the transgene could be detected only in three plants, which all

originated from experiment B (200 mg/L kanamycin) (Fig. 3). Positive lines named B2, B158 and B284 were transferred to the greenhouse for further cultivation.

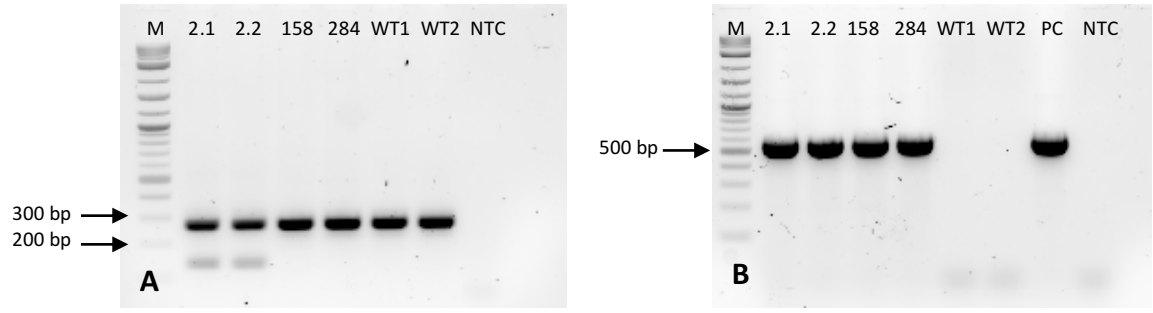


Fig. 2 PCR evaluation of the transgenic plants and WT control on gDNA level. A: Amplification of Ep*F3'H* fragment (primers EpF3'HpYesF and EpF3'HcrisprR), B: Amplification of *cas9* fragment (primers Cas9-F1 and Cas9-R1). M: molecular size standard 1 kb Plus DNA ladder (New England Biolabs, Austria), PC: positive control (plasmid), NTC: non template control. Selected fragments of ladder are labelled for better orientation

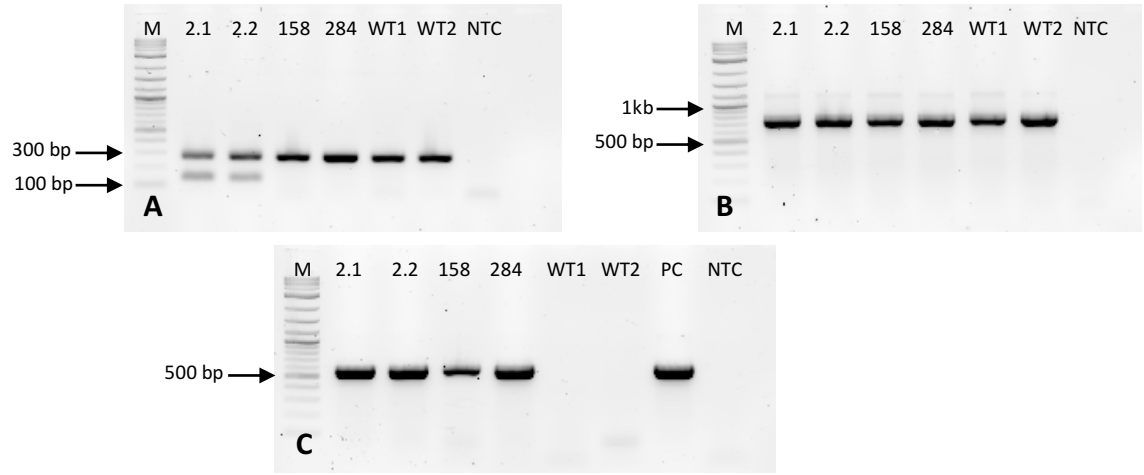


Fig. 3 PCR evaluation of the transgenic plants and WT on cDNA level. A: Amplification of Ep*F3'H* fragment (primers EpF3'HpYesF and EpF3'HcrisprR), B: Amplification of Ep*GAPDH* fragment (primers EpGAPDH-F and EpGAPDH-R), C: *cas9* fragment (primers Cas9-F1 and Cas9-R1). M: molecular size standard 1 kb Plus DNA ladder (New England Biolabs, Austria), PC: positive control (plasmid), NTC: non template control. Selected fragments of ladder are labelled for better orientation

Table 2. Regenerants screening summary

Transformation event	*Total number of plants*	*Positive DNA*	*Positive RNA*	*Dead*
A	3	0	0	0
B	4	4	3	1
C	0	0	0	0
D	0	0	0	0
E	20	4	0	2
F	25	1	0	1
G	53	5	0	1
Total	105	14	3	5

Bract analysis

Bract colouration was induced by cultivation for 8 weeks at short day conditions (11h day, 13h night). In the case of line B2, 2 plants were obtained by propagation of the regenerated shoot, of which one showed bracts with brighter colour (hereafter named B2.1) compared with the wild type (WT) (Fig 4B, 4F). The second plant (hereafter named B2.2) showed scattered points

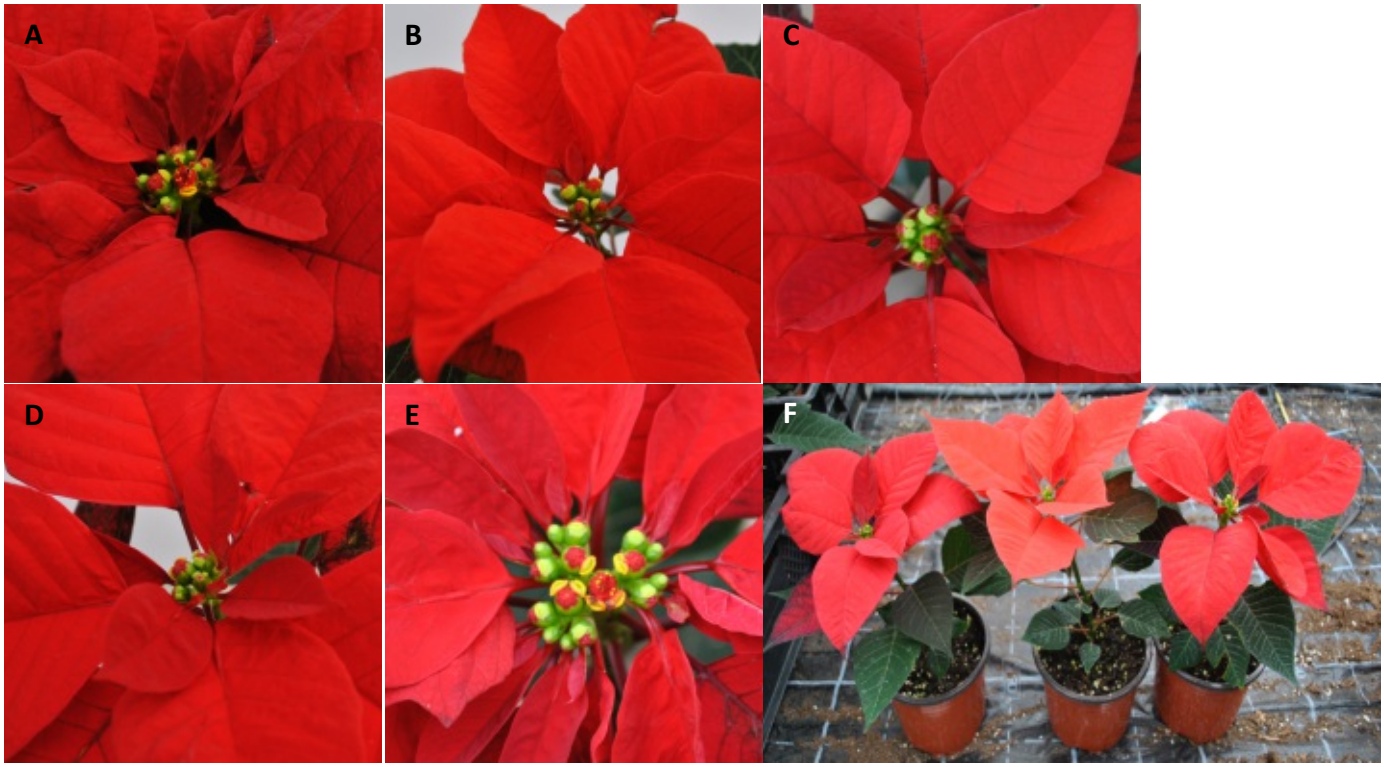


Fig. 4 Bracts colours of WT and genome-edited poinsettias. A: 'Christmas Eve WT'; B: B2.1; C: B2.2; D: B158; E: B284; F: 'Christmas Eve' WT (left), B2.1 (centre), B2.2 (right)

of brighter colour on the bracts, which could be described as chimeric phenotype (Fig 4C, 4F). For line B158, one plant that was obtained showed the same phenotype as the WT (Fig 4D). The same situation was observed for line B284, where the only plant also showed the same bract colour as WT (Fig 4E). All lines after first blooming were further propagated and analysed. The colour of propagated plants of line B2.1 was assigned to groups between 33A and 44B on the RHS charts (5th edition 2007), both are described as vivid reddish-orange, but 33A is placed in the orange colour group and 44B in the red colour group. Wild type 'Christmas Eve' was assigned to group 45B described as vivid red.

To analyse the anthocyanin content in the transgenic poinsettia bracts, HPLC analysis was performed. The total amount of anthocyanins in bracts was lower in all transgenic lines than in the WT, especially for line B2.1 with fully orange bracts (Fig. 5A). The fully orange plants of line B2.1 had a significantly lower amount of cyanidin compared with the WT (Fig. 5B). In all other lines, the cyanidin level was only slightly lower than in the WT (Fig. 5B). The

pelargonidin amount, however, in all transgenic lines did not change that much. Only for line B2.2 was the increase of the pelargonidin content significant (Fig. 5C), for lines B158 and B284, it was even a bit lower in comparison to the WT (Fig. 5C).

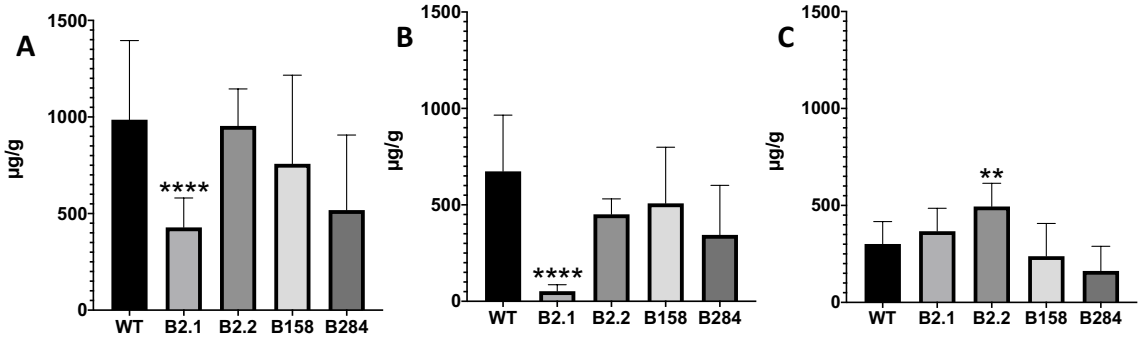


Fig. 5 Anthocyanins in bracts of poinsettia. A: Total anthocyanin content in transgenic lines and WT control, B: Cyanidin content, C: Pelargonidin content. Data were calculated from at least four biological repetitions and with error bars representing standard deviation. Statistical significance * p<0,05, ** p<0,01, ***p<0,001, ****p<0,0001

Sequence analysis

To analyse the *F3'H* sequence of transgenic plants, next generation sequencing (NGS) was performed. 24 % of the reads for line B2 orange (B2.1) and 19 % of line B2 chimera (B2.2) was modified (Table 3). For lines B158 and B284, no modifications were found. The most prevalent modification for line B2 was an insertion of a thymine (T) located three nucleotides before the PAM sequence. Nevertheless, in all transgenic lines, the most prevalent sequence was that of the WT, which suggests that the genome editing approach was not efficient enough. Even for line B2 more that 66 % reads were identical to the sequence of the WT, which explains the presence of cyanidin-type anthocyanins in the bracts. Interestingly, only a slightly higher number of WT reads was detected for line B2 chimeric plants in comparison to the fully orange B2 plant. In all analysed plants, also other sequences than the major reads were present and typically consisted of 0.5 % of total reads or less. This outcome is considered to be a result of PCR and NGS errors. Sequencing analysis confirmed that the genome editing in line B2 worked, but still the WT sequence is present, which results in just a partial shift toward pelargonidin in anthocyanin synthesis for this transgenic line.

Table 3. NGS analysis of poinsettia *F3'H* amplicons of genome-edited lines. Inserted nucleotides are marked in red, PAM sequence (TCGGGT) is marked in green

Line	Total number of reads	Number of reads	%	Targeted F3'H sequence	In/Del
WT	2327800	2185214	93.87	CAGTCAATAGCCTCCTTGGCTCGGGT	WT
B2.1	2326081	1551158	66.69	CAGTCAATAGCCTCCTTGGCTCGGGT	WT
		552471	23.75	CAGTCAATAGCCTCCTTTGGCTCGGGT	+1
B2.2	1646282	1176195	71.45	CAGTCAATAGCCTCCTTGGCTCGGGT	WT
		316765	19.24	CAGTCAATAGCCTCCTTTGGCTCGGGT	+1
B158	3096423	2935697	94.81	CAGTCAATAGCCTCCTTGGCTCGGGT	WT
B284	3097207	2925612	94.46	CAGTCAATAGCCTCCTTGGCTCGGGT	WT

Gene expression studies

The expression level of poinsettia *F3'H* and the *Cas9* gene, in the transgenic plants and the WT 'Christmas Eve' was determined by quantitative real-time PCR using *actin* and *EF1A* for normalization (primer sequences Suppl. Table S1). In general, the expression level of poinsettia *F3'H* for all transgenic lines was on a comparable level as in the WT, only for line B2.2 was it slightly lower (Fig. 6). We were able to detect *Cas9* expression in all transgenic lines, whereas in the WT it was not observed. In all lines, expression of *Cas9* between the propagated plants varied widely. The highest values were measured for line B2, whereas for the two other lines it was two or four times lower (Fig. 6). This correlates with the fact that a changed colouration could be observed only in line B2. In the other two lines (B158 and B284), the expression of *Cas9* was probably not sufficient to perform the edit of the targeted gene.

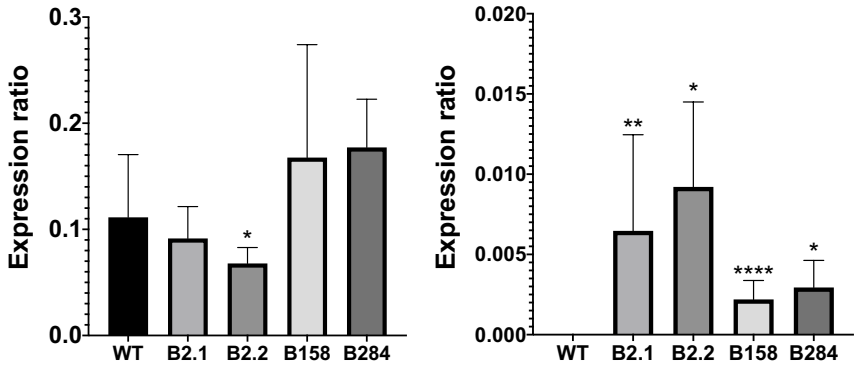


Fig. 6 Gene expression of poinsettia *F3'H* (left) and *Cas9* (right) in transgenic poinsettia and WT. Quantitative expression was normalized to *actin*. Data were calculated from at least four biological repetitions and with error bars representing standard deviation. Statistical significance * p<0,05, ** p<0,01, ***p<0,001, ****p<0,0001

F3'H cloning and heterologous expression in yeast

After PCR amplification of full size *F3'H* from transgenic plants from lines B2.1 and B2.2, two bands were detected (Fig. 7). One band with the original size present in the wild type around 1,5 kb, and second which was approx. 100 base pairs smaller. The same result was

observed during screening, when also two bands were obtained when *F3'H* fragment was amplified as endogenous control (Fig. 2A, Fig. 3A). Therefore, *F3'Hs* from all transgenic plants and the WT were isolated and heterologously expressed in yeast in order to check the recombinant proteins for functional activity.

From the line B2, three different versions of F3'H cDNA clones were isolated, version 1 (v1) with a sequence identical to that of the WT, version 2 (v2) showing an insertion of a T in position 170, 3 nucleotides upstream PAM sequence, and version 3 (v3) showing a deletion of 126 nucleotides (Suppl. Fig S1). *F3'Hs* clones isolated from lines B158 and B284 were identical to those of the WT (Suppl. Fig. S1, S2). In *F3'H* v2, the insertion of an additional T caused a shift in the open reading frame and thus, a premature termination of protein synthesis, whereas the deletion in *F3'H* v3, results in the loss of 42 amino acids between positions 37 and 79 (Suppl. Fig. S2). However, recombinant F3'Hs produced from *F3'H* v2 and v3 were not functionally active, which was tested with two main substrates of F3'H. All other recombinant F3'Hs were active, and the conversion of the substrate was on a similar level as that of the F3'Hs present in the WT (Table 4).

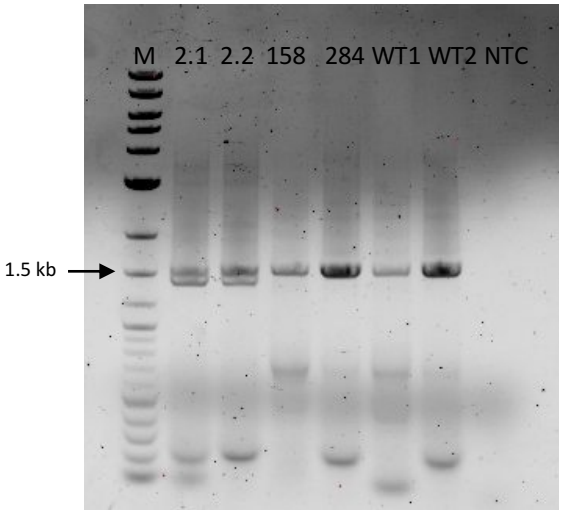


Fig. 7 PCR amplification of full-size poinsettia *F3'H* with primers EpF3'HpYesF and EpF3'HpYesR and cDNA as a template. M: molecular size standard 1 kb Plus DNA ladder (New England Biolabs, Austria), NTC: non template control. 1.5 kb fragment of the size marker is labelled for better orientation.

Table 4. Functional activity test of recombinant F3'Hs from transgenic plants

Name	*Naringenin conversion (%)*	*DHK conversion (%)*
WT	96	82
B2-v1	83	78
B2-v2	0	0
B2-v3	4	0
B158	84	81
B284	89	42

Discussion

Over more than 200 years of cultivation, the appearance of poinsettia has changed tremendously. Wild poinsettias are shrubs or small trees of up to 3 meters height, with long internodes, a few stems, and narrow leaves and bracts (Trejo et al. 2012; Lee 2000). Commercial poinsettias as we know them from flower shops and supermarkets, in contrast, are small compact potted plants with multiple branches and wide large bracts (Trejo et al. 2012). The development of modern poinsettia took place in the 20th century and the main milestones were the introduction of photoperiodic control, branching induction by transfer of phytoplasma via grafting, and the use of growth regulators (Taylor et al. 2011).

The colour spectrum of the bracts has also changed over time. Wild poinsettias are red or in a few cases white, but nowadays poinsettia are available in many different colour variations (Taylor et al. 2011), which gain more and more popularity (Barrett 2005). Apart from crossbreeding, radiation mutation is one of the basic methods to obtain novel bract colours (Broertjes and Van Harten 2013). The main drawback of this method is that mutations occur randomly, and a large number of plants has to be screened for interesting novel phenotypes (Kishi-Kaboshi et al. 2018), although in poinsettia the mutation rates leading to changes in the flower colour is relatively high (Lee et al. 2010). Genome editing methods like the CRISPR/Cas9 system offer significant improvement, as these allow precise targeting of the gene to be mutated (Liu et al. 2017). An additional advantage of CRISPR/Cas9 is that the transgene can be segregated in the progeny, so that a mutated plant without presence of a transgene can be obtained (Gao et al. 2016). CRISPR/Cas9 constructs are usually transferred to the plant cells by *Agrobacterium*-mediated transformation or particle bombardment (Erpen-Dalla Corte et al. 2019), which is a challenging process in some plant species.

Up to now, three classical poinsettia transformations mediated by *Agrobacterium* (Clarke et al. 2008) (Islam et al. 2013) (Sagvaag 2015) have been reported. To our knowledge, our approach is the first attempt to combine *Agrobacterium*-mediated transformations with CRISPR/Cas9 application in poinsettias. The approach faced problems with explant contamination due to insufficient surface sterilization. Clarke et al. (2008) suggested that the latex sap occurring at poinsettia cutting spots makes surface sterilization more challenging. The regeneration process of the chosen cultivar 'Christmas Eve' was satisfactory. The percentage of somatic embryo producing explants was, however, lower than reported by Clarke et al. (2008) for cv. 'Millennium' and is probably strongly dependent on the cultivar. The total transformation frequency during the transformation of cv. 'Millennium' (Clarke et al., 2008) was similar to the value obtained in this study and it is around 2 % (considering only explants that were not lost due to contamination).

Despite the fact that the transgene was detected in 14 regenerated plants on the genomic DNA level, only 3 of them showed transgene expression (5 were lost during the cultivation process). Of these, just one line (B2) developed bracts with brighter, orange-red colour, than the WT (Fig. 4). When plants were propagated from the original shoots of B2, chimeric types were obtained in addition to the fully bright orange-red phenotype. The chimeric plants can be described as non-pattern sectorial chimeras. This kind of mosaic phenotype is unstable (Frank and Chitwood 2016) and during further propagation, plants with different amounts of brighter areas were obtained. The occurrence of chimeric phenotypes was also reported for Japanese morning glory, in which *DFR* had been knocked out with the application of CRISPR/Cas9 system (Watanabe et al. 2017), and torenia where *F3H* was silenced (Nishihara et al. 2018). Probably chimeric tissue is a result of ongoing genome editing in line B2, despite that most of the genome edition events is considered to take place during early transformation stage (Nishihara et al. 2018). Only possibility to stop this process is crossing out *Cas9*, what is a problem in vegetatively propagated plants like poinsettia (Taylor et al. 2011). Chimerism of line B2 was confirmed by NGS data, when WT reads were detected together with mutated sequence (+1 nucleotide) in both fully orange and the chimeric plant, and by cloning of three versions of *F3'H* which were expressed in line B2. Lines B158 and B284 produced bracts with the same colour as the WT, indicating that the transgene could have been silenced. NGS data showed that only WT type reads are present in both lines indicating that the edition did not occur in those lines. This can happen, when the transgene is incorporated in a highly methylated region of the genome, or when more than one copy of the transgene is integrated (Vaucheret et

al. 1998). It can also be explained by a transgene inactivation mechanism, in which the plant inactivates the transgene if recognized as a foreign DNA (Finnegan and McElroy 1994), which could happen before the edit occurred and as a result the plants without the edition and with no transgene expression are obtained.

The total amount of anthocyanins in the orange-red plants obtained from line B2.1 was significantly lower than that in the WT. This is in agreement with the lower anthocyanin content of orange compared with dark red cultivars (Nitarska et al. 2018). The level of pelargonidin remained almost unchanged in the transgenic plants compared with the wild type, only in line B2.2 was it significantly higher. Simultaneously, line B2.1 has a significantly lower cyanidin level, which indicates reduced F3'H activity compared with the WT (Fig. 5). The presence of cyanidin-type anthocyanidins in line B2, however, suggests that unmutated *F3'H* is also expressed. The lower anthocyanin content of the orange-red line and the unchanged pelargonidin content can be explained by the low substrate specificity of the dihydroflavonol 4-reductase of poinsettia for DHK (Nitarska et al. 2018), which creates a bottleneck resulting in a slower anthocyanin formation and, therefore, in lower total anthocyanin concentrations, but because of the reduced F3'H activity an increased ratio of pelargonidin:cyanidin.
As reported for other plants (Jang et al. 2016), the expression of poinsettia *F3'H* was not affected by the CRISPR/Cas9 system. The expression level of *Cas9* was much higher in the plants obtained from line B2 compared with plants obtained from lines B158 and B284, which correlates with the mutation efficiency. A positive correlation between *Cas9* expression and mutation frequency was also reported in rice (Mikami et al. 2015; Jang et al. 2016).

Cloning of *F3'H* from transgenic plants, expression in yeast and enzyme assay revealed that in line B2 at least three versions of *F3'H* are expressed. Version 1, with a sequence identical to the WT, showed no change in protein activity, which explains the presence of cyanidin in the bracts of line B2. Version 2, with insertion of T in position 170, which causes a shift in open reading frame and premature protein termination. The encoded protein was not active, which also supports the hypothesis that the mutation in the *F3'H* gene is a cause of change in poinsettia bract colour from red to orange (Nitarska et al. 2018). There is also a third version of *F3'H*, where 42 amino acids from the N-terminal part of the protein were deleted. The obtained protein is almost not active, with only very slight activity with naringenin being observed. This version of *F3'H* was not detected during our NGS studies, probably due to big difference in the size, leading to this fragment being lost during the size selection process.

The CRISPR/Cas9 system is without doubt an interesting alternative for targeted mutagenesis in poinsettia. It should, however, be also taken into account that poinsettia transformation and regeneration is a challenging process and protocol optimization for each variety seems to be essential. Future work will concentrate on self-pollination of line B2 in order to obtain homozygous plants with more intense orange colour, which will accumulate prevalently pelargonidin. We are also planning to try to increase mutation efficiency in lines B158 and B284 by heat stress treatment as was performed by (LeBlanc et al. 2018). In this study we reported for the first time successful targeted mutagenesis with the CRISPR/Cas9 system in poinsettia. We also provided a proof of concept that mutation of F3'H is sufficient to obtain poinsettia showing orange-red bract colour.

Funding

This project gratefully received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 675657 Flower Power and the Austrian Science Fund (FWF) P32901–B. The funding bodies had no role in the design of the study, collection, analysis or interpretation of data or in writing the manuscript.

Acknowledgement

We would like to thank Christopher Schlosser for critical reading of the manuscript, Renate Paltram for technical support in HPLC analysis and Gabriel Alexander Vignolle for support during performing NGS analysis. The authors acknowledge Astrid Mach Aigner (Research Group Synthetic Biology and Molecular Biotechnology at the Institute of Chemical, Environmental and Bioscience Engineering, TU Wien) for making available the Illumina MiSeq system used in this study.

Conflict of interest/Competing interests

The authors declare that they have no conflict of interest.

Author contributions

DN, RB, TD and HH conceived the research. DN and RB designed the experiments. DN performed the experiments. RCL analysed the sequencing data. DN and HH wrote the manuscript. All authors read and approved the final manuscript.

Ethic approval

not applicable

Consent to participate

not applicable

Consent of publication

not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information file. Primary datasets are available from the corresponding author on reasonable request.

Code availability

not applicable

References

Barrett J (2005) Consumer Poinsettia Preference. Lawn and Garden Retailer 4 (2):1-4

Broertjes C, Van Harten A (2013) Applied mutation breeding for vegetatively propagated crops. Elsevier,

Chapple C (1998) Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. Annual review of plant biology 49 (1):311-343

Chiruvella KK, Liang Z, Wilson TE (2013) Repair of double-strand breaks by end joining. Cold Spring Harbor perspectives in biology 5 (5):a012757

Clarke JL, Spetz C, Haugslien S, Xing S, Dees MW, Moe R, Blystad D-R (2008) Agrobacterium tumefaciens-mediated transformation of poinsettia, Euphorbia pulcherrima, with virus-derived hairpin RNA constructs confers resistance to Poinsettia mosaic virus. Plant Cell Reports 27 (6):1027-1038. doi:10.1007/s00299-008-0526-9

Clement K, Rees H, Canver MC, Gehrke JM, Farouni R, Hsu JY, Cole MA, Liu DR, Joung JK, Bauer DE, Pinello L (2019) CRISPResso2 provides accurate and rapid genome editing sequence analysis. Nature Biotechnology 37 (3):224-226. doi:10.1038/s41587-019-0032-3

Ecke P (2004) The Ecke poinsettia manual. Ball Pub., Batavia, Ill.

Erpen-Dalla Corte L, Mahmoud LM, Moraes TS, Mou Z, Grosser JW, Dutt M (2019) Development of Improved Fruit, Vegetable, and Ornamental Crops Using the CRISPR/Cas9 Genome Editing Technique. Plants (Basel, Switzerland) 8 (12)

Finnegan J, McElroy D (1994) Transgene inactivation: plants fight back! Bio/technology 12 (9):883-888

Frank MH, Chitwood DH (2016) Plant chimeras: the good, the bad, and the 'Bizzaria'. Developmental biology 419 (1):41-53

Gao X, Chen J, Dai X, Zhang D, Zhao Y (2016) An effective strategy for reliably isolating heritable and Cas9-free Arabidopsis mutants generated by CRISPR/Cas9-mediated genome editing. Plant physiology 171 (3):1794-1800

Halbwirth H (2010) The creation and physiological relevance of divergent hydroxylation patterns in the flavonoid pathway. International journal of molecular sciences 11 (2):595-621

Halbwirth H, Kahl S, Jäger W, Reznicek G, Forkmann G, Stich K (2006) Synthesis of (14C)-labeled 5-deoxyflavonoids and their application in the study of dihydroflavonol/leucoanthocyanidin interconversion by dihydroflavonol 4-reductase. Plant Science 170 (3):587-595

Haselmair-Gosch C, Miosic S, Nitarska D, Roth BL, Walliser B, Paltram R, Lucaciu RC, Eidenberger L, Rattei T, Olbricht K (2018) Great cause—small effect: undeclared genetically engineered Orange Petunias Harbor an inefficient Dihydroflavonol 4-Reductase. Frontiers in plant science 9:149

Islam MA, Lütken H, Haugslien S, Blystad D-R, Torre S, Rolcik J, Rasmussen SK, Olsen JE, Clarke JL (2013) Overexpression of the AtSHI gene in poinsettia, Euphorbia pulcherrima, results in compact plants. PloS one 8 (1)

Jang G, Lee S, Um TY, Chang SH, Lee HY, Chung PJ, Kim J-K, Do Choi Y (2016) Genetic chimerism of CRISPR/Cas9-mediated rice mutants. Plant biotechnology reports 10 (6):425-435

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. science 337 (6096):816-821

Kishi-Kaboshi M, Aida R, Sasaki K (2017) Generation of gene-edited Chrysanthemum morifolium using multicopy transgenes as targets and markers. Plant and Cell Physiology 58 (2):216-226

Kishi-Kaboshi M, Aida R, Sasaki K (2018) Genome engineering in ornamental plants: current status and future prospects. Plant Physiology and Biochemistry 131:47-52

Labun K, Montague TG, Gagnon JA, Thyme SB, Valen E (2016) CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. Nucleic acids research 44 (W1):W272-W276

LeBlanc C, Zhang F, Mendez J, Lozano Y, Chatpar K, Irish VF, Jacob Y (2018) Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. The Plant Journal 93 (2):377-386

Lee EK, Kim WH, Kim ST, Kang SY (2010) Rooting, growth, and color mutation of poinsettias affected by gamma radiation. Journal of Radiation Industry 4 (3):253-257

Lee I-M (2000) Phytoplasma casts a magic spell that turns the fair poinsettia into a Christmas showpiece. Plant Health Progress 1 (1):16

Liu X, Wu S, Xu J, Sui C, Wei J (2017) Application of CRISPR/Cas9 in plant biology. Acta pharmaceutica sinica B 7 (3):292-302

Ma X, Zhu Q, Chen Y, Liu Y-G (2016) CRISPR/Cas9 platforms for genome editing in plants: developments and applications. Molecular plant 9 (7):961-974

Mikami M, Toki S, Endo M (2015) Parameters affecting frequency of CRISPR/Cas9 mediated targeted mutagenesis in rice. Plant cell reports 34 (10):1807-1815

Montague TG, Cruz JM, Gagnon JA, Church GM, Valen E (2014) CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic acids research 42 (W1):W401-W407

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia plantarum 15 (3):473-497

Nakatsuka T, Abe Y, Kakizaki Y, Yamamura S, Nishihara M (2007) Production of red-flowered plants by genetic engineering of multiple flavonoid biosynthetic genes. Plant cell reports 26 (11):1951-1959

Nishihara M, Higuchi A, Watanabe A, Tasaki K (2018) Application of the CRISPR/Cas9 system for modification of flower color in Torenia fournieri. BMC plant biology 18 (1):1-9

Nitarska D, Stefanini C, Haselmair-Gosch C, Miosic S, Walliser B, Mikulic-Petkovsek M, Regos I, Slatnar A, Debener T, Terefe-Ayana D (2018) The rare orange-red colored Euphorbia pulcherrima cultivar 'Harvest Orange'shows a nonsense mutation in a flavonoid 3'-hydroxylase allele expressed in the bracts. BMC plant biology 18 (1):216

Pfaffl MW (2004) Quantification strategies in real-time PCR. AZ of quantitative PCR 1:89-113

Puchta H (2005) The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. Journal of experimental botany 56 (409):1-14

Sagvaag AK (2015) Genetic transformation of Poinsettia (Euphorbia pulcherrima). Norwegian University of Life Sciences, Ås,

Schuler MA, Werck-Reichhart D (2003) Functional genomics of P450s. Annual Review of Plant Biology 54 (1):629-667

Schwinn K, Miosic S, Davies K, Thill J, Gotame TP, Stich K, Halbwirth H (2014) The B-ring hydroxylation pattern of anthocyanins can be determined through activity of the flavonoid 3′-hydroxylase on leucoanthocyanidins. Planta 240 (5):1003-1010

Slatnar A, Mikulic-Petkovsek M, Veberic R, Stampar F, Schmitzer V (2013) Anthocyanin and chlorophyll content during poinsettia bract development. Scientia Horticulturae 150:142-145

Steinert J, Schiml S, Fauser F, Puchta H (2015) Highly efficient heritable plant genome engineering using Cas9 orthologues from Streptococcus thermophilus and Staphylococcus aureus. The Plant Journal 84 (6):1295-1305

Stewart R, Asen S, Massie D, Norris K (1979) The identification of Poinsettia cultivars by HPLC analysis of their anthocyanin content. Biochemical Systematics and Ecology 7 (4):281-287

Tanaka Y (2006) Flower colour and cytochromes P450. Phytochemistry Reviews 5 (2-3):283-291

Tasaki K, Higuchi A, Watanabe A, Sasaki N, Nishihara M (2019) Effects of knocking out three anthocyanin modification genes on the blue pigmentation of gentian flowers. Scientific reports 9 (1):1-10

Taylor JM, Lopez RG, Currey CJ, Janick J (2011) The poinsettia: History and transformation. Chronica Horticulturae 51 (3):23-28

Tong CG, Wu FH, Yuan YH, Chen YR, Lin CS (2020) High-efficiency CRISPR/Cas-based editing of Phalaenopsis orchid MADS genes. Plant Biotechnology Journal 18 (4):889-891

Trejo L, Feria Arroyo TP, Olsen KM, Eguiarte LE, Arroyo B, Gruhn JA, Olson ME (2012) Poinsettia's wild ancestor in the Mexican dry tropics: Historical, genetic, and environmental evidence. American Journal of Botany 99 (7):1146-1157

Vaucheret H, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Mourrain P, Palauqui JC, Vernhettes S (1998) Transgene-induced gene silencing in plants. The Plant Journal 16 (6):651-659

Watanabe K, Kobayashi A, Endo M, Sage-Ono K, Toki S, Ono M (2017) CRISPR/Cas9-mediated mutagenesis of the dihydroflavonol-4-reductase-B (DFR-B) locus in the Japanese morning glory Ipomoea (Pharbitis) nil. Scientific reports 7 (1):1-9

Yan R, Wang Z, Ren Y, Li H, Liu N, Sun H (2019) Establishment of Efficient Genetic Transformation Systems and Application of CRISPR/Cas9 Genome Editing Technology in Lilium pumilum DC. Fisch. and Lilium longiflorum White Heaven. International journal of molecular sciences 20 (12):2920

Zhang B, Yang X, Yang C, Li M, Guo Y (2016) Exploiting the CRISPR/Cas9 system for targeted genome mutagenesis in petunia. Scientific reports 6:20315

Zhang L, He L-L, Fu Q-T, Xu Z-F (2013) Selection of reliable reference genes for gene expression studies in the biofuel plant Jatropha curcas using real-time quantitative PCR. International journal of molecular sciences 14 (12):24338-24354

Suppl. Table S1. Primers used during the study

Name	Sequence 5' – 3'	Product size, purpose
nptII-F	ACAAGATGGATTGCACGCAGG	780 bp, transgene detection
nptII-R	AACTCGTCAAGAAGGCGATAG	
Cas9-F1	ATGAAGAGGAACTACATCCTCGG	564 bp, transgene detection
Cas9-F2	CTTCTGCACCTTGAGGAGCT	
Cas9-F2	GGATAATGGACCTGTGATCAAGAAG	577 bp, transgene detection
Cas9-R2	CTTCCTCTTCTTCTTAGGATCAGC	
gRNAfull-F	ACGCGTCCTGAGGCTTTTTTTC	510 bp, transgene detection
gRNAfull-R	CAGGACGCGTAAAAATCTCGC	
EpActin	GCTCAGTCCAAGAGAGGTATTT	243 bp, transgene detection
EpActin	AGCCTGAATAGCGACATACATAG	
EpGAPDH-F	GAGACGATGTGGAGCTTGTT	766 bp, transgene detection
EpGAPDH-R	TTTCCCTCAGATTCTGCCTTTAT	
EpF3'HpYes-F	ATGTTACCACTCTTTGCGTTTACC	265 bp, sequence analysis
EpF3'Hcrispr-R	CTTTCAAGAACTGGGCAGCAAC	
qEpActin2-F	CTGTTCCAGCCATCTCTCATT	127 bp, qPCR efficiency 2.06
qEpActin2-R	AACCGCCACTCAGAACTATG	
qEpEF1A-F	AAGATGATTCCCACCAAGCCCA	72 bp, qPCR efficiency 2.01
qEpEF1A-R	CACAGCAAAACGACCCAGAGGA	
qEpF3'H2-F	AATCAACGGCTACCACATCC	91 bp, qPCR efficiency 2.02
qepF3'H2-R	CAGCGGCTCTTTCCATACTT	
qCas9-F	CAACGAGGGAAGAAGGTCTAAG	110 bp, qPCR efficiency 2.04
qCas9-R	GATCGGTGAGGAGGTTGTAATC	
EpF3'HpYes-F	ATGTTACCACTCTTTGCGTTTACC	1533 bp, F3'H cloning into
EpF3'HpYes-R	TCAAACAGGAGCAGCATAGACTA	pYES vector

```
WT-K4       ATGTTACCACTCTTTGCGTTTACCATTTTTTCTGCCATTTTCATTTCCTTTTT---CTTC   57
2-K2-v1     ATGTTACCACTCTTTGCGTTTACCATTTTTTCTGCCATTTTCATTTCCTTTTT---CTTC   57
2-K1-v2     ATGTTACCACTCTTTGCGTTTACCATTTTTTCTGCCATTTTCATTTCCTTTTT---CTTC   57
2-K3-v3     ATGTTACCACTCTTTGCGTTTACCATTTTTTCTGCCATTTTCATTTCCTTTTTTTTCTTC   60
158-K6      ATGTTACCACTCTTTGCGTTTACCATTTTTTCTGCCATTTTCATTTCCTTTTT---CTTC   57
284-K3      ATGTTACCACTCTTTGCGTTTACCATTTTTTCTGCCATTTTCATTTCCTTTTTTTTCTTC   60

WT-K4       TTCTTTTTTCGCCGTACCTCTCGCCCCCCTCTTCCTCCCGGTCCTAGACCACTGCCTGTA   117
2-K2-v1     TTCTTTTTTCGCCGTACCTCTCGCCCCCCTCTTCCTCCCGGTCCTAGACCACTGCCTGTA   117
2-K1-v2     TTCTTTTTTCGCCGTACCTCTCGCCCCCCTCTTCCTCCCGGTCCTAGACCACTGCCTGTA   117
2-K3-v3     TTCTTTTTTCGCCGTACCTCTCGCCCCCCTCTTCCTCCCGGTCCTAGACCAC--------   90
158-K6      TTCTTTTTTCGCCGTACCTCTCGCCCCCCTCTTCCTCCCGGTCCTAGACCACTGCCTGTA   117
284-K3      TTCTTTTTTCGCCGTACCTCTCGCCCCCCTCTTCCTCCCGGTCCTAGACCACTGCCTGTA   120

WT-K4       ATTGGAAACCTGCCTCATTTAGGCCCCAAACCCCACCAGTCAATAGCCTCCT-TGGCTCG   176
2-K2-v1     ATTGGAAACCTGCCTCATTTAGGCCCCAAACCCCACCAGTCAATAGCCTCCT-TGGCTCG   176
2-K1-v2     ATTGGAAACCTGCCTCATTTAGGCCCCAAACCCCACCAGTCAATAGCCTCCTTTGGCTCG   177
2-K3-v3     ------------------------------------------------------------   90
158-K6      ATTGGAAACCTGCCTCATTTAGGCCCCAAACCCCACCAGTCAATAGCCTCCT-TGGCTCG   176
284-K3      ATTGGAAACCTGCCTCATTTAGGCCCCAAACCCCACCAGTCAATAGCCTCCT-TGGCTCG   179

WT-K4       GGTTTATGGCCCCCTTATGCACCTCCGTATGGGCTTTGTCGACGTCGTTGTGGCGGCGTC   236
2-K2-v1     GGTTTATGGCCCCCTTATGCACCTCCGTATGGGCTTTGTCGACGTCGTTGTGGCGGCGTC   236
2-K1-v2     GGTTTATGGCCCCCTTATGCACCTCCGTATGGGCTTTGTCGACGTCGTTGTGGCGGCGTC   237
2-K3-v3     ------------------------------------------------------------C   113
158-K6      GGTTTATGGCCCCCTTATGCACCTCCGTATGGGCTTTGTCGACGTCGTTGTGGCGGCGTC   236
284-K3      GGTTTATGGCCCCCTTATGCACCTCCGTATAGGCTTTGTCGACGTCGTTGTGGCGGCGTC   239

WT-K4       GGCGTCCGTTGCTGCCCAGTTCTTGAAAGCTCATGACGCTAATTTCTCGAGCCGGCCGCC   296
2-K2-v1     GGCGTCCGTTGCTGCCCAGTTCTTGAAAGCTCATGACGCTAATTTCTCGAGCCGGCCGCC   296
2-K1-v2     GGCGTCCGTTGCTGCCCAGTTCTTGAAAGCTCATGACGCTAATTTCTCGAGCCGGCCGCC   297
2-K3-v3     GGCGTCCGTTGCTGCCCAGTTCTTGAAAGCTCATGACGCTAATTTCTCGAGCCGGCCGCC   173
158-K6      GGCGTCCGTTGCTGCCCAGTTCTTGAAAGCTCATGACGCTAATTTCTCGAGCCGGCCGCC   296
284-K3      GGCGTCCGTTGCTGCCCAGTTCTTGAAAGCTCATGACGCTAATTTCTCGAGCCGGCCGCC   299

WT-K4       TAATTCGGGTGCTAAGTATGTTGCTTATAATTACCAAGATCTTGTTTTTGCCCCGTACGG   356
2-K2-v1     TAATTCGGGTGCTAAGTATGTTGCTTATAATTACCAAGATCTTGTTTTTGCCCCGTACGG   356
2-K1-v2     TAATTCGGGTGCTAAGTATGTTGCTTATAATTACCAAGATCTTGTTTTTGCCCCGTACGG   357
2-K3-v3     TAATTCGGGTGCTAAGTATGTTGCTTATAATTACCAAGATCTTGTTTTTGCCCCGTACGG   233
158-K6      TAATTCGGGTGCTAAGTATGTTGCTTATAATTACCAAGATCTTGTTTTTGCCCCGTACGG   356
284-K3      TAATTCGGGTGCTAAGTATGTTGCTTATAATTACCAAGATCTTGTTTTTGCCCCGTACGG   359

WT-K4       ACCTCGCTGGCGCATGCTCAGGAAAATCAGTGCCGTGCATCTCTTCTCGGCTAAGGCCTT   416
2-K2-v1     ACCTCGCTGGCGCATGCTCAGGAAAATCAGTGCCGTGCATCTCTTCTCGGCTAAGGCCTT   416
2-K1-v2     ACCTCGCTGGCGCATGCTCAGGAAAATCAGTGCCGTGCATCTCTTCTCGGCTAAGGCCTT   417
2-K3-v3     ACCTCGCTGGCGCATGCTCAGGAAAATCAGTGCCGTGCATCTCTTCTCGGCTAAGGCCTT   293
158-K6      ACCTCGCTGGCGCATGCTCAGGAAAATCAGTGCCGTGCATCTCTTCTCGGCTAAGGCCTT   416
284-K3      ACCTCGCTGGCGCATGCTCAGGAAAATCAGTGCCGTGCATCTCTTCTCGGCTAAGGCCTT   419

WT-K4       GGATGATTTCCGCCATGTTAGACAGGAAGAAGTGGCAATCCTTGTACGGTCTCTAGTAAG   476
2-K2-v1     GGATGATTTCCGCCATGTTAGACAGGAAGAAGTGGCAATCCTTGTACGGTCTCTAGTAAG   476
2-K1-v2     GGATGATTTCCGCCATGTTAGACAGGAAGAAGTGGCAATCCTTGTACGGTCTCTAGTAAG   477
2-K3-v3     GGATGATTTCCGCCATGTTAGACAGGAAGAAGTGGCAATCCTTGTACGGTCTCTAGTAAG   353
158-K6      GGATGATTTCCGCCATGTTAGACAGGAAGAAGTGGCAATCCTTGTACGGTCTCTAGTAAG   476
284-K3      GGATGATTTCCGCCATGTTAGACAGGAAGAAGTGGCAATCCTTGTACGGTCTCTAGTAAG   479

WT-K4       TTCCGGGCACGAAAGAGCGGTCAATTTAGGGCATCTGGTGAACCTGTGCGCCACAAATGC   536
2-K2-v1     TTCCGGGCACGAAAGAGCGGTCAATTTAGGGCATCTGGTGAACCTGTGCGCCACAAATGC   536
2-K1-v2     TTCCGGGCACGAAAGAGCGGTCAATTTAGGGCATCTGGTGAACCTGTGCGCCACAAATGC   537
2-K3-v3     TTCCGGGCACGAAAGAGCGGTCAATTTAGGGCATCTGGTGAACTTGTGCGCCACAAATGC   413
158-K6      TTCCGGGCACGAAAGAGCGGTCAATTTAGGGCATCTGGTGAACCTGTGCGCCACAAATGC   536
284-K3      TTCCGGGCACGAAAGAGCGGTCAATTTAGGGCATCTGGTGAACCTGTGCGCCACAAATGC   539
```

```
WT-K4       ACTGGCACGCGTAATGATTGGCAGAAGAGTATTCAGCGACAGCGGTGATCCGAAGGCCGA   596
2-K2-v1     ACTGGCACGCGTAATGATTGGCAGAAGAGTATTCAGCGACAGCGGTGATCCGAAGGCCGA   596
2-K1-v2     ACTGGCACGCGTAATGATTGGCAGAAGAGTATTCAGCGACAGCGGTGATCCGAAGGCCGA   597
2-K3-v3     ACTGGCACGCGTAATGATTGGCAGAAGAGTATTCAGCGACAGCGGTGATCCGAAGGCCGA   473
158-K6      ACTGGCACGCGTAATGATTGGCAGAAGAGTATTCAGCGACAGCGGTGATCCGAAGGCCGA   596
284-K3      ACTGGCACGCGTAATGATTGGCAGAAGAGTATTCAGCGACAGCGGTGATCCGAAGGCCGA   599

WT-K4       CGAGTTCAAGTCAATGGTGGTGGAACTGATGAGACTTGCCGGAGTATTCAATATAGGGGA   656
2-K2-v1     CGAGTTCAAGTCAATGGTGGTGGAACTGATGAGACTTGCCGGAGTATTCAATATAGGGGA   656
2-K1-v2     CGAGTTCAAGTCAATGGTGGTGGAACTGATGAGACTTGCCGGAGTATTCAATATAGGGGA   657
2-K3-v3     CGAGTTCAAGTCAATGGTGGTGGAACTGATGAGACTTGCCGGAGTATTCAATATAGGGGA   533
158-K6      CGAGTTCAAGTCAATGGTGGTGGAACTGATGAGACTTGCCGGAGTATTCAATATAGGGGA   656
284-K3      CGAGTTCAAGTCAATGGTGGTGGAACTGATGAGACTTGCCGGAGTATTCAATATAGGGGA   659

WT-K4       TTTTATTCCGGCACTGGAGTGGCTGGATTTACAGCGAGTAGCAGCTAAAATGAAGAAACT   716
2-K2-v1     TTTTATTCCGGCACTGGAGTGGCTGGATTTACAGCGAGTAGCAGCTAAAATGAAGAAACT   716
2-K1-v2     TTTTATTCCGGCACTGGAGTGGCTGGATTTACAGCGAGTAGCAGCTAAAATGAAGAAACT   717
2-K3-v3     TTTTATTCCGGCACTGGAGTGGCTGGATTTACAGCGAGTAGCAGCTAAAATGAAGAAACT   593
158-K6      TTTTATTCCGGCACTGGAGTGGCTGGATTTACAGCGAGTAGCAGCTAAAATGAAGAAACT   716
284-K3      TTTTATTCCGGCACTGGAGTGGCTGGATTTACAGCGAGTAGCAGCTAAGATGAAGAAACT   719

WT-K4       CCATAAGAGATTCGATGCGTTTTTGACTGAAATCGTCGAGGAACACAAGAGTAACAAAGG   776
2-K2-v1     CCATAAGAGATTCGATGCGTTTTTGACTGAAATCGTCGAGGAACACAAGAGTAACAAAGG   776
2-K1-v2     CCATAAGAGATTCGATGCGTTTTTGACTGAAATCGTCGAGGAACACAAGAGTAACAAAGG   777
2-K3-v3     CCATAAGAGATTCGATGCGTTTTTGACTGAAATCGTCGAGGAACACAAGAGTAACAAAGG   653
158-K6      CCATAAGAGATTCGATGCGTTTTTGACTGAAATCGTCGAGGAACACAAGAGTAACAAAGG   776
284-K3      CCATAAGAGATTCGATGCGTTTTTGACTGAAATCGTCGAGGAACACAAGAGTAACAGAGG   779

WT-K4       AGAGTCAACTCACAGAGACATGTTGACTACTTTAATCTCGTTAAAGGAGGAAGAAGCTGA   836
2-K2-v1     AGAGTCAACTCACAGAGACATGTTGACTACTTTAATCTCGTTAAAGGAGGAAGAAGCTGA   836
2-K1-v2     AGAGTCAACTCACAGAGACATGTTGACTACTTTAATCTCGTTAAAGGAGGAAGAAGCTGA   837
2-K3-v3     AGAGTCAACTCACAGAGACATGTTGACTACTTTAATCTCGTTAAAGGAGGAAGAAGCTGA   713
158-K6      AGAGTCAACTCACAGAGACATGTTGACTACTTTAATCTCGTTAAAGGAGGAAGAAGCTGA   836
284-K3      AGAGTCAACTCACAGAGACATGTTGACTACTTTAATCTCGTTAAAGGAGGAAGAAGCTGA   839

WT-K4       TGACGGTGAGGGAGGGAAAATCACTGACACCGAAATTAAAGCCCTGCTTCTGAACATGTT   896
2-K2-v1     TGACGGTGAGGGAGGGAAAATCACTGACACCGAAATTAAAGCCCTGCTTCTGAACATGTT   896
2-K1-v2     TGACGGTGAGGGAGGGAAAATCACTGACACCGAAATTAAAGCCCTGCTTCTGAACATGTT   897
2-K3-v3     TGACGGTGAGGGAGGGAAAATCACTGACACCGAAATTAAAGCCCTGCTTCTGAACATGTT   773
158-K6      TGACGGTGAGGGAGGGAAAATCACTGACACCGAAATTAAAGCCCTGCTTCTGAACATGTT   896
284-K3      TGACGGTGAGGGAGGGAAAATCACTGACACCGAAATTAAAGCCCTGCTTCTGAACATGTT   899

WT-K4       TGCAGCAGGCACCGACACTACATCAAGCACGGTTGAGTGGGCCATTGCTGAGCTCATCAG   956
2-K2-v1     TGCAGCAGGCACCGACACTACATCCAGCACAGTTGAGTGGGCCATTGCTGAGCTCATCAG   956
2-K1-v2     TGCAGCAGGCACCGACACTACATCCAGCACAGTTGAGTGGGCCATTGCTGAGCTCATCAG   957
2-K3-v3     TGCAGCAGGCACCGACACTACATCAAGCACGGTTGAGTGGGCCATTGCTGAGCTCATCAG   833
158-K6      TGCAGCAGGCACCGACACTACATCAAGCACGGTTGAGTGGGCCATTGCTGAGCTCATCAG   956
284-K3      TGCAGCAGGCACCGACACTACATCAAGCACGGTTGAGTGGGCCATTGCTGAGCTCATCAG   959

WT-K4       GCACCCCAAAATACTAACCAAACTCCGGCGAGAACTCGACTCCGTCGTCGGCGCCGATTG   1016
2-K2-v1     GCACCCCAAAATACTAACCAAACTCCGGCGAGAACTCGACTCCGTCGTCGGCGCCGATTG   1016
2-K1-v2     GCACCCCAAAATACTAACCAAACTCCGGCAAGAACTCGACTCCGTCGTCGGCGCCGATTG   1017
2-K3-v3     GCACCCCAAAATACTAACCAAACTCCGGCAAGAACTCGACTCCGTCGTCGGCGCCGATTG   893
158-K6      GCACCCCAAAATACTAACCAAACTCCGGCAAGAACTCGACTCCGTCGTCGGCGCCGATTG   1016
284-K3      GCACCCCAAAATACTAACCAAACTCCGGCAAGAACTCGACTCCGTCGTCGGCGCCGATTG   1019

WT-K4       TCTCGTAACCGAGCTAGACATCACTCAACTCCCCTACCTCCAAGCCGTCGTCAAAGAAAC   1076
2-K2-v1     TCTCGTAACCGAGCTAGACATCACTCAACTCCCCTACCTCCAAGCCGTCGTCAAAGAAAC   1076
2-K1-v2     TCTCGTAACCGAGCTAGACATCACTCAACTCCCCTACCTCCAAGCCGTCGTCAAAGAAAC   1077
2-K3-v3     TCTCGTAACCGAGCTAGACATCACTCAACTCCCCTACCTCCAAGCCGTCGTCAAAGAAAC   953
158-K6      TCTCGTAACCGAGCTAGACATCACTCAACTCCCCTACCTCCAAGCCGTCGTCAAAGAAAC   1076
284-K3      TCTCGTAACCGAGCTAGACATCACTCAACTCCCCTACCTCCAAGCCGTCGTCAAAGAAAC   1079
```

```
WT-K4       CTTCCGCCTCCACCCATCAACTCCCCTCTCTCTCCCTCGAATGGCGGCCGAAAGCTGCGA   1136
2-K2-v1     CTTCCGCCTCCACCCATCAACTCCCCTCTCTCTCCCTCGAATGGCGGCCGAAAGCTGCGA   1136
2-K1-v2     CTTCCGCCTCCACCCATCAACTCCCCTCTCTCTCCCTCGAATGGCGGCCGAAAGCTGCGA   1137
2-K3-v3     CTTCCGCCTCCACCCATCAACTCCCCTCTCTCTCCCTCGAATGGCGGCCGAAAGCTGCGA   1013
158-K6      CTTCCGCCTCCACCCATCAACTCCCCTCTCTCTCCCTCGAATGGCGGCCGAAAGCTGCGA   1136
284-K3      CTTCCGCCTCCACCCATCAACTCCCCTCTCTCTCCCTCGAATGGCGGCCGAAAGCTGCGA   1139

WT-K4       AATCAACGGCTACCACATCCCAAAAGGCGCCACGCTTCTGGTCAACGTGTGGGCAATAGC   1196
2-K2-v1     AATCAACGGCTACCACATCCCAAAAGGCGCCACGCTTCTGGTCAACGTGTGGGCAATAGC   1196
2-K1-v2     AATCAACGGCTACCACATCCCAAAAGGCGCCACGCTTCTGGTCAACGTGTGGGCAATAGC   1197
2-K3-v3     AATCAACGGCTACCACATCCCAAAAGGCGCCACGCTTCTGGTCAACGTGTGGGCAATAGC   1073
158-K6      AATCAACGGCTACCACATCCCAAAAGGCGCCACGCTTCTGGTCAACGTGTGGGCAATAGC   1196
284-K3      AATCAACGGCTACCACATCCCAAAAGGCGCCACGCTTCTGGTCAACGTGTGGGCAATAGC   1199

WT-K4       TCGCGATCCAGAAGTATGGAAAGAGCCGCTGGAGTTTCGACCGGAGAGGTTTCTCGCCGG   1256
2-K2-v1     TCGCGATCCAGAAGTATGGAAAGAGCCGCTGGAGTTTCGACCGGAGAGGTTTCTCGCCGG   1256
2-K1-v2     TCGCGATCCAGAAGTATGGAAAGAGCCGCTGGAGTTTCGACCGGAGAGGTTTCTCGCCGG   1257
2-K3-v3     TCGCGATCCAGAAGTATGGAAAGAGCCGCTGGAGTTTCGACCGGAGAGGTTTCTCGCCGG   1133
158-K6      TCGCGATCCAGAAGTATGGAAAGAGCCGCTGGAGTTTCGACCGGAGAGGTTTCTCGCCGG   1256
284-K3      TCGCGATCCAGAAGTATGGAAAGAGCCGCTGGAGTTTCGACCGGAGAGGTTTCTCGCCGG   1259

WT-K4       CGGAGAAAGGCCGAACGCCGACGTGAAAGGGACGGATTTTGAGGTGATTCCGTTTGGGGC   1316
2-K2-v1     CGGAGAAAGGCCGAACGCCGACGTGAAAGGGACGGATTTTGAGGTGATTCCGTTTGGGGC   1316
2-K1-v2     CGGAGAAAGGCCGAACGCCGACGTGAAAGGGACGGATTTTGAGGTGATTCCGTTTGGGGC   1317
2-K3-v3     CGGAGAAAGGCCGAACGCCGACGTGAAAGGGACGGATTTTGAGGTGATTCCGTTTGGGGC   1193
158-K6      CGGAGAAAGGCCGAACGCCGACGTGAAAGGGACGGATTTTGAGGTGATTCCGTTTGGGGC   1316
284-K3      CGGAGAAAGGCCGAACGCCGACGTGAAAGGGACGGATTTTGAGGTGATTCCGTTTGGGGC   1319

WT-K4       AGGGCGGAGAATTTGCGCAGGGATGAATTTAGGGTTAGTAATGGTTCATCTGCTTATTGC   1376
2-K2-v1     AGGGCGGAGAATTTGCGCAGGGATGAATTTAGGGTTAGTAATGGTTCATCTGCTTATTGC   1376
2-K1-v2     AGGGCGGAGAATTTGCGCAGGGATAAATTTAGGGTTAGTAATGGTTCATCTGCTTATTGC   1377
2-K3-v3     AGGGCGGAGAATTTGCGCAGGGATGAATTTAGGGTTAGTAATGGTTCATCTGCTTATTGC   1253
158-K6      AGGGCGGAGAATTTGCGCAGGGATGAATTTAGGGTTAGTAATGGTTCATCTGCTTATTGC   1376
284-K3      AGGGCGGAGAATTTGCGCAGGGATGAATTTAGGGTTAGTAATGGTTCATCTGCTTATTGC   1379

WT-K4       GAGTTTAGTACAAGGATTTGAATGGGAATTGGAAAGGGAAAAACCAGAGAAATTGAACAT   1436
2-K2-v1     GAGTTTAGTACAAGGATTTGAATGGGAATTGGAAAGGGAAAAACCAGAGAAATTGAACAT   1436
2-K1-v2     GAGTTTAGTACAAGGATTTGAATGGGAATTGGAAAGGGAAAAACCAGAGAAATTGAACAT   1437
2-K3-v3     GAGTTTAGTACAAGGATTTGAATGGGAATTGGAAAGGGAAAAACCAGAGAAATTGAACAT   1313
158-K6      GAGTTTAGTACAAGGATTTGAATGGGAATTGGAAAGGGAAAAACCAGAGAAATTGAACAT   1436
284-K3      GAGTTTAGTACAAGGATTTGAATGGGAATTGGAAAGGGAAAAACCAGAGAAATTGAACAT   1439

WT-K4       GGAGGAAGCTTATGGGCTGACCTTACAACGACTTGAGCCATTGATGGTGTACCCAAACCC   1496
2-K2-v1     GGAGGAAGCTTATGGGCTGACCTTACAACGACTTGAGCCATTGATGGTGTACCCAAACCC   1496
2-K1-v2     GGAGGAAGCTTATGGGCTGACCTTACAACGACTTGAGCCATTGATGGTGTACCCAAACCC   1497
2-K3-v3     GGAGGAAGCTTATGGGCTGACCTTACAACGACTTGAGCCATTGATGGTGTACCCAAACCC   1373
158-K6      GGAGGAAGCTTATGGGCTGACCTTACAACGACTTGAGCCATTGATGGTGTACCCAAACCC   1496
284-K3      GGAGGAAGCTTATGGGCTGACCTTACAACGACTTGAGCCATTGATGGTGTACCCAAACCC   1499

WT-K4       TAGGTTGTCGTCTCTAGTCTATGCTGCTCCTGTTTGA    1533
2-K2-v1     TAGGTTGTCGTCTCTAGTCTATGCTGCTCCTGTTTGA    1533
2-K1-v2     TAGGTTGTCGTCTCTAGTCTATGCTGCTCCTGTTTGA    1534
2-K3-v3     TAGGTTGTCGTCTCTAGTCTATGCTGCTCCTGTTTGA    1410
158-K6      TAGGTTGTCGTCTCTAGTCTATGCTGCTCCTGTTTGA    1533
284-K3      TAGGTTGTCGTCTCTAGTCTATGCTGCTCCTGTTTGA    1536
```

Suppl. Fig. S1 Multiple nucleotide sequence alignment of *F3'H*s from transgenic poinsettia and WT. Black highlight marks insertion of T in position 170 in 2-K1-v2

```
WT-K4       MLPLFAFTIFSAIFISFFF-FFFRRTSRPPLPPGPRPLPVIGNLPHLGPKPHQSIASLAR   59
2-K2-v1     MLPLFAFTIFSAIFISFFF-FFFRRTSRPPLPPGPRPLPVIGNLPHLGPKPHQSIASLAR   59
2-K1-v2     MLPLFAFTIFSAIFISFFF-FFFRRTSRPPLPPGPRPLPVIGNLPHLGPKPHQSIASFGS   59
2-K3-v3     MLPLFAFTIFSAIFISFFFFFFFRRTSRPPLPPGPRPP----------------------   38
158-K6      MLPLFAFTIFSAIFISFFF-FFFRRTSRPPLPPGPRPLPVIGNLPHLGPKPHQSIASLAR   59
284-K3      MLPLFAFTIFSAIFISFFFFFFFRRTSRPPLPPGPRPLPVIGNLPHLGPKPHQSIASLAR   60

WT-K4       VYGPLMHLRMGFVDVVVAASASVAAQFLKAHDANFSSRPPNSGAKYVAYNYQDLVFAPYG   119
2-K2-v1     VYGPLMHLRMGFVDVVVAASASVAAQFLKAHDANFSSRPPNSGAKYVAYNYQDLVFAPYG   119
2-K1-v2     GLWPPYAPPYGLCRRRCGGVGVRCCPVLESS------------------------------   90
2-K3-v3     --------------------ASVAAQFLKAHDANFSSRPPNSGAKYVAYNYQDLVFAPYG   78
158-K6      VYGPLMHLRMGFVDVVVAASASVAAQFLKAHDANFSSRPPNSGAKYVAYNYQDLVFAPYG   119
284-K3      VYGPLMHLRIGFVDVVVAASASVAAQFLKAHDANFSSRPPNSGAKYVAYNYQDLVFAPYG   120

WT-K4       PRWRMLRKISAVHLFSAKALDDFRHVRQEEVAILVRSLVSSGHERAVNLGHLVNLCATNA   179
2-K2-v1     PRWRMLRKISAVHLFSAKALDDFRHVRQEEVAILVRSLVSSGHERAVNLGHLVNLCATNA   179
2-K1-v2     ------------------------------------------------------------   90
2-K3-v3     PRWRMLRKISAVHLFSAKALDDFRHVRQEEVAILVRSLVSSGHERAVNLGHLVNLCATNA   138
158-K6      PRWRMLRKISAVHLFSAKALDDFRHVRQEEVAILVRSLVSSGHERAVNLGHLVNLCATNA   179
284-K3      PRWRMLRKISAVHLFSAKALDDFRHVRQEEVAILVRSLVSSGHERAVNLGHLVNLCATNA   180

WT-K4       LARVMIGRRVFSDSGDPKADEFKSMVVELMRLAGVFNIGDFIPALEWLDLQRVAAKMKKL   239
2-K2-v1     LARVMIGRRVFSDSGDPKADEFKSMVVELMRLAGVFNIGDFIPALEWLDLQRVAAKMKKL   239
2-K1-v2     ------------------------------------------------------------   90
2-K3-v3     LARVMIGRRVFSDSGDPKADEFKSMVVELMRLAGVFNIGDFIPALEWLDLQRVAAKMKKL   198
158-K6      LARVMIGRRVFSDSGDPKADEFKSMVVELMRLAGVFNIGDFIPALEWLDLQRVAAKMKKL   239
284-K3      LARVMIGRRVFSDSGDPKADEFKSMVVELMRLAGVFNIGDFIPALEWLDLQRVAAKMKKL   240

WT-K4       HKRFDAFLTEIVEEHKSNKGESTHRDMLTTLISLKEEEADDGEGGKITDTEIKALLLNMF   299
2-K2-v1     HKRFDAFLTEIVEEHKSNKGESTHRDMLTTLISLKEEEADDGEGGKITDTEIKALLLNMF   299
2-K1-v2     ------------------------------------------------------------   90
2-K3-v3     HKRFDAFLTEIVEEHKSNKGESTHRDMLTTLISLKEEEADDGEGGKITDTEIKALLLNMF   258
158-K6      HKRFDAFLTEIVEEHKSNKGESTHRDMLTTLISLKEEEADDGEGGKITDTEIKALLLNMF   299
284-K3      HKRFDAFLTEIVEEHKSNRGESTHRDMLTTLISLKEEEADDGEGGKITDTEIKALLLNMF   300

WT-K4       AAGTDTTSSTVEWAIAELIRHPKILTKLRRELDSVVGADCLVTELDITQLPYLQAVVKET   359
2-K2-v1     AAGTDTTSSTVEWAIAELIRHPKILTKLRRELDSVVGADCLVTELDITQLPYLQAVVKET   359
2-K1-v2     ------------------------------------------------------------   90
2-K3-v3     AAGTDTTSSTVEWAIAELIRHPKILTKLRQELDSVVGADCLVTELDITQLPYLQAVVKET   318
158-K6      AAGTDTTSSTVEWAIAELIRHPKILTKLRQELDSVVGADCLVTELDITQLPYLQAVVKET   359
284-K3      AAGTDTTSSTVEWAIAELIRHPKILTKLRQELDSVVGADCLVTELDITQLPYLQAVVKET   360

WT-K4       FRLHPSTPLSLPRMAAESCEINGYHIPKGATLLVNVWAIARDPEVWKEPLEFRPERFLAG   419
2-K2-v1     FRLHPSTPLSLPRMAAESCEINGYHIPKGATLLVNVWAIARDPEVWKEPLEFRPERFLAG   419
2-K1-v2     ------------------------------------------------------------   90
2-K3-v3     FRLHPSTPLSLPRMAAESCEINGYHIPKGATLLVNVWAIARDPEVWKEPLEFRPERFLAG   378
158-K6      FRLHPSTPLSLPRMAAESCEINGYHIPKGATLLVNVWAIARDPEVWKEPLEFRPERFLAG   419
284-K3      FRLHPSTPLSLPRMAAESCEINGYHIPKGATLLVNVWAIARDPEVWKEPLEFRPERFLAG   420

WT-K4       GERPNADVKGTDFEVIPFGAGRRICAGMNLGLVMVHLLIASLVQGFEWELEREKPEKLNM   479
2-K2-v1     GERPNADVKGTDFEVIPFGAGRRICAGMNLGLVMVHLLIASLVQGFEWELEREKPEKLNM   479
2-K1-v2     ------------------------------------------------------------   90
2-K3-v3     GERPNADVKGTDFEVIPFGAGRRICAGMNLGLVMVHLLIASLVQGFEWELEREKPEKLNM   438
158-K6      GERPNADVKGTDFEVIPFGAGRRICAGMNLGLVMVHLLIASLVQGFEWELEREKPEKLNM   479
284-K3      GERPNADVKGTDFEVIPFGAGRRICAGMNLGLVMVHLLIASLVQGFEWELEREKPEKLNM   480

WT-K4       EEAYGLTLQRLEPLMVYPNPRLSSLVYAAPV  510
2-K2-v1     EEAYGLTLQRLEPLMVYPNPRLSSLVYAAPV  510
2-K1-v2     --------------------------------  90
2-K3-v3     EEAYGLTLQRLEPLMVYPNPRLSSLVYAAPV  469
158-K6      EEAYGLTLQRLEPLMVYPNPRLSSLVYAAPV  510
284-K3      EEAYGLTLQRLEPLMVYPNPRLSSLVYAAPV  511
```

Suppl. Fig. S2 Multiple amino acid sequences alignment of F3'H from transgenic poinsettia and WT

2.4 Publication 4

Molecular breeding for blue poinsettia

D. Nitarska, R. Boehm, T. Debener, H. Halbwirth

Publication 4

Molecular breeding for blue poinsettia

Daria Nitarska[1], Robert Boehm[2], Thomas Debener[3], and Heidi Halbwirth[1*]

[1]Institute of Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, 1060, Vienna, Austria

[2]Klemm + Sohn GmbH & Co., 70379, Stuttgart, Germany

[3]Institute of Plant Genetics, Leibniz Universität Hannover, 30419, Hannover, Germany

[*]corresponding author, Tel. (43) 1 58801166559, Fax. (43) 1 5880117399, heidrun.halbwirth@tuwien.ac.at

Key words

Poinsettia (*Euphorbia pulcherrima*), *Agrobacterium*-mediated transformation flavonoid 3'5'-hydroxylase, delphinidin, blue bracts colour

Abstract

Poinsettia (*Euphorbia pulcherrima*) is well known for its intense red bracts, which accumulate prevalently cyanidin-type anthocyanins. Despite numerous attempts, classical breeding approaches never resulted in creation of blue flowering poinsettia. Due to lack of flavonoid 3'5'-hydroxylase (F3'5'H) activity, poinsettia is not able to form blue, delphinidin-type pigments. This obstacle could be overcome by molecular breeding techniques. During this work, the possibility of introducing flavonoid 3'5'-hydroxylase activity into poinsettia flavonoid 3'-hydroxylase was evaluated as a promising approach for breeding blue poinsettia with application of genome editing. Introduced mutations did not result in the creation of an enzyme that was able to perform 3' and 5' hydroxylation. Therefore, *Cyclamen persicum F3'5'H* was introduced into the poinsettia cultivar 'Premium Red' with *Agrobacterium*-mediated transformation in a classical transgenic approach. As a result, a transgenic plant was obtained that expressed the transgene and accumulated significantly higher amounts of delphinidin. Nevertheless, a shift in bract colour towards blue was not observed, due to insufficient amounts of delphinidin in comparison to other anthocyanin types. This work is a first step in breeding blue poinsettia with a biotechnological approach.

Introduction

Poinsettia is one of the most economically important potted ornamental plants, mainly associated with the Christmas period. The most popular are red poinsettias, but also white, pink or marble varieties are in big demand (Taylor et al. 2011). One of the bract colour options that poinsettia is missing is blue. Similar to roses, carnations and chrysanthemum, poinsettia lacks the ability to accumulate delphinidin-type anthocyanins, pigments that can mediate blue flower colouration (Sasaki and Nakayama 2015). In general, blue flowers are often regarded as being more rare and exquisite. The principle market interest for blue poinsettia is convincingly demonstrated by the success of artificially stained blue plants available on the US market (Barrett 2005).

Anthocyanins are one of the main classes of secondary metabolites responsible for flower colours. Their biosynthesis has been extensively investigated in many plant species. The important factor determining flower colour is the B-ring hydroxylation pattern of the dihydroflavonols, which are intermediates in anthocyanidin biosynthesis. The two main enzymes that are responsible for the addition of the second and third hydroxyl group(s) on the B-ring are flavonoid 3'-hydroxylase (F3'H) and flavonoid 3'5'-hydroxylase (F3'5'H) (Tanaka 2006). F3'H introduces a second hydroxyl group in position 3', which leads to formation of cyanidin-type pigments and thereby to red or pink hues. F3'5'H introduces two hydroxyl groups in position 3' and 5', which results in the formation of delphinidin-type pigments that have violet to blue colours (Fig. 1). Both enzymes belong to the cytochrome P450 dependent monooxygenases and are membrane bound proteins. F3'H and F3'5'H are closely related proteins and the latter probably evolved from the former as an evolutionary adaptation to insect pollinators (Seitz et al. 2015). According to Seitz et al. (2007), the protein sequence at the C-terminus, particularly substrate recognition site 6 (SRS6) (Gotoh 1992), determines whether the enzyme is able to perform only 3' or 3' and 5'-hydroxylation. Even a single amino acid mutation in this region in the gerbera F3'H sequence was reported to introduce partial F3'5'H activity.

In poinsettia, the prevalent anthocyanin pigments are cyanidin-based, which are responsible for the red colour of the bracts (Asen 1958). Only traces of delphinidin were found in poinsettia bracts (Slatnar et al. 2013). In principle, this points at a lack of F3'5'H activity in poinsettia, and it remains unclear how the delphinidin traces are formed. Consequently, biotechnological breeding for blue flower colour in poinsettia would involve the introduction of heterologous active F3'5'H, in order to obtain precursors for delphinidin synthesis (Yoshida et al. 2009). An

interesting alternative could be introduction of F3'5'H activity into poinsettia F3'H, by the substitution of a single amino acid, as previously proposed (Seitz et al. 2007). This would be particularly attractive, if this modification could be achieved by a genome editing approach like CRISPR/Cas9.

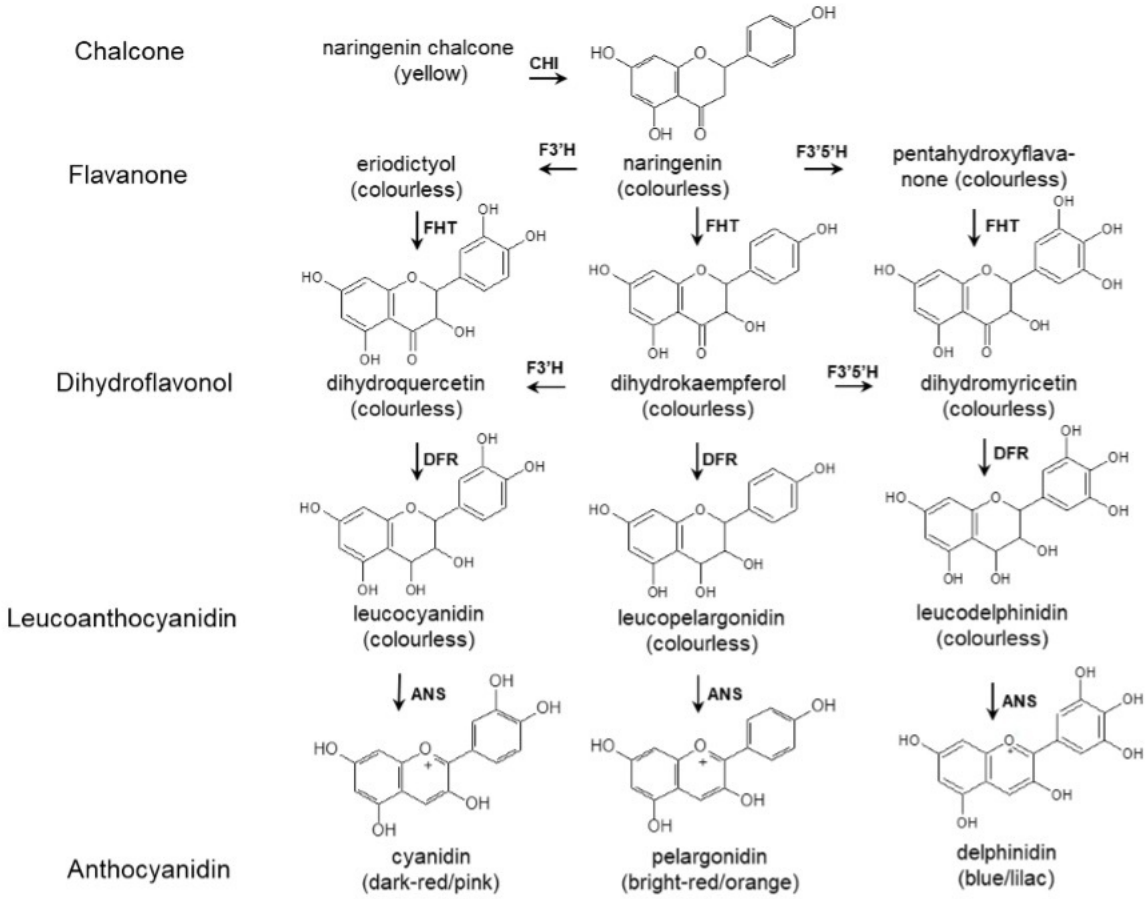


Fig. 1 Simplified overview of the anthocyanin synthesis pathway. Abbrev: ANS: anthocyanidin synthase, CHI: chalcone isomerase, CHS: chalcone synthase, DFR: dihydroflavonol 4-reductase, FHT: flavanone 3-hydroxylase, F3′H: flavonoid 3′-hydroxylase, F3′5′H: flavonoid 3′,5′-hydroxylase

In this study, we investigated the possibility of breeding for blue poinsettia. We first focused on the strategy suggested by Seitz et al. (2007), as a promising opportunity for genome editing. Thereafter, we performed *Agrobacterium*–mediated transformation of poinsettia with *Cyclamen persicum F3'5'H* in order to obtain plants that accumulate delphinidin in the bracts. This work is a first step in the breeding of blue poinsettia with application of biotechnological approach.

Material and Methods

Chemicals

(2-^{14}C)-Malonyl-coenzyme A (55 mCi/mmol) was purchased from New England Nuclear Corp. GmbH (Vienna, Austria). Synthesis of radiolabelled substrates was performed as described (Halbwirth et al. 2006). Reference substances (cyanidin, pelargonidin, delphinidin, malvidin, peonidin, petunidin) were purchased from Extrasynthese (Genay, France).

Cloning

The *F3'H* gene of poinsettia (*Euphorbia pulcherrima*) (NCBI accession number KY273440.1), apple (*Malus domestica*) (NCBI accession number FJ919633.1) and *Arabidopsis thaliana* (NCBI accession number AF271651.1) were cloned into the pYSG IBA103 (IBA, Germany). Additionally, *F3'H*s from poinsettia and apple were cloned into pYES2.1/V5-His-TOPO (Invitrogen, US) vector according to the manufacturer's protocol. Site-directed mutagenesis was performed on the vectors harbouring the different cDNA clones.

Table 1. Primer list.

Name	***Primer sequence 5'-3'***	***Temp.***	***Purpose***
EpF3'HpYes-F	ATGTTACCACTCTTTGCGTTTACC	61 °C	Cloning
EpF3'HpYes-R	TCAAACAGGAGCAGCATAGACTA		
EpF3'HpYSG-F	AGCGGCTCTTCAATGTTACCACTCTTTGCGTTTAC*C	59 °C	Cloning
EpF3'HpYSG-R	AGCGGCTCTTCTCCCAACAGGAGCAGCATAGACTA*G		
AtF3'HpYSG-F	AGCGGCTCTTCAATGGCAACTCTATTTCTCACAATC*C	59 °C	Cloning
AtF3'HpYSG-R	AGCGCTCTTCTCCCACCCGACCCGAGTCCATA*A		
MdF3'HpYes-F	ATGTTTGTTCTCATATTCTTCACCGTTG	61 °C	Cloning
MdF3'HpYes-R	TCAAGGTGATGACGCATTATATGC		
MdF3'HpYSG-F	AGCGGCTCTTCAATGTTTGTTCTCATATTCTTCACC*G	58 °C	Cloning
MdF3'HpYSG-R	AGCGGCTCTTCTCCCAGGTGATGACGCATTATATG*C		
EpF3'H_486A-F	TTATGGGCTGGCCTTACAACG	62 °C	Mutagenesis
EpF3'H_486A-R	GCTTCCTCCATGTTCAATTTC		
EpF3'H_486S-F	TATGGGCTGAGCTTACAACGAC	63 °C	Mutagenesis
EpF3'H_486S-R	AGCTTCCTCCATGTTCAATTTC		
EpF3'H_ost1-F	GTGCCCCCAAGGCCGAGGTTAGCCCCCCCCACATATATAGGGAGCGCTTGGAGCCAC	69 °C	Mutagenesis

The list is continued on the next page

EpF3'H_ost1-R	CATCAAGGGCTCAGCCTTTTGAA CGCTTATCCCAAACTCTTCCTCC ATGTTCAATTTCTCTGGTTTTTCC		
EpF3'H_ost2-F	GATTGGGAACTGGCTAATGGGT TAGACCCCGAGAGACTCAACAT GGAGGAAGAGTTTGGGATAAG	60 °C	Mutagenesis
EpF3'H_ost2-R	AAAGGCTTGAATCAATGTTGCA ATGAGCAAATGGACCATTTTCA ACCCTAAATTCATCCCTGC		
AtF3'H_488A-F	TTATGGGCTTGCACTGCAAAG	59 °C	Mutagenesis
AtF3'H_488A-F	CTCTCCTCCATATTCAGC		
AtF3'H_488S-F	TATGGGCTTAGTCTGCAAAGAG CG	58 °C	Mutagenesis
AtF3'H_488S-R	ACTCTCCTCCATATTCAGC		
AtF3'H_Ost1-F	GTGCCCCCAAGGCCGAGGTTAG CCCCCCCCCACATATATAGGGAG CGCTTGGAGCCAC	68 °C	Mutagenesis
AtF3'H_Ost1-R	CATCAAGGGCTCAGCCTTTTGAA CGCTTATCCCAAACTCCTCCTCC ATATTCAGCTTCTCCG		
AtF3'H_Ost2-F	ACCTTTGACTGGGAATTGGCTAA CGGGGTACTACCGGAGAAGCTG AATATG	59 °C	Mutagenesis
AtF3'H_Ost2-R	TTGGACTAGCGTAGCGACAAGC AACTGGACCATACGTAACCCTA AACTTAAAC		
MdF3'H_486A-F	TTATGGGCTCGCACTACAAAG	61 °C	Mutagenesis
MdF3'H_486A-R	GCCTCGTCCATGTTCAATTTC		
MdF'3H_486S-F	TTATGGGCTCTCACTACAAAGA G	61 °C	Mutagenesis
MdF'3H_486S-R	GCCTCGTCCATGTTCAATTTC		
MdF3'HII_Ost1-F	ATGGTGCCCCCAAGGCCGAGGT TAGCCCCCCCCCACATATATAGG GAGCGCTTGGAGCCAC	69 °C	Mutagenesis
MdF3'HII_Ost1-F	CAAGGGCTCAGCCTTTTGAACG CTTATCCCAAACTCTTCTTCCAT GTTCAATTTCTCAGGTGTGAGCC		
MdF3'HII_Ost2-F	GATTGGGAACTGGCTAATGGGT TAGACCCCGAGAGACTCAACAT GGAAGAAGAGTTTGGGATAAGC GTTCAAAAG	69 °C	Mutagenesis
MdF3'HII_Ost2-R	AAAGGCTTGAATCAATGTTGCA ATGAGCAAATGGACCATTTTCA ACCCAAGGGTCATCCCGGC		
CpF3'5'H-F	ATGGCACTAGACATGGTC	58 °C	Cloning
CpF3'5'H-R	TTAAGCAACATAAGCACTTGGG		
M13-F	GTAAAACGACGGCCAGT	55 °C	Cloning
M13-R	CAGGAAACAGCTATGAC		

The list is continued on the next page

nptII-F	ACAAGATGGATTGCACGCAGG	60 °C	Screening
nptII-R	AACTCGTCAAGAAGGCGATAG		
CpF3'5'H600-F	GGAATCGAACGTGGGATGAA	62 °C	Screening
CpF3'5'H600-R	TCCAGCTCCAAACGGAATAAG		
CpF3'5'H500-F	ATGGCACTAGACATGGTC	62 °C	Screening
CpF3'5'H500-R	CATAGAGTATGCCAACATCTC		
EpActin-F	GCTCAGTCCAAGAGAGGTATTT	62 °C	Screening
EpActin-R	AGCCTGAATAGCGACATACATA G		
EpGAPDH-F	GAGACGATGTGGAGCTTGTT	62 °C	Screening
EpGAPDH-R	TTTCCCTCAGATTCTGCCTTTAT		
EpF3'Hf-F	ATGTTACCACTCTTTGCGTTTAC C	62 °C	Screening
EpF3'Hf-R	CTTTCAAGAACTGGGCAGCAAC		
qEpF3'H2-F	AATCAACGGCTACCACATCC	62 °C	qPCR – primer
qEpF3'H2-R	CAGCGGCTCTTTCCATACTT		efficiency 2,02
qEpActin2-F	CTGTTCCAGCCATCTCTCATT	62 °C	qPCR – primer
qEpActin2-R	AACCGCCACTCAGAACTATG		efficiency 2,06
qEpEF1a-F	AAGATGATTCCCACCAAGCCCA	62 °C	qPCR – primer
qEpEF1a-R	CACAGCAAAACGACCCAGAGGA		efficiency 2,01
qCpF3'5'H-F	TCTTCATCATCACCCACTTCTTT	62 °C	qPCR – primer
qCpF3'5'H-R	GAGGGTAGAGAGCCGATTAGT		efficiency 2.05

* indicates phosphorothioate nucleotide

Site-directed mutagenesis

Mutagenesis was performed with the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Austria). Mutagenesis primers were designed with the NEBase Changer™ v 1.25 provided at http://nebasechanger.neb.com (primer sequences listed in table 1). Success of mutation was confirmed with sequencing (Microsynth, Austria).

Heterologous expression in yeast

Plasmids carrying mutated *F3'H* clones were transferred into the yeast strain INVSc1 using the *Sc*. EasyComp Transformation Kit (Invitrogen, US) and heterologously expressed as described (Nitarska et al. 2018). Protein fractions were shock frozen in liquid nitrogen and stored at -80 °C until further analysis.

Enzyme Assay

F3'H enzyme assays were performed as described before (Nitarska et al. 2018).

Plant material

For the transformation of *E. pulcherrima,* the cultivar 'Premium Red' (Dümmen Orange GmbH, Germany) was used. Plants were cultivated in the greenhouse under long day conditions (16 h day/ 8 h night). Around 2 - 3 cm internode stem segments were harvested and used as a source of explants for transformation. Surface sterilization was performed by washing

excised stems for 10 minutes in 1,5 % solution of sodium hypochlorite with one drop of Tween 20, followed by washing two times for 10 minutes in sterile water. Then stems were cut in around 1-2 mm slices and placed on the CIM (Callus Induction Media: CIM – MS media (Murashige and Skoog 1962) supplemented with 0,2 mg/L 4-chlorophenoxy acetic acid (CPA) and 0,2 mg/L 6-Benzylaminopurine (BAP)) for 4-5 days before transformation.

Transgenic poinsettias were cultivated in the greenhouse under long day conditions. For induction of bract colouration, plants were grown under short day conditions (11h day, 13 h night) for 8 weeks. Leaves and bracts for nucleic acids extraction and pigment analysis were shock frozen in liquid nitrogen and stored at -80 °C.

_F3'5'H_ cloning into p9N35s vector

A *Cyclamen persicum F3'5'H* (NCBI accession number GQ891056.1) cDNA clone was first cloned into the entry vector pCR™8 TOPO® TA according to the manufacturer's protocol (Invitrogen, US). In the next step, the cDNA clone was transferred to the destination vector p9N-35s (DNA Cloning Service, Hamburg, Germany) by Gateway LR reaction (Thermo Fisher Scientific) according to the manufacturer's protocol with one modification. The LR reaction was modified due to the fact that the entry and acceptor vector had the same antibiotic resistance gene (spectinomycin). Instead of using the entry vector for the reaction, the gene of interest flanked by attL1 and attL2 was amplified with M13 primers and then around 150 ng of PCR product was used in LR reaction. Cloning success was confirmed by sequencing (Microsynth, Austria). The p9N-35s vector carrying cyclamen *F3'5'H* under the control of the constitutive CaMV 35s promoter was transferred into the *Agrobacterium tumefaciens* strain GV3101 and used for poinsettia transformation.

Poinsettia transformation

Agrobacterium strain GV3101 carrying p9N-35s vector was cultivated for 24 h in SOB medium (2 % w/v tryptone, 0.5 % w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) supplemented with rifampicin (50 mg/L), spectinomycin (100 mg/L) and gentamycin (30 mg/L). Subsequently, 50 mL of minimal A medium (10.5 g/L K_2HPO_4, 4.5 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 0.52 g/L $Na_3C_6H_5O_7 \times 2\ H_2O$, 0.1 % w/v glucose, 0. 005 % $MgSO_4 \times 7\ H_20$, 0.00025 % thiamine) was inoculated with 5 mL of this culture. When OD of the culture reached 0.5, it was used for poinsettia stem explant transformation. Explants were incubated directly in 10 mL of bacteria inoculum, with addition of three drops of Tween 20, for 30 minutes with gentle shaking, then dried on sterile paper and placed on CIM for two days of co-cultivation. As a control explants were incubated with 10 mL of Minimal A Medium and then placed on

the CIM media. Afterwards, explants were washed in sterile water with 250 mg/L cefotaxim and 150 mg/L timentin for 30 minutes, dried for a moment on sterile paper and placed on CIM supplemented with 250 mg/L cefotaxim and 150 mg/L timentin for callus induction. After 21 days on CIM, explants were transferred to SEIM – MS medium (with 0.2 mg/L 1-Naphthaleneacetic acid (NAA) and 0.1 mg/L isopentenyl adenine (2ip)) supplemented with 250 mg/L cefotaxim, 150 mg/L timentin and 2.5 mg/L kanamycin for 3 to 6 weeks for induction of embryo formation. When first green embryos appeared, explants were transferred to somatic embryo maturation medium (SEMM – MS medium with 0.05 mg/L BAP) supplemented with 250 mg/L cefotaxim, 150 mg/L timentina and 50 mg/L kanamycin for selection. Fully regenerated plants were transferred to the MS media, propagated and moved to the greenhouse for further cultivation.

Screening

Genomic DNA was extracted from leaves of regenerated poinsettia with InVisorb Plant mini kit (Stratec, Germany). The presence of the transgene was detected by PCR amplification of the *nptII* gene or cyclamen *F3'5'H* (Primer sequences Table 1). The screening was performed using Go *Taq* polymerase (Promega) in the following reaction mixture: 4 µL 5X Go*Taq* Green Reaction Buffer, 0.4 µL dNTPs, 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 2 µL DNA, 0.2 µL *Taq*. Reaction conditions were: 2 minutes 94 °C initial denaturation, 40 cycles (98 °C, 30 seconds denaturation, 60/62 °C, 30 seconds primer annealing, 72 °C, 45 seconds extension), 10 minutes 72 °C final extension. Positive plants were further cultivated in the greenhouse.

Total RNA was extracted with mirPremier Kit (Sigma Aldrich, Austria) in order to check the transgene expression. cDNA synthesis was performed with RevertAid H Minus Reverse Transcriptase (Thermo Scientific, US) according to the manufacturer's protocol. The expression was detected using PCR in the same condition as above. The integrity of DNA and cDNA was confirmed by amplification of poinsettia *actin*, *GAPDH* or *F3'H* fragment.

HPLC analysis

Poinsettia bracts pigment analysis was perform by HPLC as was described before (Haselmair-Gosch et al. 2018). For the extraction, 0.5 g of shock frozen poinsettia bracts was used in 1.5 mL of 2M HCl in methanol.

qPCR

To quantify gene expression, mRNA from poinsettia bracts was extracted using the µMACS mRNA isolation Kit (Miltenyi Biotec, Germany) and the cDNA was synthesized with RevertAid H Minus Reverse Transcriptase (Thermo Scientific, US). Expression of the transgene and poinsettia *F3'H* was measured on the StepOnePlus System (Applied Biosystems, Germany). Luna® Universal qPCR Master Mix (New England Biolabs) was used according to the manufacturer's protocol. Measurements were performed in triplicates and results were normalized to two housekeeping genes, *actin* and *translation elongation factor 1-alpha* (*EF1A*) (Zhang et al. 2013). The relative gene expression and primer efficiency was calculated according to Pfaffl (Pfaffl 2004). Product specificity was evaluated by analyzing melting curves.

Statistical analysis

The statistical analysis was performed in GraphPad Prims version 8.4.3 for Mac. Normality of data was evaluated with the Shapiro-Wilk test. Statistical significance was calculated using a double-tailed, unpaired *t*-test when the variances were equal or unpaired *t*-test with Welch's correction when variances were different. The Mann-Whitney test was applied for not normally distributed data.

Results

Introducing F3'5'H activity into the F3'H of poinsettia

The possibility of changing the activity of F3'H towards at least partial F3'5'H activity (Seitz et al. 2007) was tested with recombinant F3'Hs of poinsettia, *Arabidopsis thaliana* and apple (*Malus domestica*).

```
petunia        IVMVEYILGTLVHSFDWKLPSEV--IELNMEEAFGLALQKAVPLEAMVTPRLQLDVYVP--- 506
arabidopsis    LRTIQFLTATLVQGFDWELAGGVTPEKLNMEESYGLTLQRAVPLVVHPKPRLAPNVYGLGSG 513
malus          LRMVSLMIATLVHGFDWTLADGLTPEKLNMDEAYGLTLQRAAPLMVHPRNRLAPHAYNASSP 511
gerbera        LRMVQLLTATLIHAFDWELADGLNPKKLNMEEAYGLTLQRAAPLVVHPRPRLAPHVYETTKV 512
poinsettia     LVMVHLLIASLVQGFEWELER-EKPEKLNMEEAYGLTLQRLEPLMVYPNPRLSSLVYAAPV- 511
osteospermum   LKMVHLLIATLIQAFDWELANGLDPERLNMEEEFGISVQKAEPLMVHPRPRLAPHTYI---- 508
```

Fig. 2. Multiple alignment of *Arabidopsis thaliana* (AF271651.1), *Malus domestica* (FJ919633.1), *Gerbera hybrida* (ABA64468.1) and poinsettia (KY273440.1) F3'H amino acid sequence, with *Petunia* x *hybrida* (AY245545.1) and *Osteospermum hybrid* (ABB43031.1) F3'5'Hs. Position 8 of SRS6 is highlighted in black, sequence of *Osteospermum hybrid* F3'5'H that was exchanged with C-terminal end of F3'Hs is highlighted in grey.

A single nucleotide exchange in position 8 of substrate recognition site 6 (exchange T/A or T/S) (Fig. 2) did not change the functional activity of the F3'Hs from poinsettia (*Ep*F3'H_T486A, *Ep*F3'H_T486S, Table 2) and *Arabidopsis* kept their F3'H activity (*At*F3'H_T488A, *At*F3'H_T488S, Table 2). In none of the assays, formation of a 3'4'5'-

hydroxylated product (pentahydroxyflavanone (PHF) or dihydromyricetin (DHM)) could be observed (Table 2). The conversion rate for *Arabidopsis* F3'H mutants was on almost the same level as that of not mutated protein. Poinsettia F3'H mutants had conversion rates lower than not mutated protein, but still it was quite high (especially for DHK). The *Md*F3'H _T486S mutant was able to convert DHK to some extent, but not naringenin (Table 2), whereas the *Md*F3'H _T486A mutant was not active. The second approach suggested by Seitz et al. (2007) was an exchange of the C-terminus of F3'H with the C-terminus of an F3'5'H. For technical reasons, the exchange of these 58 amino acids was performed in two steps. In the first step, 26 amino acids were exchanged with the corresponding sequence of the *Osteospermum hybrida* F3'5'H (ost1 - EFGISVQKAEPLMVPPRPRLAPPTYI) at the C- terminus of the F3'H, and in the second step further 32 amino acids (ost2 – LKMVHLLIATLIQAFDWELANGLDPER-LNMEE) (Fig. 2) were exchanged. The resulting chimeric proteins were, however, not active. This revealed that also this approach is not suitable for introduction of F3'5'H activity to F3'H of the investigated species.

Table 2. Enzymatic activity of mutated recombinant enzymes.

	% conversion rate	
Name	**Naringenin/PHF**	**DHK/DHM**
EpF3'H (control)	83/ 0	96/ 0
EpF3'H_T486A	30/ 0	49 / 0
EpF3'H_T486S	48/ 0	70 / 0
EpF3'H_ost1	0/ 0	0/ 0
EpF3'H_ost2	0/ 0	0/ 0
AtF3'H (control)	88/ 0	85/ 0
AtF3'H_T486A	85/ 0	86/ 0
AtF3'H_T486S	63/ 0	83/ 0
AtF3'H_ost1	0/ 0	0/ 0
AtF3'H_ost2	0/ 0	0/0
MdF3'H_T486A	0/ 0	0/ 0
MdF3'H_T486S	0/ 0	12%/ 0
MdF3'H_ost1	0/ 0	0/ 0
MdF3'H_ost2	0/ 0	0/ 0

Poinsettia transformation

To obtain poinsettia plants, which accumulate delphinidin-type anthocyanins in their bracts, we decided to overexpress cyclamen *F3'5'H* (Cp*F3'5'H*). The cultivar 'Premium Red' was chosen for transformation, because of its lower cyanidin content, which indicates a potential

for reducing competition between the endogenous F3'H and the *Cp*F3'5'H to be introduced (Nitarska et al. 2018).

During the transformation process serious contamination problems occurred. Surface sterilisation of stems was not always sufficient, which resulted in the loss of many explants during the first few days after transformation. To eliminate contaminated explants before transformation, we placed them on the CIM medium for 4-5 days, but this also did not result in detection of all contaminated explants. Callus formation on the remaining explants was very efficient.

Table 3. Regeneration summary

Transformation event	No of explants	No of lost explants	No of embryo-genic explants	No of regenerated plants
A	168	149	14	18
B	95	22	20	25
C	221	174	2	6
D	124	55	16	22
E	280	24	30	25
Total	888	424	82	96

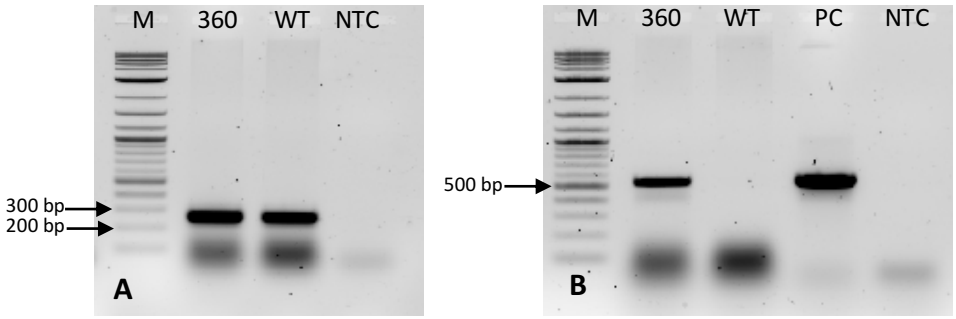


Fig 3. PCR analysis of the transgenic plants and WT control on gDNA level. A: amplification of *EpF3'H* fragment (primers EpF3'Hf-F and EpF3'Hf-R), B: amplification of Cp*F3'5'H* fragment (primers CpF3'5'H500-F and CpF3'5'H500-R). M: molecular size standard 1kb Plus DNA ladder (New England Biolabs, Austria), PC: positive control (plasmid), NTC: non template control. Selected fragments of ladder are labelled for better orientation

Unfortunately, embryos did not form on each explant. Only around 20 % of explants produced embryos, but while on each of those that did, usually 5-10 embryos were formed, most of them did not develop into plantlets. In total, 96 putatively transgenic plants were obtained from all the transformation events (Table 3). Only 9 of them showed positive signals for *nptII* and Cp*F3'5'H* in the first step of screening (Table 3, Fig. 3). During the transfer to the greenhouse six plants were lost and RNA could be extracted from only 3 of the remaining plants. Of these,

just one was positive (line E360) (Table 4, Fig. 4). The positive line was propagated and transferred to the greenhouse. In the first step 3 propagated plants from line E360 were obtained. After further propagation additional 12 plants were obtained and were used for subsequent analysis.

Table 4. Transformation summary

Transformation event	Total plants	Positive DNA	Negative DNA	Positive RNA	Negative RNA	Lost plants
A	18	0	18	0	0	0
B	25	3	22	0	0	3
C	6	0	6	0	0	0
D	22	2	20	0	1	1
E	25	4	21	1	1	2
Total	96	9	87	1	2	6

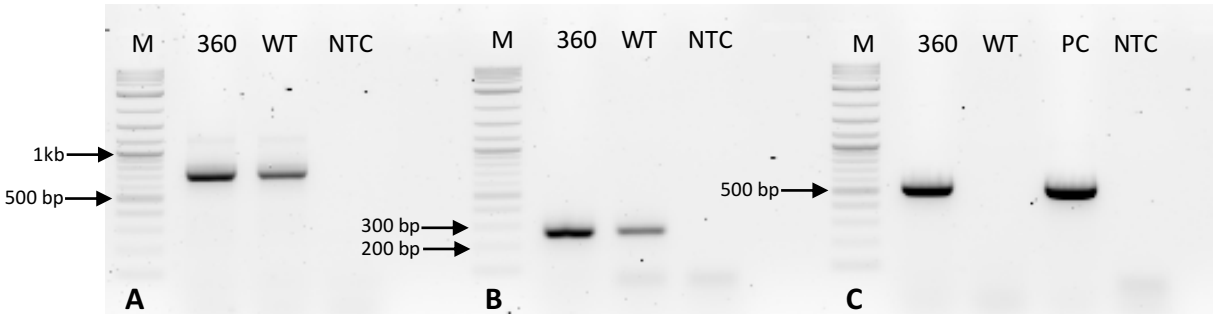


Fig 4. PCR analysis of the transgenic plants and WT on cDNA level. A: amplification of Ep*F3'H* fragment (primers EpF3'Hf-F and EpF3'Hf-R), B: amplification of Ep*GAPDH* fragment (primers EpGAPDH-F and EpGAPDH-R), C: amplification of Cp*F3'5'H* fragment (primers CpF3'5'H600-F and CpF3'5'H500-R). M: molecular size standard 1kb Plus DNA ladder (New England Biolabs, Austria), PC: positive control (plasmid), NTC: non template control. Selected fragments of ladder are labelled for better orientation

Pigment analysis

After 8 weeks of growing at short day conditions, bract colour was developed. The line expressing the transgene (E360) did, however, not differ in colour from the WT (Fig. 5). The total anthocyanin content in the transgenic line was slightly lower than in the WT (Fig. 6A). HPLC analysis of anthocyanins revealed that in line E360, the delphinidin level was significantly higher than that in the WT (Fig. 6B), but obviously too low for phenotypic changes of the bract colour. The cyanidin level in the transgenic line was lower in comparison to the WT (Fig. 6C), but around 10 times higher than delphinidin content in the transgenic line. The lower level of cyanidin suggests a strong competition between the endogenous F3'H and

the exogenous F3'5'H. The level of pelargonidin in the transgenic plant did not differ from the level in WT control (Fig. 6D).

Fig 5. Bracts colours of poinsettia cultivar 'Premium Red' WT (left) and transgenic line E360 (right)

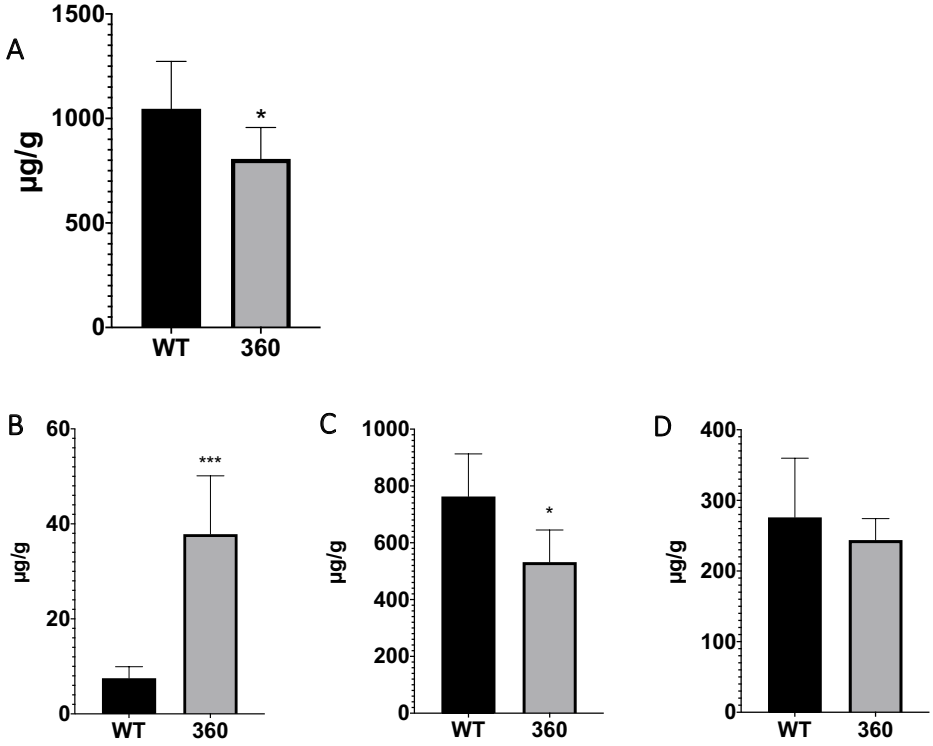


Fig 6. Anthocyanins in bracts of poinsettia, HPLC analysis. A: total anthocyanins content in transgenic line and WT control, B: delphinidin content, C: cyanidin content, D: pelargonidin content. Data were calculated from at least four biological repetitions and with error bars representing standard deviation. Statistical significance * p<0,05, ** p<0,01, ***p<0,001, ****p<0,0001

Gene expression

The expression levels of poinsettia *F3'H* and the overexpressed cyclamen *F3'5'H* was detected in the transgenic line by qPCR. The expression level of poinsettia *F3'H* was not significantly different between the WT and line E360 (Fig. 7A). Expression of introduced cyclamen *F3'5'H* was detected only in the transgenic plant. The expression level of cyclamen *F3'5'H* varied

among all propagated plants obtained from line E360 (Fig. 7B). Importantly, expression of the transgene was around 10 times lower than that of endogenous poinsettia *F3'H* (Fig. 7).

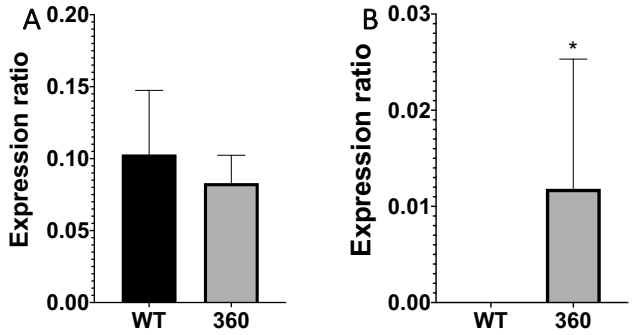


Fig 7. Gene expression of poinsettia *F3'H* (A) and cyclamen *F3'5'H* (B) in transgenic poinsettia and WT. Quantitative expression was normalized to *actin*. Data were calculated from at least four biological repetitions and with error bars representing standard deviation. Statistical significance * p<0,05, ** p<0,01, ***p<0,001, ****p<0,0001

Discussion

For people around the world, poinsettia is a mandatory element of Christmas decorations. Breeders try to satisfy consumers by providing cultivars with the best shape, most excellent colour and best stress and disease resistance. Colour is one of the most important traits of poinsettia. Nowadays we can buy poinsettia in almost all possible shades of red, pink, yellow or white, but blue colour is still missing among cultivars obtained by crossbreeding or radiation mutations (Taylor et al. 2011). The only "blue" poinsettias that are available on the market are obtained with application of a special dye or spray, applied to white poinsettia bracts. This demonstrates existing demand for blue poinsettia cultivars and that there is a customer group that would be highly interested in buying such novel colour option (Barrett 2005).

Poinsettia, similar to rose and carnation, does not accumulate delphinidin-type pigments, which are responsible for blue flower colouration due to lack of active F3'5'H (Tanaka et al. 2005). As it was showed by (Seitz et al. 2015) F3'5'H evolved from F3'H at least four times in dicotyledonous plants. Only in Asteraceae family it evolved independently three times in all main subfamilies (Seitz et al. 2015). In rice and *Medicago* it was showed that enzymes that are classified as CYP75B are able to perform hydroxylation in position 5' during synthesis of tricin (Lam et al. 2019; Lo 2020). Seitz at al. (2007) introduced F3'5'H activity into gerbera F3'H, by exchange of single amino acid in position 8 of SRS6 (Fig. 2). The exchange of threonine, which is characteristic for F3'Hs to alanine (typical for regular F3'5'Hs) or serine (typical for Asteraceae F3'5'Hs) allowed them to introduce F3'5'H activity in gerbera F3'H. The importance of position 8 in SRS6 was also showed in *Medicago* where all flavonoid B-ring

hydroxylases that belong to CYP75B family and can perform just 3' hydroxylation have threonine in this position while enzymes that can perform 3' and 5' hydroxylation have glycine (Lo 2020). We tried to introduce F3'5'H activity into poinsettia F3'H according to Seitz et al. (2007). Apple and *Arabidopsis thaliana* F3'Hs were included for a sufficiently broad evaluation. Exchange of a single amino acid in SRS6 was particularly interesting because, if successful, this could be a promising target for the application of genome editing in order to obtain blue poinsettia. So far, genome editing was successfully performed in a few ornamental plants, mostly as a tool for gene knockout by non-homologous end joining (NHEJ) (Zhang et al. 2016; Watanabe et al. 2017; Kishi-Kaboshi et al. 2017; Nishihara et al. 2018; Tasaki et al. 2019; Wang et al. 2017), but targeted change of gene sequence by homology-directed repair (HDR) was also successfully performed in rice and tomato (Wang et al. 2017; Čermák et al. 2015). Nevertheless, the exchange of threonine in position 8 SRS6 of poinsettia, apple and *Arabidopsis* F3'Hs for alanine or serine did not result in F3'5'H activity of the F3'Hs (Table 2). The three mutated proteins showed only F3'H activity. For *Arabidopsis* activity was on similar level like in the WT enzymes, for poinsettia it was a bit lower, only for apple it decreased significantly (Table 2). We also tried to exchange the C- terminus of the three investigated F3'Hs with *Osteospermum hybrida* F3'5'H (Fig. 2), but we obtained inactive chimeric proteins only (Table 2). This prompted us to change our strategy for the attempts at molecular breeding of blue flowering poinsettia.

The most promising possibility for creating blue poinsettia at this stage was the introduction of an active F3'5'H from another species by classical *Agrobacterium*-mediated transformation. By this approach, we expected to obtain poinsettia that accumulate delphinidin-type blue pigments. The first attempt to introduce petunia F3'5'H to poinsettia via *Agrobacterium*-mediated transformation was made by Sagvaag (2015)(Sagvaag 2015), but in the process of regeneration, no transgenic plants were obtained. This indicates that poinsettia transformation is a very challenging and convoluted process. So far, two successful poinsettia transformations have been reported (Clarke et al. 2008; Islam et al. 2013). The success of transformation seems to depend greatly on the cultivar used, due to different abilities for regeneration. In this study we chose cultivar 'Premium Red', because of its high pelargonidin/cyanidin ratio (Nitarska et al. 2018). We also considered cultivar 'Harvest Orange', which accumulates prevalently pelargonidin-type pigment (Nitarska et al. 2018), but due to its low regeneration rate (data not shown) we decided to use 'Premium Red'. An additional obstacle during poinsettia transformation is the high contamination rate due to insufficient surface sterilization of the stem

segments used as an explant source (Clarke et al. 2008). In total, during this work, we lost more than 50 % of transformed explants due to this problem. The same trouble was encountered by Clarke et al (2008) and Sagvaag (2015)(Clarke et al. 2008; Sagvaag 2015). During this study we tried to increase the percentage of NaClO, but it did not result in a decrease of contamination (data not shown). The number of embryogenic explants varied between experiments and depended on the number of explants that were not lost due to contamination, but on average reached 20 %, which is lower than for the cultivar 'Millennium' used by Clark et al. (2008). In total we managed to obtain 96 regenerated plants, of which nine gave a positive signal in the PCR screening (Table 3, Table 4). However, six plants were lost during cultivation and transfer to the greenhouse. Among the three surviving plants, just one showed transgene expression on an RNA level.

After transfer to the greenhouse and bract colour development, no change in flower colour was observed (Fig. 5). HPLC analysis revealed that delphinidin-type anthocyanins in line E360 were present at a significantly higher level than in the WT, but, in comparison to total cyanidin and pelargonidin contents, it was not enough for a discernible blue shift in bract colour (Fig. 6). In chrysanthemum, it was observed that a delphinidin content of around 15 % was enough to produce the desired blue shift in flower colour, and when the level reached at least 30 % the change was significant (Brugliera et al. 2013; Noda et al. 2013). The chrysanthemum cultivar used for transformation had rather pale pink petals, whereas 'Premium Red' bract colour is quite strong, albeit not of the same intensity compared with most popular poinsettia cultivars. This would suggest that delphinidin concentrations needed to produce discernibly blue bract colouration in 'Premium Red' are higher compared with what is needed in chrysanthemum. The expression level of Cp*F3'5'H* in the transgenic line was rather low and almost 10 times lower than that of endogenous poinsettia *F3'H* (Fig. 7). As was noticed in chrysanthemum, higher expression of exogenous *F3'5'H* is correlated with higher delphinidin content (Noda et al. 2017). In the future, we are planning to use line E360 for crossing with other cultivars that are identified as potentially having better properties for delphinidin production and that were not suitable for transformation. In particular, cultivars with low F3'H activity may prove promising, as was shown in chrysanthemum and roses, where silencing of endogenous *F3'H* had a positive impact on the delphinidin accumulation (Katsumoto et al. 2007; Brugliera et al. 2013). Other possibilities for achieving higher delphinidin concentrations could be the use of another source of F3'5'H. It was shown in roses that expression of petunia, gentian or butterfly pea *F3'5'H* didn't result in high delphinidin concentrations, whereas expression of pansy

F3'5'H gave much better results (Tanaka et al. 2009). During our attempts to obtain blue poinsettia, we tried to also use *Sollya heterophylla F3'5'H*, but no transgenic plants were obtained (data not shown). Another option could be the use of a promoter that is more gene specific than the constitutive CaMV 35s promoter, as was shown in chrysanthemum (Noda et al. 2013). Also, overexpression of some gene responsible for decoration of anthocyanins, together with *F3'5'H*, could prove conducive to the production of true blue bracts. Polyacetylalated and polyglucosylated anthocyanins have more intense blue colouration (Sasaki and Nakayama 2015). The first truly blue chrysanthemum was obtained by overexpression of canterbury bells *F3'5'H* and butterfly pea *A3'5'AT* (*UDP glucose:anthocyanin 3'5'-glucosyltransferase*), and it was shown that expression of *A3'5'AT* was necessary to obtain true blue colour, while plants that expressed only *F3'5'H* produced purple flowers (Noda et al. 2017).

The results presented here are a first step in breeding blue flowering poinsettia. We obtained a transgenic line that shows the expression of the transgene and accumulates delphinidin. However, it's cyanidin and pelargonidin contents are too high, so it is not sufficient to change the bracts' colour. In the next steps, we are planning to use the transgenic line in the crossing with other poinsettia cultivars to determine if the transgene can be inherited and used to produce progeny with increased delphinidin content. Another alternative would be to systematically test F3'5'H from different sources under the control of various promoters to determine the optimal combination. Also, co-expression of additional genes responsible for decoration is an interesting strategy to obtain truly blue poinsettia that could become a new symbol of Christmas.

Funding

This project gratefully received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 675657 Flower Power and the Austrian Science Fund (FWF) P32901–B. The funding bodies had no role in the design of the study, collection, analysis or interpretation of data or in writing the manuscript.

Acknowledgement

We would like to thank Christopher Schlosser for critical reading of the manuscript, Renate Paltram for technical support in HPLC and Jürgen Greiner for technical assistance.

Conflict of interest/Competing interests

The authors declare that they have no conflict of interest.

Author contributions

DN, RB, TD and HH conceived the research. DN and RB designed the experiments. DN performed the experiments. DN and HH wrote the manuscript. All authors read and approved the final manuscript.

Ethic approval

not applicable

Consent to participate

not applicable

Consent of publication

not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information file. Primary datasets are available from the corresponding author on reasonable request.

Code availability

not applicable

Refernces

Asen S (1958) Anthocyanins in Bracts of Euphorbia Pulcherrima as Revealed by Paper Chromatographic and Spectrophotometric Methods. Plant physiology 33 (1):14

Barrett J (2005) Consumer Poinsettia Preference. Lawn and Garden Retailer 4 (2):1-4

Brugliera F, Tao G-Q, Tems U, Kalc G, Mouradova E, Price K, Stevenson K, Nakamura N, Stacey I, Katsumoto Y (2013) Violet/blue chrysanthemums—metabolic engineering of the anthocyanin biosynthetic pathway results in novel petal colors. Plant and Cell Physiology 54 (10):1696-1710

Čermák T, Baltes NJ, Čegan R, Zhang Y, Voytas DF (2015) High-frequency, precise modification of the tomato genome. Genome biology 16 (1):232

Clarke JL, Spetz C, Haugslien S, Xing S, Dees MW, Moe R, Blystad D-R (2008) Agrobacterium tumefaciens-mediated transformation of poinsettia, Euphorbia pulcherrima, with virus-derived hairpin RNA constructs confers resistance to Poinsettia mosaic virus. Plant Cell Reports 27 (6):1027-1038. doi:10.1007/s00299-008-0526-9

Gotoh O (1992) Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. Journal of Biological Chemistry 267 (1):83-90

Halbwirth H, Kahl S, Jäger W, Reznicek G, Forkmann G, Stich K (2006) Synthesis of (14C)-labeled 5-deoxyflavonoids and their application in the study of dihydroflavonol/leucoanthocyanidin interconversion by dihydroflavonol 4-reductase. Plant Science 170 (3):587-595

Haselmair-Gosch C, Miosic S, Nitarska D, Roth BL, Walliser B, Paltram R, Lucaciu RC, Eidenberger L, Rattei T, Olbricht K (2018) Great cause—small effect: undeclared genetically engineered Orange Petunias Harbor an inefficient Dihydroflavonol 4-Reductase. Frontiers in plant science 9:149

Islam MA, Lütken H, Haugslien S, Blystad D-R, Torre S, Rolcik J, Rasmussen SK, Olsen JE, Clarke JL (2013) Overexpression of the AtSHI gene in poinsettia, Euphorbia pulcherrima, results in compact plants. PloS one 8 (1)

Katsumoto Y, Fukuchi-Mizutani M, Fukui Y, Brugliera F, Holton TA, Karan M, Nakamura N, Yonekura-Sakakibara K, Togami J, Pigeaire A (2007) Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. Plant and Cell Physiology 48 (11):1589-1600

Kishi-Kaboshi M, Aida R, Sasaki K (2017) Generation of gene-edited Chrysanthemum morifolium using multicopy transgenes as targets and markers. Plant and Cell Physiology 58 (2):216-226

Lam PY, Lui AC, Yamamura M, Wang L, Takeda Y, Suzuki S, Liu H, Zhu FY, Chen MX, Zhang J (2019) Recruitment of specific flavonoid B-ring hydroxylases for two independent biosynthesis pathways of flavone-derived metabolites in grasses. New Phytologist 223 (1):204-219

Lo C (2020) Convergent recruitment of 5 0-hydroxylase activities by CYP75B flavonoid B-ring hydroxylases for tricin biosynthesis in Medicago legumes. New Phytologist:1

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia plantarum 15 (3):473-497

Nishihara M, Higuchi A, Watanabe A, Tasaki K (2018) Application of the CRISPR/Cas9 system for modification of flower color in Torenia fournieri. BMC plant biology 18 (1):1-9

Nitarska D, Stefanini C, Haselmair-Gosch C, Miosic S, Walliser B, Mikulic-Petkovsek M, Regos I, Slatnar A, Debener T, Terefe-Ayana D (2018) The rare orange-red colored

Euphorbia pulcherrima cultivar 'Harvest Orange'shows a nonsense mutation in a flavonoid 3'-hydroxylase allele expressed in the bracts. BMC plant biology 18 (1):216
Noda N, Aida R, Kishimoto S, Ishiguro K, Fukuchi-Mizutani M, Tanaka Y, Ohmiya A (2013) Genetic engineering of novel bluer-colored chrysanthemums produced by accumulation of delphinidin-based anthocyanins. Plant and cell physiology 54 (10):1684-1695
Noda N, Yoshioka S, Kishimoto S, Nakayama M, Douzono M, Tanaka Y, Aida R (2017) Generation of blue chrysanthemums by anthocyanin B-ring hydroxylation and glucosylation and its coloration mechanism. Science advances 3 (7):e1602785
Pfaffl MW (2004) Quantification strategies in real-time PCR. AZ of quantitative PCR 1:89-113
Sagvaag AK (2015) Genetic transformation of Poinsettia (Euphorbia pulcherrima). Norwegian University of Life Sciences, Ås,
Sasaki N, Nakayama T (2015) Achievements and perspectives in biochemistry concerning anthocyanin modification for blue flower coloration. Plant and Cell Physiology 56 (1):28-40
Seitz C, Ameres S, Forkmann G (2007) Identification of the molecular basis for the functional difference between flavonoid 3′-hydroxylase and flavonoid 3′, 5′-hydroxylase. FEBS letters 581 (18):3429-3434
Seitz C, Ameres S, Schlangen K, Forkmann G, Halbwirth H (2015) Multiple evolution of flavonoid 3′, 5′-hydroxylase. Planta 242 (3):561-573
Slatnar A, Mikulic-Petkovsek M, Veberic R, Stampar F, Schmitzer V (2013) Anthocyanin and chlorophyll content during poinsettia bract development. Scientia Horticulturae 150:142-145
Tanaka Y (2006) Flower colour and cytochromes P450. Phytochemistry Reviews 5 (2-3):283-291
Tanaka Y, Brugliera F, Chandler S (2009) Recent progress of flower colour modification by biotechnology. International journal of molecular sciences 10 (12):5350-5369
Tanaka Y, Katsumoto Y, Brugliera F, Mason J (2005) Genetic engineering in floriculture. Plant cell, tissue and organ culture 80 (1):1-24
Tasaki K, Higuchi A, Watanabe A, Sasaki N, Nishihara M (2019) Effects of knocking out three anthocyanin modification genes on the blue pigmentation of gentian flowers. Scientific reports 9 (1):1-10
Taylor JM, Lopez RG, Currey CJ, Janick J (2011) The poinsettia: History and transformation. Chronica Horticulturae 51 (3):23-28
Wang M, Lu Y, Botella JR, Mao Y, Hua K, Zhu J-k (2017) Gene targeting by homology-directed repair in rice using a geminivirus-based CRISPR/Cas9 system. Molecular plant 10 (7):1007-1010
Watanabe K, Kobayashi A, Endo M, Sage-Ono K, Toki S, Ono M (2017) CRISPR/Cas9-mediated mutagenesis of the dihydroflavonol-4-reductase-B (DFR-B) locus in the Japanese morning glory Ipomoea (Pharbitis) nil. Scientific reports 7 (1):1-9
Yoshida K, Mori M, Kondo T (2009) Blue flower color development by anthocyanins: from chemical structure to cell physiology. Natural product reports 26 (7):884-915
Zhang B, Yang X, Yang C, Li M, Guo Y (2016) Exploiting the CRISPR/Cas9 system for targeted genome mutagenesis in petunia. Scientific reports 6:20315
Zhang L, He L-L, Fu Q-T, Xu Z-F (2013) Selection of reliable reference genes for gene expression studies in the biofuel plant Jatropha curcas using real-time quantitative PCR. International journal of molecular sciences 14 (12):24338-24354

2.5 Publication 5

Event-specific qualitative polymerase chain reaction analysis for two T-DNA copies in genetically modified orange Petunia

C. Haselmair-Gosch*, D. Nitarska*, B. Walliser, H. Flachowsky, S. Marinovic and H. Halbwirth

* shared co-first authorship

Plant Cell, Tissue and Organ Culture (PCTOC), *142*(2), pp.415-424.
doi.org/10.1007/s11240-020-01871-w

Plant Cell, Tissue and Organ Culture (PCTOC) (2020) 142:415–424
https://doi.org/10.1007/s11240-020-01871-w

ORIGINAL ARTICLE

Event-specific qualitative polymerase chain reaction analysis for two T-DNA copies in genetically modified orange *Petunia*

Christian Haselmair-Gosch[1] · Daria Nitarska[1] · Benjamin Walliser[1] · Henryk Flachowsky[2] · Silvija Marinovic[1] · Heidi Halbwirth[1]

Received: 5 April 2020 / Accepted: 14 June 2020 / Published online: 19 June 2020

Abstract
In 2017, various orange coloured petunia on the market turned out to be genetically modified (GM) without an official authorization for commercialization. Sequence analysis suggested these undeclared plants most probably originated from a plant transformation experiment performed in the 1980s. For a deeper understanding how GM petunia entered classical breeding programmes worldwide, and whether they originated from a single source or not, we undertook a molecular genetic characterization of the T-DNA integration sites in different GM petunia cultivars and breeding lines. By means of genome walking, we isolated different T-DNA sequences, which are located at the junctions between the T-DNA(s) and the petunia DNA. Based on the results obtained we conclude that there are at least two T-DNA copies of different lengths. This is supported by Southern blot analysis. For T-DNA1, the 3′-junction sequence was isolated, whereas the 5′-junction remained unclear. In contrast, for T-DNA2, the 5′-junction sequence was isolated, whereas the sequence isolated from the 3′-region consists only of T-DNA, but did not include the junction from the T-DNA to the petunia DNA. We developed primers for event-specific PCRs and screened a set of three orange GM petunia cultivars and 126 GM offspring from a commercial breeding program. We show that both T-DNA copies are present in all our tested GM petunia samples, which underpins the assumption of a single transgenic origin of the undeclared GM petunia. Most likely, the two T-DNAs are integrated in close proximity into the petunia genome.

Key message
The recently escaped genetically modified orange petunia contain at least two T-DNAs, which have the same integration sites in all tested plant samples, indicating a single transgenic origin. Both T-DNAs can be unambiguously identified via event-specific PCRs.

Keywords *Petunia* × *hybrida* · Event-specific transgene detection · Anthocyanin · Orange flower colour · Transgenic plant

Communicated by Maria Margarida Oliveira.

Christian Haselmair-Gosch and Daria Nitarska contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11240-020-01871-w) contains supplementary material, which is available to authorized users.

✉ Heidi Halbwirth
heidrun.halbwirth@tuwien.ac.at

[1] Institute of Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, Getreidemarkt 9, 1060 Vienna, Austria

[2] Institute for Breeding Research on Fruit Crops, Julius Kühn-Institut, Pillnitzer Platz 3a, 01326 Dresden, Germany

Introduction

Petunias are economically important balcony and bedding plants, which are available in many different growing shapes and flower colours. However, like some other species (e.g. cyclamen, African violet, *Cymbidium*, *Angelonia*), petunia do not naturally produce orange/bright-red flowers because they lack the ability to synthesize pelargonidin-type anthocyanin pigments (Forkmann and Ruhnau 1987; Gosch et al. 2014; Johnson et al. 1999). This is because of the substrate specificity of petunia dihydroflavonol 4-reductase (DFR), which does not accept the essential pelargonidin-precursor dihydrokaempferol as a substrate during anthocyanin biosynthesis. Starting in the 1980s, transgenic petunia that

produce pelargonidin-type anthocyanins were created to achieve orange flower colour, by introducing either a maize *DFR* encoded by the *A1* coding sequence (Elomaa et al. 1995; Meyer et al. 1987), a gerbera *DFR* (Elomaa et al. 1995; Helariutta et al. 1993), a *Calibrachoa DFR* (Chu et al. 2015) or a rose *DFR* (Chu et al. 2015; Tsuda et al. 2004). However, no transgenic orange petunia has been officially commercialized. More than 30 years later, different orange coloured petunia appeared on the market, which were assumed to originate from classical breeding programs and not from biotechnological approaches. In 2017, it turned out that most of the orange petunia contain foreign DNA from maize and plant transformation vectors (Bashandy and Teeri 2017; Haselmair-Gosch et al. 2018). Based on the current information, the transformation construct of the first petunia transformation experiments performed by Meyer et al. (1987) can be assumed to be the source of those undeclared genetically modified (GM) orange petunia (Bashandy and Teeri 2017; Fraiture et al. 2019; Haselmair-Gosch et al. 2018). Subsequently, the cultivars, which were identified as GM, were removed from the market with some exceptions like e.g. Canada (CFIA 2020).

Initially, only PCR screening for transgenic DNA sequences (e.g. for *A1 DFR*, *nptII*, p*35S* promoter DNA elements or construct combinations thereof) was possible for the detection of those GM petunia (Bashandy and Teeri 2017; Haselmair-Gosch et al. 2018). This limits specificity, since such PCR methods do not necessarily discriminate between independent transgenic petunia events, if PCR targets the same genetic elements for e.g. promoters or selection markers (Elomaa et al. 1995; Meyer et al. 1987; Shimada et al. 1999). For petunia, several GM are published, targeting traits like flower colour, early/late flowering, plant morphology, fragrance, longevity or biotic/abiotic stress resistance (for review see Boutigny et al. 2020). Event-specific PCRs are more accurate and allow the analysis of distinct transformation events, since the method is based on the detection of junction sequences between the T-DNA(s) and the host genome, which is unique for each transformation event, even if more than one T-DNA is integrated during the transformation process (Holst-Jensen et al. 2012, 2003). Event-specific PCRs are available for various plants—mainly major crop plants like maize, soybean or canola etc. and are mainly used for screening food for genetic modifications (for review see e.g. Holst-Jensen et al. 2003; Salisu et al. 2017).

Recently, MinION sequencing technology was shown to be suitable for isolating a transgene flanking sequence part of GM petunia, which was identical in the 23 tested cultivars, suggesting a single origin of the GM plants (Fraiture et al. 2019). However, for orange GM petunia, several transgenic lines with a different number of integrated copies of the transgenic sequence were reported from the initial transformation experiment (Linn et al. 1990) but it is still unclear, which of these could be the source of the undeclared orange GM petunia.

For a deeper understanding how GM petunia entered classical breeding programmes worldwide and whether they originated from a single source or not, we aimed for the molecular genetic characterization of the T-DNA integration sites in different GM petunia cultivars and breeding lines.

Materials and methods

Chemicals

Primers for Southern blot probe amplification were synthesized by Eurofins (Germany). All other oligonucleotide primers were synthesized by Sigma-Aldrich (Austria). dNTPs were ordered from Fermentas (Germany). Standard chemicals (e.g. Tris, solvents, agarose, CTAB) were purchased from Sigma-Aldrich (Austria), Merck (Austria) or VWR (Austria). Ultrapure water was used (Ultrapure Direct-Q 3 UV equipped with a Millipak Express 20 filter; Merck, Austria).

Plant samples

Young flowers or young leaves of different *Petunia* × *hybrida* cultivars or breeding lines were used. The GM cultivars were 'Salmon Ray' (Danziger, Moshav Mishmar Hashiva, Israel), 'Viva Orange' (Florensis, Ambacht, The Netherlands) and 'Electric Orange' (Selecta One, Germany). Non-GM control plants of *Petunia* cv. 'Baby Doll' were obtained from Selecta One (Germany), those of cv. 'Blackberry' were purchased from Austrosaat (Vienna, Austria). Moreover we used 154 individual crossings of a commercial breeding program, which involved 179 different parental lines in various combinations. From those 154 crossings, 126 were GM and 28 were not. GM was tested by means of PCR with two primer pairs targeting for fragments of the *A1 DFR* coding sequence of maize and the *nptII* gene as described earlier (Haselmair-Gosch et al. 2018). Plant material was harvested, shock-frozen and kept at − 80 °C until analysis. Plant material was ground in liquid nitrogen and genomic DNA was isolated either with the Invisorb Spin Plant Mini Kit (Invitek Molecular, Germany) or the CTAB method (Lipp et al. 1999).

Genome walking for T-DNA integration site analysis

The GenomeWalker Universal Kit (Clontech, Takara Bio Inc., USA) was used according to the manufacturer's instructions to determine the 5′- and 3′-junction sequences spanning the T-DNA and the adjacent plant DNA. In brief, four GenomeWalker DNA libraries were constructed with genomic DNA of cv. 'Viva Orange' using the restriction

enzymes *Dra*I, *Eco*RV, *Pvu*II and *Stu*I with subsequent GenomeWalker adaptor ligation. Three gene specific (nested) reverse and forward primers were designed close to the 5′- and 3′-end of the NCBI genbank sequences MF521566 (Haselmair-Gosch et al. 2018) and KY964325 (Bashandy and Teeri 2017), representing the transgene construct sequence of orange GM petunia. Adaptor primers AP1 and AP2 were provided by the GenomeWalker Kit. All primer sequences are listed in Table 1. Genome walking PCR reactions were performed in a Mastercycler ep Gradient PCR device (Eppendorf, Germany) in a final volume of 20 µl consisting of: 1 × Go*Taq* Green reaction buffer, 0.4 µl dNTPs (10 mM), 1 µl forward and reverse primer each (10 µM), 1 µl GenomeWalker library DNA and 0.2 µl DNA polymerase (5 U/µl, Go*Taq* DNA Polymerase Kit, Promega, Germany). In the primary PCR the primers p35S-R6 (5′-junction) or gm-ocs-F1 (3′-junction) were used in combination with the genome walker primer AP1 and the following cycling conditions: 94 °C 1 min; 40 cycles (94 °C 30 s, 67 °C 30 s, 72 °C 2 min) and a final extension at 72 °C for 7 min. For the secondary PCR the primers p35S-R5 (5′-junction) or gm-ocs-F2 (3′-junction) were used in combination with the genome walker primer AP2 (double nested) at the same cycling conditions as for the primary PCR. 1 µl of a 1:10 dilution of the primary PCR reaction was used as template despite no amplification products being detected by agarose gel analysis of the primary PCR reaction. Single amplification products of the secondary PCR were cut out and eluted after 1% agarose gel electrophoresis and used in a 1:10 dilution as template (1 µl) for the tertiary PCR. Tertiary PCR was performed with the primers p35S-R4 (5′-junction) or gm-ocs-F3 (3′-junction) in combination with the genome walker primer AP2 (single nested), again at the same cycling conditions as mentioned for the primary PCR. Amplification products were cut and eluted after 1% agarose gel electrophoresis and used for direct PCR sequencing, which was performed by Microsynth (Switzerland).

Event-specific and element-specific qualitative PCR

Primers were designed based upon the sequences obtained by genome walking and are listed in Table 1 and shown in Fig. 1. Event-specific PCR primers for T-DNA1 were rc-ocs-k-R2 and gm-P-R6 (60 °C PCR annealing temperature) and those for T-DNA2 were gm-P-F3 and p35S-R4 (65 °C PCR annealing temperature), which allow the amplification of the event-specific junctions between the T-DNAs and the petunia DNA. Additionally, element-specific PCR primers for the *octopine synthase* gene terminator sequence (t*OCS*) of T-DNA2 were designed (rc-ocs-k-R2, ocs-l-R1, 60 °C PCR annealing temperature). To verify the integrity of petunia DNA samples, primers specific for a petunia *DFR* gene were used (Haselmair-Gosch et al. 2018), resulting in an amplicon of 565 bp. Water and DNA of non-GM petunia were used as negative controls. The PCR reactions were performed in a final volume of 20 µl consisting of: 1 × Go*Taq* Green reaction buffer, 0.4 µl dNTPs (10 mM), 1 µl forward and reverse primer each (10 µM), 1 µl DNA (50 ng/µl) and 0.2 µl DNA

Table 1 Oligonucleotide primers

Primer name	Sequence (5′ > 3′)	Application
AP1	GTAATACGACTCACTATAGGGC	Genome walking (adaptor primer)
AP2	ACTATAGGGCACGCGTGGT	Genome walking (adaptor primer)
DFR_A1a_Fwd	GGAAGACGAAGCCATTGAT	Southern blot analysis (*A1* probe generation)
DFR_A1a_Rev	GTGCGAGGAGCAAACGAA	Southern blot analysis (*A1* probe generation)
gm-ocs-F1	GGTTGGGCTTCGGAATCGTTTTCCG	3′-genome walking (gene specific primer)
gm-ocs-F2	GAGATATGCGAGACGCCTATGATCGCAT	3′-genome walking (gene specific primer)
gm-ocs-F3	CCTGAGCATGTGTAGCTCAGATCCTTAC	3′-genome walking (gene specific primer)
gm-P-F3	CTCCCACAGAGATTCCAAAGGCAGTAGAC	Forward primer specific for Pet_5′T-DNA2 amplification
gm-P-R6	GTCATCAAAGGCTTGAGATGTGAACTCACC	Reverse primer specific for 3′T-DNA1_Pet amplification
nptII_F	ACAAGATGGATTGCACGCAGG	Southern blot analysis (*nptII* probe generation)
nptII_R	AACTCGTCAAGAAGGCGATAG	Southern blot analysis (*nptII* probe generation)
ocs-l-R1	GGGATCGAGCCCCTGCTGAG	Reverse primer specific for 3′T-DNA2 amplification
p35S-R4	ATCAGTTGGGTGCACGAGTGGGTTACAT	5′-genome walking (gene specific primer) and reverse primer specific for Pet_5′T-DNA2 amplification
p35S-R5	ACTTTTCGGGGAAATGTGCGCGGAACC	5′-genome walking (gene specific primer)
p35S-R6	AAGACGAAAGGGCCTCGTGATACGCCTATT	5′-genome walking (gene specific primer)
Pet-DFR-F1	TCACTTCATCTGCTGGAACTCTCGATG	Forward primer specific for petunia dihydroflavonol 4-reductase
Pet-DFR-R	GCCTCACAAAGATCATCCAAATGCACATAT	Reverse primer specific for petunia dihydroflavonol 4-reductase
rc-ocs-k-R2	CTGATTGTACCCTACTACTTATATGTACAA	Forward primer for 3′T-DNA1_Pet and 3′T-DNA2 amplification

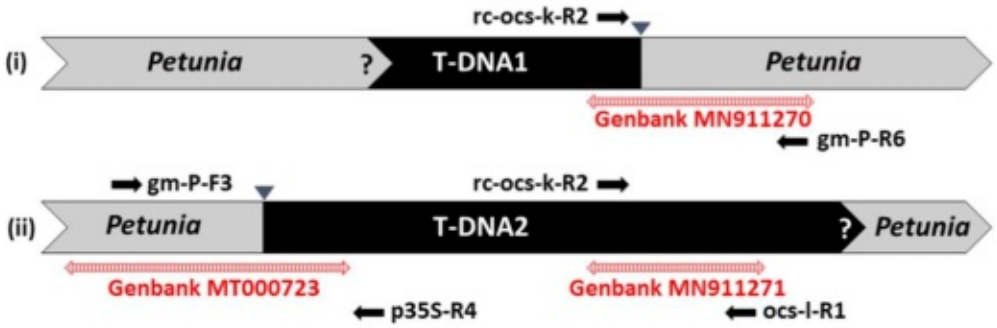


Fig. 1 Schematic overview of the situation at the genomic integration sites of (i) T-DNA1 and (ii) T-DNA2. Single arrows in black show the location and direction of primers for specific detection of the two T-DNAs. Double arrows in red indicate sequence sections identified by means of genome walking during this study. Question marks represent the unknown junctions and triangles the identified junctions from T-DNAs to petunia DNA. The drawing does not reflect exact size relations

polymerase (5 U/µl, Go*Taq* DNA Polymerase Kit, Promega, Germany). The following cycling conditions were used in a Mastercycler ep Gradient PCR device (Eppendorf, Germany): 98 °C 2 min; 40 cycles (98 °C 30 s, 50 s specific annealing temperature, 72 °C 40 s) and a final extension at 72 °C for 10 min. PCR products were separated on a 2% agarose gel and 1 × buffer TAE and stained with SERVA DNA Stain Clear G (SERVA Electrophoresis GmbH, Germany). The 1 kb Plus DNA ladder (NewEngland Biolabs, Austria) was used as a molecular size standard. Gels were scanned and evaluated with an Amersham Typhoon 5 Biomolecular Imager (GE Healthcare Bio-Sciences Corp., Austria).

Southern blot analysis

Detection of integrated T-DNA copy numbers was performed by Southern hybridization. 10 µg DNA of the orange GM petunia cvs. 'Viva Orange', 'Electric Orange' and 'Salmon Ray' as well as the wild type cv. 'Baby Doll' (used as negative control) were incubated with 100 units of *Eco*81I or *Bsp*OI (Thermo Fisher Scientific, Germany) at 37 °C over night. The cleaved DNA was separated on a 0.8% agarose gel and transferred onto a positively charged nylon membrane (Roche Diagnostics, Germany). Two PCR amplified, digoxigenin-labeled probes amplified either from the *A1 DFR* coding sequence (probe length: 641 bp) or the *nptII* marker gene (probe length: 780 bp) sequences were generated (primers listed in Table 1) using the PCR DIG Probe synthesis Kit (Roche Diagnostics, Germany) and used for hybridization. Hybridization and detection were performed using the ECF-Random-Prime-Labeling and Detection Kit (GE Healthcare Amersham™ Biosciences, UK) according to the manufacturer's manual.

Results

T-DNAs integration site analysis

Genome walking technology was used to isolate the T-DNA junction sequences between the known T-DNA sequences MF521566 and KY964325, which are available at the database of the National Center for Biotechnology Information (NCBI), and the petunia genome in the orange GM petunia cv. 'Viva Orange'. Originally, only the presence of a single T-DNA in the genome was expected. After tertiary PCRs using the four GenomeWalker libraries as template, three putative T-DNA junction sequences were obtained from two T-DNAs inserted into the petunia genome (Fig. 1). These sequences were designated as Pet_5′T-DNA2, 3′T-DNA1_Pet and 3′T-DNA2 (Fig. 2).

For T-DNA2 only the 5′ junction sequence could be isolated. This 5′T-DNA2 sequence together with the upstream petunia DNA was obtained from the GenomeWalker library digested with *Eco*RV. The 1,078 bp sequence was deposited in the NCBI database (accession No. MT000723) and comprises identically a recently isolated junction sequence of 402 bp of GM petunia (Fraiture et al. 2019). The entire sequence of the Pet_5′T-DNA2 fragment consists of 828 bp petunia DNA followed by 250 bp of a β-lactamase gene (*bla*) in antisense direction. Six frame translation of the petunia DNA upstream of 5′T-DNA2 resulted in a partial open reading frame of 275 codons from a protein with highest homology to retrotransposon polyproteins found in e.g. *Coffea* (AQY61297) or *Vitis* (RVW93168).

Searching for 3′T-DNA junction sequences, two different sequences with a length of 765 bp and 605 bp, respectively, were found. The first sequence was obtained from the GenomeWalker library digested with *Stu*I, whereas the

second sequence was obtained from the library digested with *Pvu*II. Both sequences were deposited in the NCBI database under the numbers MN911270 for 3′T-DNA1_Pet and MN911271 for 3′T-DNA2. The sequence 3′T-DNA1 contains 138 bp, which are identical to the corresponding section of the longer 3′T-DNA2, which has a total size of 605 bp. 3′T-DNA2 is identical to commonly used plant transformation vector sequences (e.g. accession No. JQ974028) and consists of mainly the *octopine synthase* terminator. Six frame translation of the petunia DNA (627 bp) downstream of 3′T-DNA1 resulted in a partial open reading frame of 209 codons, again with highest homology to retrotransposon polyproteins as described before.

By performing PCRs with forward primers designed against the petunia DNA upstream of 5′T-DNA2 and reverse primers specific to 3′T-DNA2 we found, that sequence 5′T-DNA2 belongs to sequence 3′T-DNA2 of the same T-DNA (Supplemental Figure S1).

The petunia DNAs upstream of 5′T-DNA2 and downstream of 3′T-DNA1 were mapped to publically available genome sequences of *P. inflata* and *P. axillaris* (www.solgenomics.net), which represent the two ancestors of *P.* × *hybrida* (Bombarely et al. 2016; Fernandez-Pozo et al. 2015). Independent mapping of the two petunia DNAs revealed highly similar sequences in several of the same scaffolds but in a different BLAST results order. Interestingly, petunia DNA of 3′T-DNA1_Pet is located in silico in the genome closely upstream (1258 bp distance) of Pet_5′T-DNA2, suggesting a tandem insertion of T-DNA1 and T-DNA2, with a petunia DNA spacer piece of all together 2,224 bp (138 bp + 1258 bp + 828 bp) of petunia DNA in between (e.g. *P. axillaris* v1.6.2 genome Scaffold Peaxi162Scf00714). However, by means of PCR with different primer combinations (e.g. 3′T-DNA1 specific forward and 5′T-DNA2 specific reverse primers) such a tandem integration could not be verified.

The 3′T-DNA2, which was isolated by genome walking did not include the junction from the T-DNA to the petunia DNA, because *Pvu*II, which was successful for GenomeWalker library generation cuts within the sequence of 3′T-DNA2. Using the other three libraries, where the DNA was cut with either *Dra*I, *Eco*RV or *Stu*I, respectively, failed to isolate the T-DNA flanking petunia sequence.

For T-DNA1, no 5′ junction sequence could be isolated by means of two independent 5′ genome walking experiments using the primers specific for the CaMV 35S promoter p*35S*. In addition, another approach was used to identify the missing junctions of T-DNA1 and T-DNA2: Therefore, several PCR primers were designed based on sequences adjacent to the putative insertion regions, which were determined within the genomes from *P. axillaris* and *P. inflata* by using the isolated petunia DNA downstream of T-DNA1 or upstream of T-DNA2. Those primers were used together with primers specific for T-DNA1 or T-DNA2, respectively. Unfortunately, this strategy has so far failed to amplify the missing junctions (data not shown).

Southern blot analysis

Southern blot analysis was performed with three orange GM petunia cultivars and one wild type cultivar used as negative control. The restriction enzymes *Eco*81I and *Bsp*O1 and probes specific to *A1 DFR* and *nptII* sequences were used (Fig. 3). Hybridization revealed for all GM cultivars only one copy of *A1 DFR*, but two to three copies of *nptII* (Fig. 4).

Event-specific and element-specific qualitative PCR

Event-specific and element-specific qualitative PCR was developed and optimized by using DNA of cv. 'Viva Orange' as template, for which the specificity of the products was confirmed by agarose gel electrophoresis and sequencing by Microsynth (Austria). The primers (Table 1 and Fig. 2) were designed upon the sequences obtained by genome walking and showed in silico no homology to any sequence present in the publicly available genome data of *Petunia inflata* or *P. axillaris* (Bombarely et al. 2016; Fernandez-Pozo et al. 2015). Event-specific primers rc-ocs-k-R2 and gm-P-R6 were used to amplify a 682 bp sequence of the junction of 3′T-DNA1_Pet. For the junction of Pet_5′T-DNA2 the primers gm-P-F3 and p35S-R4 were used, with the expected amplicon size of 791 bp. Element-specific PCR for the rear part of T-DNA2 was done with primers rc-ocs-k-R2 and ocs-l-R1, with the expected amplicon size of 536 bp. Figure 5 shows representative results of positive and negative samples as well as negative controls. We screened the orange GM petunia cultivars 'Salmon Ray', 'Viva Orange', 'Electric Orange' and 126 GM offsprings from individual crossings of a commercial breeding program. As non-GM controls, 28 offsprings and the cultivar 'Blackberry' were used. All GM samples showed amplification products at the expected length, which are presented exemplarily in Fig. 5.

Discussion

In 1986, at the Max Planck Institute for Plant Breeding Research in Cologne, Germany, the first modification of a flower's colour by a transgenic approach was achieved (Meyer et al. 1987). Doubtless, this was a milestone for flower colour research and subsequently enabled a deeper understanding of e.g. epigenetic changes via DNA methylation, gene copy numbers or the T-DNA integration site, etc. (Linn et al. 1990; Meyer et al. 1992; Pröls and Meyer 1992). Orange GM petunia of those experiments were the first GM

◂**Fig. 2** Sequences of **a** Pet_5′T-DNA2 (1078 bp, accession No. MT000723) plus underlined sequence originating from accession No. KY964325 (Bashandy and Teeri 2017), **b** 3′T-DNA1_Pet (765 bp, accession No. MN911270) and **c** 3′T-DNA2 (605 bp, accession No. MN911271) obtained by genome walking. PCR primers derived thereof are given. Bold letters indicate T-DNA sequences, italic letters are petunia DNA

plants, which were released into the field in Germany and attracted attention far beyond the horticultural community. Although the orange GM petunia has never been officially commercialized, in 2017 different orange coloured petunia were identified on the market without GM declaration. There are several documented cases where un-authorized GM plant species or products thereof unintentionally emerged on the market or in the food chain via different ways (Holst-Jensen et al. 2012). Based on the current sequence information, the transformation construct of the first petunia transformation experiments performed by Meyer et al. (1987) can be assumed to be the source of those undeclared orange GM petunia (Bashandy and Teeri 2017; Fraiture et al. 2019; Haselmair-Gosch et al. 2018). To understand, how GM petunia could enter classical breeding programmes worldwide and whether they originated from a single source or not, we aimed for the molecular genetic characterization of the T-DNA integration sites in different GM petunia cultivars and breeding lines. By means of genome walking techniques, we isolated three putative T-DNA junction sequences from two T-DNAs (T-DNA1, T-DNA2) inserted into the petunia genome.

For T-DNA2 only the 5′ junction sequence could be isolated, which was designated as Pet_5′T-DNA2. For the rear part of T-DNA2, only a T-DNA specific sequence was isolated (designated as 3′T-DNA2), whereas the junction and adjacent petunia DNA remained unclear. Most probably, because *Pvu*II was used during GenomeWalker library construction, which was found to cut within T-DNA2 but not in the petunia DNA flanking this T-DNA sequence.

In contrast, the 3′ junction sequence of T-DNA1 (designated as 3′T-DNA1_Pet) was obtained from the GenomeWalker library digested with *Stu*I, which cuts within the petunia genome downstream of T-DNA1. However, for T-DNA1, no 5′ junction sequence could be isolated by means of two independent 5′ genome walking experiments using the primers specific for the CaMV *35S* promoter p*35S*. We assume that T-DNA1 could be truncated at the front part, and is therefore incapable of binding p*35S* specific primers during genome walking, compared to T-DNA2. This is in accordance with the results of Fraiture et al. (Fraiture et al. 2019), who used a p*35S* specific target for isolating 5′ T-DNA flanking sequences and could also only identify one T-DNA. The hypothesis of T-DNA1 being truncated in the front part is underpinned by our Southern hybridization results, where only one copy of the *A1 DFR* coding sequence (referring to T-DNA2) was found, whereas two to three copies of the *nptII* gene were detected. Most likely, T-DNA1 is truncated at the 5′-side somewhere before or within the *A1 DFR* sequence to such an extent that the *A1 DFR* probe is not able to bind for a Southern blot based detection. Southern hybridization was done with a small selection of three orange GM petunia cultivars. Despite those cultivars originate from three independent companies from different countries it cannot be excluded that other—currently undetected—T-DNAs are present in GM petunia. However, in general, uniform and stable orange colouration was commonly observed only when 1–2 intact copies of the *A1* coding sequence were integrated into the genome (Linn et al. 1990; Tsuda et al. 2004). From the initial transformation experiment (Meyer et al. 1987), several lines with a different number of integrated copies of the transgenic sequence, which ranged mainly from 1 to 8 copies, are known (Linn et al. 1990). Some of them, like No. 235/1-15 or No. 235/1-17, which both have only one intact *A1 DFR* coding sequence copy integrated, were later used as donors in classical breeding programs, to obtain petunia with improved phenotypical expression of the orange flower colour (Oud et al. 1995). Those lines could also be the source of the illegally marketed GM-petunia.

For the two T-DNAs that were isolated in the present study, we developed event-specific PCRs for the junction sequences of 3′T-DNA1_Pet and Pet_5′T-DNA and an element-specific PCR for 3′T-DNA2, targeting the *octopine synthase* gene terminator t*OCS*. Since event-specific PCRs are based on the detection of junction sequences between the T-DNA(s) and the host genome, they allow the analysis of distinct transformation events, even if more than one identical copy is integrated. We screened the orange GM petunia cultivars ‘Salmon Ray’, ‘Viva Orange’, ‘Electric Orange’ and 126 GM offspring from individual crossings of a commercial breeding program by PCR. All GM samples have both T-DNAs integrated at the same location in the host genome, which underpins the assumption of a single transgenic line as source of the undeclared GM petunia. Since previous results have shown that the presence of the *A1 DFR* coding sequence in GM petunia does not necessarily result in orange phenotypes in a common biochemical petunia background (Haselmair-Gosch et al. 2018), also non-orange petunia could have a concealed transgenic status, which can also be determined with our PCR protocols.

We mapped our two identified petunia DNA sequences adjacent to 5′T-DNA2 and 3′-TDNA1 by using the publically available genome sequences of petunia (Bombarely et al. 2016; Fernandez-Pozo et al. 2015) and identified several scaffolds, indicating an insertion into a repetitive genome region. In silico, petunia DNA of 3′T-DNA1_Pet is located in the genome closely upstream of Pet_5′T-DNA2, suggesting a tandem insertion of T-DNA1 and

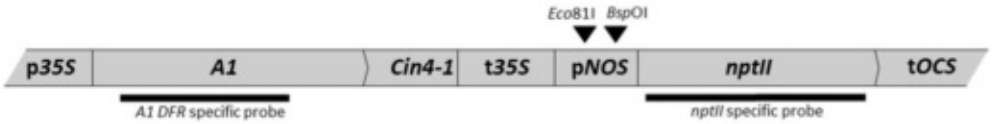


Fig. 3 Schematic representation of the position of the restriction sites *Eco*81I and *Bsp*OI and the Southern probes for *A1 DFR* and *nptII* located on the transgenic insert found in orange GM petunia (accession No. MF521566 (Haselmair-Gosch et al. 2018)). p*35S*, promoter sequence of the *35S* Cauliflower mosaic virus gene; *A1*, coding sequence of the *A1 DFR* gene; *Cin4-1*, partial *Cin4-1* transposable element present in type 2 allele of *A1 DFR* (Schwarz-Sommer et al. 1987a, b); t*35S*, terminator sequence of the *35S* Cauliflower mosaic virus gene; p*NOS*, promoter sequence of the *nopaline synthase* gene; *nptII*, coding sequence of the *neomycine phosphotransferase II* selectable marker gene; t*OCS*, terminator sequence of the *octopine synthase* gene. The drawing does not reflect exact size relations

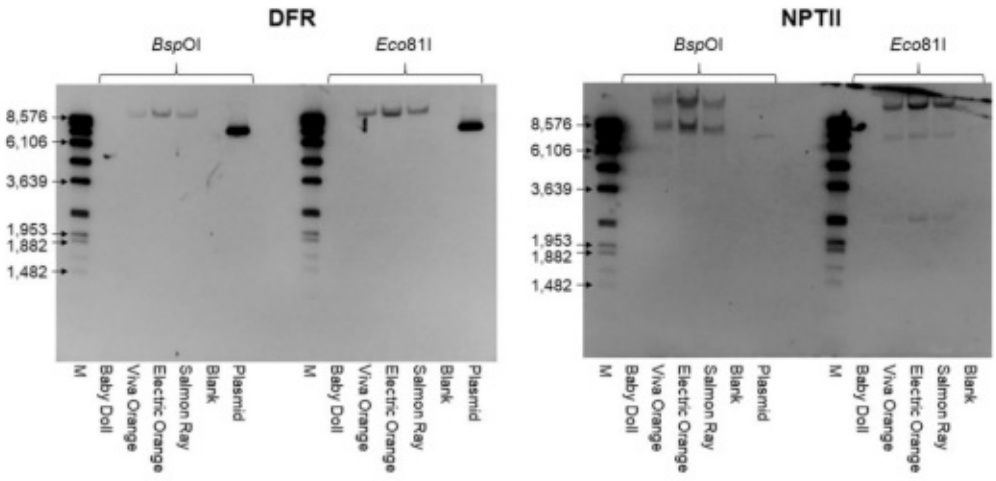


Fig. 4 Southern blot analysis of orange GM petunia cultivars 'Viva Orange', 'Electric Orange' and 'Salmon Ray' with probes for the *A1 DFR* coding sequence (left) and the *nptII* gene (right). Wild type cv. 'Baby Doll' was used as non-transgenic negative control. A plasmid harbouring the *A1 DFR* coding sequence was used as positive control for *DFR* probe. The restriction endonucleases *Bsp*OI and *Eco*81I were used. M, molecular size standard (DIG Molecular Weight Marker VII, Roche Diagnostics, Germany); Blank, H_2O used as negative control. Selected fragments of the size marker are labelled with fragment lengths in bp for better orientation. Smaller fragments of the size standard are not visible

T-DNA2, which could not be verified by means of PCR with different primer combinations. This could be because of possible insertional effects like genomic rearrangements, duplication or deletion events, which may have occurred during the transfer of the T-DNAs as a commonly known phenomenon (Schnell et al. 2015), or the assumption that the T-DNAs are inserted into a region of repetitive sequences. Nevertheless, at least the insertion in close proximity on the same chromosome can be supposed, since screening of our 129 GM petunia from individual crossings with event-specific PCRs showed 100% co-segregation of both T-DNAs.

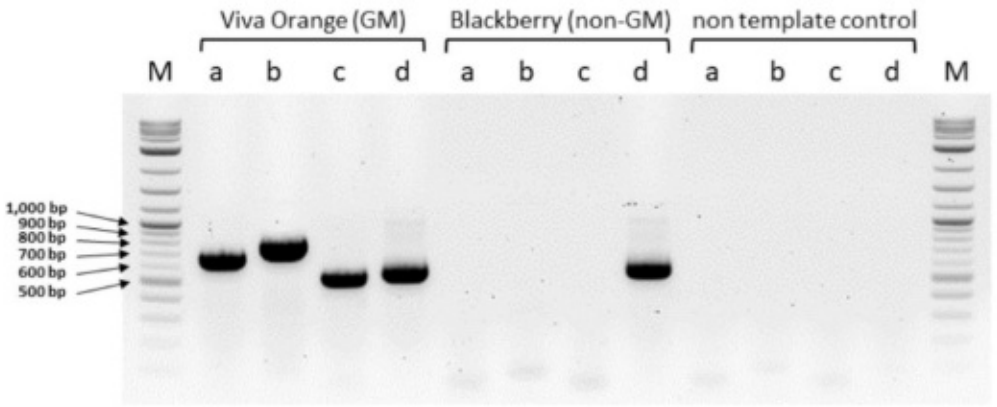


Fig. 5 PCR evaluation of petunia DNA with primers specific for 3'T-DNA1_Pet, Pet_5'T-DNA2 and 3'T-DNA2. A 2% agarose gel was used. a, primers rc-ocs-k-R2 and gm-P-R6 for 3'T-DNA1_Pet (682 bp amplicon); b, primers gm-P-F3 and p35S-R4 for Pet_5'T-DNA2 (791 bp amplicon); c, primers rc-ocs-k-R2 and ocs-l-R1 for 3'T-DNA2 (536 bp amplicon); d, primers Pet-DFR-F1 and Pet-DFR-R for a partial sequence of the petunia *DFR* (565 bp amplicon); GM, genetically modified; M, molecular size standard 2-Log DNA Ladder

Acknowledgements Open access funding provided by Austrian Science Fund (FWF). The authors acknowledge the financial support for substantial parts of this work by the Austrian Science Fund (FWF) P 28134-B25 and the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie Grant Agreement No 675657-FlowerPower. Furthermore, we gratefully acknowledge Ines Hiller for her technical assistance.

Author contributions CHG, HH and HF conceived the research. CHG and DN designed the experiments. DN, BW, SM, performed the experiments. The first draft of the manuscript was written by CHG and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This project has received funding from the Austrian Science Fund (FWF) P 28134-B25 and from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie Grant Agreement No 675657-FlowerPower.

Availability of data and material All data generated or analyzed during this study are included in this published article and its supplementary information file. Primary datasets are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Bashandy H, Teeri TH (2017) Genetically engineered orange petunias on the market. Planta 246:277–280

Bombarely A et al (2016) Insight into the evolution of the Solanaceae from the parental genomes of *Petunia hybrida*. Nat Plants 2:16074

Boutigny A-L, Dohin N, Pornin D, Rolland M (2020) Overview and detectability of the genetic modifications in ornamental plants. Hortic Res 7:11

CFIA (2020) Questions and answers: genetically engineered petunias. https://inspection.gc.ca/plant-health/plants-with-novel-traits/general-public/questions-and-answers/eng/1504228888140/1504229002939. Accessed 12 Mar 2020

Chu YX, Chen HR, Wu AZ, Cai R, Pan J (2015) Expression analysis of dihydroflavonol 4-reductase genes in *Petunia hybrida*. Genet Mol Res 14:5010–5021

Elomaa P, Helariutta Y, Kotilainen M, Teeri TH, Griesbach RJ, Seppänen P (1995) Transgene inactivation in *Petunia hybrida* is influenced by the properties of the foreign gene. MGG 248:649–656

Fernandez-Pozo N et al (2015) The sol genomics network (SGN)-from genotype to phenotype to breeding. Nucleic Acids Res 43:D1036–1041

Forkmann G, Ruhnau B (1987) Distinct substrate specificity of dihydroflavonol 4-reductase from flowers of *Petunia hybrida*. Zeitschrift für Naturforschung C 42:1146–1148

Fraiture M-A et al (2019) MinION sequencing technology to characterize unauthorized GM petunia plants circulating on the European Union market. Sci Rep 9:7141

Gosch C et al (2014) Isolation of dihydroflavonol 4-reductase cDNA clones from *Angelonia* x *angustifolia* and heterologous expression as GST fusion protein in *Escherichia coli*. PLoS ONE 9:e107755

Haselmair-Gosch C et al (2018) Great cause—small effect: undeclared genetically engineered orange petunias harbor an inefficient dihydroflavonol 4-reductase. Front Plant Sci 9:149

Helariutta Y, Elomaa P, Kotilainen M, Seppänen P, Teeri TH (1993) Cloning of cDNA coding for dihydroflavonol-4-reductase (DFR) and characterization of dfr expression in the corollas of *Gerbera hybrida* var. Regina (Compositae). Plant Mol Biol 22:183–193

Holst-Jensen A, Ronning SB, Lovseth A, Berdal KG (2003) PCR technology for screening and quantification of genetically modified organisms (GMOs). Anal Bioanal Chem 375:985–993

Holst-Jensen A et al (2012) Detecting un-authorized genetically modified organisms (GMOs) and derived materials. Biotechnol Adv 30:1318–1335

Johnson ET, Yi HK, Shin BC, Oh BJ, Cheong HS, Choi G (1999) *Cymbidium hybrida* dihydroflavonol 4-reductase does not efficiently reduce dihydrokaempferol to produce orange pelargonidin-type anthocyanins. Plant J 19:81–85

Linn F, Heidmann I, Saedler H, Meyer P (1990) Epigenetic changes in the expression of the maize *A1* gene in *Petunia hybrida*: role of numbers of integrated gene copies and state of methylation. MGG 222:329–336

Lipp M et al (1999) IUPAC Collaborative trial study of a method to detect genetically modified soy beans & maize in dried powder. J AOAC Int 82:923–928

Meyer P, Heidmann I, Forkmann G, Saedler H (1987) A new petunia flower colour generated by transformation of a mutant with a maize gene. Nature 330:677–678

Meyer P, Linn F, Heidmann I, Meyer H, Niedenhof I, Saedler H (1992) Endogenous and environmental factors influence 35S promoter methylation of a maize *A1* gene construct in transgenic petunia and its colour phenotype. MGG 231:345–352

Oud JSN, Schneiders H, Kool AJ, van Grinsven MQJM (1995) Breeding of transgenic orange *Petunia hybrida* varieties. Euphytica 85:403–409

Pröls F, Meyer P (1992) The methylation patterns of chromosomal integration regions influence gene activity of transferred DNA in *Petunia hybrida*. Plant J 2:465–475

Salisu IB, Shahid AA, Yaqoob A, Ali Q, Bajwa KS, Rao AQ, Husnain T (2017) Molecular approaches for high throughput detection and quantification of genetically modified crops: a review. Front Plant Sci 8:1670

Schnell J et al (2015) A comparative analysis of insertional effects in genetically engineered plants: considerations for pre-market assessments. Transgenic Res 24:1–17

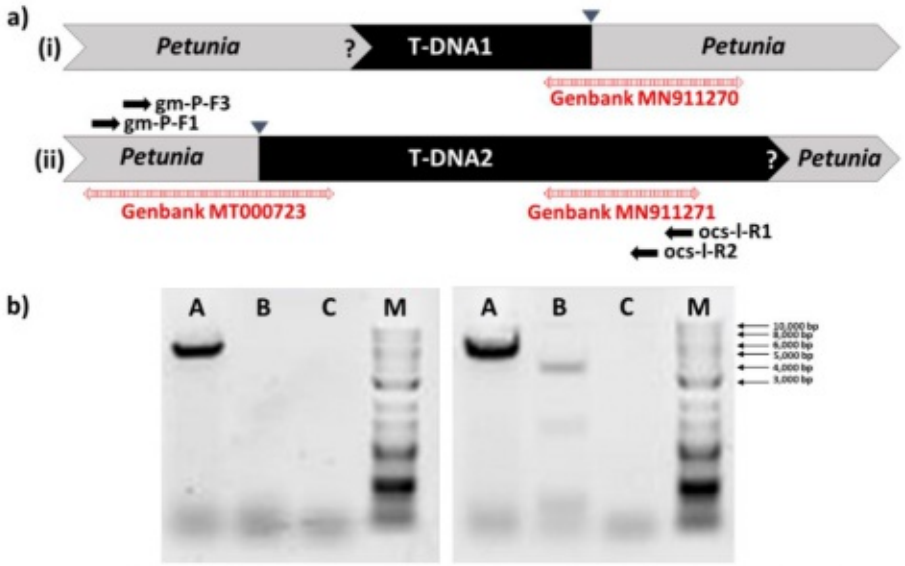
Schwarz-Sommer Z, Leclercq L, Göbel E, Saedler H (1987a) Cin4, an insert altering the structure of the *A1* gene in *Zea mays*, exhibits properties of nonviral retrotransposons. EMBO J 6:3873–3880

Schwarz-Sommer Z et al (1987b) Influence of transposable elements on the structure and function of the *A1* gene of *Zea mays*. EMBO J 6:287–294

Shimada Y, Nakano-Shimada R, Ohbayashi M, Okinaka Y, Kiyokawa S, Kikuchi Y (1999) Expression of chimeric P450 genes encoding flavonoid-3′,5′-hydroxylase in transgenic tobacco and petunia plants. FEBS Lett 461:241–245

Tsuda S et al (2004) Flower color modification of *Petunia hybrida* commercial varieties by metabolic engineering. Plant Biotechnol 21:377–386

Suppl. Fig S1 Mapping of the sequences MT000723 (5´ genome walking) and MN911271 (3´ genome walking) to T-DNA2 by means of PCR



a) Schematic overview of the situation at the genomic integration sites of (i) T-DNA1 and (ii) T-DNA2. Single arrows in black show the location and direction of primers. Double arrows in red indicate sequence sections identified by means of genome walking during this study. Question marks represent the unknown junctions and triangles the identified junctions from T-DNAs to petunia DNA. The drawing does not reflect exact size relations.

b) 1% agarose gel evaluation of PCR products. Two different primer combinations were used to show that the 5´ junction (accession No. MT000723) obtained by genome walking belongs to 3´-T-DNA2: left: gm-P-F1 (GTCACTGCCTATTTATATGGCTCGTTGGAC) + ocs-l-R1 (GGGATCGAGCCCCTGCTGAG) (expected amplicon size of 5,627 bp). right: gm-P-F3 (CTCCCACAGAGATTCCAAAGGCAGTAGAC) + ocs-l-R2 (GTTGTCGCAAAATTCGCCCTGGAC) (expected amplicon size of 5,306 bp). A, GM petunia cv. 'Viva Orange'; B, wild type petunia cv. 'Blackberry'; C, non template control (water); M, molecular size standard 2-Log DNA Ladder