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Anthochlor Biosynthesis and its application in biotechnological breeding for novel flower color

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

Institut 166-06-2

Institut für Verfahrenstechnik, Umwelttechnik und technische Biowissenschaften

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Fakultät für Technische Chemie

von

M. Sc. Benjamin Walliser

Matrikelnummer: 01634385

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Abstract

Anthochlor pigments are a group of secondary plant metabolites that, together with carotenoids and some flavonoids, produce yellow flower colour. Both, chalcones and aurones are collectively known as anthochlor pigments and serve as UV nectar guides in some plants. Especially *Asteraceae* accumulate two types of anthochlor pigments: on the one hand, the hydroxy types of chalcones and aurones, and on the other hand the deoxy types of chalcones and their corresponding aurones. Both types differ only in the presence of a hydroxyl group in the 6' position of the B-ring (chalcones) or the 4 position of the A-ring (aurones). Thus, hydroxychalcones do not readily accumulate in plants due to quick izomerization. Hence, our strategies in establishing yellow colouration involve the introduction of genes encoding proteins that lead to the accumulation of deoxy type anthochlors.

Three chapters make up this thesis: 1.) The biochemical basis of pigmentation of different coloured *Bidens ferulifolia* cultivars was unraveled. The main aspects were the pigment composition and enzyme activity assays of involved proteins. The prevalent pigment in yellow cultivars was the 6'-deoxychalcone okanin and its derivates, whereas red cultivars accumulated cyanidin. Furthermore, we concluded that missing FHT and/or DFR activity created a bottleneck in anthocyanin biosynthesis. This aspect may be highly interesting for breeders of *Bidens ferulifolia*. 2.) Genetic and biochemical analysis of the bicoloured white-yellow *Dahlia variabilis* 'Seattle'. From three putative CHRs, none were differentially expressed. Additionally, a strong difference in CHS2 expression between yellow and white petal parts may explain the bicolouration. Nonetheless, the transcriptomes that were built during the work can form the basis for further research and consolidate *Dahlia variabilis* 'Seattle' as a model plant for anthochlor research. 3.) Within this thesis, genes involved in anthochlor biosynthesis, derived from different members of the *Asteraceae* species, were cloned in large multigene constructs, utilizing the GoldenBraid vector system. These multigene constructs were introduced in model and target plants via transient and stable transformation, utilizing different transformation methods. We successfully created yellow petals with transient expression of a legume CHR and a glycosyltransferase in petals of *Pelargonium zonale*. Though the presence of transcripts was confirmed, HPLC analysis did not show traces of the expected 6'deoxychalcone isoliquiritigenin. Stable transformation of *Pelargonium zonale* and *Petunia hybrida* struggled with problems during regeneration. Only few regenerants that lacked a stable integration of the transgenes could be obtained. Other approaches to elucidate the contribution of the single genes of the anthochlor biosynthesis included the design of constructs of the novel CRISPR/Cas9 system and virus-induced gene silencing (VIGS) methods, with the same results as the stable integration of genes.

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Zusammenfassung

Die Anthochloren Pigmente sind eine Gruppe sekundärer Pflanzenmetabolite und tragen gemeinsam mit Carotenoiden und einigen Flavonoiden zur gelben Blütenfärbung bei. Chalcone und Aurone gemeinsam bilden hierbei die Klasse der Anthochlore und dienen als Blütenmal für UV-sensitive Insekten. Insbesondere Asteraceen, akkumulieren zwei Typen an Anthochloren. Zum einen die Hydroxy-Typen von Chalconen und Auronen, zum anderen die Deoxy-Typen von Chalconen und Auronen. Beide Typen unterscheiden sich lediglich im Vorhandensein einer Hydroxy-Gruppe an der Position 6‘ des B-Rings (Chalcone), bzw. an der Position 4 des A-Rings bei Auronen. Hydroxychalcone akkumulieren sich nicht in Pflanzen, da sie schnell isomerisieren. Daher entschieden wir uns für eine Strategie zur Schaffung gelber Blütenfarben, Gene einzuführen, welche Proteine kodieren, die an der Synthese von Anthochloren des Deoxytyps beteiligt sind.

Die Arbeit umfasst drei große Themenbereiche: 1.) Die biochemische Basis der Pigmentation verschieden farbiger Kultivare von *Bidens ferulifolia* wurde untersucht. Der Fokus hierbei lag auf der Ergründung der Pigmentkomposition wie auch der Untersuchungen der Aktivität beteiligter Enzyme. Die vorherrschenden Pigmente waren in den gelben Kultivaren 6‘-Deoxychalcone, in roten Blütenteilen Cyanidin. Desweiteren wurde festgestellt, dass teilweise fehlende FHT und/oder DFR Aktivität zu einem Flaschenhals der Anthocyanbiosynthese führte. Dieser Aspekt kann sich als höchst interessant für *Bidens*-Züchter erweisen. 2.) Genetische und biochemische Analysen der zweifarbigen, weiß-gelben *Dahlia variabilis* ‚Seattle‘.Eine Erklärung für die Zweifarbigkeit fand sich unter anderem in einer stark reduzierten Expression von CHS2. Nichtsdestotrotz bilden die im Rahmen der Arbeit gebildeteten Transkriptome eine gute Basis für weitere Untersuchung und sprechen für die Verwendung der *Dahlia variabilis* ‚Seattle‘ als Modellpflanze für die Forschung an Anthochloren. 3.) Im Rahmen dieser Arbeit wurden Gene, die an der Synthese von Anthochloren des Deoxytyps beteiligt sind, aus diversen Mitgliedern der Asteraceen-Familie isoliert und in große Multigen-Konstrukte des GoldenBraid-Systems geklont. Diese Multigen-Konstrukte wurden mittels verschiedener Transformationsmethoden sowohl stabil als auch transient in verschiedenen Ziel- und Modellpflanzen eingebracht. Die daraus resultierenden Pflanzen wurden sowohl auf RNA-, DNA- und Metabolit-Ebene auf das Vorhandensein der Gene und ihrer Produkte untersucht. Es gelang die Erschaffung von einzelnen gelben Petalen bei der transienten Expression von CHR mit einer Glykosyltransferase in *Pelargonium zonale*. Obwohl Transkripte durch PCR bestätigt wurden, zeigten HPLC Untersuchungen jedoch keinerlei Vorhandensein von dem zu erwarteten Pigment Isoliquiritigenin. Die stabile Transformation von *Pelargonium zonale* und *Petunia hybrida* führte zu nur wenige Stecklingen, welche jedoch keinen stabilen Einbau der Transgene aufwiesen.

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1. Introduction

1.1. Flower color

It is not on the artist's canvas, but in the gardener's flower that the greatest wealth of color may be seen... Flowers possess the next best quality of color tone to that which we see in the rainbow.

~F. Schuyler Mathews, "Comparative Colors and their Relation to Flowers," 1894

Nature created a bewildering diversity of different flower shapes and colors. Pollinators wield influence on the selection of flower color and shape, because pollination by animal interaction involves floral attributes of flower color signaling and the sensory abilities of the pollinators themselves (Chittka and Menzel 1992; Chittka and Waser 1997; Chittka and Raine 2006). The interaction of chemical, physical and morphological factors of the flowers themselves and the physiological properties of animals determines the appearance of flower colors. The chemical base for flower coloration are the different pigment classes (Clement and Mabry 1996; van der Kooi et al. 2016), their concentration, their abundance in different tissues (Miosic et al. 2013) and co-pigmentation (Goto and Kondo 1991; Yabuya et al. 2000; Mizuno et al. 2015). Physical factors of perceiving flower colors include the distance and direction to the flower, ambient weather and light conditions (Chittka and Raine 2006; Spaethe et al. 2006). Additionally, morphological factors like iridescence may influence the way color can be perceived (Kjernsmo et al. 2018).

Flower color as a pollinator attractant must contrast against the background for detectability by foraging pollinators (Menzel et al. 1997; Koski 2020) and should contrast against the flowers of co-occurring species within the same community (Chittka and Menzel 1992; Chittka et al. 1997). But how an animal perceives different colors is highly dependent on the spectral sensitivity and the number of their photo receptors. (Trunschke et al. 2021; van der Kooi et al. 2021). *Hymenoptera* vision shows a shift towards shorter wavelengths compared to the human vision with peaks at 340, 430 and 535 nm, enabling them to perceive UV light (Peitsch et al. 1992; Chittka et al. 1994). Some plants utilize the different UV absorbance of different pigments to create a UV nectar guide, attracting those UV sensitive insects as pollinators (Scogin and Zakar 1976; Harborne and Smith 1978; Koski and Ashman 2016).

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1.2. Pigment classes

As already mentioned earlier, there are three major pigment classes which contribute to flower coloration: Carotenoids, betalains and flavonoids with the yellow colorizing anthochlor pigments.

1.2.1. Betalains

Betalains are water-soluble pigments with a nitrogen-containing backbone known as betalamic acid [4-(2-oxoethylidene)-1,2,3,4-tetrahydropyridine-2,6-dicarboxylic acid]. Betalains form the groups of the violet betacyanins and the yellow colored betaxanthins. Described first in 1958 from pigments in red beet (Peterson and Joslyn 1958), betalains were classified as different to anthocyanins. Until today, about 75 betalains are identified and structurally elucidated (Khan and Giridhar 2015). Betalains are predominantly accumulated in plants of the order of Caryophyllales in which they, generally speaking, replace the in the plant kingdom more common anthocyanins (Bate-Smith 1962; Mabry 1964). However, the Molluginacaea and Caryophyllaceae, the only two families within the Caryophyllales, produce anthocyanins instead of betalains. There are multiple theories on why there are no plants with concomitant accumulation of betalains and anthocyanins. Since plants accumulating betalains also show the intact biosynthetic pathway up until leucoanthocyanins, it seems probable that the ancestors of modern Caryophyllales “lost” their anthocyanin synthase (ANS), an enzyme which catalyzes the oxidation of leucoanthocyanidins to their corresponding anthocyanidins (Clement and Mabry 1996; Grotewold 2006). More claryfiying, Shimada et al. (2007) reported that Caryophylalles do possess functional dihydroflavonol 4-reductase (DFR) and ANS when enzyme activity assays with recombinant proteins using cDNA from Caryophyllales were conducted. Furthermore, they showed that the promotor regions of *DFR* and *ANS* from anthocyanin-lacking species differ from those of anthocyanin-producing species. Thus, the difference in accumulation of anthocyanins or betalains may be explained by control on the transcriptional level, since recently the existence of a transcription factor was described, regulating betalain synthesis but not anthocyanin synthesis (Hatlestad et al. 2015).

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Fig. 1.: Portulac flowers; *Portulaca grandiflora* is an ornamental plant of the *Portulacaceae* which is known to accumulate betalains in their petals; Picture source: https://pim.selecta-one-shop.com/Pictures/Products/46_Portulaca/Mood/Hero_Sortiment_B%26B_Portulaca%20Oleracea.jpg

The base for betalain biosynthesis is the amino acid tyrosine. Tyrosine is hydroxylated by a tyrosinase resulting in precursor molecules of L-5,6-dihydroxy-phenylalanine (L-DOPA). The same tyrosinase or a PPO complex (DOPA oxidase) then forms DOPA-quinone in a ring cleavage which subsequently spontaneously converts into *cyclo*-DOPA (Delgado-Vargas and Paredes-Lopez 2002).

Cyclo-DOPA is a common intermediate in betalain producing plants. In parallel, a second molecule of L-DOPA is converted into 4,5-*seco*-DOPA by the enzyme DOPA-4,5-dioxygenase (DOD) in an extradiol aromatic ring cleavage. Again, 4,5-*seco*-DOPA subsequently converts non-enzymatically into betalamic acid, which then can undergo a condensation reaction with the imino group of *cyclo*-DOPA resulting in betanidin or the imino and amino groups of amino acids, resulting in the yellow betaxanthins. Glycosylation can occur either on betanidin, resulting in betanin, or prior of the condensation reaction on *cyclo*-DOPA with the intermediate of *cyclo*-DOPA-5-glycoside. Betanidin is the backbone of all betacyanins. A thorough overview of betalain biosynthesis can be found in the review by Khan and Giridhar (2015).

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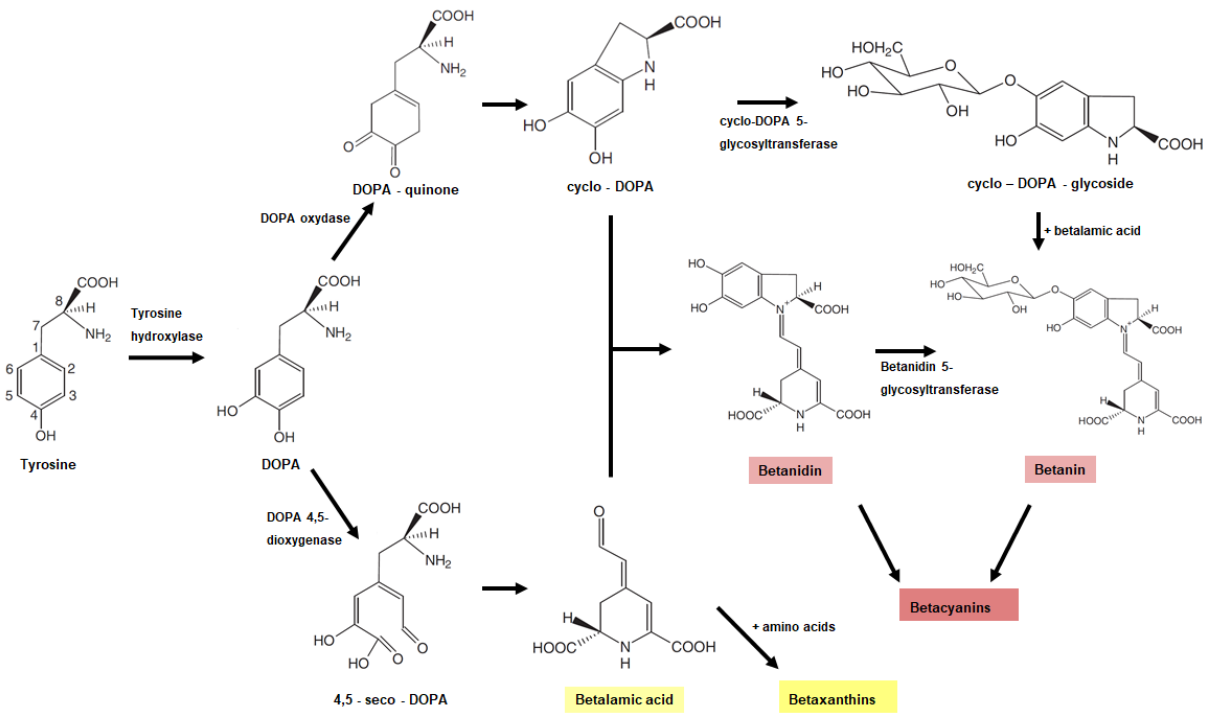

Fig. 2.: Schematic overview of Betalain biosynthetic pathway (Khan and Giridhar 2015)

In planta, betalains are accumulated in leafs, stems, roots, leafs, fruits, bracts and seeds where they seem to serve the same purpose as anthocyanins. However, the exact roles remain unclear (Brockington et al. 2011). There are contrary reports about light as a factor on the betalain accumulation. Ibdah et al. (2002) and Portillo-Nava et al. (2021) report enhanced induction of betalain biosynthesis upon high photosynthetic active radiation (PAR) and UV light or light in the blue and red light spectra, respectively. In contrast, there are studies stating a degrading effect of blue light on betacyanins (Chang-Quan and Tao 2006; Sawut et al. 2021) and improved betalain accumulation when plants are grown under dark conditions (Wang et al. 2006). This makes sense, since betalains are often found in tubers like red beet. Betalains are known to be heat sensitive. In general, thermal degradation at temperatures above 70 °C (Havlkov et al. 1983) or above 20 °C (S K Reshmi et al. 2012) is associated with color loss or browning due to isomerization, cleavage or dehydrogenation. The pH does not play such a vital role in betalain stability. However, pH below 2 and above 9 may have negative influence on the stability (S K Reshmi et al. 2012). Additionally, a bathochromatic shift can be observed above pH 8, with a peak absorbance at 400 – 460 nm (S K Reshmi et al. 2012).

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1.2.2. Carotenoids

Carotenoids are lipophilic C_{40} isoprenoid compounds which can carry epoxyl, hydroxyl and keto groups and may contain up to 15 conjugated C-C double bonds. More than 1,100 carotenoids are known today to be accumulated in plants, fungi, algae and animals (Avalos and Carmen Limón 2015; Maoka 2020; Quian-Ulloa and Stange 2021). Different configurations are possible due to the potential isomerism around the C-C double bonds. Additionally, rotation is possible about C-C single bonds, enabling carotenoids a vast number of potential configurations (Britton 1995). Carrying a tetraterpene backbone with an ionone ring at the end, carotenoids absorb visible light of the short wavelengths ranging from violet to green light. The number and position of the C-C double bonds determines the absorbed wavelength and thus the different carotenoids appear from yellow over orange to red. The length, shape, and *cis/trans* configuration of the polyene chain as well as the size and shape of the end groups have great impact on the properties and thus on the function of carotenoids in planta. Carotenoids can be divided in two subgroups: Carotenes (hydrocarbons lacking oxygen-containing functional groups) and xantophylls (hydrocarbons carrying oxygen-containing functional groups).

Fig. 3.: Autumn leaves and *Tagetes erecta*, two examples of carotenoids as source for different red and yellow color hues; Picture source autumn leaves: https://commons.wikimedia.org/wiki/File:Indian_Summer_im_Odenwald_-_panoramio.jpg; Picture source Tagetes erecta: https://cdn.shopify.com/s/files/1/0972/6282/products/crackerjack-marigold-flowers-pinetree-garden-seeds-953.jpg?v=1603980747

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Thanks to many studies in the last years, the carotenoid biosynthetic pathway is well understood (Howitt and Pogson 2006; Quian-Ulloa and Stange 2021; Sathasivam et al. 2021). The initial step of carotenoid biosynthesis is the condenstation of four C_5 isopentenyl diphosphate (IPP) to form the C_{20} geranylgeranyl pyrophosphate (GGPP). A head-to-head reaction of two GGPP results in the formation of the first C_{40} carotenoid structure, the colorless phytoene. This reaction is catalyzed by the enzyme phytoene synthase (PSY). The subsequent addition of conjugated C-C double bonds and the isomerization of *cis*- to *trans*- configurations yields the red-colored all-*trans*-lycopene which is the favored substrate for the downstream processes. Involved in these reactions forming lycopene are the two desaturases phytoene desaturase (PDS) steps, ζ-carotene desaturase (ZDS), ζ-carotene isomerase (Z-ISO) and carotene isomerase (CRTISO). Starting from *trans*-lycopene, two branches open. The β,β-branch, leading to β-carotene and its derivates, and the β,ε-branch, leading to α-carotene and its derivates, respectively. On the β,β-branch, β-rings are introduced on both ends of lycopene by the enzyme lycopene β-cyclase (βLCY). On the β,ε-branch, lycopene ε-cyclase first introduces an ε-ring on one end of lycopene, forming δ-carotene which is subsequently converted into α-carotene by βLCY by introducing a β-ring on the other end. Cunningham and Gantt (2001) showed in their study, that the ability to introduce either two β-rings or single ε-rings or β-rings is only determined by a single point mutation from histidine to leucin at position 457. Up to the biosynthesis of α-carotene and β-carotene, all product belonged to the subgroup of carotenes. α-carotene and β-carotene can be further modified to produce xanthophylles which are carotenoids containing oxygen. Characteristically for xanthophylles are hydroxy groups at position C3 or epoxy groups at the position 5,6 of the ionone ring. Hydroxylation on the β-ring is catalyzed by β-hydroxylase (β-OH) and hydroxylation on the ε-ring is catalyzed by ε-hydroxylase (ε-OH), respectively. Whilst β-OH is a non-haeme diiron monooxygenase, ε-OH is a cytochrome P450 monooxygenase (Tian et al. 2004). The mutual action of both hydroxylases leads to the formation of lutein with zeinoxanthin as an intermediate when only β-OH acted before ε-hydroxylation. β-OH acting twice in the β,β-branch leads to the formation of zeaxanthin with the intermediate of β-cryptoxanthin. Lutein, a 3,3'-dihydroxy-α-carotene, is the end product of the β,ε-branch whereas zeaxanthin can be further modified to violaxanthin. Zeaxanthin epoxidase (ZEP) introduces 5,6-epoxy groups via antheraxanthin as an intermediate. It is known that under heavy light stress violaxanthin can be de-epoxidized back to zeaxanthin by

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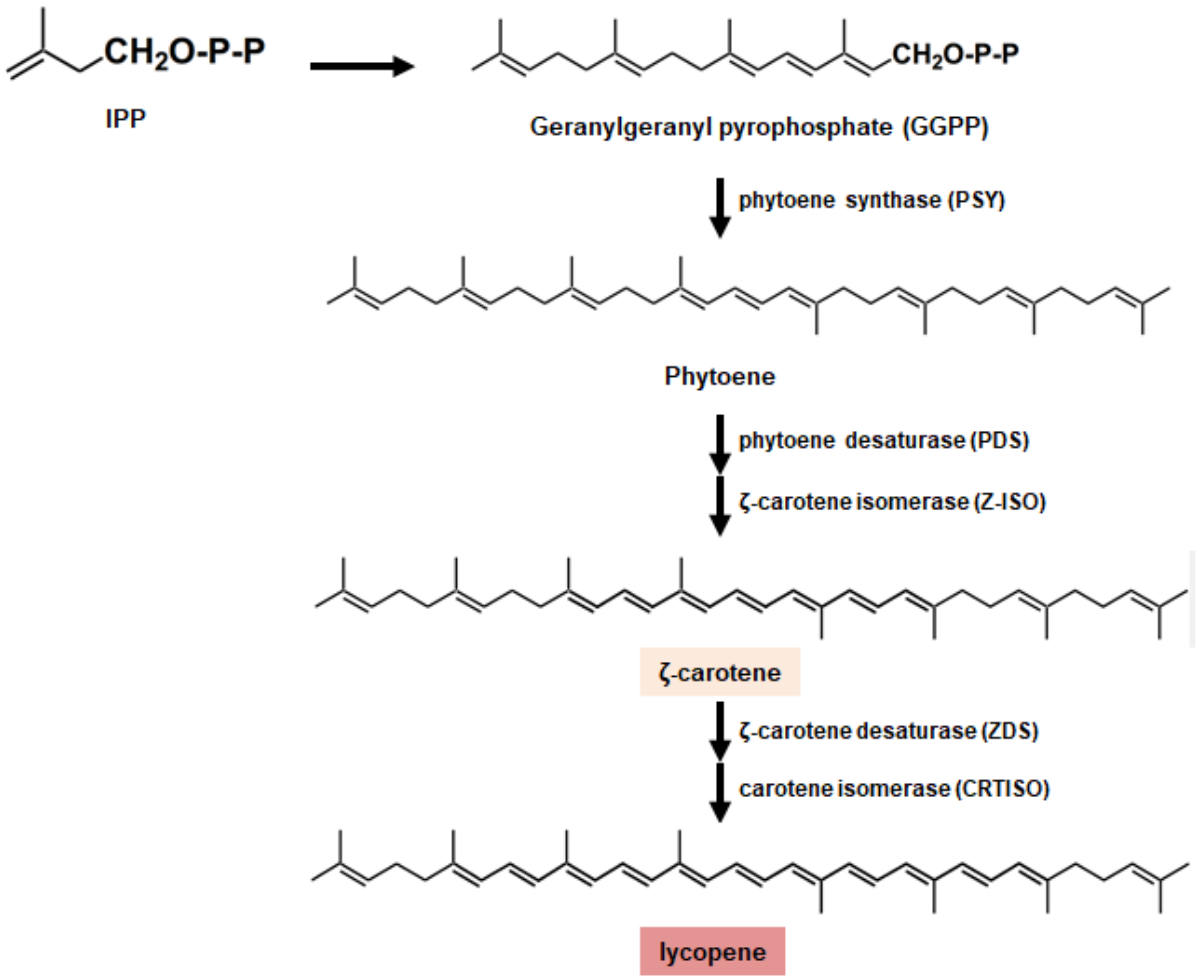
the catalytic action of violaxanthin de-epoxidase (VDE) (Pfündel and Bilger 1994). Furthermore, both violaxanthin and antheraxanthin can be converted to the red colored capsanthin and capsorubin, respectively by capsanthin-capsorubin synthase (CCS) (Hirschberg 2001). The last carotenoid in the β,β-branch, neoxanthin, is produced by the conversion of violaxanthin via neoxanthin synthase (NXS). Both neoxanthin and violaxanthin can be substrates for the biosynthesis of the plant hormone abscisic acid (ABA).



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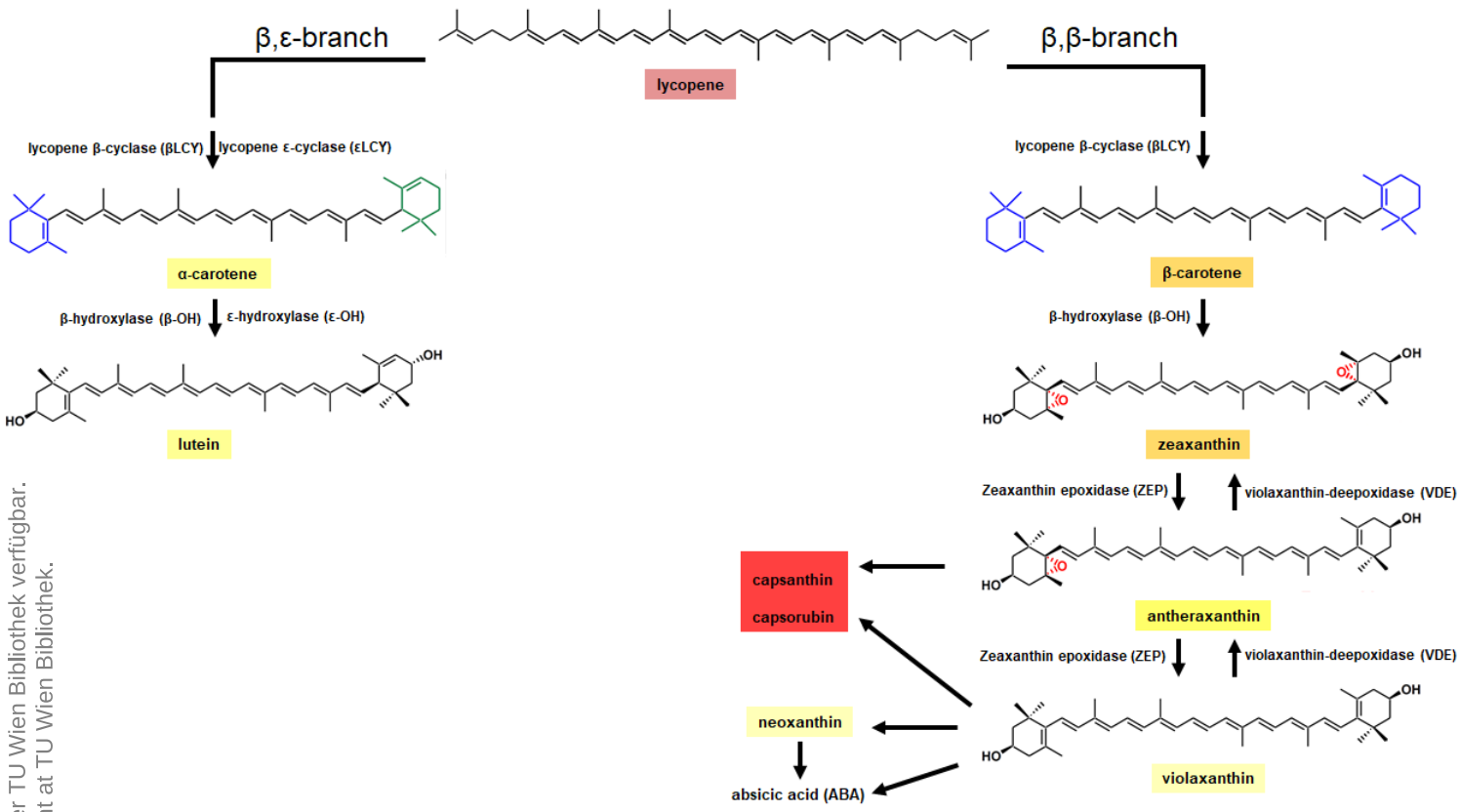

Fig. 4.: Schematic overview of carotenoid biosynthesis; note the different colored ε-rings (green) and β-rings (blue);

The main cell compartment for storing carotenoids are chromoplasts, cell organelles belonging to the plastids. However, the amounts and prevalence of specific carotenoids highly differs between plants and tissues of the same plants (Howitt and Pogson 2006). Depending on the plant and tissue, carotenoids play different roles in the organism. Alone or in combination with other (yellow) pigments, carotenoids act as coloring pigments in flowers and fruits as attractants for insects and birds as pollinators. In yellow-colored cultivars of *Bidens ferulifolia*, the flower petals accumulate both anthochlors as main pigments and carotenoids as minor pigments (Walliser et al. 2022). The local distribution of the two different pigments is separated with carotenoids (mainly lutein and β-carotene) evenly distributed over the petal and anthochlors primarily accumulated in the petal base. This distribution in combination with the different light absorbance of the different pigment classes is utilized by the plant in the creation of nectar guides (Miosic et al. 2013). But not only local distribution of different carotenoids has been reported, but also accumulation of different carotenoids depending of the developmental stage. Oliveira et al. (2003) showed that some xantophylles (violaxanthin, lueoxanthin and 5,6-epoxy-lutein) accumulate in high

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amounts in ripe grapes, whereas the same xanthophylles could not be detected prior ripening. Whilst many of the prevalent carotenoids found in flower petals are xantophylles and β-carotene (Ohmya 2011), some plants accumulate “exotic” and particular carotenoids. For example, the Asiatic hybrid lily (*Lilium* spp.) accumulates capsanthin in its red tepals, a carotenoid commonly found in the red fruits of *Capsicum anuum* (Yamagishi et al. 2010) whereas astaxanthin, a red carotenoid synthesized by algae, is also expressed in *Adonis aestivalis* (Maoka et al. 2011). Besides their function of attracting pollinators, carotenoid pigments play a vital role in photoprotection. Rate of photosynthesis is dependent on light intensity. Too high light intensity leads to production of harmful free radicals due to the limited photosynthetic capacity of excited chlorophyll molecules (Szabó et al. 2005). In order to avoid the production of harmful free radicals (such reactive oxygen species like $^1O_2$) plants utilize the xantophyll cycle to retain excess energy. As already mentioned earlier, violaxanthin-deepoxidase (VDE) reduces the diepoxide violaxanthin over the monoepoxide antheraxanthin to zeaxanthin. VDE is an enzyme with a pH optimum of 5.0 and is activated when the pH in the lumen of chloroplasts is lowered enough due to the inability of the plant under high light intensities. High light intensity leads to a surplus of protons in the lumen, which are normally used to produce ATP, since ATP synthase is inhibited due the high ATP production in contrast to its demand. Since only violaxanthin is capable of passing photons to chlorophyll, antheraxanthin and zeaxanthin disperse the energy of photons in form of heat (Baker 2008).

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1.2.3. Flavonoids

Flavonoids belong to the widely abundant class of phenylpropanoids. With over 8.000 metabolites and an estimated carbon flux of 20 %, flavonoids are by far the largest and member of this group and play an important role in the plant's metabolism (Yonekura-Sakakibara et al. 2019). As members of the polyphenol family, they carry the common C_6-C_3-C_6 diphenylpropane backbone comprised of two aromatic carbon rings (named ring A and B) combined by a three-carbon linking chain (Nabavi et al. 2020) (Fig. X). This three-carbon linking chain forms a third ring (C-ring) in many flavonoids. The absence of the C-ring is characteristic for chalcones which are precursors of the anthocyanin pathway and its intermediates. The high prevalence of flavonoids structures is based on the extensive modification of the flavonoid base structures. Additional hydroxyl groups on the rings A and B, which can be further glycosylated, acetylated and methylated, are responsible for the high abundance and differences in physical and biological functions (Halbwirth 2010). The class of flavonoids is divided into six major subgroups: chalcones (which can be also grouped into the group of anthochlor pigments), flavones, flavonols, flavandiols, anthocyanins and proanthocyanins. Less abundant are the legume isoflavonoids (Du et al. 2010), 3-deoxyanthocyanins (Dykes 2019; Liu et al. 2021) or stilbenes (Chong et al. 2009).

Fig. 5.: Different *Euphorbia pulcherrima* and *Petuni hybrida* cultivars, expressing different colored bracts and petals due to different anthocyanin composition; source of pictures of poinsettias: https://pim.selecta-one-shop.com/Pictures/Products/03_Poinsettia/Mood/Christmas%20Feelings/Poinsettia_FeelingsFamily_Mood%20%286%29.jpg; source of pictures of petunias: https://pim.selecta-one-shop.com/Pictures/Products/07_Petunia/Mood/SKYfamily/SkyFamily.jpg

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Flavonoid biosynthesis is one of the most well understood biosynthetic pathway in plants and controlled by internal (e.g. circadian clock) and external factors (e.g. light, temperature, salt stress, pathogens, etc.) (Koes et al. 2005). The structural genes involved in anthocyanin biosynthesis are mostly regulated on the transcriptional level by R2R3 MYB, basic-helix-loop-helix and WD40 repeat proteins (Schwinn et al. 2006; Ohno et al. 2011a). The precursor molecules of flavonoid biosynthesis are malonyl-CoA and *p*-coumaroyl-CoA. Whilst *p*-coumaroyl-CoA is derived from phenylalanine involving the enzymes phenylalanine ammounium lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumaroyl:CoA ligase (4CL), whereas malonyl-CoA originates from the carbohydrate metabolism. The first step of the flavonoid pathway is the condensation reaction of one molecule *p*-coumaroyl-CoA with three molecules of malonyl-CoA, catalyzed by the enzyme chalcone synthase (CHS) and located in the cytosol, forming the yellow colored tetrahydroxychalcone (THC) or naringenin chalcone. THC is not stable, so it either isomerized spontaneously in some species or enzymatically through the enzymatic action of chalcone isomerase (CHI) to the colorless flavone naringenin. A malfunctioning CHI is the cause for yellow *Dianthus caryophyllus* by blocking the downstream production of anthocyanins and their intermediates and enhanced accumulation of naringenin-chalcone-2'-glycoside (Forkmann and Dangelmayr 1980). Naringenin can now be further converted to the di-hydroxylated eriodictyol by flavonoid 3'-hydroxylase (F3'H) or to the tri-hydroxylated pentahydroxyflavanone (PHF) by flavonoid 3'5'-hydroxylase (F3'5'H). F3'H and F3'5'H are also capable of performing the hydroxylation on flavones, flavonols and leucoanthocyanins (Halbwirth 2010). As mentioned above, the hydroxylation pattern has a huge impact on the properties of the pigment. In the case of anthocyanins, the pigment color is the most relevant property (Nitarska et al. 2018). Flavanone 3-hydroxylase (F3H or FHT) introduces a hydroxy group at position C3, resulting in the hydroxylation of naringenin, eriodictyol and PHF to dihydrokaempferol (DHK), dihydroquercetin (DHQ) and dihydromyrecitin (DHM), respectively. The dihydroflavonols are subsequent reduced to their corresponding leucoanthocyanidins by dihydroflavonol 4-reductase (DFR). The NAPDH dependent oxidoreductase mostly accepts all dihydroflavonols as substrates, but studies showed that there are DFR that do not accept DHK, leading to a lack of bright red flowers in *Petunia hybrida* (Winkel-Shirley 2001; Haselmair-Gosch et al. 2018). Leucoanthocyanidins are then converted to their corresponding anthocyanidins through the enzymatic activity of anthocyanidin

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synthase / leucoanthocyanidin dioxygenase (ANS / LDOX). Since anthocyanidins are unstable under normal conditions, anthocyanidins can be modified by glycosylation, acetylation, methylation or sulfation reactions. Especially the glycosylation of the hydroxyl group at position C3 is regarded as an essential step in anthocyanidin biosynthesis, since it stabilizes the pigment and allows transportation and storage in the vacuole (Mazza and Brouillard 1987; Gosch et al. 2010). The described biosynthetic pathway only shows the steps in anthocyanidin production. There are many branches leading to the production of other flavonoid compounds and related classes such as anthochlor pigments, isoflavonoids, flavones, flavanols, catechins and others. Flavones are products of flavone synthase (FNS), which converts naringenin, eriodictyol or PHF to apigenin, luteolin or tricetin, respectively. DHK, DHQ or DHM are converted to kaempferol, quercetin or myricetin by flavonol synthase (FLS). The direct competition between FLS and DFR for the same substrates has direct impact on content of anthocyanidins. Thrill et al. (2012) showed that a decline in FLS activity results in higher anthocyanidin accumulation.

Flavonoids play multiple important roles in plants. The biological activity of flavones depends highly on their molecular structure. As one of the most obvious functions for humans is their role in attracting pollinators as colored pigments in flowers and bracts. In contrast to chalcones and anthocyanins, which are colored pigments, flavones, flavanones and flavonols are colorless and act as stabilizers for anthocyanins as co-pigments, since anthocyanins are susceptible to oxidation and hydroxylation (Brouillard and Dangles 1994/1988). There is a multitude of literature on flavonoids in general and anthocyanins in special as primary colorizing pigments in ornamental plants such as *Dahlia variabilis* (Halbwirth et al. 2008; Ohno et al. 2011a; Ohno et al. 2011b; Thill et al. 2012; Ohno et al. 2018b), *Euphorbia pulcherrima* (Horn et al. 2012; Nitarska et al. 2018) and *Petunia hybrida* (Meyer et al. 1987; Davies et al. 1998; Koseki et al. 2005; Haselmair-Gosch et al. 2018) or food plants like apples (Espley et al. 2007; Wang et al. 2015), tomatoes (Slimestad and Verheul 2009), pineapples (Hossain and Rahman 2011) and many more (Review by Terahara (2015). The hydroxylation patterns of the B-ring are leading to different absorption of light and thus to different perception of the resulting coloration. The common hydroxylation patterns are 4'-, 3'4'- or 3'4'5'-hydroxylation, corresponding to the anthocyanins pelargonidin (bright red),

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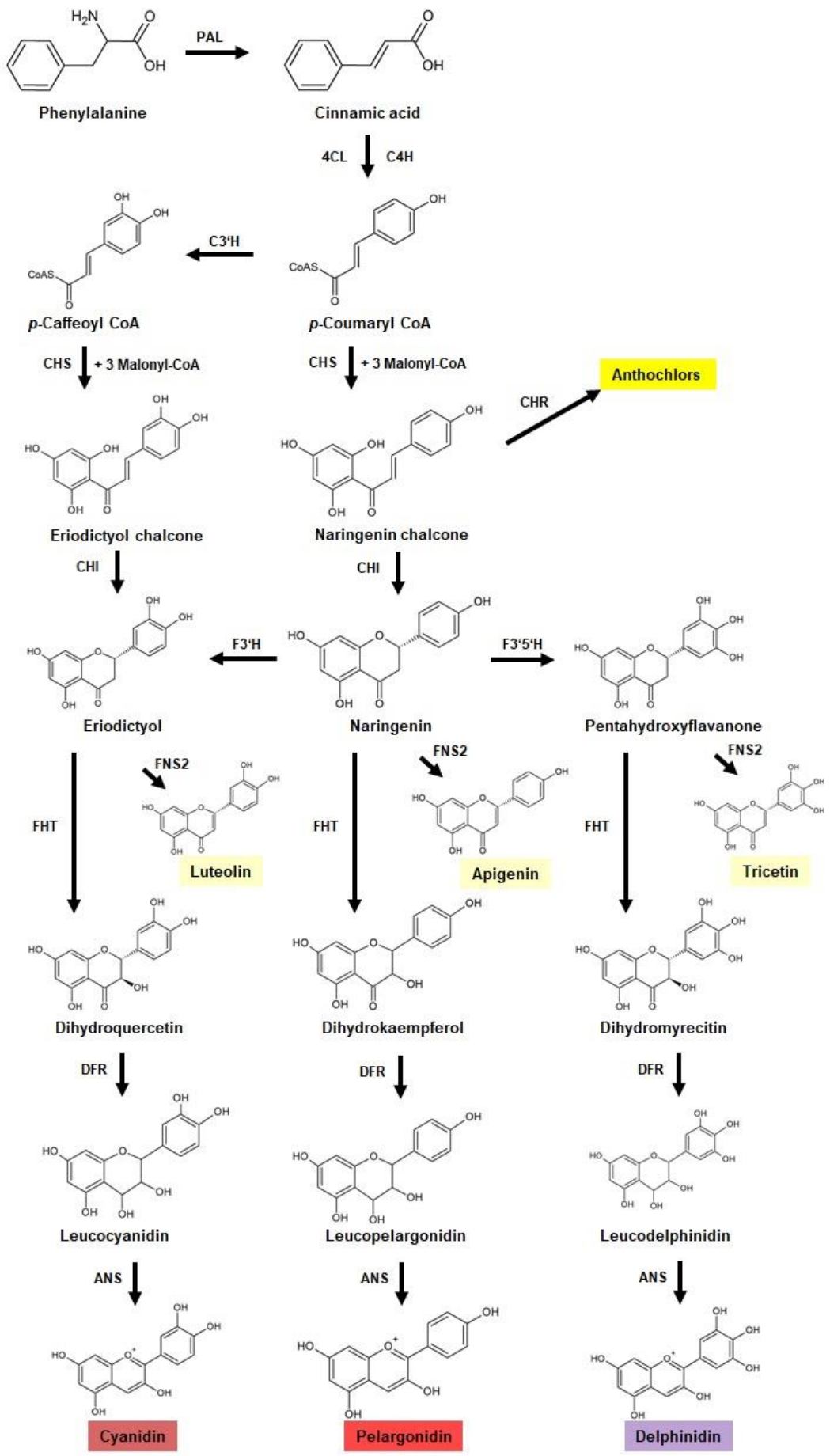

Fig. 6.: Overview of the flavonoid biosynthetic pathway

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cyanidin (dark red) and delphinidin (purple). Though, carotenoids are the most common yellow flower pigment, some flavonols beside the related anthochlor pigments contribute to yellow flowers in plants of the *Asteraceae* family. The cytochrome P450 dependent monooxygenase flavonol 6-hydroxylase, found in the yellow petals of *Tagetes erecta* and *Tagetes patula*, is capable of introduction a hydroxy group at position 6 of the colorless flavonol quercetin (Halbwirth et al. 2004).

Flavonoids also act as signaling molecules in the communication between plants and soil bacteria symbiosis (Fisher and Long 1992; Abdel-Lateif et al. 2012). Additionally, flavonoids play an important role in warding off pathogenic microorganisms in plants as phytoalexins (Ndakidemi and Dakora 2003; Bhattacharya et al. 2010). Depending on the molecular structure and hydroxylation, flavonoids show efficient antioxidative properties and UV light protection. Most harmful is the energy-rich UV-B light, affecting photosynthesis and transpiration and causing DNA and cellular damage (Reboredo and J. C. Lidon 2012). Flavonoids located in the outer epidermal cell layers provide an effective filter against UV-B radiation without hindering photosynthesis (Caldwell et al. 1983). Furthermore, due to their antioxidant capacity owed to their structural features, flavonoids can directly scavenge harmful free radicals such as H_2O_2, OH- or other reactive oxygen species. To show in an exemplary way, the flavonol quercetin features a 3',4'-dihydroxy structure at the B-ring, a 2,3-double bond in the C-ring (allows electron delocalization) and a total of 5 OH-groups resulting in significant electron donor ability (Amalesh Samanta et al. 2011). Further functions of flavonoids include involvement in seed germination, temperature acclimatization and pathogen and herbivore protection (Amalesh Samanta et al. 2011; Sharma et al. 2019).

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1.2.4. Anthochlor pigments

As already mentioned in chapter 1.2.2 (carotenoids), some plants, especially species from the Asteraceae family, accumulate a second class of yellow-colored pigments, the so-called anthochlors or anthochlor pigments. Anthochlor pigment is an umbrella term for the sub-classes of chalcones and aurones. Anthochlors share the same precursor molecules with pigments of the class of flavonoid and therefore are considered as part of the flavonoid family. However, whereas flavonoids mostly contribute colorless co-pigments and red or at least pale-yellow pigments to flower coloration, anthochlors are bright yellow. Anthochlors can generally be distinguished into two groups, defined by the presence or absence of a hydroxyl-group at the 6'-position of the A-ring for chalcones or at position 4 at the B-ring for aurones, respectively (Fig. 8).

Fig. 7.: *Bidens ferulifolia* and *Dahlia variabilis*, two model plants for anthochlor biosynthesis

The exact pathway of anthochlor biosynthesis was a topic of research for long time and has not been completely elucidated until today (reviewed in Boucherle et al. (2017). Since (hydroxy-)chalcones are the intermediates for flavonoid biosynthesis, involved enzymes are identical to those described in chapter 1.iii. Subsequent to the condensation reaction of one molecule *p*-coumaroyl-CoA with three molecules of malonyl-CoA catalyzed by the enzyme chalcone synthase (CHS) the unstable naringenin chalcone (2',4',6',4-tetrahydroxychalcone) is formed. This naringenin chalcone, light yellow to the naked eye, is highly unstable and quickly is isomerized

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either chemically or enzymatically through CHI to the colorless naringenin. Due to the chemical instability of naringenin chalcone, it is unlikely that it accumulates *in planta* in sufficient quantities to contribute to the flower coloration. Besides isomerization, the hydroxy-halcones can be either directly processed its corresponding hydroxytype aurone or converted to stable deoxy-type chalcones with potential subsequent conversion to their corresponding deoxy-type aurones. The reaction step from hydroxychalcones to deoxychalcones is not fully understood up today. Mutual activity of CHS with chalcone reductase (CHR), a rare enzyme belonging to the aldo-keto reductase (AKR) 4A superfamily and first described in plants of the Leguminosae family, leads to the production of 6'-deoxychalcones (Ayabe et al. 1988; Welle and Grisebach 1988; Bomati et al. 2005). The proposed mode of action includes the reduction of the non-linear polyketide intermediate during the enzymatic reactions of CHS. Bomati et al. (2005) concluded in their study, based on x-ray crystallography, kinetic analyses and binding studies, that the linear coumaryl-derived polyketide-CoA intermediate may be a substrate for CHR, but the coumaryl-trione of the non-linear intermediate favors a better docking in the active site of CHR. Regardless of the mode of action, the final product of mutual activity of CHS/CHR is the chemically stable 6'-deoxychalcone isoliquiritigenin (2',4,4'-trihydroxychalcone). In legumes, isoliquritigenin is an intermediate for isoflavone biosynthesis, so CHR activity is an important branch point for the production of phytoalexins and communication with symbiotic bacteria, leading to a variety of different structures even within one legume species (Bomati et al. 2005; Sepiol et al. 2017). As a stable deoxychalcone, isoliquiritigenin can accumulated in plants and thus can contribute to flower coloration. Enzymatic isomerization as it happens of 6'-hydroxychalcones requires a CHI with broad substrate specific. Such a CHI was so far only reported in plants of the *Fabacae* family (Kimura et al. 2001). Additionally, chemical isomerization occurs at a much lower rate compared to 6'-hydroxychalcones (Miles and Main 1985, 1989). Together with isoliquiritigenin, the deoxychalcone butein (2′,3,4,4′-tetrahydroxychalcone) contributes to yellow flower coloration mostly in *Asteraceae* (Bohm 1988). The conversion from isoliquiritigenin to butein includes the introduction of a second hydroxy group at position 3 in the B-ring by the catalytic action of the cytochrome P450 monooxygenase chalcone 3-hydroxylase (CH3H). In *Dahlia variabilis*, an allelic variation of F3'H shows chalcone 3-hydroxylase activity, suggesting the evolution of CH3H from F3'H (Schlangen et al. 2010a; Schlangen et al. 2010b). Besides chalcones, aurones

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complete the class of anthochlors. Aurone formation was primarily studied in *Antirrhinum majus*, which accumulates 4-hydroxyaurones. It has been shown that in *Antirrhinum majus,* naringenin chalcone is converted to the 4-hydroxyaurone aureusin by B-ring hydroxylation and oxidative cyclization catalyzed by the enzyme aureusidin or aurone synthase (AUS) (Nakayama et al. 2000; Nakayama et al. 2001; Sato et al. 2001; Ono et al. 2006a). Additionally, *Am*AUS shows a higher preference for eriodyctiol chalcone (2',4',6',3,4-pentahydroxychalcone) compared to naringenin chalcone (Nakayama et al. 2001). The conversion of eriodyctiol chalcone leads to the formation of the aurone bracteatin. Furthermore, *Am*AUS shows a higher preference for 6'-hydroxychalcones than to 6'-deoxychalcones. Recently, 4-deoxyaurone formation is adopted primarily in *Asteraceae* and was center of research in *Coreopsis grandiflora* (Kaintz et al. 2014) and *Bidens ferulifolia* (Miosic et al. 2013). Molitor et al. (2015) characterized the AUS from *Coreopsis grandiflora* (*Cg*AUS) first as a catechol oxidase, but was later found to be a tyrosinase-like enzyme with hydroxylase activity parallel to *Am*AUS (Molitor et al. 2016). However, in contrast to *Am*AUS which seems to be localized in vacuoles *Cg*AUS is located in the chloroplasts as structural analysis suggest (Molitor et al. 2016). Additionally, activity of *Am*AUS reaches its optimum around pH 5, whereas *Cg*AUS is allosterically activated at physiological pH (Molitor et al. 2015). Chalcones and aurones can undergo subsequent modification including glycolyzation, methylation or methoxylation (Boucherle et al. 2017).

In the past, yellow colors have been mostly contributed to carotenoids but in 1920, Gustav Klein firstly described yellow pigments which he named anthochlors and which differed from known yellow pigments by their color shift when exposed to an alkaline environment. Later, more yellow pigments with the same properties were described in *Cosmos sulphureus* (Geissman 1942; Geissman and Jurd 1954), *Coreopsis grandiflora* (Geissman and Heaton 1943), *Antirrhinum majus* (Geissman et al. 1954). Except for *Antirrhinum*, most of the plants in which anthochlors were detected belong to the family of *Asteraceae*. Other *Asteraceae* accumulating anthochlors include plants of the genus *Bidens* (Scogin and Zakar 1976; Deba et al. 2008; Miosic et al. 2013; Walliser et al. 2022), *Dahlia variabilis* (Nordström and Swain 1956; Harborne et al. 1990; Wimmer et al. 1998; Halbwirth et al. 2008; Thill et al. 2012; Ohno et al. 2018a; Walliser et al. 2021; Ohno et al. 2021), *Helianthus anuus* (Schilling et al. 1987; Alfatafta and Mullin 1992), *Viguiera*, *Baeria* (Shimokoryama and Geissman 1960), *Mussaenda* or *Zinnia* (Harborne et al. 1983). But also, in plant genus other than *Asteraceae,*

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anthochlors have been reported. Ahmed et al. (2011) reported, that the otherwise *Asteraceae* exclusive sulfuretin and its aglycone sulfurein were found in *Butea monosperma,* a member of the *Fabaceae* family. *Dianthus caryophyllus* , a member of the *Caryophyllaceae*, accumulates hydroxychalcones due to a defective CHI and glycolyzation of naringenin chalcone (Forkmann and Dangelmayr 1980). The production 4'-hydroxyaurones was also achieved by (Hoshino et al. 2019) in *Ipomoea nil* by insertion of *Am*AUS and a chalcone 4'-glucosyltransferase from *Antirrhinum majus*.

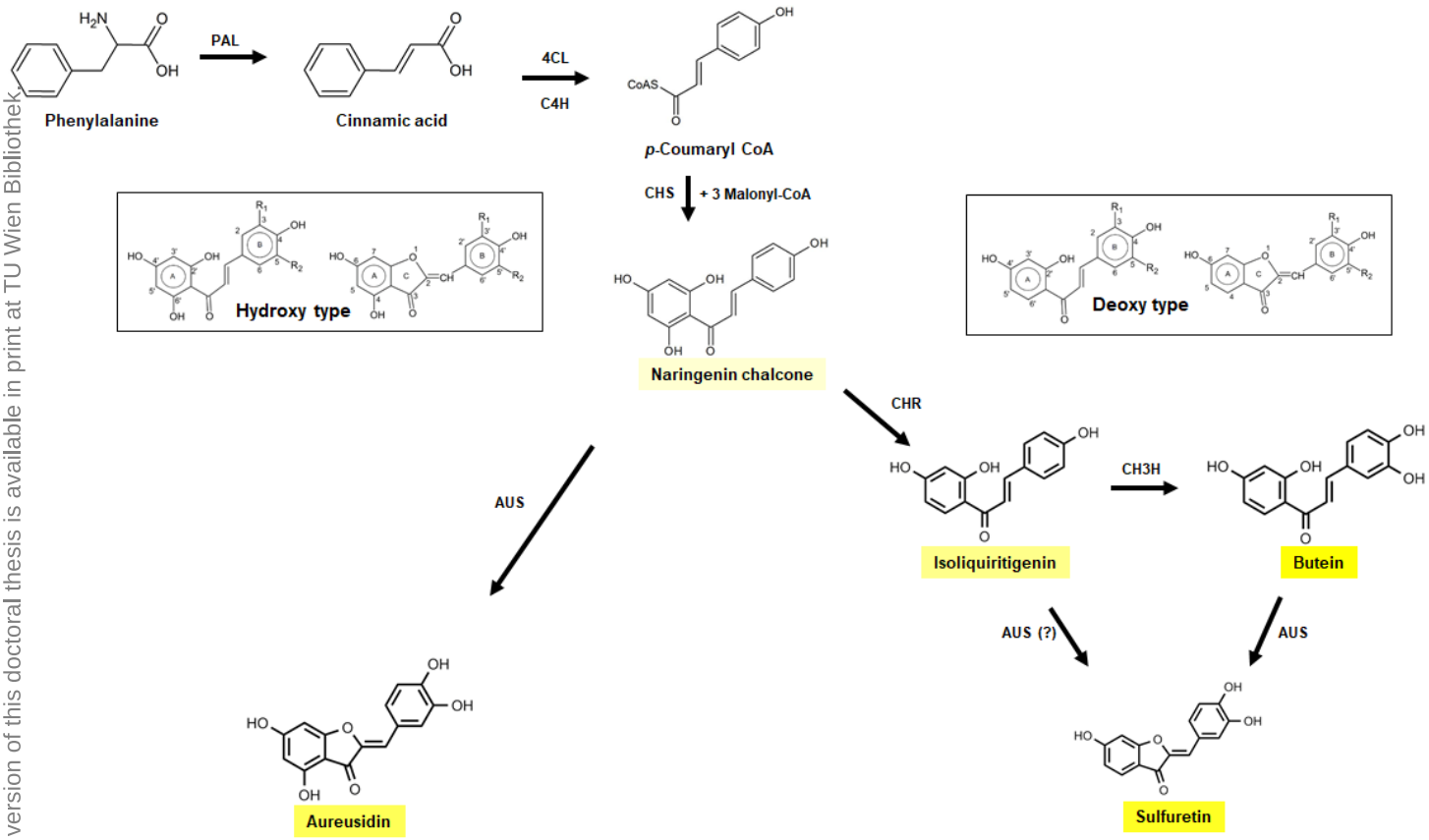

Fig. 8.: Schematic overview of the anthochlor biosynthetic pathway; please note the different hydroxylation at position C6' for chalcones and position 4 for aurones, respectively

Aurones are the brightest yellow pigments derived from the flavonoid family compared to chalcones (still a bold yellow), flavonols (pale yellow), flavones, isoflavones, flavanones (colorless) or anthocyanins (red to purple) and plays a vital role in attracting pollinators. Yet, this property alone is shared with other yellow pigments such as carotenoids which are more common throughout the plant kingdom as attracting pigment. Though anthochlors and carotenoids are often accumulated concomitantly

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and may appear uniform yellow to the human eye, the “hidden” synergy of aurones and carotenoids is a key feature of anthochlor accumulation. Characteristic to anthochlors is their high UV-absorbance (Mabry et al. 1970). For example, the difference in UV absorbance maxima lies between maritimetin (~413 nm) and okanin (~382 nm) (the in *Bidens ferulifolia* predominant aurones (Miosic et al. 2013; Walliser et al. 2022)) on the one hand and lutein (~445 nm) on the other hand (Scott 2001; Boucherle et al. 2017). This bathochromatic shift leads to a distinctive perception of UV reflection for UV sensitive organisms. Plants utilize this phenomenon to create nectar guides, sharply contrasted patterns in flowers which are known to attract pollinators (Harborne and Smith 1978; Schlangen et al. 2009; Rieseberg and Schilling 1985). Some plants such as *Antirrhinum majus* combine UV nectar guides with colorful patterns also visible to human eyes (Davies et al. 2006) or can express both phenotypes depending on genetics in different varieties of the same species such in *Rudbeckia hirta* (Schlangen et al. 2009) *Coreopsis gigantea* (Scogin et al. 1977) or *Bidens ferulifolia* (Walliser et al. 2022). Besides their role in attracting UV-sensitive pollinators, anthochlors can act as effective protection against photo-oxidative damage caused by reactive oxygen species. Since their structure is similar to other flavonoids which are known to play vital roles in UV protection (such as flavonols and flavanols), 3’,4’-dihydroxylated aurones like aureusidin, sulfuretin or maritimetin possess a comparable radical scavenging potential. This thesis was confirmed by studies performed by Nenadis and Sigalas (2011), Vagánek et al. (2012), Lin and Chang (2013) or Luo et al. (2014). Furthermore, aurones were found in photosynthetic active tissue of *Artocarpus altilis* (Mai et al. 2012), *Flemengia strobilifera* (Nigam and Saxena 1975) and *Cyperus scariosus* (Bhatt et al. 1981) and AUS activity was verified in raw extracts of leaves from *Bidens ferulifolia* and *Antirrhinum majus* (Miosic et al. 2013). Aurones and phytoalexins derived from aurones, such as hispiridol or cephalocerone, have been reported with antifeedant, anti-bacterial, anti-fungal and anti-inflammatory properties (Treutter 2006; Sutton et al. 2017; Kumar et al. 2020; Sui et al. 2021; Mazziotti et al. 2021).

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1.3. Molecular Breeding

1.3.1. Desired traits in ornamentals

In 2018, 191.7 million hectars of genetically modified crops were grown, with maize, soybeans, cotton as the “global” favorites (ISAAA 2018). Agricultural field crops take the lion’s share both for the area grown with it and the number of approvals. Indeed, from 4,411 approvals scattered among 409 events in 30 crops accumulated between 1992 and 2018 in 43 countries, only 62 approvals from 22 events in three crops belong to ornamentals. Nonetheless, genetic modification of ornamentals rises attention. Customers demand for new and exciting plants delivers the stimulation for breeders to improve genotypes with better post-harvest life, altered morphology, new flower color or other novel traits. Furthermore, additional traits specially interesting for growers are production related traits such as increased resistance to pests, pathogens or environmental factors or shorter production times (Milosevic et al. 2015).

Flower color, structure and fragrance belong to the most important traits of ornamental plants in the eye of customers and hence in ornamental plant breeding. However, the color range of flower color is subjected to the genetics of the plant species (Debener and Winkelmann 2010). *Petunia hybrida* was the first ornamental plant which was subjected to genetic engineering (Horsch et al. 1985). Meyer et al. (1987) introduced a dihydroflavonol 4-reductase (DFR) from maize, resulting in transgenic plants with orange-colored flowers. This coloration is a result of pelargonidin accumulation, which naturally does not occur in wild-type petunias since their DFR does not accept dihydrokaempferol (DHK), a precursor molecule to pelargonidin, as a substrate. Years later, a novel orange petunia appeared on the market and quickly found its way into private and public gardening in Europe and the US. Recently, the transgenic background and their origin of the Meyer transformation was enlighten (Bashandy and Teeri 2017; Haselmair-Gosch et al. 2018; Haselmair-Gosch et al. 2020). The elucidation of the biosynthetic pathways of pigments like anthocyanins and other flavonoids (Tanaka et al. 2005; Tanaka et al. 2008; Tohge et al. 2017), carotenoids (Hirschberg 2001; Tanaka et al. 2005; Ohmya et al. 2019) and betalains (Brockington et al. 2011; Khan and Giridhar 2015) opened the door for alterations in pigment composition by biotechnological methods. Potential approaches include the introduction of novel genes and complete biosynthetic pathways independent of the presence in the own species (Tanaka et al. 2010; Ohno et al. 2022; Ono et al. 2006a),

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over upregulating genes or suppressing biosynthetic pathways which are already present in the target plant (Fukusaki et al. 2004; Chen et al. 2011) and altering present pathways by genome editing of involved genes (Nitarska et al. 2021). For example, Ohno et al. (2022) introduced a chalcone reductase from *Dahlia variabilis*, a chalcone 4'-glycosyltransferase from *Antirrhinum majus* and the MYBA transcription factor from *Capsicum anuum* into *Nicotiana benthamiana*, which resulted in the induction of the 6'-deoxychalcone biosynthetic pathway. A similar approach did we adopt when we conceptualized this thesis (Chapter 4d: Introduction of genes involved in anthochlor biosynthesis). Tsuda et al. (2004) published in their study multiple approaches. By suppressing chalcone synthase A by antisense RNA in *Petunia hybrida*, they received white phenotypes. Suppression of flavanone 3-hydroxylase and dihydroflavanol 4-reductase resulted in star-shaped and spotted phenotypes. Furthermore, suppression of flavonoid 3'5'-hydroxylase, normally necessary for delphinidin-type (blue) flowers, resulted in red petunias with a cyanidin-type pigment profile. Similar to Meyer et al. (1987), the introduction of a DFR with dihydrokaempferol-acceptance from roses in combination with suppressing flavonoid 3' hydroxylase via RNAi lead to bright red petunias. Another approach resulted in pale flowers after the endogenous flavonol synthase was suppressed, creating a bottleneck for intermediates of the downstream anthocyanin pathway. Lastly, in order to examine the effect of altered co-pigmentation, a *Torenia* flavone synthase 2 was introduced, elevating flavone/anthocyanin ratio and ultimately resulting in bluer petunias. Summarizing, Tsuda et al. (2004) delivered a great overview of the possible ways in single or mutual approaches of introducing new genes and suppressing endogenous genes.

1.3.2. Plant transformation

In 1866, a Czech friar achieved one of the greatest milestones in the history of agriculture (Mendel 1866). Unnoticed by the people of his time, Gregor Mendel's work in his monastery garden laid the foundation to modern plant breeding. Nearly 90 years later, Watson, Crick and Franklin unraveled the enigma of biochemical genetics, endowing scientists with one of the most powerful tools in breeding (Watson and Crick 1953; Klug 1968). Besides conventional techniques such as selection breeding, combination breeding, heterosis breeding, hybrid breeding or mutation breeding, molecular approaches gained more relevance in the past thirty years (Schlegel 2018).

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One of the most limiting factors of conventional breeding, besides the time- and labor-intensive work and randomness of the outcome within the Mendelian laws, are species boundaries. Only in two species which show crossability, an exchange or combination of traits is possible. Using molecular breeding, we are now capable of interchanging genes crossing species boundaries. Simplified, molecular breeding can be divided into two sub-areas which are inherently different but also are dependent on each other to lead to considerable results. On the one hand, there are the typical molecular biological methods like polymerase chain reactions (PCR), cloning, isolation of nuclein acids, etc. which are needed to isolate and characterize genes which then on the other hand need to be introduced into new host organisms utilizing the areas of transformation and regeneration of transgenic plants using *in-vitro* tissue culture. Transformation, as the gene transfer is also called, can be directly or indirectly achieved. Direct gene transfer is mostly performed using physical principles, whereas indirect gene transfer relies on biological principles. Direct gene transfer approaches include particle bombardment via gene gun, electroporation, micro-injection and protoplast fusion. First introduced 1987 by Klein et al. (1987), the gene gun quickly became the tool of choice for introducing nucleic acids into cells of monocots since *Agrobacterium*-mediated transformation was not feasible in those plants. During particle bombardment, microprojectiles of dense material such as tungsten or gold are coated with DNA and accelerated at velocities which enables the projectiles to enter the target tissue. Since then, many protocols emerged for transformation of different plant species such as papaya (Yue et al. 2022), rice (Sarangi et al. 2019; Mukhtar 2018), sugarcane (Gao et al. 2018), wheat (Hamada et al. 2017) and many more (Ozyigit and Yucebilgili Kurtoglu 2020). Besides stable introduction, particle bombardment can be used for inducing transient gene expression (Fig. 9).

Fig. 9.: Fluorescence picture of *Dahlia variabilis* petals after particle bombardment with a construct contaiing the reporter gene dsRED

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Electroporation is widely used for transformation of bacteria (Mattanovich et al. 1989) but can also be used for gene transfer in plants. The principle of electroporation is based on the temporary permeability of cell membranes for macro molecules such as DNA when treated with a pulse strong electrical current (Kotnik et al. 2015). However, since this method is highly stressful for organisms, electroporation finds rarely use in plant transformation. Micro injection is a very labor-intensive method for introducing DNA directly and manually into a cell using a thin glass capillary. Routine with eukaryotes, micro-injection of DNA in plant cells is only used in exceptional cases (Dirk Börstling 2001). Protoplasts are plant cells whose cell walls were removed enzymatically. This “naked” cell is stable in a suitable osmotic environment and can be used for multiple transformation methods (Fig. 10). Protoplast transformation can be achieved by electroporation or polyethylene glycol-mediated (PEG) transformation (Sant'Ana et al. 2020). Besides creating transgenic plants, genetically different protoplasts can be fused in somatic hybridization to overcome sexual barriers (Melchers et al. 1978; Tiwari et al. 2018).

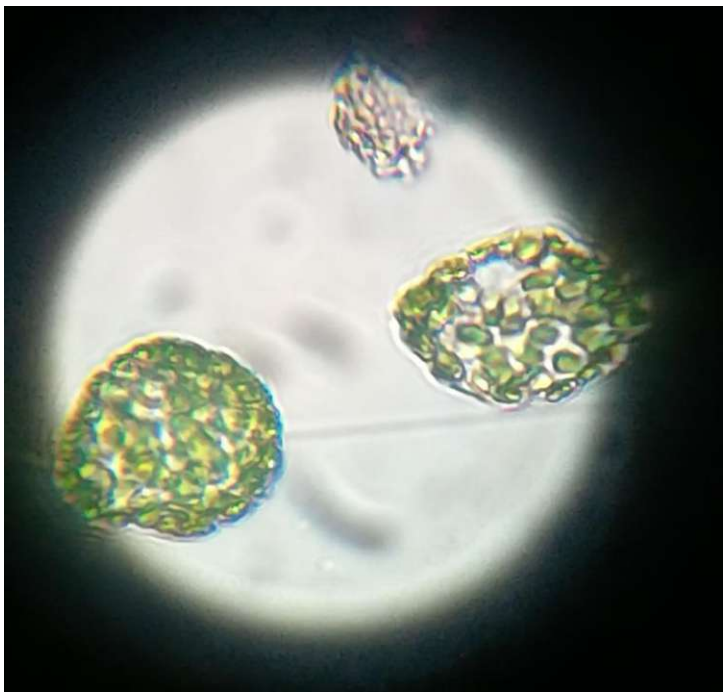

Fig. 10.: Picture of *Pelargonium zonale* protoplasts after removal of the cell walls with pectinase and cellulase

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Besides the direct gene transfer (introduction of DNA directly into the cell, hence its name), DNA can also be introduced into plant cells by indirect gene transfer, using bacteria, viruses and virus-like particles as vectors. Gene transfer mediated via viral vectors is often not stable since the genes aren't integrated into the genome of the target plant. A joined approach of combining viral vectors with *Agrobacterium*-mediated transformation is called Agroinfection and can lead to integration of the (viral) gene of interest (GOI) into the genome, which then can spread systemically throughout the plant. Agroinfection is a suitable method when high expression quantities are desired in a relative short time frame (Giritch et al. 2006; Walliser 2015).

Agrobacterium-mediated transformation is the most important and widely utilized tool for plant transformation. Agrobacteria are gram-negative, ground-dwelling and plant pathogenic bacteria. The genus *Agrobacterium* includes several species, each separated from its kin by the structure of their plasmids, disease symptology and host range. Thus, *A. tumefaciens* causes crown gall disease, *A. rhizogenes* causes hairy root disease, *A. rubi* causes cane gall disease, whereas *A. radiobacter* does not cause any diseases to plants. Due to their relevance in agriculture, the ability of *A. tumefaciens* and *A. rhizogenes* to genetically modify their host plant was an early subject of research (Hwang et al. 2017; Joos et al. 1983). The genes introduced into the host plant induce growth hormon biosynthesis, leading to an alteration of auxin-cytokinin ratio and subsequent gall growth. Additionally, genes encoding enzymes for biosynthesis of opines which serves the agrobacteria as nutrition. Different *A. tumefaciens* strains are known to use different opines as nitrogen source.

In nature, agrobacteria infect their host plant through wounds on the stem base. Attractants to *Agrobacterium* include acetosyringon, vanillin, catechol, sinapic acid or ferulic acid (Gelvin 2003). Those compounds activate to so-called *vir* gene of the agrobacterium. The *vir* gene lies, like all genes which are involved in altering the genome of the host plant, on a single tumor-inducing (Ti) plasmid (or rhizogenic (Ri) plasmid for *A. rhizogenes*, respectively) and determine the range of plant species which can be transformed. For example, the *vir*H locus seems to be responsible for the ability of *Agrobacterium* to transform maize (Jarchow et al. 1991), whereas *vir*G is related with supervirulence of certain *A. tumefaciens* strains (Chen et al. 1991). Some of the *vir* genes encode enzymes like endonucleases which themselves are needed for the incorporation of the transferred DNA (T-DNA). Other *vir* genes like *vir*A or *vir*G

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play roles in sensory-signal regulatory system, whereas *vir*D2 and *vir*E2 form a T-complex with the T-DNA und function as a navigation system (virD2) for leading the T-DNA to the plant's nucleus and protection against nucleolytic degradation (*vir*E2), respectively (Gelvin 2003).

T-DNA is the region of the bacterial Ti plasmid which contains all genes necessary for inducing tumor growth and opine synthese (T-region) and represents usually less than 10 % of the Ti plasmid. With an approximately size of 10 to 30 kbp, Ti plasmids usually carry one T-region, but also multiple T-regions on one Ti plasmid are known (Suzuki et al. 2000). The T-region is flanked by left and right T-DNA border sequences. These borders, 25 bp in length and highly homologous, represent the target sites for *vir*D1/*vir*D2 border-specific endonucleases which process the T-DNA from the Ti plasmid. Soon after the discovery that the Ti plasmid is responsible for the oncogenicity and elucidation of the involved genes, scientists were able to "disarm" the Ti plasmid by removing the *onc* genes (Zambryski et al. 1983). However, since Ti plasmids are very large (200 – 800 kbp) and do not contain internal unique endonuclease recognition sites, it's not possible to simply integrate a gene of interest into the T-region and return it into the agrobacterium. There were multiple approaches to overcome this problem (Gelvin 2003), but the most common until today involves T-DNA binary vectors. Developed by Hoekema et al. (1983), binary vectors benefit from their smaller size (5-17 kbp) and thus easier handling during cloning. Between left and right border, binary vectors generally contain a marker gene (often mediating resistance against kanamycine or hygromycine) and a multiple cloning site (MCS) for easy integration of a gene of interest. Additionally, the prokaryotic origin of replication (ORI) RK2 is embed into the backbone of the binary vector, allowing the replication of the binary vector both in *E. coli* and *Agrobacterium*. For transformation, both the binary vector containing the gene of interest and a selection marker and the *vir* helper plasmid, which is often completely cured of the T-region, must be present in the *Agrobacterium*. Over the past years, labs and companies provided many different strains with different combinations and constructions of binary and helper plasmids. An overview of many *A. tumefaciens* strains and plasmids commonly used for plant genetic engineering was written by Hellens et al. (2000).

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1.3.3. The GoldenBraid cloning system

Rising interests in metabolomic research, complex protein production or genome engineering highlighted the demand for introducing multiple genes or transcription complexes into living organisms. Known methodologies involve whole genome synthesis (Gibson et al. 2010), sequential crossing between and/or re-transformation of already existing transgenic lines or co-transformation of multiple constructs in a single event. Whilst the first is incredibly complex and requires extreme amounts of labor time and personal costs, co-transformation and re-transformations exhibit varying expression levels depending on the integration loci (Lechtenberg et al. 2003; Felipe et al. 2006). Additionally, multigene transformation makes demands to effective promoter strategies. The same promoter may be used repeatedly, but may inherit the risk of transgene instability (Peremarti et al. 2010). Potentially, a single vector construct containing multiple expression cassettes under the control of a variety of different regulatory elements may counter potential co-suppression effects due to promotor rivalry (Fagard and Vaucheret 2000; Daniell and Dhingra 2002).

The GoldenBraid (GB) cloning system provides a tool for overcoming co-suppression effects by enabling the user to combine different regulatory elements, based on the Golden Gate cloning system, one of the most effective methods for vector construction (Engler et al. 2008; Weber et al. 2011). The GoldenBraid cloning system was designed by the PGB group of the IBMPC-CSIC-UPV in Valencia, Spain. It's a modular DNA construction method specially designed for plant synthetic biology and metabolic engineering. GoldenBraid's signature feature is its modularity and efficient assembly (Sarrion-Perdigones et al. 2011; Sarrion-Perdigones et al. 2013; Vazquez-Vilar et al. 2016; Kundert et al. 2020b). Rather large constructs up to 15 – 20 kbp comprising multiple transcriptional units made of individual fabricated modules can be designed using an online tool (http://gbcloning.upv.es/) and cloned in the matter of just a few days. The biochemical base for the easy and effective cloning procedure is the use of type IIS restriction enzymes. These enzymes generate four-nucleotide sticky ends to each DNA piece which are then subsequent joined together using T4 ligase. The GB basic DNA pieces are called "GoldenBraid parts" (GBparts) and are the simplest modules in the system. These GBparts are prefabricated modules using a standardized "grammar" and involve a variety of different promotors, terminators, enhancers, coding sequences, etc. The user is enabled to introduce own desired

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sequences into the GB grammar during the so-called “Domestication”. During domestication, internal recognition sites of the used type IIS restriction enzymes are removed from the sequence, necessary 4 bp flanking restriction sites produced after cleavage with a Type IIS restriction enzyme, either BsaI or BtgZI, are added and subsequent introduced into a suitable entry vector. This specific entry vector is called pUPD2. After domestication, those GBparts can be used in the same manner as the pre-fabricated ones. GBparts can be combined in any combination to form “transcriptional units” (TU). The basic transcriptional unit contains a promoter, a coding sequence of a gene of interest after domestication and a terminator. Each of these parts is interchangeable and can be chosen from a vast library. For example, the GoldenBraid library contains 35 promoters and 14 terminators which can be combined in any possible way. Note, that promoters (which include the transcription origin and 5´UTR), CDS and terminators (which includes 3´-UTR and polyA site and transcriptional stop signal) are already supercategories, which can be further comprised of additional GBparts for more specific purposes such as coding sequences for transit peptides, secreting instructions or specific expression conditions. The single GBparts can be joined in a special destination vector as recipients of the new DNA assemblies. The GoldenBraid system comes with eight different destination vectors called pDGBα1, pDGBα2, pDGBΩ1, pDGBΩ2 and pDGBα1R, pDGBα2R, pDGBΩ1R and pDGBΩ2R, respectively. GB destination vectors are binary plasmids containing a specially designed cloning structure name the “GB cassette”. This cassette contains the blue/white selection lacZ gene flanked by recognition sites of the Type IIS restriction enzymes BsmBI and BsaI. During a GB reaction, the GB cassette of the GB destination vector is replaced by the new DNA assembly. All destination plasmids are labeled as “pDGBs”. The numbers and the Greek letters serve to identify each destination plasmid according to the flanking overhangs left by BsaI and BsmBI digestion respectively. The α-level destination vectors are used in BsaI reactions, whereas the Ω-level destination vectors are used in BsmBI reactions, respectively. Each of those TUs is the minimal instruction which can be introduced into an organism. However, it is possible to combine several TUs into larger constructs in Multipartite GB assembly reactions. An important feature of GB assembly is that TUs can be combined indefinitely to grow multigene structures. This is due to the special pDGBs design, which ensures that any newly assembled composite part (a TUs or a group of TUs) ends up being flanked by target sites of the alternative restriction enzyme. During these

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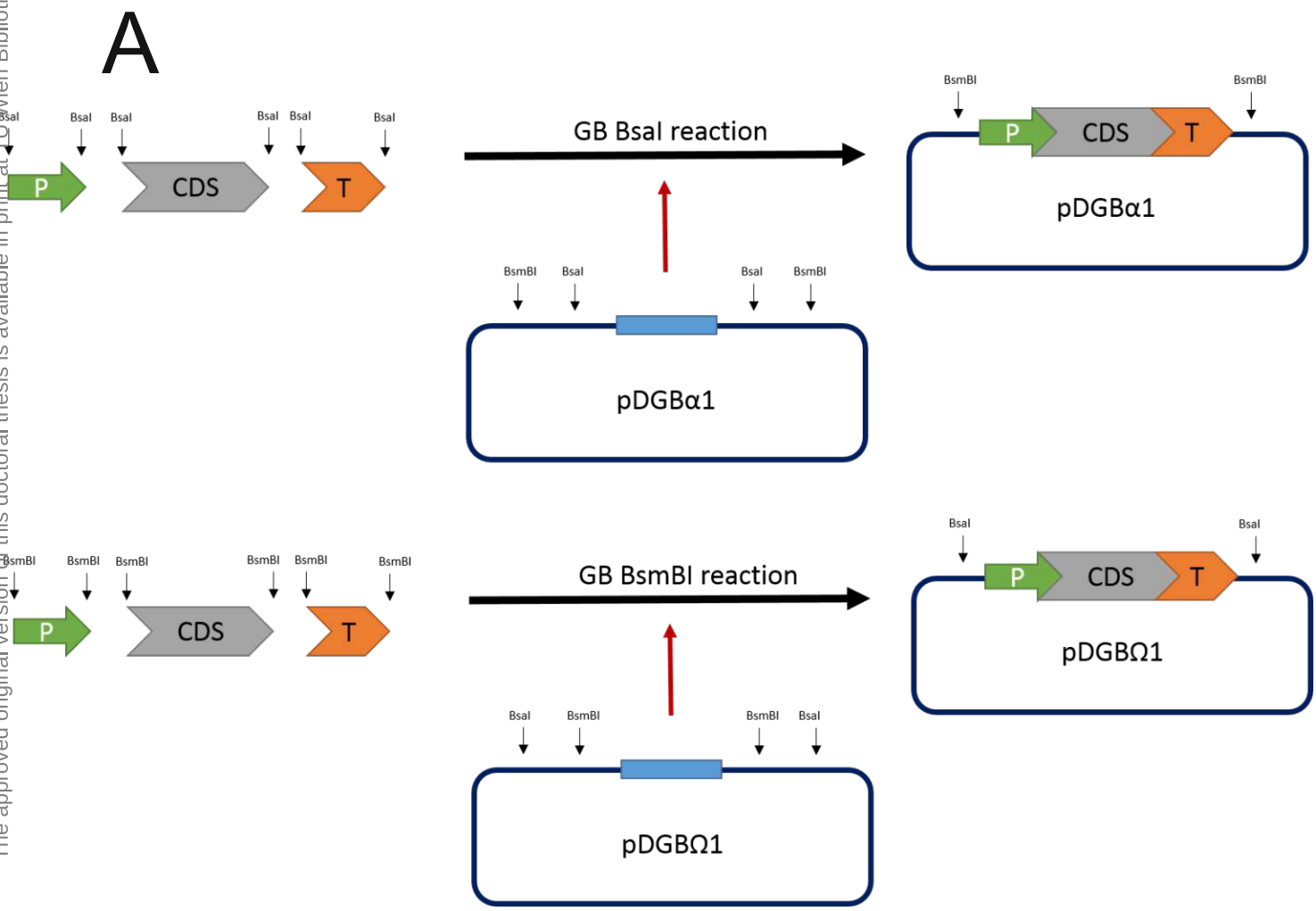
binary assembly reactions, pDGBα1 and pDGBα2 are linked together into an Ω-level destination plasmid and vice versa (Fig. 2B). Upon ligation, the recognition sites of the enzymes used in the assembly will disappear, therefore the composite part cannot be broken apart anymore. A group of helper plasmids (Twister-plasmids) containing small intergenic stuffer sequences can act as an “empty” TU, avoiding getting stuck in the assembly process. Through continuous assembly reactions with complement pDGBs, an endless iterative assembling loop (braid) can be created. pDGBs are themselves binary plasmids, and therefore the resulting composite parts are ready for plant transformation.



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B

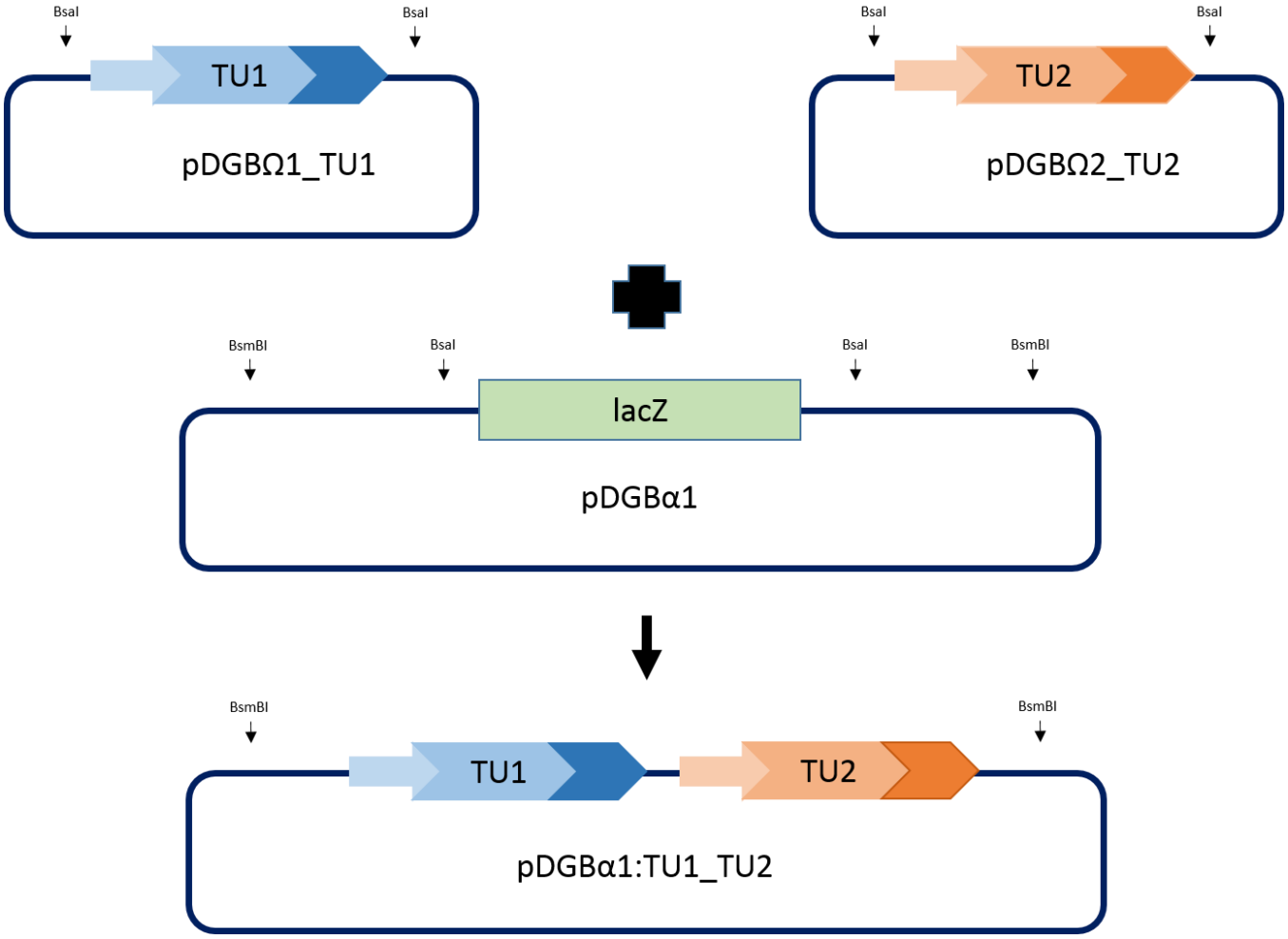

Fig. 11.: Schematic overview of GoldenBraid vector construction: **A**.: Construction of two transcriptional units (TU), containing three GBparts (promoter, CDS, terminator) and an alpha-level or beta-level destination plasmid; note the different Type IIS restriction enzyme recognition sites; **B**.: Binary assembly of two independent transcriptional units into one destination plasmid; note the complementary levels of the plasmids

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1.4. Goal of the Thesis

Anthochlor pigments are a group of secondary plant metabolites and with carotenoids and some flavonoids produce yellow flower colour. Both, chalcones and aurones are collectively known as anthochlor pigments and serve as UV nectar guides in some plants, mainly from the *Asteraceae* species. Though anthochlors are frequently ranked among flavonoids, their structure cannot be derived from the flavonoid skeleton. Some plants (especially *Asteraceae*) accumulate two types of anthochlor pigments. On the one hand, the hydroxytypes of chalcones and aurones, on the other hand the deoxy-types of chalcones and their corresponding aurones. Both types differ only in the presence of a hydroxyl group in the 6' position of the B-ring (chalcones) or the 4 position of the A-ring (aurones), respectively. Hydroxychalcones are intermediates of the subsequent biosynthesis of flavonoids and quickly isomerize to flavanones either chemically or enzymatically. Thus, hydroxychalcones cannot be accumulated in plants. Hence, our strategies in establishing yellow coloration involve the introduction of genes encoding proteins which lead to the accumulation of anthochlors of the deoxytype.

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 the introduction of the anthochlor biosynthetic pathway in plants which naturally do not produce those pigment class.

This should be achieved with the following sub steps:

1. Evaluation of the pigment composition and involved genes in the model plants *Bidens ferulifolia* and *Dahlia variabilis*

2. Design and creation of multigene vector constructs containing single or groups of genes involved in anthochlor biosynthesis. The origin of those genes should not only include one plant species, but multiple of the *Asteraceae*. Additionally, CRISPR/Cas9 and VIGS constructs should be designed for silencing single genes involved in anthochlor biosynthesis

3. Introduction the vector constructs of sub step 2 in model and target plants such as *Pelargonium zonale* and *Petunia hybrida* utilizing transient and stable expression. Transgenic plant will undergo further characterization and analysis.

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2. Methodology

2.1. Materials

2.1.1. Chemicals

Name	*Company*
Acetic acid	VWR
Acetonitrile	VWR
Acetosyringone	Sigma-Aldrich
Ammonium nitrate	Roth
Ampicillin	Duchefa
Agar	Roth
Agarose	VWR
*Bsa*I	New England Biolabs
BbsI	New England Biolabs
*Bam*HI	New England Biolabs
BsmBI	New England Biolabs
6-Benzylaminopurine	Duchefa
Boric acid	Roth
Calcium chloride	Roth
4-chlorophenoxy acetic acid	Duchefa
Cefotaxim	Duchefa
Cobalt chlorid 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

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Dihydrokaempferol	Sigma-Aldrich
Dihydromyrecitin	Extrasynthese
Dihydroquercetin	Extrasynthese
D-Sorbitol	Sigma-Aldrich
dNTPs	Thermo Fisher Scientific
DNA Stain Clear G	Serva
*Dra*I	New England Biolabs
*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
Indol acetic acid (IAA)	Duchefa
Isopentenyl adenine	Duchefa
Isopropanol	Merck
Kaempferol	Extrasynthese
Kanamycin sulphate	Duchefa
L-Tryptophan	Sigma-Aldrich
LunaR Universal qPCR Master Mix	New England Biolabs
LR Clonase II Enzyme Mix	Thermo Fisher Scientific

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Magnesium sulphate	Roth
Malvidin	Extrasynthese
Methanol	VWR
2-Mercaptoethanol	Sigma-Aldrich
2-(N-Morpholino)ethansuphon acid (MES)	
Myo-Inositol	Roth
Myricetin	Extrasynthese
NADPH	Boehringer Mannheim
1-Naphthaleneacetic acid	Duchefa
Naringinase	Sigma-Aldrich
Nicotinic Acid	Roth
One Shot TOP10- Chemically Competent Cells	Invitrogen
p-Coumaroyl-CoA	TransMIT
Pelargonidin	Extrasynthese
Peonidin	Extrasynthese
Petunidin	Extrasynthese
Pepton	Merck
PEG 4,000	Sigma-Aldrich
Plant Preservative Mixture	Plant Cell Technology
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

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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
*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
Zeatin	Duchefa
Zinc sulphate heptahydrate	Roth

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2.1.2. Equipment

Device	*Company*
Allegra™21 R Centrifuge	Beckmann
Autoclave Systec V-55	Systec
AvantiR J-26xP Centrifuge	Beckmann Coulter™
Balance PJ 3000	Mettler
Balance Research R 160 P	Sartorius Research
Berthold LB 2842 Linear Analyzer	Berthold
CertomatR 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
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 A	mersham

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UV-working bench Reprostar II	Camag
Vacuum pump Laboport	KNF Neuberger, Inc.
Vortex-Genie 2	Scientific Industries
Water bath MP-PA/KU	Julabo

2.1.3. Kits used in this research

Name	*Company*
DNeasy plant mini kit	Qiagen
GenElute Plasmid Mini Prep Kit	Sigma-Aldrich
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

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2.1.4. Vectors used in this research

Vector	*Company*
pCR2.1-TOPO	Invitrogen
pCR™8 TOPO. TA	Invitrogen
pDGB1α1	Sarrion-Perdigones et al. (2011)
pDGB1α2	Sarrion-Perdigones et al. (2011)
pDGB3α1	Sarrion-Perdigones et al. (2011)
pDGB3α2	Sarrion-Perdigones et al. (2011)
pDGB1Ω1	Sarrion-Perdigones et al. (2011)
pDGB1Ω2	Sarrion-Perdigones et al. (2011)
pDGB3Ω1	Sarrion-Perdigones et al. (2011)
pDGB3Ω2	Sarrion-Perdigones et al. (2011)
pDe-Sa_Cas9	Steinert et al. 2015
pEN-Sa_Chimera	Steinert et al. 2015
pENTRY-IBA	Iba
pGEX-6P-1	GE Healthcare
pUPD2	Sarrion-Perdigones et al. (2011)

2.1.5. Media

2.1.5.1. LB-Medium

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 MgSO4 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.

2.1.5.2. SOB Media

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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 100 mg/L spectinomycin, 50 mg/L kanamycin, 50 mg/L rifampicin or 30 mg/L gentamicin. The medium was stored at 4 °C until use.

2.1.5.3. SOC Media

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

2.1.5.4. MS Media

Media used in the plant tissue culture. Recipe according to Murashige and Skoog (1962). Media were supplemented with 30 g/L sucrose, 5 g/L PVP-20 and 1 mL/L Plant Preservative Medium (PPM). pH was adjusted with KOH to 5.7-5.8. Phytohormones used for regeneration were 0.5 mg/L indol acetic acid and 2.2 mg/L zeatin. For the solid media 7.5 g/L of agar was added. Media were autoclaved at 121 °C for 20 minutes and stored at 4 °C. Antibiotics used against agrobacterial growth were 250 mg/L cefotaxim, 150 mg/L timentin and for selection of positive transformations 50 mg/L kanamycin.

2.1.6. Buffers and solutions

2.1.6.1. 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.

2.1.6.2. Infiltration Media

For the transformation of plants, infiltration medium was prepared, consisting of 10 mM MES-NaOH pH 5,5, 10 mM $MgSO_4$.

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2.1.6.3. McIllvaine buffer

The McIlvaine buffer was used during enzymatic hydrolysis of HPLC samples. Composition: 0.1 M Na2HPO4, 0.4 % Na-ascorbate. Set up the pH to 4 with citric acid. The buffer was prepared fresh every day.

2.1.6.4. 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 L Eppendorf tubes, stored in -20 °C.

2.1.6.5. Plant hormone stock solutions

For all hormones (IAA, Zeatin), 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.

2.1.7. Plant material

Targeted plants for stable and transient transformation are five Pelargonium cultivars and one Petunia hybrida cultivar from the company Selecta One (Stuttgart, Germany). The petunia cultivar ‘Lilac Glo’ was cultivated in vitro, whereas the pelargonia were cultivated in a green house. The Pelargonium cultivars ‘Sailing ‘12’ (white), ‘Sunrise’ (white), ‘Goesta’ (pink), ‘Michelle’ (salmon) were obtained from Selecta One (Stuttgart, Germany) and ‘French Vanilla’ (yellow-creamish) was obtained from PAC-Elsner, respectively.

Samples of the Bidens ferulifolia cultivars ‘Firewheel’ (Florsaika, Netherlands), ‘Beedance Painted Red’ (MNP Suntory, Netherlands), ‘Eldoro Red’, ‘Eldoro Yellow’, ‘Giant Yellow’, ‘Megacharm’ (Danziger, Israel) and ‘Beedance White’ (MNP Suntory,

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Netherlands) were collected from the collection of Selecta One GmbH, Stuttgart (Germany), samples of the cultivars '9163', '9177' and 'Tiki Taku' were obtained from Volmary GmbH, Münster (Germany). Petals from opened flowers were harvested and separated in a bottom part and a tip part. The intermediate area was discarded in order to avoid cross contamination. The separated parts were shock frozen in liquid nitrogen and stored at -80 °C.

2.2. Methods

2.2.1. 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 µ-MACS 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)23VN).

2.2.2. Polymerase Chain Reaction (PCR)

For standard PCR, such as colony PCR, transgenic plants screening or nother qualitative PCR, GoTaq polymerase (Promega, Germany) was used. The reaction mix contained in a total volume of 20 µL: 4 µL of 5 x GoTaq 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 GoTaq 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

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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).

2.2.3. Golden Braid Cloning

All cloning steps were guided and planned using the "Domestication", "GB TU Assembler" and "GB Binary Assembler" tools from the GoldenBraid homepage (https://gbcloning.upv.es/). DNA patches were obtained by PCR amplification using suitable templates.

For domestication, 40 – 75 ng of each amplicon and 75 ng of the domestication vector (pUPD) were mixed with *Bsm*BI (New England Biolabs) and T4 DNA ligase in 1x T4 DNA ligase Buffer (Promega) in a 25-cycle restriction-ligation reaction (2 min at 37 °C, 5 min at 16 °C). One microliter of the reaction was used for *E. coli* transformation and positive clones were selected through blue/white selection and PCR. Plasmid DNA was extracted using the GenElute™ HP Plasmid Miniprep Kit (Sigma-Aldrich). Correct assemblies were confirmed by sequencing (Microsynth, Vienna, Austria).

Transcriptional Unit and Binary Assemblies were carried out similar to the domestication reactions, unless otherwise noted. For assembling the initial or subsequent transcriptional units in α-level vectors, 75 ng each of the individual GBparts (promotor, GOI and terminator) or TU in Ω-level vectors were mixed with 75 ng of the desired α-level, *Bsa*I (New England Biolabs) and T4 DNA ligase in 1x T4 DNA ligase Buffer (Promega) in a 25-cycle restriction-ligation reaction (2 min at 37 °C, 5 min at 16 °C), followed by *E. coli* transformation. For assembling the transcriptional units in Ω-level vectors, 75 ng each of the individual GBparts or TU in α-level vectors were mixed with 75 ng of the desired Ω-level vector, *Bsm*BI (New England Biolabs) and T4 DNA ligase in 1x T4 DNA ligase Buffer (Promega) in a 25-cycle restriction-ligation reaction (2 min at 37 °C, 5 min at 16 °C), again followed by *E. coli* transformation. Initial and

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process cloning were carried out using pDGB1 vectors, the final assembly of the finished constructs were cloned in pDGB3 α-level vectors or Ω-level vectors.

2.2.4. 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.

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 LB plates with the appropriate antibiotic and incubated for two to four days at 28 °C.

2.2.5. 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.

2.2.6. Site-directed mutagenesis

Mutations were generated by use of the Q5 R Site-Directed Mutagenesis Kit (NewEngland Biolabs, Vienna, Austria). Primers were designed using the NEBase ChangerTM provided at http://nebasechanger.neb.com. The integrity of the constructs was confirmed by commercial sequencing (Microsynth, Vienna, Austria).

2.2.7. Gene expression studies

Gene expression was evaluated by qPCR using the StepOnePlus system (Applied Biosystems, Germany) and the LunaR Universal qPCR Master Mix (New England

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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 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative expression ratio was calculated according to Pfaffl (2004). Product specificity was confirmed by analysis of melting curves. Primer efficiency was calculated according to Pfaffl (2004).

2.2.8. HPLC analysis

Analysis for flavonoid class/anthocyanin type composition was done according to (Haselmair-Gosch, et al., 2018). 0.25 – 0.5 g of frozen petal material was extracted with 1 mL 2 M hydrochloric acid in methanol. After centrifugation, 40 µL supernatant were incubated with 160 µL 4N HCl for 60 min at 95 °C for anthocyanin analysis. For enzymatic hydrolysis of flavones, flavonols and anthochlors, 20 µL of the supernatant were subjected to enzymatic hydrolysis by 10 U Naringinase (Sigma-Aldrich, Vienna, Austria) and incubated for 20 min at 40 °C in 0.1 M McIlvaine buffer pH 4.0. The reaction was stopped by addition with 30 µL methanol and used in HPLC analysis according to Thill, et al. (2012). A Thermo Scientific Dionex UltiMate(R) 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 x 150 mm (Dionex bonded Silica Products: No. 071399) was operated at 25 °C. For analysis of flavones, flavonols and anthochlor pigments, 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). The compounds were detected at 290 nm and identified by retention times and comparison of the 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.

2.2.9. Stable transformation and regeneration of Pelargonium and Petunia

Leaves and petiols were incubated in 500 mL sodium hypochloride with 1.5 % available chlorine with one drop of Tween20 for 10 min, followed by three washing steps in sterile

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water for 10 minutes each. Petiols were cut into segments of 4 – 6 mm length and were placed horizontally on the medium in 6 cm Petri dishes containing 10 mL MS medium.

Leaf blade explants were cut into 5 x 5 mm discs and were incubated with the adaxial side up. The Petri dishes were sealed with parafilm and all cultures were kept at 23 – 25 °C under 16 h of light (20-40 µmol m^{-2} s^{-1}) for three days.

Petiole and leaf disc explants were incubated for 10 min in *A. tumefaciens* infiltration culture, then blotted dry on sterile filter paper and placed horizontally (leaf discs with the adaxial facing up, respectively) on the medium in 6 cm Petri dishes containing 10 mL MS medium. After two and four days of co-cultivation, the explants were washed three times in 150 mg/L timentin, followed by three washing steps in sterile water for 10 minutes and transferred to basal medium containing 500 mg/L cefotaxim, 150 mg/L timentin and 50 mg/L kanamycin. The Petri dishes were sealed with Parafilm and cultures were kept at 23 – 25 °C under 16 h of light (20-40 µmol m^{-2} s^{-1}) for four weeks. Every four weeks or when visible contamination occurred, the non-contaminated explants were transferred onto new plates.

Small plantlets were excised and placed into boxes containing MS medium as described above. Leaves were harvested, shock-frozen in liquid nitrogen, and stored at -80 °C.

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2.2.10. Transient transformation

For transient transformation, two strategies were used. One was the introduction of *A. tumefaciens* using a needleless syringe and the other was vacuum infiltration. For the syringe method, ~ 1 mL of the infiltration culture of *A. tumefaciens* is injected into petals using a needleless syringe after carefully scratching the abaxial side of a petal using a scalpel. The plants are either kept under greenhouse conditions or flower heads were harvested and placed in plastic containers containing 10 g/mL water agar. Color changes and/or fluorescence was checked optically.

Vacuum infiltration was accomplished according to Leuzinger et. al unless otherwise stated. The flower heads were submerged upside down into the *A. fumefaciens* solution and placed inside an exsiccator. A pressure of 100 mbar was applied and hold for two to five minutes to allow the bacteria to infiltrate the intercellular space. After those two to five minutes, pressure compensation was done over the course of additional five minutes with only minor changes in air pressure at a time to exclude additional damaging of the petals. Infiltrated plants were kept in the dark overnight before placing them again under light.

Infiltrated petals showing alterations were harvested 7 to 13 days post infiltration (dpi), shock-frozen in liquid nitrogen and stored and -80 °C.

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Fig. 13.: *Dahlia variabilis* after and during vacuum infiltration. Notice the translucent petals, proving that all air has been replaced with *A. tumefaciens* containing infiltration media

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3. Scientific contribution of the PhD thesis

Independent of global crisis, the ornamental flower market is a booming business. With a calculated growth of 28.98 billion $ until 2024, the people are willing to spend money on beautiful looking flowers and plants with new and remarkable features, putting pressure on breeders worldwide to match the demand in time (Technavio Report 2022). In these times, genetic modification of ornamentals rises attention. Customers demand for new and exciting plants delivers the stimulation for breeders to improve genotypes with better post-harvest life, altered morphology, new flower color or other novel traits. Furthermore, additional traits specially interesting for growers are production related traits such as increased resistance to pests, pathogens or environmental factors or shorter production times. Flower color, structure and fragrance belong to the most important traits of ornamental plants in the eye of customers and hence in ornamental plant breeding (Milosevic et al. 2015). However, the color range of flower color is subjected to the genetics of the plant species (Debener and Winkelmann 2010). So, a deep understanding of the biochemical and physiological background is mandatory for successful breeding, especially in molecular breeding and genetic engineering.

Anthochlor pigments are a group of secondary plant metabolites and with carotenoids and some flavonoids produce yellow flower colour. Both, chalcones and aurones are collectively known as anthochlor pigments and serve as UV nectar guides in some plants, mainly from the *Asteraceae* species (Scogin and Zakar 1976; Harborne and Smith 1978; Brouillard and Dangles 1994). Though anthochlors are frequently ranked among flavonoids, their structure cannot be derived from the flavonoid skeleton. Some plants (especially *Asteraceae*) accumulate two types of anthochlor pigments. On the one hand, the hydroxy types of chalcones and aurones, on the other hand the deoxy-types of chalcones and their corresponding aurones (Boucherle et al. 2017). Both types differ only in the presence of a hydroxyl group in the 6’ position of the B-ring (chalcones) or the 4 position of the A-ring (aurones), respectively. Hydroxychalcones are intermediates of the subsequent biosynthesis of flavonoids and quickly isomerize

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to flavanones either chemically or enzymatically. Thus, hydroxychalcones are rearely accumulated in plants (Forkmann and Dangelmayr 1980).

Although anthochlor biosynthesis generally involves only a handful of enzymes, the exact mode of action is not completely understood yet (Boucherle et al. 2017; Walliser et al. 2021). The first parts of this PhD thesis address this problem by characterizing two model plants of the *Asteraceae* family on molecular and biochemical level.

Secondly, multigene constructs containing single or groups of genes involved in anthochlor biosynthesis will be designed and transformed stably and transiently in model and target plants in order to establish the biosynthetic pathway of anthochlor in plants which naturally do not produce these pigments. An additional approach addressing the individual contribution of the single genes involved in anthochlor biosynthesis will be silencing of said genes in model plants, both transiently with virus induced gene silencing and stable using the CRISPR/Cas9 system.

3.1. Unravelling the biochemical base of *Bidens ferulifolia* pigmentation

Bidens ferulifolia is a yellow flowering, popular ornamental plant from the *Asteraceae* family. Best known for its pure yellow coloration, the past few years saw an emergence of different colored cultivars, such as yellow-red, white-yellow or pure white or pure purple varieties (Dwyer 2021). In this study, we investigated 16 *B. ferulifolia* genotypes (cultivars and breeding lines) exhibiting different colour combinations to shed light on the underlying chemical and biochemical base of colour formation in this increasingly popular ornamental. To our knowledge, this is the first study dealing with these colour combinations in *Bidens ferulifolia*.

In *Bidens ferulifolia,* the four main pigment classes determining flower color are anthocyanins, flavones, anthochlors and carotenoids. The most prevalent anthocyanidin in the red and purple varieties was cyanidin. The production of cyanidin requires a full set of enzymes of the pathway, so the lack of anthocyanins in most lines can be explained by missing activity of FHT and/or DFR. Generally, the yellow-creamish and white cultivars were lacking DFR activity, resulting in a bottleneck and thus in elevated flavone and dihydroflavonol accumulation. Notably, in a pure-yellow cultivar, a high DFR activity was observable. So, this cultivar carries the potential to be

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a valuable breeding partner for crossing with DFR-lacking line to gain new and striking colour combinations.

In accordance with findings by Miosic et al. (2013), the yellow *B. ferulifolia* we examined also showed anthochlors as their main pigment. However, whilst Miosic et al. reported of aurones as main anthochlor pigment, we showed that the chalcone Okanin and its derivates were the most abundant. Most interestingly, okanin possess an additional hydroxyl group at position 3', in addition to the common 2'4' hydroxylation. Due to the free rotation of the A-ring, a 3' hydroxylation of chalcones corresponds both to the position 6 and 8 of 5-hydroxyflavonols or position 8 of 5-deoxyflavonols. Although rare, such configurations have been reported in *Chrysanthemum segetum* (Harborne 1967) and *Gossypium hirsutum* (Berim and Gang 2013). Whereas the establishment of the B-ring hydroxylation pattern of chalcones is well investigated and involves a cytochrome P450 dependent monooxygenase (Schlangen et al. 2010b), the introduction of a hydroxy group in position 3′ has not been investigated so far. We reported for the first time the presence of a CH3'H, catalyzing the introduction of a hydroxy group at position 3' of 6'-deoxychalcones. Accepted substrates were the 6'-deoxychalcones isoliquiritigenin and butein. Since the reaction required NADPH and was inhibited by ketoconazole and tetcyclacis, its highly possible that a cytochrome-P450-dependent monooxygenase is involved. Furthermore, characterizing the *B. ferulifolia* cultivars depending on the presence of anthochlors, we can conclude that anthochlors are primarily located at the petal base and in the veins of the outer petal parts. This is more relevant for the creamish tips, where anthochlors were exclusively accumulated in the veins. Pure white cultivars do not accumulate anthochlors at all.

Carotenoids have been reported in various Asteraceae species (Ohmya 2011). However, until now, *B. ferulifolia* extracts have not been analysed by modern analytical techniques. Comparing with anthochlors, carotenoids are the minor yellow pigments in *Bidens* petals. Lutein and β-carotene, which have both been found before in *B. ferulifolia* extracts (Valadon and Mummery 1971). In addition, various xanthophylls were identified in *B. ferulifola* for the first time, namely violaxanthin, luteoxanthin, antheraxanthin, lutein 5,6-epoxide and zeaxanthin. For some of those carotenoids, the *cis* isomers were detected, which can be a results of high light intensities, heat or an acidic environment (Rodriguez-Amaya and Kimura 2004). Interestingly, the content of carotenoids strongly depends on the genotype. High light-intensities are also known to

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be important for the induction and regulation of anthocyanin biosynthesis (Weiss 2000; Zhao and Tao 2015).

One of the goals of this study was the search for candidates for breeding new color combinations. Potential breeding partners are the yellow-red cv. Painted Red, the pure yellow cv. Giant Yellow and the pure white genotypes. Whilst the first two genotypes possess a functional DFR, a lack of FHT activity prevents the synthesis of anthocyanins and vice versa in the pure white genotypes. Introducing a functional enzyme may lead to new colour combinations such as red-white or pure red. Follow-up studies with these genotypes may include investigations of the gene level such as expression studies and sequencing. Nonetheless, this study shows that biochemistry can provide useful information for plant breeders.

3.2. *Dahlia variabilis* cultivar 'Seattle' as a model plant for anthochlor biosynthesis

The yellow-white *Dahlia variabilis* var. 'Seattle' exhibits bright yellow petals with pure white tips. This plant accumulates anthochlor pigments in its inner petal parts, whereas only low amounts of pigments without traces of anthochlor pigments can be found in the outer petal parts. Bicolored varieties can be powerful models to identify the key enzymes of the pathways leading to the formation of the different pigment types (Ohno et al. 2016, 2018a, 2018b). We used this cultivar as a potential model plant for studying anthochlor biosynthesis and identified the biosynthetic bottleneck in the formation of anthochlors.

Regarding anthocyanin formation, no traces of anthocyanins were detected irrespective of petal position. However, the white tips showed elevated FHT, DFR and ANS expression. Additionally, the presence of flavones and other intermediates upstream of FHT suggest a bottleneck in the biosynthetic pathway following FHT. Additionally, besides FHT, CHS1 and the transcription factor IVS showed low expression. It was previously shown, that the bHLH transcription factor IVS regulates anthocyanin synthesis by activation of CHS1, FHT, DFR, ANS and MaT (Ohno 2011a), which correlates partly with our findings, since DFR, ANS and MaT were not downregulated. Other previous studies reported that neither pure white nor pure yellow

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cultivars exhibit such high expression ratios for genes of anthocyanin synthesis are we showed in our finding between both colored petal parts (Thrill et al. 2012).

Anthochlors were the most prevalent pigments found in the petal base, with the 6'-deoxychalcones isoliquiritin and butein and the 4-deoxyaurone sulfuretin. Furthermore, CHS expression varied highly between the different colored petal parts. Whereas CHS1, a chalcone synthase reported to be correlated with anthocyanidin biosynthesis (Ohno et al. 2018b), is almost not expressed in both petal parts, CHS2, which is expressed regardless of flower coloration, showed a high relative expression in the yellow base that correlated with the high concentrations of anthochlors and flavones. The low-level expression of CHS in the white petal tips provides a good explanation for the observed low concentration of flavones and anthochlors in the white tips.

Until only recently (Ohno et al. 2021), no chalcone reductase candidate genes in *Asteraceae* species were known. Transcriptomic data on *D. variabilis* 'Seattle' gave hints to three candidate sequences for a putative CHR. Phylogenetic analysis showed that those CHR cluster within the chalcone reductase and codeinone reductase subfamily (AKR4A and AKR4B, respectively), a clade which they have in common with known legume CHR (Hyndman, et al. 2013). However, the rather distant relationship in sequence homology is no surprise since this phenomenon is known from other enzymes and subfamilies between *Asteraceae* and other plant families (Seitz et al. 2006; Molitor et al. 2015; Yonekura-Sakakibara et al. 2019). Gene expression studies and RNA seq of those three putative CHR revealed only low expression ratios which were on the same level as other genes of the flavonoid pathway. A clear difference in expression between the yellow and white petal parts was not observable. On the other hand, the expression ratios were comparable to those of AUS, CH3H or F3'H. Additionally, the expression ratio of the three CHR was low compared to CHS. Existing literature about legume CHR misses information about optimal CHS/CHR ratios since both enzyme coact together (Bomati et al 2005; Boucherle et al. 2017).

In summary, it was shown that the bicolouration of cv. 'Seattle' is most likely caused by a bottleneck of CHS in the white tip rather than caused by a differentially expressed CHR. However, a difference in expression of an enzyme catalyzing a chalcone reductase-like reaction could not be excluded, in addition to the different CHS expression. The relative prevalence of flavones compared to the

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anthochlors in the white tips could be an indication that the presumable key enzyme for 6′-deoxychalcone formation is less expressed in white tips than in the yellow base.

3.3. Introduction of Genes involved in anthochlor biosynthesis into plants using the GoldenBraid system

Based on previous studies, chalcone reductase CHR (Bomati et al. 2005; Miosic et al. 2013; Ohno et al. 2022), chalcone 3-hydroxylase (CH3H) (Schlangen et al. 2010a; Schlangen et al. 2010b), aurone synthase (AUS) (Nakayama et al. 2001; Molitor et al. 2015; Molitor et al. 2016) and flavonoid glycosyltransferases (GT) (Gosch et al. 2010; Ohno et al. 2022; Nakayama et al. 2000) were identified as crucial for anthochlor biosynthesis. However, the interaction and individual contribution on accumulation of specific anthochlors was not elucidated yet in a holistic approach. The aim of this thesis was the introduction of those genes singly and in varying groups to examine the influence of each single gene in anthochlor biosynthesis in plants lacking this pathway. We chose to use different colored *Pelargonium zonale* cultivars as host plants. Previous analysis on pigment composition showed that all tested varieties accumulated flavonoids, so the essential substrates were present. Additionally, our industrial partner Selecta Klemm had great interest to present the world-first bold yellow colored pelargonia. As vector system we chose the novel GoldenBraid cloning system since its modularity and flexibility regarding plasmid size were perfectly for our approaches.

In total, twelve constructs were designed, carrying different combinations of the involved genes including kanamycin-selection markers, ranging from carrying only a chalcone reductase (either from a legume or from an *Asteraceae*) with a glycosyltransferase up to the full set of enzymes. Additional to those twelve, we designed a handful of vectors carrying GFP and dsRED genes for optical tracing when used in transient transformation experiments. However, since stable transformation experiments require lots of worktime, we limited the constructs to test to three in total: BA_A, carrying the full set of anthochlor biosynthesis related genes, BA_F, carrying CHR, CH3H and GT and BA_L, carrying a legume CHR and a GT, with every gene under its own promoter-terminator combination.

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Transient transformation assays were performed in *Dianthus caryophyllus*, *Dahlia variabilis, Pelargonium zonale* and *Petunia hybrida*. We relied on manual injection of *A. tumefaciens* infiltration solutions using the syringe method. When using vacuum infiltration, the physical stress lead to necrosis of the petals in all species except *Dianthus*. Syringe-infiltrated petals survived long enough to observe both transcript expression and GFP / dsRED activity. The presence of transcripts was confirmed by PCR, following mRNA extraction and cDNA synthesis. In the case of *Pelargonium,* we were able to induce pale yellow coloration in different cultivars when introducing BA_L. Although transcripts were found, presence of anthochlors couldn't be detected. The yellow coloration seemed to be induced by elevated flavonol accumulation. This may be explained by the dual infiltration with dsRED and GFP. Since both marker genes are observed using UV light, the irradiated tissue induced anthocyanin (as observed in white carnations) and flavonol biosynthesis as photoprotection (Takeda and Abe 1992; Halbwirth 2010).

Stable transformation proved itself to be more complicated. Since *Pelargonium* donor plants were grown in an open greenhouse, we had to fight against endogenous bacteria and fungi. The petunia we used for stable transformation was already established in tissue culture and therefore was sterile. It took a while until we had a functional disinfection protocol (modified after Winkelmann et al. (2005) which keeps endogenous infestation in check but in parallel keeps the explants alive. Nonetheless we lost 30 % of explants to endogenous contamination over the course of the first days prior transformation. Additional to endogenous contamination in *Pelargonium*, the used *A. tumefaciens* strain seemed to be contaminated with additional resistances to different antibiotics we used for selection, leading to continuous overgrowth of agrobacteria. And since all good things come in three, the Plant Preservative Mixture© we used in the media to keep fungal contamination in check, proved to interfere with the regeneration of the explants. This phenomenon and other adverse effects of PPM have been reported in multiple studies and coincide with our experiences (Compton and Koch 2001; Paul et al. 2001; Miyazaki et al. 2010). Furthermore, the used phytohormone Thidiazuron (TDZ) seemed to play an important role in elucidating the problems in plant regeneration. Despite positive regenerations of other pelargonium genotypes in the past, the phenotypes of regenerates of the used pelargonium genotypes are in agreement with other publications. A common side effect is hyperhydricity, which has been shown in *Petunia* and other plant species when using

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TDZ as cytokinin (Kadota and Niimi 2003; Dewir et al. 2006; Ivanona and van Staden 2013).

Nonetheless, in the end of the project we were able to raise three putative transgenic petunias, carrying the transcripts of the legume CHR and GT, whereas none of the *Pelargonium* explants survived since they failed to root. When screened for integration into the genome, neither the structural genes nor *nptII* could be detected.

In parallel to the introduction experiments, we worked on silencing genes involved in anthochlor biosynthesis transiently utilizing virus-induced gene silencing (VIGS) and stably using the CRISPR/Cas9 system. Vector have been designed and produced. However, due to the problems in regeneration, the application of CRISPR/Cas9 was postponed in favor of additional introduction transformations. Equally, VIGS experiments were only tested so far but never utilized in silencing genes in target organisms. Yet, the constructs are finished and ready to use.

There are no bad results, just results. Indeed, we failed to introduce the anthochlor biosynthesis into plants, but we gained important insights in the obstacles during this process. We identified the problems and found solutions to overcome them. Unfortunately, due to the expired project time, we were not able to repeat the experiments with the updated protocols. These updates include: i) more deeply characterization of promoter-terminator interaction; ii) characterization of recombinant proteins produced *in planta* in combination with viral vectors for rapid production of protein of interests; iii) continuously performing transient transformation prior stable transformation in order to elucidate the fate of the constructs in model plants (Walliser et al. 2015); iv) using fresh and different *A. tumefaciens* strains; v) utilization of additional model plant species in combination with proven transformation methods such as *Arabidopsis thaliana* with the floral dip method; vi) use improved regeneration protocols based on our findings regarding alternative cytokinins and replacement of PPM; .

3.4. Outlook

The creation of genetically modified plants is never an easy task, especially when it's not routine in the lab. This work showed some of the obstacles which can occur during the cloning of novel vectors, during plant transformation and plant regeneration. Although we did not obtain bright yellow pelargonia at the end of the project time, we

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laid the foundation for follow-up researches in achieving this goal. The research we performed on *Bidens ferulifolia* is highly valuable for breeders in creating new and striking varieties and its findings were received favorably by the *Bidens* breeder at Selecta One. And as already mentioned before, the experiences we achieved in transformation and regeneration of *Pelargonium* provides the foundation for potential follow-up experiments in this field. Even though the political environment in Europe is not friendly to genetic modified organisms, the potential for creating novel flower coloration through biotechnological breeding remains valuable for breeders.

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References

Abdel-Lateif, K., Bogusz, D., and Hocher, V. (2012), 'The role of flavonoids in the establishment of plant roots endosymbioses with arbuscular mycorrhiza fungi, rhizobia and Frankia bacteria', *Plant Signaling & Behavior*, 7/6: 636–641.

Advances in Applied Biotechnology.

Ahmed, F. A., Kim, S. Y., Kurimoto, S. I. et al. (2011), *Biflavonoids from flowers of Butea monosperma (Lam.) Taub* (Heterocycles, 83).

Alfatafta, A. A., and Mullin, C. A. (1992), 'Epicuticular terpenoids and an aurone from flowers of Helianthus annuus', *Phytochemistry*, 31/12: 4109–4113.

Amalesh Samanta, Gouranga Das, and Sanjoy Kumar Das (2011), *Roles of flavonoids in Plants* (6) <https://www.researchgate.net/profile/sanjoy-das-6/publication/279499208_roles_of_flavonoids_in_plants>.

Avalos, J., and Carmen Limón, M. (2015), 'Biological roles of fungal carotenoids', *Current genetics*, 61/3: 309–324.

Avila, E. M., and Day, A. (2021), 'Stable Plastid Transformation of Petunia for Studies in Basic Research', *Methods in molecular biology (Clifton, N.J.)*, 2317: 229–245.

Ayabe, S.-I., Udagawa, A., and Furuya, T. (1988), 'NAD(P)H-dependent 6'-deoxychalcone synthase activity in Glycyrrhiza echinata cells induced by yeast extract', *Archives of Biochemistry and Biophysics*, 261/2: 458–462.

Baker, N. R. (2008), 'Chlorophyll fluorescence: a probe of photosynthesis in vivo', *Annual review of plant biology*, 59: 89–113.

Bashandy, H., and Teeri, T. H. (2017), 'Genetically engineered orange petunias on the market', *Planta*, 246/2: 277–280.

Bate-Smith, E. C. (1962), 'The phenolic constituents of plants and their taxonomic significance. I. Dicotyledons', *Journal of the Linnean Society of London, Botany*, 58/371: 95–173.

Berim, A., Gang, D.R. (2013), 'The roles of a flavone 6-hydroxylase and 7-O-demethylation in the flavone biosynthetic network of sweet basil', *J. Biol. Chem.*, 288, 1795-1805

Bhatt, S. K., Saxena, V. K., and Singh, K. V. (1981), 'A leptosidin glycoside from leaves of Cyperus scariosus', *Phytochemistry*, 20/11: 2605.

Bhattacharya, A., Sood, P., and Citovsky, V. (2010), 'The roles of plant phenolics in defence and communication during Agrobacterium and Rhizobium infection', *Molecular Plant Pathology*, 11/5: 705–719.

Boase, M. R., Bradley, J. M., and Borst, N. K. (1998), 'An improved method for transformation of regal pelargonium (Pelargonium Xdomesticum Dubonnet) by Agrobacterium tumefaciens', *Plant Science*, 139/1: 59–69.

Bohm, AB (1988), *The minor flavonoids the flavonoids advances in research since 1980 (p. 352)*.

Bomati, E. K., Austin, M. B., Bowman, M. E. et al. (2005), 'Structural elucidation of chalcone reductase and implications for deoxychalcone biosynthesis', *The Journal of biological chemistry*, 280/34: 30496–30503.

Boucherle, B., Peuchmaur, M., Boumendjel, A. et al. (2017), 'Occurrences, biosynthesis and properties of aurones as high-end evolutionary products', *Phytochemistry*, 142: 92–111.

Britton, G. (1995), 'Structure and properties of carotenoids in relation to function', *The FASEB Journal*, 9/15: 1551–1558.

Brockington, S. F., Walker, R. H., Glover, B. J. et al. (2011), 'Complex pigment evolution in the Caryophyllales', *The New phytologist*, 190/4: 854–864.

Die approbierte gedruckte Originalversion dieser Dissertation ist an der TU Wien Bibliothek verfügbar.
The approved original version of this doctoral thesis is available in print at TU Wien Bibliothek.
TU WIEN Bibliothek Your knowledge hub

Broderick, S. R., and Jones, M. L. (2014), 'An Optimized Protocol to Increase Virus-Induced Gene Silencing Efficiency and Minimize Viral Symptoms in Petunia', *Plant molecular biology reporter*, 32: 219–233.

Brouillard, R., and Dangles, O. (1988), 'Flavonoids and flower color', in J. B. Harborne (ed.), *The Flavonoids - Advances in Research*, 565–86.

Caldwell, M. M., Robberecht, R., and Flint, S. D. (1983), 'Internal filters: Prospects for UV-acclimation in higher plants', *Physiol Plant*, 58/3: 445–450.

Chabannes, M., Marazuela, E., and Borja, M. (2009), 'EXPRESSION OF AN ARABIDOPSIS ASPARTIC PROTEASE IN PELARGONIUM', *Acta Hortic.*, 2009: 271–275.

Chang-Quan, W., and Tao, L. (2006), 'Cryptochrome 2 is involved in betacyanin decomposition induced by blue light in Suaeda salsa', *Functional Plant Biol.*, 33/7: 697–702 <https://www.publish.csiro.au/fp/fp06073>.

Chen, C. Y., Wang, L., and Winans, S. C. (1991), 'Characterization of the supervirulent virG gene of the Agrobacterium tumefaciens plasmid pTiBo542', *Molecular & general genetics : MGG*, 230/1-2: 302–309.

Chen, W.-H., Hsu, C.-Y., Cheng, H.-Y. et al. (2011), 'Downregulation of putative UDP-glucose: flavonoid 3-O-glucosyltransferase gene alters flower coloring in Phalaenopsis', *Plant cell reports*, 30/6: 1007–1017.

Chetty, V. J., Ceballos, N., Garcia, D. et al. (2013), 'Evaluation of four Agrobacterium tumefaciens strains for the genetic transformation of tomato (Solanum lycopersicum L.) cultivar Micro-Tom', *Plant cell reports*, 32/2: 239–247.

Chittka, L., Gumbert, A., and Kunze, J. (1997), 'Foraging dynamics of bumble bees: correlates of movements within and between plant species', *Behavioral Ecology*, 8/3: 239–249.

Chittka, L., and Menzel, R. (1992), 'The evolutionary adaptation of flower colours and the insect pollinators' colour vision', *J Comp Physiol A*, 171/2.

Chittka, L., and Raine, N. E. (2006), 'Recognition of flowers by pollinators', *Current opinion in plant biology*, 9/4: 428–435.

Chittka, L., Shmida, A., Troje, N. et al. (1994), 'Ultraviolet as a component of flower reflections, and the colour perception of hymenoptera', *Vision Research*, 34/11: 1489–1508.

Chittka, L., and Waser, N. M. (1997), 'WHY RED FLOWERS ARE NOT INVISIBLE TO BEES', *Israel J Plant Sci*, 45/2-3: 169–183.

Chong, J., Poutaraud, A., and Hugueney, P. (2009), 'Metabolism and roles of stilbenes in plants', *Plant Science*, 177/3: 143–155.

Clement, J. S., and Mabry, T. J. (1996), 'Pigment Evolution in the Caryophyllales: a Systematic Overview*', *Botanica Acta*, 109/5: 360–367.

Compton, M. E., and Koch, J. M. (2001), 'Influence of plant preservative mixture (PPM)TM on adventitious organogenesis in melon, petunia, and tobacco', *In Vitro Cell.Dev.Biol.-Plant*, 37/2: 259–261.

Conner, A. J., Albert, N. W., and Deroles, S. C. (2009), 'Transformation and Regeneration of Petunia', in , *Petunia* (Springer, New York, NY), 395–409 <https://link.springer.com/chapter/10.1007/978-0-387-84796-2_19>.

Cunningham, F. X., and Gantt, E. (2001), 'One ring or two? Determination of ring number in carotenoids by lycopene epsilon-cyclases', *Proceedings of the National Academy of Sciences of the United States of America*, 98/5: 2905–2910.

Da Lin, and O'Callaghan, C. A. (2018), 'MetClo: methylase-assisted hierarchical DNA assembly using a single type IIS restriction enzyme', *Nucleic acids research*, 46/19: e113.

Damalas, S. G., Batianis, C., Martin-Pascual, M. et al. (2020), 'SEVA 3.1: enabling interoperability of DNA assembly among the SEVA, BioBricks and Type IIS restriction enzyme standards', *Microbial Biotechnology*, 13/6: 1793–1806.

Die approbierte gedruckte Originalversion dieser Dissertation ist an der TU Wien Bibliothek verfügbar.
The approved original version of this doctoral thesis is available in print at TU Wien Bibliothek.
TU WIEN Bibliothek Your knowledge hub

Daniell, H., and Dhingra, A. (2002), 'Multigene engineering: dawn of an exciting new era in biotechnology', *Current Opinion in Biotechnology*, 13/2: 136–141 <https://www.sciencedirect.com/science/article/pii/s0958166902002975>.

Davies, K. M., Bloor, S. J., Spiller, G. B. et al. (1998), 'Production of yellow colour in flowers: redirection of flavonoid biosynthesis in Petunia', *The Plant Journal*, 13/2: 259–266.

—— (2002), 'Production of yellow colour in flowers. Redirection of flavonoid biosynthesis in Petunia', *The Plant Journal*, 13/2: 259–266.

Davies, K. M., Marshall, G. B., Bradley, J. M. et al. (2006), 'Characterisation of aurone biosynthesis in Antirrhinum majus', *Physiologia Plantarum*, 128/4: 593–603.

Deba, F., Xuan, T. D., Yasuda, M. et al. (2008), 'Chemical composition and antioxidant, antibacterial and antifungal activities of the essential oils from Bidens pilosa Linn. var. Radiata', *Food Control*, 19/4: 346–352.

Debener, T., and Winkelmann, T. (2010), 'Ornamentals', in F. Kempken and C. Jung (eds.), *Genetic Modification of Plants* (Biotechnology in Agriculture and Forestry, Berlin, Heidelberg: Springer Berlin Heidelberg), 369–91.

Delgado-Vargas, F., and Paredes-Lopez, O. (2002), *Natural Colorants for Food and Nutraceutical Uses* (CRC Press).

Dewir, Y. H., Chakrabarty, D., Ali, M. B. et al. (2006), 'Lipid peroxidation and antioxidant enzyme activities of Euphorbia millii hyperhydric shoots', *Environmental and Experimental Botany*, 58/1-3: 93–99.

Dewir, Y. H., Nurmansyah, Naidoo, Y. et al. (2018), 'Thidiazuron-induced abnormalities in plant tissue cultures', *Plant cell reports*, 37/11: 1451–1470.

Dirk Börstling (2001), 'Transformation von Zierpflanzen. Methoden und Anwendungen', Dissertation (Weihenstephan, TU München).

Du, H., Huang, Y., and Tang, Y. (2010), 'Genetic and metabolic engineering of isoflavonoid biosynthesis', *Applied microbiology and biotechnology*, 86/5: 1293–1312.

Dwyer, M., (2019), 'Bidens are biding their time no longer', https://rotarybotanicalgardens.org/bidens-are-biding-their-time-no-longer/, accessed on 18.07.2022

Dykes, L. (2019), 'Sorghum Phytochemicals and Their Potential Impact on Human Health', *Methods in molecular biology (Clifton, N.J.)*, 1931: 121–140.

Engler, C., Kandzia, R., and Marillonnet, S. (2008), 'A one pot, one step, precision cloning method with high throughput capability', *PLOS ONE*, 3/11: e3647 <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0003647>.

Espley, R. V., Hellens, R. P., Putterill, J. et al. (2007), 'Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10', *The Plant Journal*, 49/3: 414–427.

Fagard, M., and Vaucheret, H. (2000), '(TRANS)GENE SILENCING IN PLANTS: How Many Mechanisms?', *Annual review of plant physiology and plant molecular biology*, 51: 167–194.

Felipe, P. de, Luke, G. A., Hughes, L. E. et al. (2006), 'E unum pluribus: multiple proteins from a self-processing polyprotein', *Trends in Biotechnology*, 24/2: 68–75 <https://www.sciencedirect.com/science/article/pii/s0167779905003288>.

Fisher, R. F., and Long, S. R. (1992), 'Rhizobium--plant signal exchange', *Nature*, 357/6380: 655–660 <https://www.nature.com/articles/357655a0>.

Forkmann, G., and Dangelmayr, B. (1980), 'Genetic control of chalcone isomerase activity in flowers of Dianthus caryophyllus', *Biochem Genet*, 18/5-6: 519–527 <https://link.springer.com/article/10.1007/bf00484399>.

Fraley, R. T., Horsch, R. B., Matzke, A. et al. (1984), 'In vitro transformation of petunia cells by an improved method of co-cultivation with A. tumefaciens strains', *Plant molecular biology*, 3/6: 371–378 <https://link.springer.com/article/10.1007/bf00033384>.

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Fukusaki, E., Kawasaki, K., Kajiyama, S. et al. (2004), 'Flower color modulations of Torenia hybrida by downregulation of chalcone synthase genes with RNA interference', *Journal of biotechnology*, 111/3: 229–240.

Gao, S., Yang, Y., Xu, L. et al. (2018), 'Particle Bombardment of the cry2A Gene Cassette Induces Stem Borer Resistance in Sugarcane', *International journal of molecular sciences*, 19/6.

García-Sogo, B., Pineda, B., Roque, E. et al. (2012), 'Production of engineered long-life and male sterile Pelargonium plants', *BMC plant biology*, 12: 156.

Gehl, C., Wamhoff, D., Schaarschmidt, F. et al. (2018), 'Improved leaf and flower longevity by expressing the etr1-1 allele in Pelargonium zonale under control of FBP1 and SAG12 promoters', *Plant Growth Regul*, 86/3: 351–363.

Geissman, T. A. (1942), 'Anthochlor Pigments. III. The Pigments of Cosmos Sulphureus', *J. Am. Chem. Soc.*, 64/7: 1704–1707.

Geissman, T. A., and Heaton, C. D. (1943), 'Anthochlor Pigments. IV. The Pigments of Coreopsis grandiflora, Nutt. I', *J. Am. Chem. Soc.*, 65/4: 677–683.

Geissman, T. A., Jorgensen, E. C., and Johnson, B. (1954), 'The chemistry of flower pigmentation in Antirrhinum majus. Color genotypes. I. The flavonoid components of the homozygous P, M, Y color types', *Archives of Biochemistry and Biophysics*, 49/2: 368–388.

Geissman, T. A., and Jurd, L. (1954), 'Anthochlor Pigments. IX. The Structure of the Aurone Pigment of Cosmos sulfureus, "Orange Flare" and "Yellow Flare"', *J. Am. Chem. Soc.*, 76/17: 4475–4476.

Gelvin, S. B. (2003), 'Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool', *Microbiology and molecular biology reviews : MMBR*, 67/1: 16-37, table of contents.

George, M. W., and Tripepi, R. R. (2001), 'Plant Preservative Mixture™ Can Affect Shoot Regeneration from Leaf Explants of Chrysanthemum, European Birch, and Rhododendron', *HortSci*, 36/4: 768–769.

Gibson, D. G., Glass, J. I., Lartigue, C. et al. (2010), 'Creation of a bacterial cell controlled by a chemically synthesized genome', *Science (New York, N.Y.)*, 329/5987: 52–56.

Giritch, A., Marillonnet, S., Engler, C. et al. (2006), 'Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors', *Proceedings of the National Academy of Sciences of the United States of America*, 103/40: 14701–14706 <https://www.pnas.org/content/103/40/14701.short>.

Gosch, C., Halbwirth, H., Schneider, B. et al. (2010), 'Cloning and heterologous expression of glycosyltransferases from Malus x domestica and Pyrus communis, which convert phloretin to phloretin 2'-O-glucoside (phloridzin)', *Plant Science*, 178/3: 299–306 <https://www.sciencedirect.com/science/article/pii/s016894521000004x>.

Goto, T., and Kondo, T. (1991), 'Structure and Molecular Stacking of Anthocyanins—Flower Color Variation', *Angew. Chem. Int. Ed. Engl.*, 30/1: 17–33.

Grotewold, E. (2006), 'The genetics and biochemistry of floral pigments', *Annual review of plant biology*, 57: 761–780.

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., Forkmann, G., and Stich, K. (2004), 'The A-ring specific hydroxylation of flavonols in position 6 in Tagetes sp. is catalyzed by a cytochrome P450 dependent monooxygenase', *Plant Science*, 167/1: 129–135.

Halbwirth, H., Muster, G., and Stich, K. (2008), 'Unraveling the Biochemical Base of Dahlia Flower Coloration', *Natural Product Communications*, 3/8: 1934578X0800300.

Hamada, H., Linghu, Q., Nagira, Y. et al. (2017), 'An in planta biolistic method for stable wheat transformation', *Scientific reports*, 7/1: 11443.

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The approved original version of this doctoral thesis is available in print at TU Wien Bibliothek.
TU WIEN Bibliothek Your knowledge hub

Harborne, J. B. (1967), 'Comparative biochemistry of the flavonoids-VI', *Phytochemistry*, 6/12: 1643–1651.

Harborne, J. B., Girija, A. R., Devi, H. et al. (1983), 'Anthochlor pigments from the petals of Mussaenda hirsutissima and Zinnia linearis', *Phytochemistry*, 22/12: 2741–2742.

Harborne, J. B., Greenham, J., and Eagles, J. (1990), 'Malonylated chalcone glycosides in Dahlia', *Phytochemistry*, 29/9: 2899–2900.

Harborne, J. B., and Smith, D. M. (1978), 'Anthochlors and other flavonoids as honey guides in the compositae', *Biochemical Systematics and Ecology*, 6/4: 287–291.

Haselmair-Gosch, C., Miosic, S., Nitarska, D. et al. (2018), 'Great Cause-Small Effect: Undeclared Genetically Engineered Orange Petunias Harbor an Inefficient Dihydroflavonol 4-Reductase', *Frontiers in plant science*, 9: 149.

Haselmair-Gosch, C., Nitarska, D., Walliser, B. et al. (2020), 'Event-specific qualitative polymerase chain reaction analysis for two T-DNA copies in genetically modified orange Petunia', 2020.

Hassanein, A., Chevreau, E., and Dorion, N. (2005), 'Highly efficient transformation of zonal (Pelargonium x hortorum) and scented (P. capitatum) geraniums via Agrobacterium tumefaciens using leaf discs', *Plant Science*, 169/3: 532–541.

Hatlestad, G. J., Akhavan, N. A., Sunnadeniya, R. M. et al. (2015), 'The beet Y locus encodes an anthocyanin MYB-like protein that activates the betalain red pigment pathway', *Nat Genet*, 47/1: 92–96 <https://www.nature.com/articles/ng.3163>.

Havlkov, L., Mikov, K., and Kyzlink, V. (1983), 'Heat stability of betacyanins', *Z Lebensm Unters Forch*, 177/4: 247–250 <https://link.springer.com/article/10.1007/bf01082487>.

Hellens, R., Mullineaux, P., and Klee, H. (2000), 'Technical Focus:A guide to Agrobacterium binary Ti vectors', *Trends in Plant Science*, 5/10: 446–451.

Hirschberg, J. (2001), 'Carotenoid biosynthesis in flowering plants', *Current opinion in plant biology*, 4/3: 210–218.

Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J. et al. (1983), 'A binary plant vector strategy based on separation of vir- and T-region of the Agrobacterium tumefaciens Ti-plasmid', *Nature*, 303/5913: 179–180.

Horn, J. W., van Ee, B. W., Morawetz, J. J. et al. (2012), 'Phylogenetics and the evolution of major structural characters in the giant genus Euphorbia L. (Euphorbiaceae)', *Molecular phylogenetics and evolution*, 63/2: 305–326.

Horsch, R. B., Fry, J. E., Hoffmann, L. N. et al. (1985), 'A simple and general method for transferring genes into plants', *Science (New York, N.Y.)*, 227/4691: 1229–1231.

Hoshino, A., Mizuno, T., Shimizu, K. et al. (2019), 'Generation of Yellow Flowers of the Japanese Morning Glory by Engineering Its Flavonoid Biosynthetic Pathway toward Aurones', *Plant & cell physiology*, 60/8: 1871–1879.

Hossain, M. A., and Rahman, S. M. (2011), 'Total phenolics, flavonoids and antioxidant activity of tropical fruit pineapple', *Food Research International*, 44/3: 672–676.

Howitt, C. A., and Pogson, B. J. (2006), 'Carotenoid accumulation and function in seeds and non-green tissues', *Plant, Cell & Environment*, 29/3: 435–445.

Hutchinson, M. J., and Saxena, P. K. (1996), 'Acetylsalicylic acid enhances and synchronizes thidiazuron-induced somatic embryogenesis in geranium (Pelargonium x hortorum Bailey) tissue cultures', *Plant cell reports*, 15/7: 512–515.

Hwang, H.-H., Yu, M., and Lai, E.-M. (2017), 'Agrobacterium-mediated plant transformation: biology and applications', *The arabidopsis book*, 15: e0186.

Hyndman, D., Bauman, D.R., Heradia, V.V., Penning, T.M., (2013), 'The aldo-keto reductase superfamily homepage', *Chem. Biol. Interact.*, 621-631

Die approbierte gedruckte Originalversion dieser Dissertation ist an der TU Wien Bibliothek verfügbar.
The approved original version of this doctoral thesis is available in print at TU Wien Bibliothek.
TU WIEN Bibliothek Your knowledge hub

Ibdah, M., Krins, A., Seidlitz, H. K. et al. (2002), 'Spectral dependence of flavonol and betacyanin accumulation in Mesembryanthemum crystallinum under enhanced ultraviolet radiation', *Plant, Cell & Environment*, 25/9: 1145–1154.

ISAAA (2018), *Global Status of Commercialized Biotech/GM Crops in 2018: Biotech Crops Continue to Help Meet the Challenges of Increased Population and Climate Change* <https://www.isaaa.org/resources/publications/briefs/54/download/isaaa-brief-54-2018.pdf>, accessed 19 May 2022.

Ivanova, M., and van Staden, J. (2008), 'Effect of ammonium ions and cytokinins on hyperhydricity and multiplication rate of in vitro regenerated shoots of Aloe polyphylla', *Plant Cell Tiss Organ Cult*, 92/2: 227–231.

—— (2011), 'Influence of gelling agent and cytokinins on the control of hyperhydricity in Aloe polyphylla', *Plant Cell Tiss Organ Cult*, 104/1: 13–21.

Jarchow, E., Grimsley, N. H., and Hohn, B. (1991), 'virF, the host-range-determining virulence gene of Agrobacterium tumefaciens, affects T-DNA transfer to Zea mays', *Proceedings of the National Academy of Sciences of the United States of America*, 88/23: 10426–10430.

Joos, H., Timmerman, B., Montagu, M. V. et al. (1983), 'Genetic analysis of transfer and stabilization of Agrobacterium DNA in plant cells', *The EMBO journal*, 2/12: 2151–2160.

Kadota, M., and Niimi, Y. (2003), 'Effects of cytokinin types and their concentrations on Effects of cytokinin types and their concentrations on shoot proliferation and hyperhydricity in in vitro pear cultivar shoots', *Plant Cell Tiss Organ Cult*, 72/3: 261–265.

Kaintz, C., Molitor, C., Thill, J. et al. (2014), 'Cloning and functional expression in E. coli of a polyphenol oxidase transcript from Coreopsis grandiflora involved in aurone formation', *FEBS letters*, 588/18: 3417–3426.

Kasai, M., Matsumura, H., Yoshida, K. et al. (2013), 'Deep sequencing uncovers commonality in small RNA profiles between transgene-induced and naturally occurring RNA silencing of chalcone synthase-A gene in petunia', *BMC genomics*, 14: 63.

Khan, M. I., and Giridhar, P. (2015), 'Plant betalains: Chemistry and biochemistry', *Phytochemistry*, 117: 267–295.

Kimura, Y., Aoki, T., and Ayabe, S. (2001), 'Chalcone isomerase isozymes with different substrate specificities towards 6'-hydroxy- and 6'-deoxychalcones in cultured cells of Glycyrrhiza echinata, a leguminous plant producing 5-deoxyflavonoids', *Plant & cell physiology*, 42/10: 1169–1173.

Kjernsmo, K., Hall, J. R., Doyle, C. et al. (2018), 'Iridescence impairs object recognition in bumblebees', *Scientific reports*, 8/1: 8095.

Klein, G. (1920), 'Studien über das Anthochlor', *Sitzungsberichte: Mineralogie, Krystallographie, Botanik, Physiologie der Pflanzen, Zoologie, Paläontologie, Geologie, physische Geographie und Reisen*, 1920: 341.

Klein, T. M., Wolf, E. D., Wu, R. et al. (1987), 'High-velocity microprojectiles for delivering nucleic acids into living cells', *Nature*, 327/6117: 70–73.

Klug, A. (1968), 'Rosalind Franklin and the discovery of the structure of DNA', *Nature*, 219/5156: 808-10 passim.

Koes, R., Verweij, W., and Quattrocchio, F. (2005), 'Flavonoids: a colorful model for the regulation and evolution of biochemical pathways', *Trends in Plant Science*, 10/5: 236–242 <https://www.sciencedirect.com/science/article/pii/s1360138505000543>.

Koseki, M., Goto, K., Masuta, C. et al. (2005), 'The star-type color pattern in Petunia hybrida 'red Star' flowers is induced by sequence-specific degradation of chalcone synthase RNA', *Plant & cell physiology*, 46/11: 1879–1883.

Koski, M. H. (2020), 'The role of sensory drive in floral evolution', *The New phytologist*, 227/4: 1012–1024.

Koski, M. H., and Ashman, T.-L. (2016), 'Macroevolutionary patterns of ultraviolet floral pigmentation explained by geography and associated bioclimatic factors', *The New phytologist*, 211/2: 708–718.

Die approbierte gedruckte Originalversion dieser Dissertation ist an der TU Wien Bibliothek verfügbar.
The approved original version of this doctoral thesis is available in print at TU Wien Bibliothek.
TU Bibliothek Your knowledge hub WIEN

Kotnik, T., Frey, W., Sack, M. et al. (2015), 'Electroporation-based applications in biotechnology', *Trends in Biotechnology*, 33/8: 480–488.

Kumar, G., Lathwal, E., Saroha, B. et al. (2020), 'Synthesis and Biological Evaluation of Quinoline-Based Novel Aurones', *ChemistrySelect*, 5/12: 3539–3543.

Kundert, P., Sarrion-Perdigones, A., Gonzalez, Y. et al. (2020a), 'A GoldenBraid cloning system for synthetic biology in social amoebae', *Nucleic acids research*, 48/8: 4139–4146.

—— (2020b), 'A GoldenBraid cloning system for synthetic biology in social amoebae', *Nucleic acids research*, 48/8: 4139–4146.

Lata, H., Chandra, S., Khan, I. A. et al. (2009), 'Propagation through alginate encapsulation of axillary buds of Cannabis sativa L. - an important medicinal plant', *Physiology and molecular biology of plants : an international journal of functional plant biology*, 15/1: 79–86.

Lechtenberg, B., Schubert, D., Forsbach, A. et al. (2003), 'Neither inverted repeat T-DNA configurations nor arrangements of tandemly repeated transgenes are sufficient to trigger transgene silencing', *The Plant Journal*, 34/4: 507–517.

Lee, J. H., Won, H. J., Oh, E.-S. et al. (2020), 'Golden Gate Cloning-Compatible DNA Replicon/2A-Mediated Polycistronic Vectors for Plants', *Frontiers in plant science*, 11: 559365.

Leuzinger, K., Dent, M., Hurtado, J. et al. (2013), 'Efficient agroinfiltration of plants for high-level transient expression of recombinant proteins', *JoVE (Journal of Visualized Experiments)*, 2013: e50521 <https://www.jove.com/t/50521/efficient-agroinfiltration-plants-for-high-level-transient-expression>.

Li, Y., Gao, Z., Piao, C. et al. (2014), 'A stable and efficient Agrobacterium tumefaciens-mediated genetic transformation of the medicinal plant Digitalis purpurea L', *Applied biochemistry and biotechnology*, 172/4: 1807–1817.

Lin, H. Y., and Chang, S. T. (2013), 'Antioxidant potency of phenolic phytochemicals from the root extract of Acacia confusa', *Industrial Crops and Products*, 49: 871–878.

Liu, W., Feng, Y., Yu, S. et al. (2021), 'The Flavonoid Biosynthesis Network in Plants', *International journal of molecular sciences*, 22/23.

Luo, Y., Li, X., He, J. et al. (2014), 'Isolation, characterisation, and antioxidant activities of flavonoids from chufa (Eleocharis tuberosa) peels', *Food Chemistry*, 164: 30–35.

Mabry, T. J. (1964), *The betacyanins, a new class of red-violet pigments, and their phylogenetic significance. 239–254* (Taxonomic biochemistry and serology).

Mabry, T. J., Markham, K. R., and Thomas, M. B. (1970), 'The Ultraviolet Spectra of Chalcones and Aurones', in T. J. Mabry, K. R. Markham, and M. B. Thomas (eds.), *The Systematic Identification of Flavonoids* (Berlin, Heidelberg: Springer Berlin Heidelberg), 227–50.

Mai, N. T. T., Hai, N. X., Phu, D. H. et al. (2012), 'Three new geranyl aurones from the leaves of Artocarpus altilis', *Phytochemistry Letters*, 5/3: 647–650.

Maoka, T. (2020), 'Carotenoids as natural functional pigments', *Journal of natural medicines*, 74/1: 1–16.

Maoka, T., Etoh, T., Kishimoto, S. et al. (2011), 'Carotenoids and their fatty acid esters in the petals of Adonis aestivalis', *Journal of oleo science*, 60/2: 47–52.

Mato, M., Onozaki, T., Ozeki, Y. et al. (2000), 'Flavonoid biosynthesis in white-flowered Sim carnations (Dianthus caryophyllus)', *Scientia Horticulturae*, 84/3-4: 333–347.

Mattanovich, D., Rüker, F., Machado, A. C. et al. (1989), 'Efficient transformation of Agrobacterium spp. by electroporation', *Nucleic Acids Res*, 17/16: 6747.

Matz, M. V., Fradkov, A. F., Labas, Y. A. et al. (1999), 'Fluorescent proteins from nonbioluminescent Anthozoa species', *Nature biotechnology*, 17/10: 969–973.

Mazza, G., and Brouillard, R. (1987), 'Recent developments in the stabilization of anthocyanins in food products', *Food Chemistry*, 25/3: 207–225.

Die approbierte gedruckte Originalversion dieser Dissertation ist an der TU Wien Bibliothek verfügbar.
The approved original version of this doctoral thesis is available in print at TU Wien Bibliothek.
TU WIEN Bibliothek Your knowledge hub

Mazziotti, I., Petrarolo, G., and La Motta, C. (2021), 'Aurones: A Golden Resource for Active Compounds', *Molecules (Basel, Switzerland)*, 27/1.

Melchers, G., Sacristán, M. D., and Holder, A. A. (1978), 'Somatic hybrid plants of potato and tomato regenerated from fused protoplasts', *Carlsberg Res. Commun.*, 43/4: 203–218.

Mendel, G. (1866), 'Versuche über Pflanzenhybride', *Verhandlungen des Naturforschenden Vereins zu Brünn*, 1866 <https://vlp.mpiwg-berlin.mpg.de/references?id=lit26745&page=p0001>, accessed 18 May 2022.

Menzel, R., Gumbert, A., Kunze, J. et al. (1997), 'POLLINATORS' STRATEGIES IN FINDING FLOWERS', *Israel J Plant Sci*, 45/2-3: 141–156.

Meyer, P., Heidmann, I., Forkmann, G. et al. (1987), 'A new petunia flower colour generated by transformation of a mutant with a maize gene', *Nature*, 330/6149: 677–678 <https://www.nature.com/articles/330677a0>.

Miles, C. O., and Main, L. (1985), 'Kinetics and mechanism of the cyclisation of 2',6'-dihydroxy-4,4'-dimethoxy-chalcone; influence of the 6'-hydroxy group on the rate of cyclisation under neutral conditions', *J. Chem. Soc., Perkin Trans. 2*, 1985: 1639–1642.

—— (1989), 'Kinetics and mechanism of the cyclisation of 2',6'-dihydroxychalcone and derivatives', *J. Chem. Soc., Perkin Trans. 2*, 1989: 1623–1632.

Milosevic, S., Cingel, A., and Subotic, A. (2015), 'Agrobacterium-mediated transformation of ornamental species. A review', *Genetika*, 47/3: 1149–1164.

Miosic, S., Knop, K., Hölscher, D. et al. (2013), '4-Deoxyaurone formation in Bidens ferulifolia (Jacq.) DC', *PloS one*, 8/5: e61766.

Miyajima, I., Maehara, T., Kage, T. et al. (1991), 'Identification of the Main Agent Causing Yellow Color of Yellow-Flowered Cyclamen Muatnt', *J. Japan. Soc. Hort. Sci.*, 60/2: 409–414 <https://www.jstage.jst.go.jp/article/jjshs1925/60/2/60_2_409/_article/-char/ja/>.

Miyazaki, J., Tan, B. H., and Errington, S. G. (2010), 'Eradication of endophytic bacteria via treatment for axillary buds of Petunia hybrida using Plant Preservative Mixture (PPMTM)', *Plant Cell Tiss Organ Cult*, 102/3: 365–372.

Mizuno, T., Uehara, A., Mizuta, D. et al. (2015), 'Contribution of anthocyanin–flavone copigmentation to grayed violet flower color of Dutch iris cultivar 'Tiger's Eye' under the presence of carotenoids', *Scientia Horticulturae*, 186: 201–206.

Molitor, C., Mauracher, S. G., Pargan, S. et al. (2015), 'Latent and active aurone synthase from petals of C. grandiflora. A polyphenol oxidase with unique characteristics', *Planta*, 242/3: 519–537.

Molitor, C., Mauracher, S. G., and Rompel, A. (2016), 'Aurone synthase is a catechol oxidase with hydroxylase activity and provides insights into the mechanism of plant polyphenol oxidases', *PNAS*, 113/13: E1806-15 <https://www.pnas.org/content/113/13/e1806.short>.

Mukhtar, Z. (2018), 'OPTIMIZATION OF PARTICLE BOMBARDMENT CONDITIONS FOR RICE (Oryza sativa L.) TRANSFORMATION', *PAKJAS*, 55/02: 271–278.

Nabavi, S. M., Šamec, D., Tomczyk, M. et al. (2020), 'Flavonoid biosynthetic pathways in plants: Versatile targets for metabolic engineering', *Biotechnology advances*, 38: 107316 <https://www.sciencedirect.com/science/article/pii/S0734975018301812>.

Nakayama, T., Sato, T., Fukui, Y. et al. (2001), 'Specificity analysis and mechanism of aurone synthesis catalyzed by aureusidin synthase, a polyphenol oxidase homolog responsible for flower coloration', *FEBS letters*, 499/1-2: 107–111.

Nakayama, T., Yonekura-Sakakibara, K., Sato, T. et al. (2000), 'Aureusidin synthase: a polyphenol oxidase homolog responsible for flower coloration', *Science (New York, N.Y.)*, 290/5494: 1163–1166.

Nausch, H., Mikschofsky, H., Koslowski, R. et al. (2012a), 'High-level transient expression of ER-targeted human interleukin 6 in Nicotiana benthamiana', *PLOS ONE*, 7/11: e48938.

Die approbierte gedruckte Originalversion dieser Dissertation ist an der TU Wien Bibliothek verfügbar.
The approved original version of this doctoral thesis is available in print at TU Wien Bibliothek.
TU WIEN Bibliothek Your knowledge hub

Nausch, H., Mikschofsky, H., Mischofsky, H. et al. (2012b), ‘Expression and subcellular targeting of human complement factor C5a in Nicotiana species’, *PLOS ONE*, 7/12: e53023.

Ndakidemi, P. A., and Dakora, F. D. (2003), ‘Legume seed flavonoids and nitrogenous metabolites as signals and protectants in early seedling development’, *Functional Plant Biol.*, 30/7: 729–745 <https://www.publish.csiro.au/fp/fp03042>.

Nenadis, N., and Sigalas, M. P. (2011), ‘A DFT study on the radical scavenging potential of selected natural 3’,4’-dihydroxy aurones’, *Food Research International*, 44/1: 114–120.

Nigam, S. S., and Saxena, V. K. (1975), ‘Isolation and study of the aurone glycoside leptosin from the leaves of Flemengia strobilifera’, *Planta medica*, 27/1: 98–100.

Nitarska, D., Boehm, R., Debener, T. et al. (2021), ‘First genome edited poinsettias: targeted mutagenesis of flavonoid 3'-hydroxylase using CRISPR/Cas9 results in a colour shift’, *Plant Cell Tiss Organ Cult*, 147/1: 49–60.

Nitarska, D., Stefanini, C., Haselmair-Gosch, C. et al. (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* (Hannover, Hannover: Gottfried Wilhelm Leibniz Universität Hannover; Technische Informationsbibliothek (TIB)).

Nordström, C. G., and Swain, T. (1956), ‘The flavonoid glycosides of Dahlia variabilis. II. Glycosides of yellow varieties “Pius IX” and “Coton”’, *Archives of Biochemistry and Biophysics*, 60/2: 329–344.

Ogata, J., Itoh, Y., Ishida, M. et al. (2004), ‘Cloning and heterologous expression of cDNAs encoding flavonoid glucosyltransferases from Dianthus caryophyllus’, *Plant Biotechnology*, 21/5: 367–375 <https://www.jstage.jst.go.jp/article/plantbiotechnology/21/5/21_5_367/_article/-char/ja/>.

Ohmya, A. (2011), ‘Diversity of Carotenoid Composition in Flower Petals’, *JARQ*, 45/2: 163–171.

Ohmya, A., Kato, M., Shimada, T. et al. (2019), ‘Molecular Basis of Carotenoid Accumulation in Horticultural Crops’, *Hort. J.*, 88/2: 135–149.

Ohno, S., Hori, W., Hosokawa, M., Tatsuzawa, F., Doi, M., (2016), 'Petal Color Is Associated with Leaf Flavonoid Accumulation in a Labile Bicoloring Flowering Dahlia (*Dahlia variabilis*) 'Yuino'', *The Hortic, J,* 85 (2), 177-186

Ohno, S., Hori, W., Hosokawa, M. et al. (2018a), ‘Identification of Flavonoids in Leaves of a Labile Bicolor Flowering Dahlia (*Dahlia variabilis*) ‘Yuino’’, *The Hortic J*, 87/1: 140–148.

—— (2018b), ‘Post-transcriptional silencing of chalcone synthase is involved in phenotypic lability in petals and leaves of bicolor dahlia (Dahlia variabilis) 'Yuino'’, *Planta*, 247/2: 413–428.

Ohno, S., Hosokawa, M., Hoshino, A. et al. (2011a), ‘A bHLH transcription factor, DvIVS, is involved in regulation of anthocyanin synthesis in dahlia (Dahlia variabilis)’, *Journal of experimental botany*, 62/14: 5105–5116.

Ohno, S., Hosokawa, M., Kojima, M. et al. (2011b), ‘Simultaneous post-transcriptional gene silencing of two different chalcone synthase genes resulting in pure white flowers in the octoploid dahlia’, *Planta*, 234/5: 945–958.

Ohno, S., Yamada, H., Maruyama, K. et al. (2022), *Identification of a novel chalcone reductase gene for isoliquiritigenin biosynthesis in dahlia (Dahlia variabilis)*.

Ohno, S., Yokota, M., Yamada, H. et al. (2021), ‘Identification of Chalcones and their Contribution to Yellow Coloration in Dahlia (Dahlia variabilis) Ray Florets’, *Hort. J.*, 90/4: 450–459.

Oliveira, C., Silva Ferreira, A. C., Mendes Pinto, M. et al. (2003), ‘Carotenoid compounds in grapes and their relationship to plant water status’, *Journal of agricultural and food chemistry*, 51/20: 5967–5971.

Ono, E., Fukuchi-Mizutani, M., Nakamura, N. et al. (2006a), ‘Yellow flowers generated by expression of the aurone biosynthetic pathway’, *Proceedings of the National Academy of Sciences of the United States of America*, 103/29: 11075–11080.

Ono, E., Hatayama, M., Isono, Y. et al. (2006b), ‘Localization of a flavonoid biosynthetic polyphenol oxidase in vacuoles’, *The Plant Journal*, 45/2: 133–143.

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The approved original version of this doctoral thesis is available in print at TU Wien Bibliothek.
TU Bibliothek Your knowledge hub WIEN

Orlikowska, T., Zawadzka, M., Zenkteler, E. et al. (2012), 'Influence of the biocides PPM tm and Vitrofural on bacteria isolated from contaminated plant tissue cultures and on plant microshoots grown on various media', *The Journal of Horticultural Science and Biotechnology*, 87/3: 223–230.

Ozyigit, I. I., and Yucebilgili Kurtoglu, K. (2020), 'Particle bombardment technology and its applications in plants', *Molecular biology reports*, 47/12: 9831–9847.

Paul, A. L., Semer, C., Kucharek, T. et al. (2001), 'The fungicidal and phytotoxic properties of benomyl and PPM in supplemented agar media supporting transgenic arabidopsis plants for a Space Shuttle flight experiment', *Applied microbiology and biotechnology*, 55/4: 480–485.

Peitsch, D., Fietz, A., Hertel, H. et al. (1992), 'The spectral input systems of hymenopteran insects and their receptor-based colour vision', *Journal of comparative physiology. A, Sensory, neural, and behavioral physiology*, 170/1: 23–40.

Peremarti, A., Twyman, R. M., Gómez-Galera, S. et al. (2010), 'Promoter diversity in multigene transformation', *Plant Mol Biol*, 73/4-5: 363–378.

Peterson, R. G., and Joslyn, M. A. (1958), 'Nature of Betanin, the Pigment of Red Beet', *Nature*, 182/4627: 45–46 <https://www.nature.com/articles/182045a0>.

Pfündel, E., and Bilger, W. (1994), 'Regulation and possible function of the violaxanthin cycle', *Photosynthesis research*, 42/2: 89–109.

Portillo-Nava, C., Guerrero-Esperanza, M., Guerrero-Rangel, A. et al. (2021), 'Natural or light-induced pigment accumulation in grain amaranths coincides with enhanced resistance against insect herbivory', *Planta*, 254/5: 101 <https://link.springer.com/article/10.1007/s00425-021-03757-3>.

Quian-Ulloa, R., and Stange, C. (2021), 'Carotenoid Biosynthesis and Plastid Development in Plants: The Role of Light', *International journal of molecular sciences*, 22/3.

Rao, P. (1994), 'In vitro plant regeneration of scented-leaved geranium Pelargonium graveolens', *Plant Science*, 98/2: 193–198.

Reboredo, F., and J. C. Lidon, F. (2012), 'UV-B radiation effects on terrestrial plants – A perspective', *EJFA*, 24/6.

Reuther, G. (1983), 'PROPAGATION OF DISEASE-FREE PELARGONIUM CULTIVARS BY TISSUE CULTURE', *Acta Hortic.*, 1983: 311–320.

Rieseberg, L. H., and Schilling, E. E. (1985), 'FLORAL FLAVONOIDS AND ULTRAVIOLET PATTERNS IN VIGUIERA (COMPOSITAE)', *American Journal of Botany*, 72/7: 999–1004.

Rodriguez-Amaya, D.B., Kimura, M., 'HarvestPlus Handbook for Carotenoid Analysis', *International Food Policy Research Institute (IFPRI): Washington, DC, USA*, Volume 2

S K Reshmi, K M Aravindhan, and P Suganya (2012), 'The effect of light, temperature, pH on stability of betacyanin pigments in Basella alba fruit', *Asian Journal of Pharmaceutical and Clinical Research*, 5/4 <https://www.researchgate.net/profile/reshmi-karthiga/publication/236462229_the_effect_of_light_temperature_ph_on_stability_of_betacyanin_pigments_in_basella_alba_fruit>.

Sant'Ana, R. R. A., Caprestano, C. A., Nodari, R. O. et al. (2020), 'PEG-Delivered CRISPR-Cas9 Ribonucleoproteins System for Gene-Editing Screening of Maize Protoplasts', *Genes*, 11/9.

Sarangi, S., Mandal, C., Dutta, S. et al. (2019), 'Microprojectile based particle bombardment in development of transgenic indica rice involving AmSOD gene to impart tolerance to salinity', *Plant Gene*, 19: 100183.

Sarrion-Perdigones, A., Falconi, E. E., Zandalinas, S. I. et al. (2011), 'GoldenBraid. An iterative cloning system for standardized assembly of reusable genetic modules', *PloS one*, 6/7: e21622.

Sarrion-Perdigones, A., Vazquez-Vilar, M., Palací, J. et al. (2013), 'GoldenBraid 2.0. A comprehensive DNA assembly framework for plant synthetic biology', *Plant physiology*, 162/3: 1618–1631.

Sathasivam, R., Radhakrishnan, R., Kim, J. K. et al. (2021), 'An update on biosynthesis and regulation of carotenoids in plants', *South African Journal of Botany*, 140: 290–302.

Die approbierte gedruckte Originalversion dieser Dissertation ist an der TU Wien Bibliothek verfügbar.
The approved original version of this doctoral thesis is available in print at TU Wien Bibliothek.
TU WIEN Bibliothek Your knowledge hub

Sato, T., Nakayama, T., Kikuchi, S. et al. (2001), 'Enzymatic formation of aurones in the extracts of yellow snapdragon flowers', *Plant Science*, 160/2: 229–236 <https://www.sciencedirect.com/science/article/pii/s016894520000385x>.

Sawut, R., Li, Y., Liu, Y. et al. (2021), 'Applicability of optimized hyperspectral indices for estimating Betalain content in Suaeda salsa', *International Journal of Remote Sensing*, 42/14: 5211–5231.

Schilling, E. E., Panero, J. L., and Storbeck, T. A. (1987), 'Flavonoids of Helianthus series Microcephali', *Biochemical Systematics and Ecology*, 15/6: 671–672.

Schlangen, K., Miosic, S., Castro, A. et al. (2009), 'Formation of UV-honey guides in Rudbeckia hirta', *Phytochemistry*, 70/7: 889–898.

Schlangen, K., Miosic, S., and Halbwirth, H. (2010a), 'Allelic variants from Dahlia variabilis encode flavonoid 3'-hydroxylases with functional differences in chalcone 3-hydroxylase activity', *Archives of Biochemistry and Biophysics*, 494/1: 40–45.

Schlangen, K., Miosic, S., Thill, J. et al. (2010b), 'Cloning, functional expression, and characterization of a chalcone 3-hydroxylase from Cosmos sulphureus', *Journal of experimental botany*, 61/12: 3451–3459.

Schlegel, R. H. J. (2018), *History of plant breeding* (Boca Raton, FL: CRC Press, Taylor & Francis Group).

Schwinn, K., Venail, J., Shang, Y. et al. (2006), 'A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus Antirrhinum', *The Plant cell*, 18/4: 831–851.

Scogin, R., Young, D. A., and Jones, C. E. (1977), 'Anthochlor Pigments and Pollination Biology. II. The Ultraviolet Floral Pattern of Coreopsis gigantea (Asteraceae)', *Bulletin of the Torrey Botanical Club*, 104/2: 155.

Scogin, R., and Zakar, K. (1976), 'Anthochlor pigments and floral UV patterns in the genus Bidens', *Biochemical Systematics and Ecology*, 4/3: 165–167.

Scott, K. J. (2001), 'Detection and Measurement of Carotenoids by UV / VIS Spectrophotometry', *Current Protocols in Food Analytical Chemistry*, 00/1.

Seitz, C., Eder, C., Deiml, B., Kellner, S., Martens, S., Forkmann, G., (2006), 'Cloning, functional identificalion 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 Mol. Bio.*, 365-381

Sepiol, C. J., Yu, J., and Dhaubhadel, S. (2017), 'Genome-Wide Identification of Chalcone Reductase Gene Family in Soybean. Insight into Root-Specific GmCHRs and Phytophthora sojae Resistance', *Frontiers in plant science*, 8: 2073.

Sharma, A., Shahzad, B., Rehman, A. et al. (2019), 'Response of Phenylpropanoid Pathway and the Role of Polyphenols in Plants under Abiotic Stress', *Molecules (Basel, Switzerland)*, 24/13: 2452.

Sheikh, A. H., Raghuram, B., Eschen-Lippold, L. et al. (2014), 'Agroinfiltration by cytokinin-producing Agrobacterium sp. strain GV3101 primes defense responses in Nicotiana tabacum', *Molecular plant-microbe interactions : MPMI*, 27/11: 1175–1185.

Shimada, N., Nakatsuka, T., Nishihara, M. et al. (2006), 'Isolation and characterization of a cDNA encoding polyketide reductase in Lotus japonicus', *Plant Biotechnology*, 23/5: 509–513 <https://www.jstage.jst.go.jp/article/plantbiotechnology/23/5/23_5_509/_article/-char/ja/>.

Shimada, S., Otsuki, H., and Sakuta, M. (2007), 'Transcriptional control of anthocyanin biosynthetic genes in the Caryophyllales', *Journal of experimental botany*, 58/5: 957–967.

Shimokoryama, M., and Geissman, T. A. (1960), 'Anthochlor Pigments. XIV. The Pigments of Viguiera multiflora (Nutt.) and Baeria chrysostoma (F. and M.)', *J. Org. Chem.*, 25/11: 1956–1959.

Slimestad, R., and Verheul, M. (2009), 'Review of flavonoids and other phenolics from fruits of different tomato (Lycopersicon esculentum Mill.) cultivars', *J. Sci. Food Agric.*, 89/8: 1255–1270.

Spaethe, J., Chittka, L., and Skorupski, P. (2006), 'Visual Search and Decision Making in Bees: Time, Speed, and Accuracy', *International Journal of Comparative Psychology*, 2006: 342–357 <https://escholarship.org/uc/item/8jc9v56f>.

Die approbierte gedruckte Originalversion dieser Dissertation ist an der TU Wien Bibliothek verfügbar.
The approved original version of this doctoral thesis is available in print at TU Wien Bibliothek.
TU Bibliothek Your knowledge hub WIEN

Steinert, J., Schiml, S., Fauser, F. et al. (2015), 'Highly efficient heritable plant genome engineering using Cas9 orthologues from Streptococcus thermophilus and Staphylococcus aureus', *The Plant journal : for cell and molecular biology*, 84/6: 1295–1305.

Sui, G., Li, T., Zhang, B. et al. (2021), 'Recent advances on synthesis and biological activities of aurones', *Bioorganic & medicinal chemistry*, 29: 115895.

Sutton, C. L., Taylor, Z. E., Farone, M. B. et al. (2017), 'Antifungal activity of substituted aurones', *Bioorganic & medicinal chemistry letters*, 27/4: 901–903.

Suzuki, K., Hattori, Y., Uraji, M. et al. (2000), 'Complete nucleotide sequence of a plant tumor-inducing Ti plasmid', *Gene*, 242/1-2: 331–336.

Swamy, K. N., and Rao, S. S. R. (2006), 'Influence of Brassinosteroids on Rooting and Growth of Geranium (Pelargonium sp.) Stem Cuttings', *Asian J. of Plant Sciences*, 5/4: 619–622.

Szabó, I., Bergantino, E., and Giacometti, G. M. (2005), 'Light and oxygenic photosynthesis: energy dissipation as a protection mechanism against photo-oxidation', *EMBO reports*, 6/7: 629–634.

Takeda, J., and Abe, S. (1992), 'LIGHT-INDUCED SYNTHESIS OF ANTHOCYANIN IN CARROT CELLS IN SUSPENSION—IV. THE ACTION SPECTRUM', *Photochem Photobiol*, 56/1: 69–74.

Tanaka, Y., Brugliera, F., Kalc, G. et al. (2010), 'Flower color modification by engineering of the flavonoid biosynthetic pathway. Practical perspectives', *Bioscience, biotechnology, and biochemistry*, 74/9: 1760–1769.

Tanaka, Y., Katsumoto, Y., Brugliera, F. et al. (2005), 'Genetic engineering in floriculture', *Plant Cell Tiss Organ Cult*, 80/1: 1–24.

Tanaka, Y., Sasaki, N., and Ohmya, A. (2008), 'Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids', *The Plant journal : for cell and molecular biology*, 54/4: 733–749.

Technavio (2022), 'Flower and Ornamental Plants Market Scope', < https://www.prnewswire.com/news-releases/flower-and-ornamental-plants-market-size-to-grow-by-usd-28-98-billion--1-800-flowerscom-inc-and-astra-fund-holland-bv-among-key-vendors------technavio-301545137.html>, accessed 18 July 2022

Terahara, N. (2015), 'Flavonoids in Foods: A Review', *Natural Product Communications*, 10/3: 1934578X1501000.

Thill, J., Miosic, S., Ahmed, R. 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 biology*, 12: 225.

Thirukkumaran, G., Ntui, V. O., Khan, R. S. et al. (2009), 'Thidiazuron: an efficient plant growth regulator for enhancing Agrobacterium-mediated transformation in Petunia hybrida', *Plant Cell Tiss Organ Cult*, 99/1: 109–115 <https://link.springer.com/article/10.1007/s11240-009-9581-3>.

Tian, J., Pei, H., Zhang, S. et al. (2014), 'TRV-GFP. A modified Tobacco rattle virus vector for efficient and visualizable analysis of gene function', *Journal of experimental botany*, 65/1: 311–322.

Tian, L., Musetti, V., Kim, J. et al. (2004), 'The Arabidopsis LUT1 locus encodes a member of the cytochrome p450 family that is required for carotenoid epsilon-ring hydroxylation activity', *Proceedings of the National Academy of Sciences of the United States of America*, 101/1: 402–407.

Tiwari, J. K., Devi, S., Ali, N. et al. (2018), 'Progress in somatic hybridization research in potato during the past 40 years', *Plant Cell Tiss Organ Cult*, 132/2: 225–238.

Tohge, T., Souza, L. P. de, and Fernie, A. R. (2017), 'Current understanding of the pathways of flavonoid biosynthesis in model and crop plants', *Journal of experimental botany*, 68/15: 4013–4028.

Treutter, D. (2006), 'Significance of flavonoids in plant resistance: a review', *Environ Chem Lett*, 4/3: 147–157.

Trunschke, J., Lunau, K., Pyke, G. H. et al. (2021), 'Flower Color Evolution and the Evidence of Pollinator-Mediated Selection', *Front. Plant Sci.*, 12: 617851.

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Tsuda, S., Fukui, Y., Nakamura, N. et al. (2004), 'Flower color modification of Petunia hybrida commercial varieties by metabolic engineering', *Plant Biotechnology*, 21/5: 377–386.

Tyurin, A. A., Suhorukova, A. V., Kabardaeva, K. V. et al. (2020), 'Transient Gene Expression is an Effective Experimental Tool for the Research into the Fine Mechanisms of Plant Gene Function. Advantages, Limitations, and Solutions', *Plants (Basel, Switzerland)*, 9/9.

Vagánek, A., Rimarčík, J., Lukeš, V. et al. (2012), 'On the energetics of homolytic and heterolytic OH bond cleavage in flavonoids', *Computational and Theoretical Chemistry*, 991: 192–200.

Valadon, L., and Mummery, R. S. (1971), 'Carotenoids of compositae flowers', *Phytochemistry*, 10/10: 2349–2353.

van der Kooi, C. J., Elzenga, J. T. M., Staal, M. et al. (2016), 'How to colour a flower: on the optical principles of flower coloration', *Proceedings. Biological sciences*, 283/1830.

van der Kooi, C. J., Stavenga, D. G., Arikawa, K. et al. (2021), 'Evolution of Insect Color Vision: From Spectral Sensitivity to Visual Ecology', *Annual review of entomology*, 66: 435–461.

Vazquez-Vilar, M., Bernabé-Orts, J. M., Fernandez-del-Carmen, A. et al. (2016), 'A modular toolbox for gRNA-Cas9 genome engineering in plants based on the GoldenBraid standard', *Plant Methods*, 12/1: 10 <https://plantmethods.biomedcentral.com/articles/10.1186/s13007-016-0101-2>.

Walliser, B. (2015), 'Characterization of the expression of the fusionprotein GFP:CphB in Nicotiana benthamiana', Masterarbeit (Rostock, Universität Rostock).

Walliser, B., Lucaciu, C. R., Molitor, C. et al. (2021), 'Dahlia variabilis cultivar 'Seattle' as a model plant for anthochlor biosynthesis', *Plant physiology and biochemistry : PPB*, 159: 193–201.

Walliser, B., Marinovic, S., Kornpointner, C. et al. (2022), 'The (Bio)chemical Base of Flower Colour in Bidens ferulifolia', *Plants (Basel, Switzerland)*, 11/10: 1289.

Wang, C.-Q., Zhao, J.-Q., Chen, M. et al. (2006), 'Identification of betacyanin and effects of environmental factors on its accumulation in halophyte Suaeda salsa', *Zhi wu sheng li yu fen zi sheng wu xue xue bao = Journal of plant physiology and molecular biology*, 32/2: 195–201 <https://pubmed.ncbi.nlm.nih.gov/16622319/>.

Wang, X., Li, C., Liang, D. et al. (2015), 'Phenolic compounds and antioxidant activity in red-fleshed apples', *Journal of Functional Foods*, 18: 1086–1094.

Watson, J. D., and Crick, F. H. (1953), 'Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid', *Nature*, 171/4356: 737–738.

Weber, E., Engler, C., Gruetzner, R. et al. (2011), 'A Modular Cloning System for Standardized Assembly of Multigene Constructs', *PLOS ONE*, 6/2: e16765 <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0016765>.

Weiss, D., (2000), 'Regulation of flower pigmentation and growth: Multiple signaling pathways control anthocyanin synthesis in expanding petals', *Physiol. Plant*, 110, 152-157

Welle, R., and Grisebach, H. (1988), 'Isolation of a novel NADPH-dependent reductase which coacts with chalcone synthase in the biosynthesis of 6'-deoxychalcone', *FEBS letters*, 236/1: 221–225 <https://www.sciencedirect.com/science/article/pii/0014579388803181>.

Wimmer, G., Halbwirth, H., Wurst, F. et al. (1998), 'Enzymatic hydroxylation of 6'-deoxychalcones with protein preparations from petals of Dahlia variabilis', *Phytochemistry*, 47/6: 1013–1016.

Winkelmann, T., Kaviani, K., and Serek, M. (2005), 'Development of a shoot regeneration protocol for genetic transformation in Pelargonium zonale and Pelargonium peltatum hybrids', *Plant Cell Tiss Organ Cult*, 80/1: 33–42.

Winkelmann, T., Kayiani, K., and Serek, M. (2006), 'AGROBACTERIUM-MEDIATED TRANSFORMATION OF PELARGONIUM (PELARGONIUM ZONALE HYBRIDS AND PELARGONIUM PELTATUM HYBRIDS)', *Acta Hortic.*, 2006: 737–746.

Winkel-Shirley, B. (2001), 'Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology', *Plant physiology*, 126/2: 485–493.

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Wojtania, A. (2011), 'Effect of Meta-topolin on in vitro propagation of Pelargonium × hortorum and Pelargonium × hederaefolium cultivars', *Acta Soc Bot Pol*, 79/2: 101–106.

Yabuya, T., Saito, M., Iwashina, T. et al. (2000), 'Stability of flower colors due to anthocyanin-flavone copigmentation in Japanese garden iris, Iris ensata', *Euphytica*, 115/1: 1–5.

Yamagishi, M., Kishimoto, S., and Nakayama, M. (2010), 'Carotenoid composition and changes in expression of carotenoid biosynthetic genes in tepals of Asiatic hybrid lily', *Plant Breeding*, 129/1: 100–107.

Yonekura-Sakakibara, K., Higashi, Y., and Nakabayashi, R. (2019), 'The Origin and Evolution of Plant Flavonoid Metabolism', *Front. Plant Sci.*, 10: 943.

Yue, J., VanBuren, R., Liu, J. et al. (2022), 'SunUp and Sunset genomes revealed impact of particle bombardment mediated transformation and domestication history in papaya', *Nat Genet*, 54/5: 715–724.

Zambryski, P., Joos, H., Genetello, C. et al. (1983), 'Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity', *The EMBO journal*, 2/12: 2143–2150.

Zhao, D., Tao, J., (2015), 'Recent advances on the development and regulation of flower color in ornamental plants', *Front. Plant Sci.*, 6, 261

3.5. Brief summaries of publications and own contribution

.

Publication 1

Benjamin Walliser, Silvija Marinovic, Christoph Kornpointner, Christopher Schlosser, Mustafa Abouelnasr, Olly Sanny Hutabarat, Christian Haselmair-Gosch, Christian Molitor, Karl Stich and Heidi Halbwirth (2022); The (Bio)chemical Base of Flower Colour in *Bidens ferulifolia*; *Plants 2022, 11, 1289*

Summary

In this publication, 16 different colored cultivars of *Bidens ferulifolia* were analyzed for their pigment composition and the underlying enzymatic background. The *Bidens* coloration ranged from pure yellow over yellow-red up to yellow-white, pure white and purple. Anthochlors were the most abundant pigments in all cultivars showing yellow colors. Remarkably, the most prevalent anthochlor was a derivate of okanin, carrying

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an unusual 2'3'4'-hydroxylation pattern in ring A. Furthermore, we demonstrated for the first time an enzyme catalyzing the introduction of a 3' hydroxyl group at the A-ring of isoliquiritigenin and butein. Furthermore, all varieties accumulated the flavone luteolin, red and purple varieties additionally accumulated cyanidin-type anthocyanins. We analyzed two cultivats for carotenoids, other yellow pigments, which were also found accompanying anthochlors, but in much lower concentrations. Most interestingly, in some acyanic cultivars, flavanone 3-hydroxylase (FHT) and /or dihydroflavonol 4-reductase (DFR) activity was lacking, creating a bottleneck in anthocyanin biosynthesis. Breeding efforts in this direction may results in new and interesting color combinations.

Own contribution

Heidi Halbwirth and Karl Stich conceived the research and Heidi Halbwirth and I designed the experiments. I was responsible for tissue collection and preparation and, together with Mustafa Abouelnasr and Olly Hutabarat, performed nucleic acid extraction. Pigment analysis and enzyme activity assays was performed by Mustafa Abouelnasr, Olly Hutabarat and Christian Molitor. The provided data were analyzed by Heidi Halbwirth, Christoph Kornpointer, Christian Haselmair-Gosch and myself. Heidi Halbwirth and I wrote the manuscript which was subsequent proofread for correct English by Christopher Schlosser.

Publication 2

Benjamin Walliser, Calin Rares Lucaciu, Christian Molitor, Silvija Marinovic, Daria Agata Nitarska, Didem Aktas, Thomas Rattei, Ioannis Kampatsikas, Karl Stich, Christian Haselmair-Gosch and Heidi Halbwirth (2021); Dahlia variabilis cultivar 'Seattle' as a model plant for anthochlor biosynthesis; *Plant Physiology and Biochemistry 159 (2021) 193–201*

Summary

In this publication, we analyzed the bicolored *Dahlia variabilis* cultivar 'Seattle' for it's potential use as a model plant for anthochlor pigment research. We showed that the yellow basal part of the petals mainly accumulated 6'-deoxy chalcones, the corresponding 4-deoxy aurones and flavones, whereas only traces of anthochlors and flavones were detectable in the white colored petal tip. Newly assembled transcriptomes of both petal parts delivered three potential chalcone reductase (CHR) candidate genes. However, neither of them was differentially expressed in the single

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petal parts. However, chalcone synthase 2 (CHS2) expression was highly elevated in the yellow petal parts compared to the expression in the white tip, correlating with the accumulation ratios of found flavonoid compounds. This indicates that the difference in anthochlor accumulation is underlies the different expressed CHS2. Additionally, due to a lack of flavanone 3-hydroxylase (FHT) activity in the white petal tip, to anthocyanin accumulation was measured. Expression of other genes involved in anthocyanidin biosynthesis such as dihydroflavonol 4-reductase (DvDFR) and anthocyanidin synthase (DvANS) was not affected.

Own contribution

Heidi Halbwirth, Karl Stich and Thomas Rattei conceived the research and Heidi Halbwirth, Thomas Rattei and I designed the experiments. I performed tissue preparation, nucleic acid extraction, cloning and qPCR with subsequent statistical analysis. Additionally, I supported Silvija Marinovic in pigment analysis. Calin Rares Lucaciu, Thomas Rattei and Christian Molitor provided the transcriptomic data and phylogenetic research. Together with Heidi Halbwirth and Christian Haselmair-Gosch, I wrote the manuscript which was subsequent proofread for correct English by Christopher Schlosser.

Manuscript 3

Benjamin Walliser, Marika Takacs, Robert Boehm, Silvija Marinovic, Renate Peltram, Christian Haselmair-Gosch, Christopher Schlosser, Karl Stich and Heidi Halbwirth (2022); Introduction of Genes involved in anthochlor biosynthesis into plants using the GoldenBraid system

Summary

In this manuscript, we designed and cloned complex multigene constructs using the GoldenBraid ® cloning system. These constructs carried different combinations of genes involved in anthochlor biosynthesis, derived from several members of the *Asteraceae* family and or marker genes for optical observation of successful transformation. Following cloning, the constructs were used for transient and stable transformation methods of model and target plants. Model plants, such as *Dianthus caryophyllus, Dahlia hybrida* and *Pelargonium zonale,* showed differing susceptibility to the transformation process. Whilst dahlia petals rapidly died after infiltration of *Agrobacterium tumefaciens* carrying the constructs, *Dianthus* and *Pelargonium* proved

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themselves to be robust enough to survive the transformation process long enough to exhibit visible expression of introduced marker genes. Although the presence of introduced genes involved in anthochlor biosynthesis were verified by PCR, no accumulation of the desired pigments was detectable when subjected to HPLC. Furthermore, target plants for stable transformation using *Agrobacterium tumefaciens* were *Pelargonium zonale* and *Petunia hybrida*. However, due to serious problems with contamination of endogenous pathogens despite strong desinfection and strong reactions to ingredients of the regeneration medium, no regenerants of *Pelargonium* and only a low number of *Petunia* could be obtained at the end of the project time. Additionally, the regenerated petunias did not show integration of the introduced genes into the genome. Nonetheless, this work highlighted the potential of the GoldenBraid system for inserting multiple genes in plants, outlined potential obstacles and gave advice for overcoming those obstacles for potential repetitions of this approach.

Own contribution

Heidi Halbwirth and Robert Boehm conceived the research and together with myself, we designed the experiments. I performed all required steps from extracting nucleic acids up to cloning the multigene GoldenBraid constructs including introduction in *A. tumefaciens* and subsequent transformation of the plants. During the stable transformation and plant regeneration processes, I was supported by Marika Takacs. The follow-up analysis of transformed plants including tissue collection and PCR was performed by myself, whereas pigment analysis and HPLC were done by Silvija Marinovic and Renate Peltram. I analyzed the data and wrote the manuscript together with Heidi Halbwirth and Christian Haselmair-Gosch which was subsequent proofread for correct English by Christopher Schlosser.

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4. Publications

4.1. Publication 1

The (Bio)chemical Base of Flower Colour in *Bidens ferulifolia*

Benjamin Walliser, Silvija Marinovic, Christoph Kornpointner, Christopher Schlosser, Mustafa Abouelnasr, Olly Sanny Hutabarat, Christian Haselmair-Gosch, Christian Molitor, Karl Stich and Heidi Halbwirth

Plants 2022, 11, 1289.

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Article

The (Bio)chemical Base of Flower Colour in *Bidens ferulifolia*

Benjamin Walliser [1], Silvija Marinovic [1], Christoph Kornpointner [1], Christopher Schlosser [1], Mustafa Abouelnasr [1], Olly Sanny Hutabarat [1,2], Christian Haselmair-Gosch [1], Christian Molitor [1], Karl Stich [1] and Heidi Halbwirth [1,*]

[1] Institute of Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, 1060 Vienna, Austria; benjamin.walliser@tuwien.ac.at (B.W.); silvija.miosic@tuwien.ac.at (S.M.); christoph.kornpointner@tuwien.ac.at (C.K.); christopher.schlosser@tuwien.ac.at (C.S.); abouelnasr.mustafa@gmail.com (M.A.); olly_hutabarat@yahoo.com (O.S.H.); christian.gosch@tuwien.ac.at (C.H.-G.); christian.molitor@tuwien.ac.at (C.M.); karl.stich@tuwien.ac.at (K.S.)
[2] Department of Agricultural Technology, Hasanuddin University, Makassar 90245, Indonesia
* Correspondence: heidrun.halbwirth@tuwien.ac.at

Abstract: *Bidens ferulifolia* is a yellow flowering plant, originating from Mexico, which is increasingly popular as an ornamental plant. In the past few years, new colour combinations ranging from pure yellow over yellow-red, white-red, pure white and purple have emerged on the market. We analysed 16 *Bidens ferulifolia* genotypes to provide insight into the (bio)chemical base underlying the colour formation, which involves flavonoids, anthochlors and carotenoids. In all but purple and white genotypes, anthochlors were the prevalent pigments, primarily derivatives of okanin, a 6′-deoxychalcone carrying an unusual 2′3′4′-hydroxylation pattern in ring A. The presence of a cytochrome-P450-dependent monooxygenase introducing the additional hydroxyl group in position 3′ of both isoliquiritigenin and butein was demonstrated for the first time. All genotypes accumulate considerable amounts of the flavone luteolin. Red and purple genotypes additionally accumulate cyanidin-type anthocyanins. Acyanic genotypes lack flavanone 3-hydroxylase and/or dihydroflavonol 4-reductase activity, which creates a bottleneck in the anthocyanin pathway. The carotenoid spectrum was analysed in two *Bidens* genotypes and showed strong variation between the two cultivars. In comparison to anthochlors, carotenoids were present in much lower concentrations. Carotenoid monoesters, as well as diesters, were determined for the first time in *B. ferulifolia* flower extracts.

Keywords: *Bidens ferulifolia*; Asteraceae; anthochlors; anthocyanins; okanin; carotenoid esters; lutein; yellow flower colour; UV-honey guides; flavonoid biosynthesis; chalcone 3′-hydroxylase

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Citation: Walliser, B.; Marinovic, S.; Kornpointner, C.; Schlosser, C.; Abouelnasr, M.; Hutabarat, O.S.; Haselmair-Gosch, C.; Molitor, C.; Stich, K.; Halbwirth, H. The (Bio)chemical Base of Flower Colour in *Bidens ferulifolia*. *Plants* **2022**, *11*, 1289. https://doi.org/10.3390/plants11101289

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1. Introduction

Bidens L. is a globally spread genus belonging to Asteraceae [1], which is one of the largest and economically most important families of flowering plants [2]. Comprising more than 240 species [3], *Bidens* forms together with the closely related *Coreopsis* L. the two largest genera of tribe Coreopsidae [1]. *Bidens ferulifolia* (beggar tick, burr marigold) and hybrids thereof are the most commonly used *Bidens* species in horticulture [4].

B. ferulifolia is an upright growing bushy plant native to Mexico and Southern US [5], which has been used for almost three decades for growing in gardens, containers and mixed ornamental hanging baskets [4]. Despite being a perennial plant, it is typically discarded after the end of the season. Its heavy flower production makes it particularly attractive for pollinators. The first commercial *B. ferulifolia* cultivars were highly vigorous plants with limited market potential and exclusively yellow flowering. Since 2005, more compact cultivars were bred and in 2010, Florsaika from Japan obtained the first red and orange *B. triplinervia* through introgression [4,6]. *B. triplinervia* was crossable with *B. ferulifolia*, resulting in F2 plants combining the coloration of *B. triplinervia* with the fitness of *B. ferulifolia*. The past five years, however, have seen an explosion in the number of

commercial *Bidens* cultivars, extending the available colour spectrum from yellow to white, red and different types of bicoloured patterns [4]. While the pure white flowering cultivars are more popular in Northern Europe, the demand for orange and red flowering cultivars is rising in Southern Europe.

The most common *B. ferulifolia* cultivars are pure yellow flowering as a result of the accumulation of two yellow pigments, the widespread carotenoids and the rare anthochlors (chalcones and aurones), which can be found only in a limited number of species [7]. Whilst carotenoids are distributed uniformly across the whole petal, anthochlors concentrate at the petal base and in the veins of the outer petal parts [8–10]. Due to the different UV absorbance of anthochlor pigments and carotenoids in combination with differences in the petal surface microarchitecture of the inner and outer parts (Schulte et al. 2019), this results in the presence of UV-honey guides attracting UV-sensitive insects as pollinators [11,12]. To the human eye, those patterns are not visible, and the petals appear monochromatic. Anthochlors can be, however, easily detected by exposing petals to ammonia or the alkaline vapor of a cigarette [13]. A bathochromatic shift of approximately 100 nm from the violet to the blue range of the spectrum is caused by the pH-dependent transition of the undissociated phenol groups to phenolates. The human eye perceives this shift of the wavelengths as a colour switch from yellow to red-orange [10] (Figure 1A,B). This is a rapid and anthochlor-specific detection method because carotenoids do not show such a colour shift. In the case of flavones and flavonols, which do not show absorption in the visible spectrum, only a faint yellow coloration may occur as a result of pH-dependent phenol dissociation (Figure 1C,D).

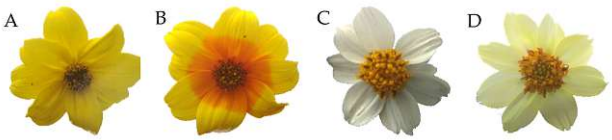

Figure 1. Effect of alkaline vapor on flower pigments. Left pair: Yellow *Bidens* cultivar accumulating carotenoids across whole petals and anthochlors in the basal parts of the petals, exemplified by cv. Mega Charm. Right pair: White *Bidens* cultivar accumulating flavones, exemplified by cv. Beedance White. (**A**) and (**C**) Native flowers. (**B**) After treatment with alkaline vapor, anthochlors in the basal part turn orange, while yellow carotenoids in the outer parts remain unaffected. (**D**) After treatment with alkaline vapor, flavones present in the petals turn a faint yellow colour.

The formation of anthochlors (chalcones and aurones) in *B. ferulifolia* has been studied earlier [10]. Anthochlors can be distinguished into two types, hydroxy and deoxy, defined by the presence or absence of a hydroxyl group at position 6′ of the A-ring (chalcones) or at position 4 of the B-ring (aurones) (Figure 2).

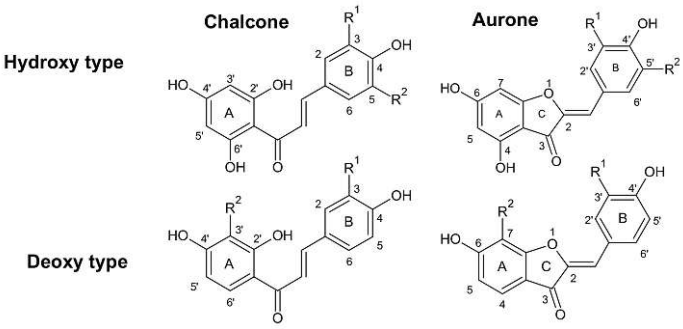

Figure 2. The general structure of anthochlors. Please note the divergent ring numbering in the chalcone and aurone structures. R^1 = H or OH, R^2 = H or OH.

Chalcones are formed by the enzyme chalcone synthase (CHS), which, acting alone, forms 6′-hydroxychalcones (Figure 3). These chalcones quickly isomerize either enzymat-

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ically or chemically to the corresponding colourless flavanones. The chemically stable 6′-deoxychalcones are formed by a mutual co-action of CHS and putative chalcone reductase (CHR) [14]. Accumulation of 6′-deoxychalcones is known to provide the base pigmentation for species of the Asteraceae family such as *Dahlia variabilis* [15], *Cosmos sulphureus*, *Coreopsis grandiflora* or *B. ferulifolia* [16,17].

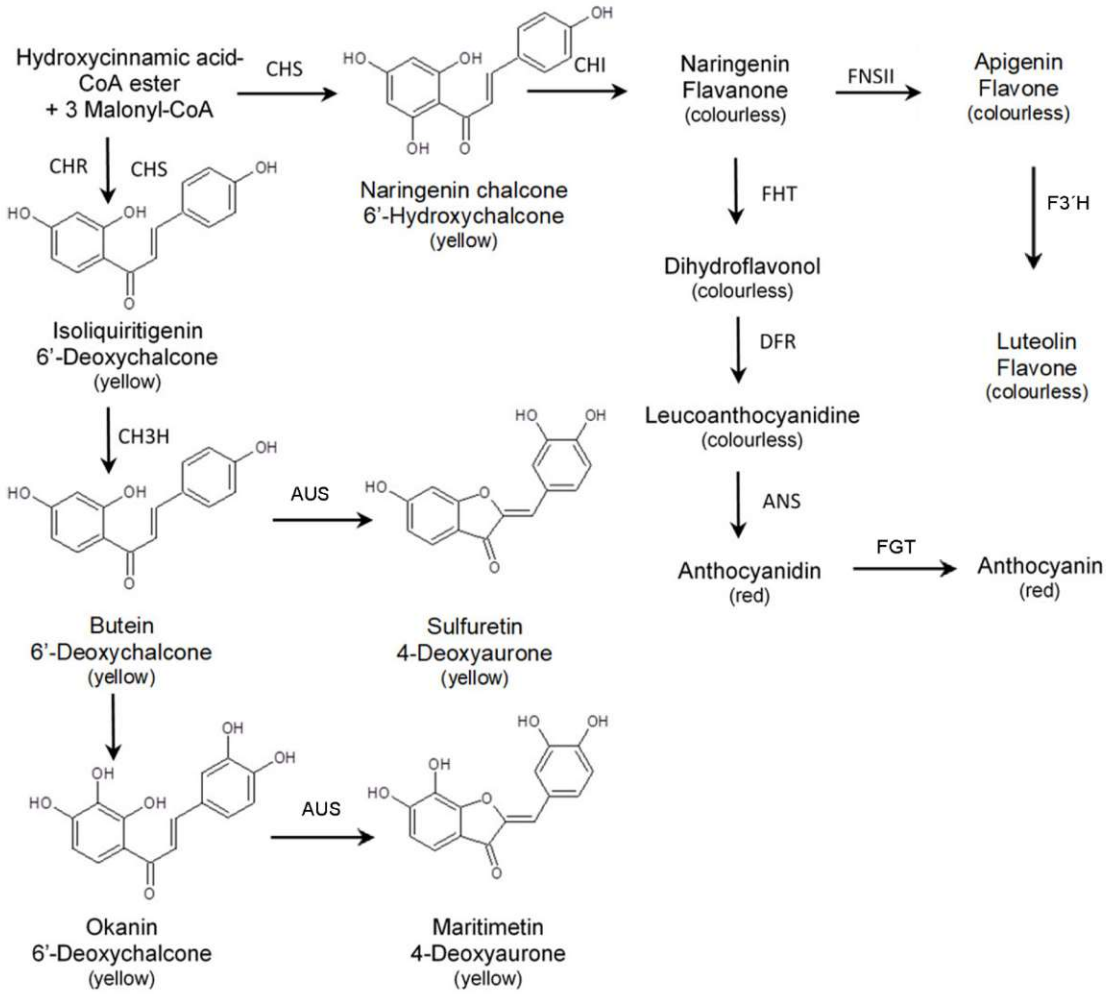

Figure 3. Overview of the biosynthetic pathway of flavonoids in *Bidens ferulifolia* and the general structure of chalcones and aurones. ANS: anthocyanidin synthase, AUS: aurone synthase, CH3H: chalcone 3-hydroxylase, CH3′H: chalcone 3′-hydroxylase, CHI: chalcone isomerase, CHR: chalcone reductase, CHS: chalcone synthase, DFR: dihydroflavonol 4-reductase, F3′H: flavonoid 3′-hydroxylase, FHT: flavanone 3-hydroxylase, FNSII: flavone synthase II.

Chalcones are immediate precursors for the formation of aurones, which can therefore also be distinguished into hydroxy and deoxy types (Figure 3) [18].

Whilst the formation of 4-hydroxyaurones by the enzyme aureusidin synthase was intensively studied in *Antirrhinum majus* [19–23], the biosynthesis of 4-deoxyaurones was studied in different Asteraceae species, and addressed aurone formation [10,24,25] as well as the establishment of the B-ring hydroxylation pattern by an Asteraceae-specific chalcone 3-hydroxylase [26,27] or by aurone synthase (AUS) [28].

In this study, we investigated 16 *B. ferulifolia* genotypes (cultivars and breeding lines) exhibiting different colour combinations (Figure 4) to shed light on the underlying chemical and biochemical base of colour formation in this increasingly popular ornamental.

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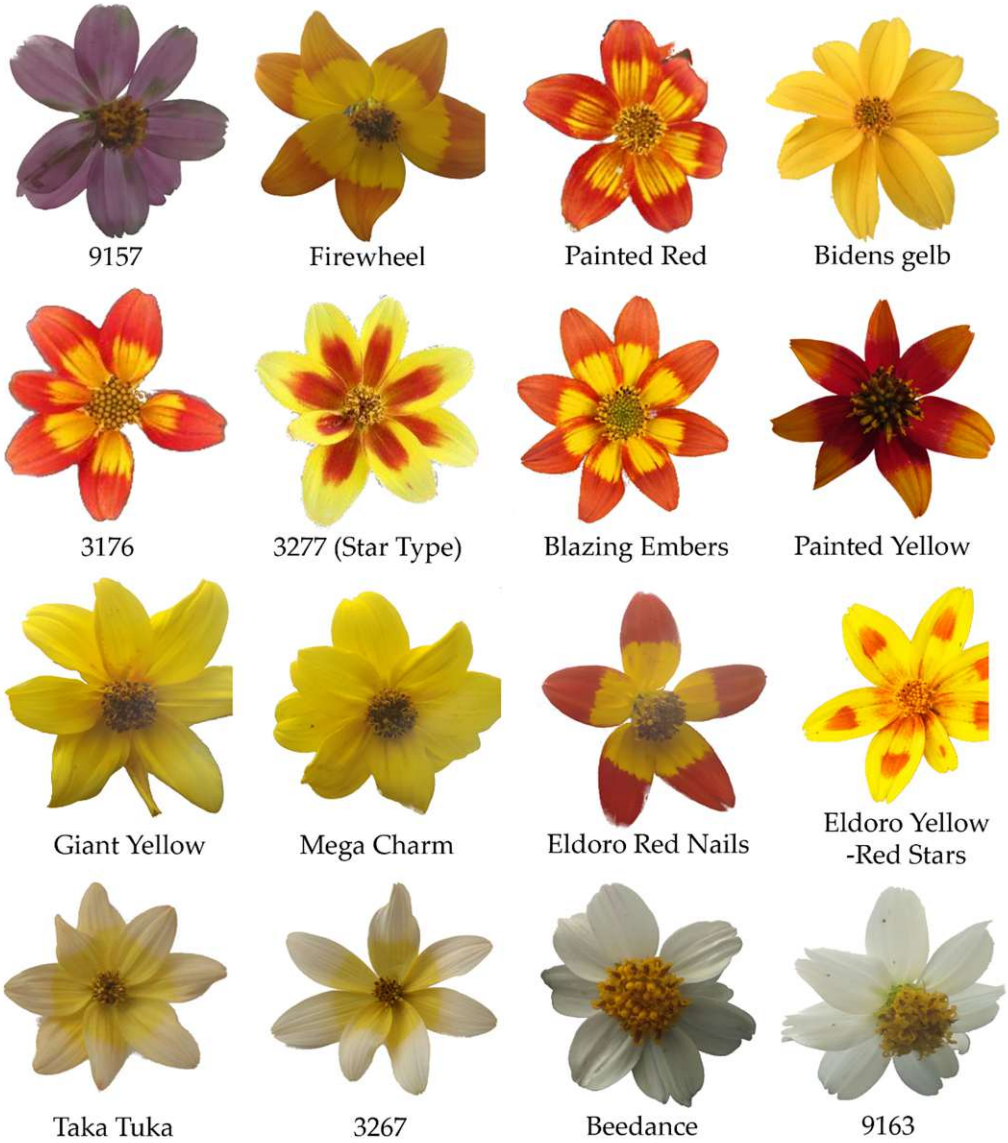

Figure 4. Overview of the *Bidens ferulifolia* genotypes analysed.

2. Results

2.1. Anthochlor and Flavonoid Pigment Composition

The pigment composition of the basal (base) and outer petal parts (tips) of the *Bidens ferulifolia* genotypes was analysed by HPLC after removal of glycoside moieties by acidic and enzymatic hydrolysis. Anthochlors, anthocyanidins and flavones were the predominantly present flavonoid pigments. In a few genotypes, low amounts of dihydroflavonols and flavonols were also found and are included in the column 'Total' (Table 1). The full spectrum present, including the individual compounds for each class, are provided in Supplementary Table S1. The pigment concentrations in the outer part were frequently lower than those in the basal parts.

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Table 1. Content of anthochlors and flavonoids (**µg/g fresh weight**), quantified as aglycones after acidic hydrolysis, in *B. ferulifolia* cultivars. Subtotals for anthochlors and each flavonoid class are shown. The column 'Total' includes anthochlors, flavones, anthocyanins and dihydroflavonols. Qualitative and quantitative composition within classes is shown in Supplementary Table S1. Same letters (a–m) indicate no statistically significant differences according to Duncan ($p < 0.05$) between different genotypes, n.d.: not detected.

Cv./Line	Petal Part	Colour	Anthochlors	Flavones	Anthocyanins	Total
			µg/g fresh weight			
9157	entirety	Purple	n.d.^{l}	2944^{b}	345^{d}	4632
Firewheel	base	Red	6184^{efgh}	787^{fg}	249^{e}	7381
	tip	Red	5272^{ghi}	1090^{e}	1806^{a}	8753
Painted Red	base	Red-Yellow	9223^{abcd}	594^{hi}	19^{f}	9841
	tip	Red	8334^{bcde}	559^{hi}	1597^{b}	10560
Bidens gelb	entirety	Yellow	1987^{kl}	152^{lm}	n.d.^{f}	2139
3176	base	Yellow	3556^{ijk}	338^{jk}	n.d.^{f}	3894
	tip	Red	11095^{a}	1287^{d}	n.d.^{f}	12382
3277 (Star type)	edge	Yellow	6269^{efgh}	193^{kl}	n.d.^{f}	6461
	star	Red	8315^{bcde}	794^{fg}	n.d.^{f}	9109
Blazing Embers	base	Yellow	7202^{cdefg}	685^{gh}	n.d.^{f}	7886
	tip	Red	3253^{ijk}	176^{klm}	n.d.^{f}	3428
Painted Yellow	base	Red	2015^{kl}	n.d.^{m}	517^{c}	2717
	tip	Yellow	1577^{kl}	n.d.^{m}	250^{e}	1843
Giant	base	Yellow	8110^{bcdef}	533^{hi}	5^{f}	8651
	tip	Yellow	1593^{kl}	39^{lm}	28^{f}	1665
Mega Charm	base	Yellow	6829^{defgh}	764^{fg}	n.d.^{f}	7593
	tip	Yellow	2003^{kl}	170^{lm}	n.d.^{f}	2173
Eldoro Red Nails	base	Yellow	4514^{hij}	458^{ij}	n.d.^{f}	4972
	tip	Red	4788^{ghij}	421^{ij}	1627^{b}	7300
Eldoro Y. Red Star	star	Red	10000^{ab}	583^{hi}	353^{d}	10966
	edge	Yellow	1545^{kl}	46^{lm}	n.d.^{f}	1591
Taka Tuka	base	Yellow	9513^{abc}	901^{f}	n.d.^{f}	35998
	tip	Cream	2543^{jkl}	112^{lm}	n.d.^{f}	11088
3267	base	Yellow	9291^{abcd}	1371^{d}	n.d.^{f}	41143
	tip	Cream	5627^{fghi}	416^{ij}	n.d.^{f}	18807
Beedance White	entirety	White	n.d. ^{l}	3252^{a}	7^{f}	3484
9163	entirety	White	n.d. ^{l}	1806^{c}	n.d.^{f}	1852

Anthochlors were present in all *Bidens* genotypes with the exception of the faint purple line 9157 and the pure white genotypes, cv. Beedance White and line 9163, in which no anthochlor pigments were found. Okanin was the most abundant pigment within the anthochlors, reaching up to 11 mg/g FW in some of the genotypes, whereas the concentrations of butein and maritimetin were below 1 mg/g FW. Flavones were found in all but one genotype. In the majority, only luteolin, but no apigenin could be detected (Supplementary Table S1). In comparison to anthochlors, flavone concentrations were much lower, reaching a maximum of 2.9 mg/g FW in the pure white and the faint purple genotypes. In these, however, small amounts of apigenin were also present, in addition to luteolin. In yellow and red *Bidens* petals, luteolin concentrations were lower, ranging from 0.06–1 mg/g FW. Red petal parts also accumulated anthocyanins, particularly cyanidins, and in one case also traces of peonidin (Supplementary Table S1). The concentrations of anthocyanins were comparable to those of flavones and, thus, much lower than the anthochlor concentrations. The highest anthocyanin concentrations were observed in the red outer parts of cv. Firewheel, cv. Eldoro Red Nails and cv. Painted Red. In red-yellow flowering genotypes, a sharp separation of yellow and red tissues was not always possible, as the areas were often intermingled (Figure 4). Of the class of dihydroflavonols, dihydroquercetin (DHQ) was almost exclusively present, but only in the faint lilac and the white genotypes and in some of the red tissues. The highest concentrations were found in the faint purple line 9157, where small amounts of dihydrokaempferol (DHK) in addition to DHQ were also present.

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2.2. Enzyme Activities of the Flavonoid and Anthochlor Pathway

The activities of selected enzymes involved in flavonoid biosynthesis were measured. An overview is provided in Table 2. All genotypes showed comparable CHS activity regardless of the flower colour, though the CHS activity was two to three times higher in monochromatic white and purple genotypes compared with the yellow and yellow-red cultivars. A lack or low activity of flavanone 3-hydroxylase (FHT) and/or dihydroflavonol 4-reductase (DFR) was found in all cultivars and tissues, respectively, in which no anthocyanins were formed (Table 2). Both enzymes were affected in the yellow cv. Mega Charm, the yellow edge of cv. Eldoro Yellow Red Star, in the yellow parts of the cultivars Blazing Embers and Eldoro Red Nails and in the cream parts of cv. Taka Tuka and line 3267. DFR activity, but no FHT activity, was detected in the red-yellow parts of cv. Painted Red, the yellow edge of line 3277, the yellow tips of cv. Painted Yellow and the yellow cv. Giant. In all other acyanic genotypes, the absence of DFR provided a sufficient explanation for the lack of anthocyanins. Flavone synthase II (FNSII) activity could be detected in enzyme preparations from all lines and showed the highest activity in the red-yellow cultivars. CH3 H and CH3′H could also be detected in all genotypes.

Table 2. Selected enzyme activities of the flavonoid and anthochlor pathways detected from enzyme preparations of *B. ferulifolia* cultivars. Same letters (a–i) in a column indicate no statistically significant differences according to Duncan ($p < 0.05$) between different genotypes, n.d.: not detected. An extended version was included as Supplementary Table S2.

Cv./Line	Petal Section	Colour	CHS	FHT	DFR	FNSII	CH3H	CH3′H
			Specific Activity (nmols^{-1}kg^{-1})					
9157	entirety	Purple	852 ^{bcd}	321 ^{b}	1286 ^{a}	183 ^{de}	2121 ^{a}	1642 ^{ghi}
Firewheel	base	Red	383 ^{ghi}	90 ^{ef}	473 ^{cd}	417 ^{ab}	3663 ^{a}	7472 ^{a}
	tip	Red	253 ^{i}	145 ^{de}	892 ^{b}	469 ^{a}	4463 ^{a}	3367 ^{defgh}
Painted Red	base	Red-Yellow	774 ^{cde}	n.d. ^{g}	605 ^{c}	435 ^{a}	3568 ^{a}	4701 ^{bcd}
	tip	Red	658 ^{cdefg}	546 ^{a}	975 ^{b}	391 ^{abc}	3704 ^{a}	5989 ^{ab}
Bidens gelb	entirety	Yellow	465 ^{fghi}	69 ^{fg}	n.d. ^{h}	162 ^{de}	2988 ^{a}	3414 ^{defgh}
3176	base	Yellow	219 ^{i}	219 ^{c}	11 ^{h}	400 ^{abc}	3519 ^{a}	4000 ^{cdef}
	tip	Red	250 ^{i}	249 ^{c}	188 ^{fg}	418 ^{ab}	5589 ^{a}	3980 ^{cdef}
3277 (Star type)	edge	Yellow	489 ^{efghi}	n.d. ^{g}	55 ^{gh}	383 ^{abc}	4386 ^{a}	4762 ^{bcd}
	star	Red	734 ^{cdef}	381 ^{b}	240 ^{ef}	315 ^{abcd}	3781 ^{a}	5619 ^{bc}
Blazing Embers	base	Yellow	226 ^{i}	n.d. ^{g}	20 ^{g}	118 ^{de}	2310 ^{a}	3159 ^{defghi}
	tip	Red	276 ^{hi}	242 ^{c}	153 ^{fgh}	179 ^{de}	1996 ^{a}	2184 ^{fghi}
Painted Yellow	base	Red	446 ^{fghi}	180 ^{cd}	377 ^{de}	231 ^{bcde}	2619 ^{a}	2454 ^{efghi}
	tip	Yellow	483 ^{cde}	n.d. ^{g}	18 ^{h}	131 ^{de}	2870 ^{a}	3101 ^{cdef}
Giant	base	Yellow	480 ^{fghi}	n.d. ^{g}	255 ^{ef}	172 ^{de}	3640 ^{a}	3958 ^{cdef}
	tip	Yellow	403 ^{ghi}	n.d. ^{g}	559 ^{c}	104 ^{e}	1769 ^{a}	3505 ^{defgh}
Mega Charm	base	Yellow	430 ^{ghi}	n.d. ^{g}	n.d. ^{h}	46 ^{e}	2795 ^{a}	4135 ^{cde}
	tip	Yellow	217 ^{i}	n.d. ^{g}	n.d. ^{h}	70 ^{e}	1730 ^{a}	1366 ^{i}
Eldoro Red Nails	base	Yellow	412 ^{ghi}	n.d. ^{g}	n.d. ^{h}	218 ^{cde}	3880 ^{a}	3126 ^{defghi}
	tip	Red	436 ^{ghi}	375 ^{b}	1028 ^{b}	221 ^{cde}	3617 ^{a}	3400 ^{defgh}
Eldoro Yellow Red	star	Red	561 ^{efghi}	187 ^{cd}	435 ^{d}	184 ^{de}	2878 ^{a}	2437 ^{efghi}
Star	edge	Yellow	490 ^{defgh}	n.d. ^{g}	n.d. ^{h}	45 ^{e}	1363 ^{a}	1751 ^{ghi}
Taka Tuka	base	Yellow	827 ^{bcd}	30 ^{fg}	n.d. ^{h}	137 ^{de}	2683 ^{a}	2736 ^{efghi}
	tip	Cream	786 ^{bcd}	n.d. ^{g}	n.d. ^{h}	60 ^{e}	1732 ^{a}	3376 ^{defgh}
3267	base	Yellow	1663 ^{a}	43 ^{fg}	n.d. ^{h}	131 ^{de}	3371 ^{a}	4167 ^{cde}
	tip	Cream	923 ^{bcd}	n.d. ^{g}	n.d. ^{h}	55 ^{e}	1578 ^{a}	3347 ^{defgh}
Beedance W.	entirety	White	1707 ^{a}	56 ^{fg}	n.d. ^{h}	69 ^{e}	1609 ^{a}	2157 ^{fghi}
9163	entirety	White	1064 ^{b}	101 ^{ef}	n.d. ^{h}	159 ^{de}	3273 ^{a}	1543 ^{hi}

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In the frame of our investigations, chalcone 3′-hydroxylase activity was demonstrated for the first time. Incubation of butein with enzyme preparations from *Bidens* petals in the presence of NADPH led to the formation of okanin, which was identified by comparison with the authentic reference compound by HPLC and LC-MS (Figure 5, top). When isoliquiritigenin was used as a substrate, simultaneous activities of CH3H and CH3′H were observed, resulting in the formation of butein, 3′-hydroxyisoliquiritigenin and okanin (Figure 5, bottom). All products were identified by LC-MS (Supplementary Table S3).

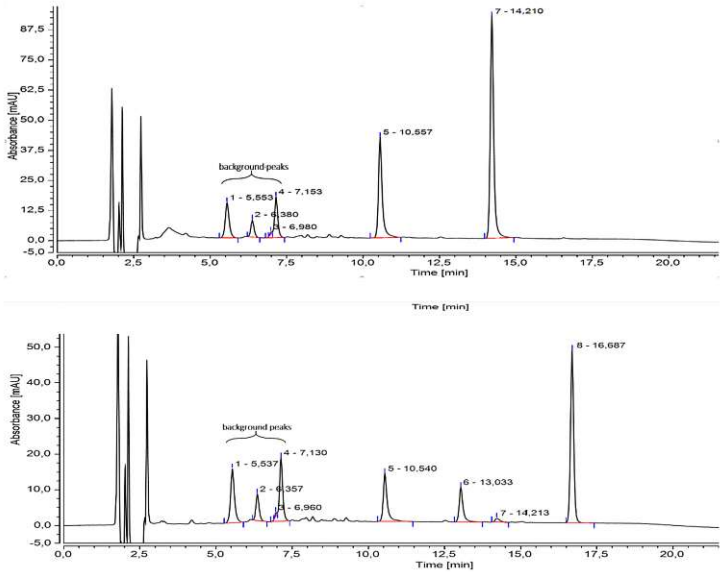

Figure 5. HPLC chromatograms at 385 nm from incubation of enzyme preparations from *Bidens* petals in the presence of NADPH using butein (top) and isoliquiritigenin (bottom) as substrates. Peaks 1–4 are background peaks resulting from remnants of anthochlor and flavonoid glycosides left by the enzyme preparation. 5: okanin, 6: 3′-hydroxyisoliquiritigenin, 7: butein, 8: isoliquiritigenin. Please note: the numbers after the retention labels 1-8 are the retention times in seconds, with a decimal comma instead of a period.

2.3. Carotenoids

Apart from flavonoids and anthochlors, the carotenoids present in *B. ferulifolia* flowers were analysed in two selected cultivars, the uniformly yellow flowering cv. Bidens gelb and the yellow cream cv. Taka Tuka. As for flavonoids and anthochlors, basal (base) and outer parts (tips) of the petals were analysed separately. The concentrations and compositions of carotenoids, which were extracted after freeze drying, varied strongly between the two analysed cultivars. Cv. Bidens gelb mainly accumulated esterized carotenoids, whereas in cv. Taka Tuka more than 90% free carotenoids were found (Figure 6 and Supplementary Table S4). Examples for HPLC chromatograms and an overview of the structures identified in the *B. ferulifolia* petals is provided in Supplementary Figures S1 and S2.

In both cultivars, the predominant xanthophyll was (all-*E*)-lutein, reaching up to 0.50 mg/g DW in cv. Taka Tuka (Figure 6, Supplementary Tables S4 and S5). In addition, high yields of the isomer (13*Z*)-lutein were found (up to 0.11 mg/g DW) in the extracts of cv. Bidens gelb. Thus, the total free carotenoid content consisted of 65% (base) and 75% (tip) lutein isomers in cv. Taka Tuka and 83% (base) and 84% (tip) in cv. Bidens gelb. In addition, further minor xanthophylls were determined in both cultivars, namely (all-*E*)-zeaxanthin, (all-*E*)-violaxanthin and (all-*E*)-antheraxanthin, whereas (all-*E*)-β-carotene was only found in cv. Taka Tuka. Concentrations were, however, only in the range of µg/g DW.

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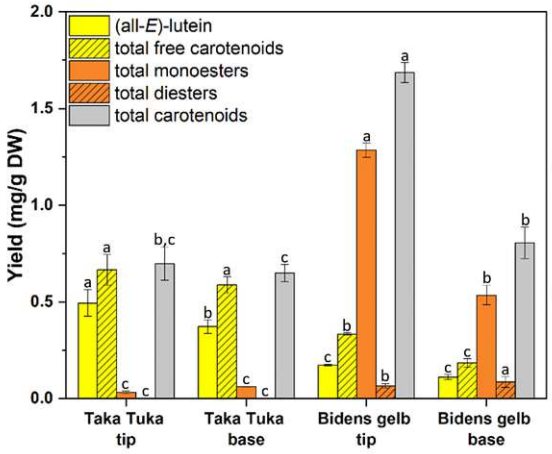

Figure 6. Carotenoid yields (mg/g DW) in cultivars Taka Tuka and Bidens gelb (n = 3, SD). The same letter (a–c) indicates no statistically significant difference across species for the same carotenoid according to Duncan ($p < 0.05$).

Some of the carotenoids were present as monoesters and diesters (Figure 6, Supplementary Table S5). In cv. Bidens gelb, up to 1.29 mg/g DW of carotenoid monoesters were found, predominantly lutein palmitates (base: 45%; tip: 52%) and lutein myristates (base: 28%; tip: 25%), but also smaller amounts of violaxanthin monoesters (base: 8%; tip: 6%) (Figure 6 and Supplementary Table S4). The total diester yields were 87 µg/g DW (base) and 66 µg/g DW (tip), consisting mainly of lutein diesters (base: 73%; tip: 68%) and violaxanthin diesters (base: 27%; tip: 32%) (Figure 6 and Supplementary Table S4). In cv. Taka Tuka, in contrast, no diesters could be detected. Carotenoid monoesters reached only a maximum of 59 µg/g DW in this cultivar and were predominantly lutein myristate (base: 30%; tip: 38%) esters and lutein palmitate esters (base: 65%; tip: 60%).

In cv. Taka Tuka, no significant differences were found between the bases and tips of the petals; 0.70 mg/g DW of total carotenoid in the tips and 0.65 mg/g dray weight in the base were yielded. The basal and outer flower parts seem to accumulate comparable amounts of free and bound carotenoids and were dominated by free carotenoids (Figure 6, Supplementary Table S4). In cv. Bidens gelb, in contrast, significantly higher total carotenoid contents of 1.69 mg/g DW in the tips and 0.81 mg/g DW in the base were found. The relative contents of free carotenoids and carotenoid esters were, however, comparable. For both parts, the total carotenoid content consists of approx. 20% free carotenoids and 80% carotenoid esters.

3. Discussion

In *B. ferulifolia*, flower colour is determined by four main pigment classes, anthochlors, flavones, anthocyanins and carotenoids. The biosynthetic pathway of the first three shares common intermediates, and therefore, the formation of anthocyanins, anthochlors and flavones is competitive. As well as contributing to flower coloration, anthochlors are also responsible for the formation of UV-honey guides, making *Bidens* a model plant for the study of anthochlor-based UV-honey guides. The biosynthesis of anthochlors and anthocyanins starts both from *p*-coumaroyl-CoA and involves CHS (Figure 3). The simultaneous action of CHR initiates the formation of anthochlors of the deoxy type, which is found in some members of the Asteraceae family. The pathway leading to flavones and anthocyanins diverges downstream at the level of flavanones (Figure 3). The flux into the individual pathways depends not only on the presence or absence of enzymes but also of relative activities of enzymes competing for the same substrate.

In this study, we measured the activities of selected enzymes involved in flavonoid and anthochlor formation in order to elucidate potential bottlenecks in the pathway. CHS as the key enzyme of the flavonoid pathway is essential for the formation of all three pigment

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types (Figure 3). FHT and/or DFR are further important enzymes for the formation of anthocyanins, whereas FNSII is the key enzyme for flavone formation and competing with FHT for the same substrate. CH3H and CH3′H are part of the anthochlor pathway determining the hydroxylation pattern of the prevalently present anthochlor okanin. Anthocyanidin synthase (ANS), which is the last enzyme in anthocyanidin formation, and chalcone reductase (CHR), which interacts with CHS to start the 6′-deoxychalcone formation, were not included, as no reliable assays for their activities are available so far.

3.1. Anthocyanins

Whilst in the early yellow cultivars, carotenoids, anthochlors and flavones were exclusively present, anthocyanins have entered the scene with the introduction of red flowering cultivars. Meanwhile a large number of anthocyanin-accumulating cultivars exist. Most of them, however, have bicolored flowers, where anthocyanins are located in the outer parts of the flower and predominantly in the adaxial epidermis, although cultivars exist where anthocyanins are concentrated at the base or appear as red stars on a yellow background. In some cultivars, there is a sharp clear border between red and yellow sections (e.g., Eldoro Red Nails, Beedance, Firewheel), but in many cultivars the borders are blurred (Figure 4). In addition, the intensity and distribution of the red colour of a cultivar depends on the cultivation and specifically requires high light intensities [29,30]. The importance of light for the induction of anthocyanin formation has been previously reported [31].

From a biochemical point of view, the formation of anthocyanins requires the presence of the full set of enzyme activities of the pathway to form the basic anthocyanidin structure. In all genotypes, except line 3176, the absence of anthocyanins was clearly reflected by a lack of the activities of one or both of the enzymes FHT and DFR, with relatively balanced distribution of lines in which DFR or FHT was affected. In the yellow base of line 3176 the absence of anthocyanins could not be completely explained, because apart from FHT activity, DFR could also be detected, albeit only in low activities. In this case, a lack of ANS activity could additionally contribute. In the white genotypes, the absence of anthocyanins is based on the lack of DFR activity, which creates a bottleneck resulting in the accumulation of flavones and dihydroflavonols, which was rarely observed in the analysed genotypes. In the yellow-cream genotypes, DFR activity was generally missing, but in the cream-colored outer parts, FHT was affected as well.

Interestingly, high DFR activity comparable to the yellow-red genotypes was measured in the monochromatically yellow cv. Giant Yellow. Such a genotype carries a high potential for breeding new lines, especially in combination with the yellow-cream and pure white DFR-lacking genotypes, potentially leading to new and striking colour combinations, such as red-white or pure, intense red, by reinstalling the pathway to anthocyanins.

The highest DFR activity was detected in the faint purple line 9157, which strongly contrasted with the highest concentrations of dihydroflavonols found in this genotype (Supplementary Table S1). This points at a bottleneck located downstream of DFR, creating a tailback of dihydroflavonol intermediates, which is in line with the low anthocyanin concentrations resulting in the faint purple colour.

3.2. Anthochlors

In the yellow-red and yellow flowering genotypes, anthochlor pigments were found in the basal and outer parts of the flowers. In the outer parts, however, concentrations were much lower,and were restricted to the veins as reported earlier [24], which is also exemplarily shown for cvs. Giant Yellow, Mega Charm and Taka Tuka in Supplementary Figure S3. This is also particularly relevant in the yellow cream genotypes, in which the outer cream parts show significantly lower concentrations of anthochlors and flavones than the yellow basal parts, as expected. However, in comparison with pure white genotypes, which completely lack anthochlors, relatively high concentrations of anthochlors are found in the cream parts. Due to the presence exclusively in the veins (Supplementary Figure S3),

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the outer part of these varieties appear cream. The absence of anthochlors in the pure white and the faint purple genotypes points at the absence of CHR activity, which cannot be proven, as the formation of 6′-deoxychalcones in the Asteraceae species is not sufficiently understood so far [32].

In contrast to earlier studies [10], we found in the current genotypes primarily chalcones and only low amounts of aurones (Supplementary Table S1). Okanin was prevalently present, a 6′-deoxychalcone exhibiting a rare hydroxylation pattern in the A-ring, because in addition to the commonly found hydroxy groups in position 2′ and 4′, a hydroxy group in position 3′ is present. This corresponds to an additional hydroxy group in position 8 of 5-deoxyflavonoids. In the case of the common 6′-hydroxychalcones, position 3′ corresponds to both positions 6 and 8 of 5-hydroxyflavonoids, respectively, because of the free rotation of ring A. Flavonoids carrying additional hydroxy groups in the A-ring are rare, but have been reported for a few Asteraceae species, e.g., gossypetin (8-hydroxyquercetin) in *Chrysanthemum segetum* and *Gossypium hirsutum* [13,33]. Cytochrome-P450-dependent monooxygenases are frequently responsible, but also FAD dependent enzymes [34]. Whereas the establishment of the B-ring hydroxylation pattern of chalcones is well investigated and involves a cytochrome P450 dependent monooxygenase [26], the introduction of a hydroxy group in position 3′ has not been investigated so far. Using enzyme preparations of *Bidens* petals, we report here for the first time the presence of an enzyme catalysing the introduction of a hydroxyl group in position 3′ of 6′-deoxychalcones. The reaction was dependent on NADPH, and conversion rates were not further increased by FAD. Both isoliquiritigenin and butein are accepted as substrates, but in the case of isoliquiritigenin, the reaction cannot be observed independently from the CH3H reaction, which is also present in the enzyme preparation and also uses NADPH as a cofactor. In the presence of the cytochrome-P450-specific inhibitors ketoconazole and tetcyclacis, the hydroxylation rate in position 3′ decreased by 90%, thereby pointing at the involvement of a cytochrome-P450-dependent monooxygenase.

3.3. Carotenoids

Carotenoids have been reported in various Asteraceae species [35]; however, for *B. ferulifolia* little is known. Valadon et al. [36] carried out a study to determine carotenoids in *B. ferulifolia* flowers and described the presence of carotenes (α, β) and epoxycarotenes, as well as three xanthophylls, namely lutein, auroxanthin and flavoxanthin. However, until now, *B. ferulifolia* extracts have not been analysed by modern analytical techniques. For this purpose, the flower petals were freeze dried to improve the extractability of carotenoids by the removal of water, and to enhance the ratio of the amount of carotenoids to the initial plant weight. Thus, carotenoid concentrations are provided on a DW base and can, therefore, not be directly compared with flavonoid and anthochlor concentrations in Table 1. It seems clear, however, that in comparison with anthochlors, carotenoids are the minor yellow pigments in *Bidens* petals.

Mass spectrometric analysis led to the identification of lutein and β-carotene, which have both been found before in *B. ferulifolia* extracts [36]. In addition, various xanthophylls were identified for the first time, namely violaxanthin, luteoxanthin, antheraxanthin, lutein 5,6-epoxide and zeaxanthin (Supplementary Table S5). Those carotenoids have been described in other yellow flowering Asteraceae species before and are known as part of the carotenoid biosynthesis [35]. For some carotenoids, e.g., lutein or violaxanthin, among others, several *cis* isomers were determined, which can be the result of light exposure, heat or acids [37].

Apart from that, carotenoid monoesters, as well as diesters, were determined for the first time in *B. ferulifolia* flower extracts. Mainly, myristate and palmitate esters of lutein or violaxanthin were characterized (Supplementary Table S4). Several identified xanthophyll esters have been shown to be accumulated in flowers of marigold (*Tagetes* sp.) [38].

In addition, a greater variety of carotenoid esters was found in cv. Bidens gelb when compared with cv. Taka Tuka, where mainly lutein esters were characterized (Figure 6,

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Supplementary Tables S4 and S5). It can be noted that xanthophyll diesters were solely determined in cv. Bidens gelb, whereas β-carotene was only found in cv. Taka Tuka. Hence, the content of carotenoids strongly depends on the genotype.

4. Materials and Methods

4.1. Plant Material

The investigations were carried out on 16 *Bidens ferulifolia* L. genotypes (cultivars and lines) which are listed in Figure 4 and Tables 1 and 2. Petals of the cultivars Firewheel (Florsaika, Netherlands), Painted Red (Beedance Painted Red, MNP Suntory, Netherlands), Eldoro Red, Eldoro Yellow, Giant Yellow, Megacharm (Danziger, Israel) and Beedance White (MNP Suntory, Netherlands) were harvested from the collection of Selecta One GmbH, Stuttgart (Germany); samples of the cultivars 9163, 9177 and Taka Tuka were obtained from Volmary GmbH, Münster (Germany). Petals from fully developed flowers were harvested and separated into the base (lower 40%) and the tip (upper 40%). The intermediate area (central 20%) was discarded in order to avoid cross contamination. The separated parts were shock frozen in liquid nitrogen and stored at −80 °C.

4.2. Chemicals

Isoliquiritigenin, butein, apigenin, luteolin, naringenin, eriodictyol, dihydrokaempferol, dihydroquercetin, marein, maritimein, peonidin and cyanidin were purchased from Extrasynthese (Genay, France). Okanin and maritimetin were obtained from marein and maritimein by enzymatic hydrolysis, as described previously [32]. Lutein (≥95%) and β-carotene (≥98%) were obtained from Extrasynthese (Genace, France). For HPLC mobile phases, acetonitrile hypergrade LC-MS (and formic acid 98–100% and ultra-pure water (Type 1, Direct-Q® 3UV)) were purchased from Merck (Darmstadt, Germany). NADPH was purchased from LabConsulting (Vienna, Austria).

4.3. Anthochlor and Flavonoid Analysis

Analysis for flavonoid class and anthochlors was conducted according to [39]. An amount of 0.5 g (fresh weight) of frozen petal material was macerated in a mortar with 1 mL 2 M hydrochloric acid in methanol and extracted for 5 min at room temperature. After centrifugation, 40 µL supernatant was incubated with 160 µL 4 M HCl for 60 min at 95 °C for anthocyanin analysis. For enzymatic hydrolysis of flavones, flavonols and anthochlors, 20 µL of the supernatant was subjected to enzymatic hydrolysis by 10 U Naringinase (Sigma-Aldrich, Vienna, Austria) and incubated for 20 min at 40 °C in 0.1 M McIlvaine buffer pH 4.0. The reaction was stopped by addition of 30 µL methanol and used in HPLC analysis according to [40]. A Thermo Scientific Dionex UltiMate(R) 3000 RSLC System with DAD-3000RS Photodiode Array Detector (Thermo Scientific, Vienna, Austria) using an Acclaim™ column RSLC 120 C18, 2.2 µm, 120 Å, 2.1 mm × 150 mm (Dionex bonded Silica Products: No. 071399) was operated at 25 °C. For analysis of flavones, flavonols and anthochlor pigments, elution solvents were (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile (gradient: 3 min pre-run at 20% B, 0–15 min 20–53% B, 15–20 min 53–95% B, 20–31 min 95% B, 31–35 min 20% B, flow rate 0.2 mL/min). The compounds were detected at 385 nm and identified by retention times and comparison of the UV-VIS spectra from 190 to 800 nm using authentic reference compounds. The concentrations were calculated from the peak areas of samples and standard lines obtained with the respective reference compounds.

4.4. Carotenoid Analysis

For carotenoid analysis, part of the separated flower petals of the cultivars Taka Tuka and Bidens gelb were freeze dried until weight consistency. Subsequently, ultrasound assisted extraction (UAE) with a Bandelin SONOREX RK 100 SH (35 KHz, 100 W, VWR, Vienna, Austria) was performed at room temperature for 2 h. Thereafter, 0.05–0.14 g dried and ground flower powder was extracted with 0.9–1.4 mL MeOH. The samples were

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centrifuged, filtered through a 0.22 µm filter and stored at −80 °C under dark conditions until analysis.

Identification of carotenoids was performed by UHPLC-DAD-APCI-MS. The methanolic extracts were injected to a 1290 Infinity LC System (Agilent Technologies, USA) equipped with an Acclaim C30 column (3 µm, 2.1 mm × 100 mm, Dionex bonded silica products: NO. 01834074, Thermo Fisher Scientific, Vienna, Austria) coupled to a G7117C diode array detector (450 nm) and subsequently followed by a 6545 LC/Q-TOF (Agilent Technologies, Santa Clara, USA) with a multimode ion source operating in APCI positive mode (range: 100–1700 *m/z*, scan rate: 2 spectra/sec). A combination of buffer A: 91% MeOH, 5% methyl *tert*-butyl ether (MTBE), 3.9% H_2O and 0.1% formic acid; and buffer B: 46% MeOH, 50% MTBE, 3.9% H_2O and 0.1% formic acid was used as mobile phase. Following gradient program was applied: 3 min pre-run at 1% B, 0–5.5 min at 1% B, 5.5–19 min to 1–75% B, 19–20 min to 75–100% B, 20–30 min 100% B, 30–31 min 1% B, 31–34 min 1% B, flow 10 min at 95 vol% (B), 1 min to 20 vol% (B) and post-run 10 min 20 vol% (B). A solvent cut was performed for 0.5 min. The flow rate was set to 0.35 mL/min and the column oven was operated at 18 °C. The recently described method [41] was adapted and modified, and identification of carotenoids was performed based on their fragmentation, retention time and the substance-specific absorbance [38,42–45]. A list of all identified carotenoids for both cultivars is presented in the Supplement Materials (Supplementary Table S4).

Quantification of carotenoids was performed by the same HPLC system as described in Section 4.3. The same C30 column and separation parameters as previously described for UHPLC-DAD-APCI-MS were carried out. External calibration was performed with lutein and β-carotene. Additional identified xanthophylls and xanthophyll esters are expressed as lutein equivalents. Results are expressed as mg per gram dried flower petals (mg/g DW).

4.5. Enzyme Assays

Assays for CHS, CH3H, FNSII, DFR and FHT were performed as described earlier [10]. For CH3′H assays, the reaction contained in the final volume of 100 µL: 40 µL enzyme preparation from petals, 10 µM isoliquiritigenin or butein, 0.05 nM 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 20 µL of 20% acetic acid in acetonitrile. After centrifugation at 16,000 × *g* for 5 min the reaction solution was filtered through a 0.22 µm PTFE membrane. The CH3′H substrates and reaction products isoliquiritigenin, butein, 3′-hydroxyisoliquiritigenin and okanin were identified by high-performance liquid chromatography as described in Section 4.2. For product identification, liquid chromatography coupled to mass spectrometry (HPLC-MS-MS) was performed. An amount of 6 µL of the assay was injected on a 1290 Infinity II LC System (Agilent Technologies, Santa Clara, CA, USA) equipped with a 1260 Infinity II diode array detector (DAD). The separation was performed on a ZORBAX Eclipse Plus C18 Rapid Resolution 1.8 µm, 2.1 mm × 150 mm column (Agilent, Santa Clara, USA) at 35 °C and 0.3 mL/min. The mobile phases were (A) H_2O with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The gradient was set as follows: 0−5 min, 5−15% B; 5−11 min, 15−53% B; 11−15 min, 53−100% B; 21.5 min, 100% B; 21–22 min 100–5% B, 22–26 min 5% B.

Mass detection was performed using an Agilent High-Resolution-y MS 6545 Q-TOF with a Multimode Ion Source (Agilent Technologies, Santa Clara, USA). The main instrumental conditions were as follows: negative electrospray ionization mode, MS scan range was from m/z 100 to 1000, product ion scan range from m/z 50 to 300, capillary voltage 2.0 kV; gas temperature 350 °C; vaporizer temperature 220 °C; gas flow 5 L/min; nebulizer 60 psi; fragmentor voltage 180 V; skimmer 75 V. Nitrogen was used as nebulizer and auxiliary gas. The collision energy for the fragmentation was set on 15 eV.

Data acquisition was carried out using Agilent Mass Hunter Workstation Data Acquisition (Agilent Technologies, Santa Clara, CA, USA) and evaluated using Agilent MassHunter Qualitative Analysis 10.0. Identifications were based on chromatographic elution time, accurate Mass, MS/MS fragmentation pattern, and comparisons with available standards.

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4.6. Statistical Analysis

The statistical analysis of HPLC and enzyme activity assays was performed using RStudio2022.02.0 + 443 and R v 4.1.2 with the library package "agricolae" [46]. Group-wise comparison of enzyme activity or flavonoid contents between different genotypes was calculated using Duncan's new multiple range test (MRT) [47]. The significance level was set to α = 0,05 (5%).

5. Conclusions

B. ferulifolia exhibiting other colours than yellow are relatively new to the market and the biochemical base behind the coloration has not been given a great deal of attention. In this study, we took a closer look at differently coloured *B. ferulifolia* cultivars ranging from pure yellow over red-yellow to white-yellow bicolored and pure white cultivars. To our knowledge, this is the first study dealing with these colour combinations in *B. ferulifolia*.

One of the goals of this study was the search for candidates for breeding new colour combinations. Potential breeding partners are the yellow-red cv. Painted Red, the pure yellow cv. Giant Yellow and the pure white genotypes. Whilst the first two genotypes possess a functional DFR, a lack of FHT activity prevents the synthesis of anthocyanins and vice versa in the pure white genotypes. Introducing a functional enzyme may lead to new colour combinations such as red-white or pure red. Follow-up studies with these genotypes may include investigations of the gene level such as expression studies and sequencing. Nonetheless, this study shows that biochemistry can provide useful information for plant breeders.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11101289/s1, Supplementary Table S1. Content of anthochlors and flavonoids, quantified as aglycones after acidic hydrolysis, in *B. ferulifolia* cultivars. Supplementary Table S2. Selected enzyme activities of the flavonoid and anthochlor pathways detected from enzyme preparations of *B. ferulifolia* cultivars. Supplementary Table S3. Mass spectrometric data of substrates and enzymatically derived products after incubation of isoliquiritigenin or butein in the presence of enzyme preparations of *B. ferulifolia* and NADPH. Supplementary Table S4. Carotenoid yields (mg/g) in extracts of flowers of cultivars Taka Tuka and Bidens gelb (n = 3, SD). Supplementary Table S5. Identified compounds by HR-APCI-MS in *B. ferulifolia* cultivars Taka Tuka (T) and Bidens gelb (G). Supplementary Figure S1. Photos showing the undersides of cv. Giant Yellow, cv. Mega Charm and cv. Taka Tuka with and without alkaline vapor treatment. Supplementary Figure S2. Chemical HPLC chromatograms of the methanolic extracts of petal tips of *B. ferulifolia* cv. Bidens gelb and cv. Taka Tuka. Supplementary Figure S3. Chemical structures of carotenoids identified in *B. ferulifolia*.

Author Contributions: H.H. conceived the research. B.W. and H.H. designed the experiments. B.W., S.M., C.K., M.A. and O.S.H. performed the experiments. C.S., K.S., C.M. and C.H.-G. analysed the data. B.W. and H.H. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Crawford, D.J.; Mort, M.E. Phylogeny of Eastern North American *Coreopsis* (Asteraceae-Coreopsideae): Insights from nuclear and plastid sequences, and comments on character evolution. *Am. J. Botany* **2005**, *92*, 330–336. [CrossRef] [PubMed]
2. Kim, K.-J.; Choi, K.-S.; Jansen, R.K. Two chloroplast DNA inversions originated simultaneously during the early evolution of the sunflower family (Asteraceae). *Mol. Biol. Evol.* **2005**, *22*, 1783–1792. [CrossRef] [PubMed]
3. Crowe, D.R.; Parker, W.H. Hybridization and agamospermy of *Bidens* in northwestern Ontario. *Taxon* **1981**, *30*, 749–760. [CrossRef]
4. Dwyer, M. Bidens are biding their time no longer. Available online: https://rotarybotanicalgardens.org/bidens-are-biding-their-time-no-longer/ (accessed on 11 November 2021).
5. García-Mendoza, A.; del Castillo, J.M. *Diversidad Florística de Oaxaca: De Musgos a Angispermas*; Universidad Nacional Autónoma de México: Mexico City, Mexico, 2011.
6. First registered non-yellow Bidens available in Europe. Available online: http://www.newplantsandflowers.com/first-registered-non-yellow-bidens-available-in-europe/ (accessed on 11 November 2021).
7. Brouillard, R.; Dangles, O. Flavonoids and flower colour. In *The Flavonoids—Advances in Research*; Chapman & Hall: London, UK, 1994; pp. 565–586.
8. Scogin, R.; Zakar, K. Anthochlor pigments and floral UV patterns in the genus *Bidens*. *Biochem. Syst. Ecol.* **1976**, *4*, 165–167. [CrossRef]
9. Harborne, J.B.; Smith, D.M. Anthochlors and other flavonoids as honey guides in the Compositae. *Biochem. Syst. Ecol.* **1978**, *6*, 287–291. [CrossRef]
10. Miosic, S.; Knop, K.; Hölscher, D.; Greiner, J.; Gosch, C.; Thill, J.; Kai, M.; Shrestha, B.K.; Schneider, B.; Crecelius, A.C. 4-Deoxyaurone formation in *Bidens ferulifolia* (Jacq.) DC. *PLoS ONE* **2013**, *8*, e61766. [CrossRef]
11. McCrea, K.D.; Levy, M. Photographic visualization of floral colors as perceived by honeybee pollinators. *Am. J. Bot.* **1983**, *70*, 369–375. [CrossRef]
12. Briscoe, A.D.; Chittka, L. The Evolution of Color Vision in Insects. *Annu. Rev. Entomol.* **2001**, *46*, 471–510. [CrossRef]
13. Harborne, J.B. *Comparative Biochemistry of Flavonoids*; Academic Press: London, UK, 1967.
14. Bomati, A.K.; Austin, M.B.; Bowman, M.E.; Dixon, R.A.; Noel, J.P. Structural elucidation of chalcone reductase and implications for deoxychalcone biosynthesis. *J. Biol. Chem.* **2005**, *280*, 30496–30503. [CrossRef]
15. Halbwirth, H.; Muster, G.; Stich, K. Unraveling the biochemical base of dahlia flower coloration. *Nat. Prod. Commun.* **2008**, *3*, 1259–1266. [CrossRef]
16. Bohm, B.A. The minor flavonoids. In *The Flavonoids: Advances in Research Since 1980*; Chapman & Hall: London, UK, 1988.
17. Bohm, B.A. The minor flavonoids. In *The Flavonoids: Advances in Research Since 1986*; Chapman & Hall: London, UK, 1993.
18. Boucherle, B.; Peuchmaur, M.; Boumendjel, A.; Haudecoeur, R. Occurrences, biosynthesis and properties of aurones as high-end evolutionary products. *Phytochemistry* **2017**, *142*, 92–111. [CrossRef] [PubMed]
19. Nakayama, T.; Sato, T.; Fukui, Y.; Yonekura-Sakakibara, K.; Hayashi, K.; Tanaka, Y.; Kusumi, T.; Nishino, T. Aureusidin synthase: A polyphenol oxidase homolog responsible for flower coloration. *Science* **2000**, *290*, 1163–1165. [CrossRef] [PubMed]
20. Nakayama, T.; Sato, M.; Fukui, Y.; Yonekura-Sakakibara, K.; Hayashi, H.; Tanaka, Y.; Kusumi, T.; Nishino, T. Specifity analysis and mechanism of aurone synthasis catalyzed by aureusidin synthase, a polyphenol oxidase homolog responsible for flower coloration. *FEBS Lett.* **2001**, *499*, 107–111. [CrossRef]
21. Sato, T.; Nakayama, T.; Kikuchi, S.; Fukui, Y.; Yonekura-Sakakibara, K.; Ueda, T.; Nishino, T.; Tanaka, Y.; Kusumi, T. Enzymatic formation of aurones in the extracts of yellow snapdragon flowers. *Plant Sci.* **2001**, *160*, 229–236. [CrossRef]
22. Ono, E.; Hatayama, M.; Isono, Y.; Sato, T.; Watanabe, R.; Yonekura-Sakakibara, K.; Fukuchi-Mizutani, M.; Tanaka, Y.; Kusumi, T.; Nishino, T.; et al. Localization of a flavonoid biosynthetic polyphenol oxidase in vacuoles. *Plant J.* **2006**, *45*, 133–143. [CrossRef]
23. Ono, E.; Fukuchi-Mizutani, M.; Nakamura, N.; Fukui, Y.; Yonekura-Sakakibara, K.; Yamaguchi, M.; Nakayama, T.; Tanaka, T.; Kusumi, T.; Tanaka, Y. Yellow flower generated by expression of the aurone biosynthetic pathway. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11075–11080. [CrossRef]
24. Molitor, C.; Mauracher, S.G.; Pargan, S.; Mayer, R.L.; Halbwirth, H.; Rompel, A. Latent and active aurone synthase from petals of C. grandiflora: A polyphenol oxidase with unique characteristics. *Planta* **2015**, *242*, 519–537. [CrossRef]
25. Kaintz, C.; Molitor, C.; Thill, J.; Kampatsikas, I.; Michael, C.; Halbwirth, H.; Rompel, A. Cloning and functional expression in *E. coli* of a polyphenol oxidase transcript from *C. grandiflora* involved in aurone formation. *FEBS Lett.* **2014**, *588*, 3417–3426. [CrossRef]
26. 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*, 3451–3459. [CrossRef]
27. Schlangen, K.; Miosic, S.; Halbwirth, H. Allelic variants from *Dahlia variabilis* encode flavonoid 3′-hydroxylases with functional differences in chalcone 3-hydroxylase activity. *Arch. Biochem. Biophys.* **2010**, *494*, 40–45. [CrossRef]
28. Molitor, C.; Mauracher, S.G.; Rompel, A. Aurone synthase is a catechol oxidase with hydroxylase activity and provides insights into the mechanism of plant polyphenol oxidases. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 1806–1815. [CrossRef] [PubMed]
29. Zhao, D.; Tao, J. Recent advances on the development and regulation of flower color in ornamental plants. *Front. Plant Sci.* **2015**, *6*, 261. [CrossRef] [PubMed]

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30. Zhao, D.; Hao, Z.; Tao, J. Effects of shade on plant growth and flower quality in the herbaceous peony (*Paeonia lactiflora* Pall.). *Plant Physiol. Biochem.* **2012**, *61*, 187–196. [CrossRef] [PubMed]
31. Weiss, D. Regulation of flower pigmentation and growth: Multiple signaling pathways control anthocyanin synthesis in expanding petals. *Physiol. Plant.* **2000**, *110*, 152–157. [CrossRef]
32. Walliser, B.; Lucaciu, C.R.; Molitor, C.; Marinovic, S.; Nitarska, D.A.; Aktaş, D.; Rattei, T.; Kampatsikas, I.; Stich, K.; Haselmair-Gosch, C. *Dahlia variabilis* cultivar 'Seattle'as a model plant for anthochlor biosynthesis. *Plant Physiol. Biochem.* **2021**, *159*, 193–201. [CrossRef]
33. Berim, A.; Gang, D.R. The roles of a flavone-6-hydroxylase and 7-O-demethylation in the flavone biosynthetic network of sweet basil. *J. Biol. Chem.* **2013**, *288*, 1795–1805. [CrossRef]
34. Halbwirth, H.; Stich, K. An NADPH and FAD dependent enzyme catalyzes hydroxylation of flavonoids in position 8. *Phytochemistry* **2006**, *67*, 1080–1087. [CrossRef]
35. Ohmiya, A. Diversity of carotenoid composition in flower petals. *Jpn. Agric. Res. Q.: JARQ* **2011**, *45*, 163–171. [CrossRef]
36. Valadon, L.; Mummery, R.S. Carotenoids of Compositae flowers. *Phytochemistry* **1971**, *10*, 2349–2353. [CrossRef]
37. Rodriguez-Amaya, D.B.; Kimura, M. *HarvestPlus Handbook for Carotenoid Analysis*; International Food Policy Research Institute (IFPRI): Washington, DC, USA, 2004; Volume 2.
38. Rodrigues, D.B.; Mercadante, A.Z.; Mariutti, L.R.B. Marigold carotenoids: Much more than lutein esters. *Food Res. Int.* **2019**, *119*, 653–664. [CrossRef]
39. Haselmair-Gosch, C.; Miosic, S.; Nitarska, D.; Walliser, B.; Roth, B.L.; Paltram, R.; Lucaciu, R.C.; 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. [CrossRef] [PubMed]
40. Thill, J.; Miosic, S.; Ahmed, R.; Schlangen, K.; Muster, G.; Stich, K.; Halbwirth, H. '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.* **2012**, *12*, 225. [CrossRef] [PubMed]
41. Doppler, P.; Kornpointner, C.; Halbwirth, H.; Remias, D.; Spadiut, O. Tetraedron minimum, First reported member of Hydrodictyaceae to accumulate secondary carotenoids. *Life* **2021**, *11*, 107. [CrossRef] [PubMed]
42. Petry, F.C.; Mercadante, A.Z. New method for carotenoid extraction and analysis by HPLC-DAD-MS/MS in freeze-dried citrus and mango pulps. *J. Braz. Chem. Soc.* **2018**, *29*, 205–215. [CrossRef]
43. Petry, F.C.; Mercadante, A.Z. Composition by LC-MS/MS of new carotenoid esters in mango and citrus. *J. Agric. Food Chem.* **2016**, *64*, 8207–8224. [CrossRef]
44. Mercadante, A.Z.; Rodrigues, D.B.; Petry, F.C.; Mariutti, L.R.B. Carotenoid esters in foods—A review and practical directions on analysis and occurrence. *Food Res. Int.* **2017**, *99*, 830–850. [CrossRef]
45. Bonaccorsi, I.; Cacciola, F.; Utczas, M.; Inferrera, V.; Giuffrida, D.; Donato, P.; Dugo, P.; Mondello, L. Characterization of the pigment fraction in sweet bell peppers (*Capsicum annuum* L.) harvested at green and overripe yellow and red stages by offline multidimensional convergence chromatography/liquid chromatography–mass spectrometry. *J. Sep. Sci.* **2016**, *39*, 3281–3291. [CrossRef]
46. De Mendiburu, F. Agricolae: Statistical procedures for agricultural research. *R Package Version* **2014**, *1*, 1–4. Available online: https://CRAN.R-project.org/package=agricolae (accessed on 1 April 2022).
47. Duncan, D.B. Multiple range and multiple F tests. *Biometrics* **1955**, *11*, 1–42. [CrossRef]

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4.2. Publication 2

Dahlia variabilis cultivar 'Seattle' as a model plant for anthochlor biosynthesis

Benjamin Walliser, Calin Rares Lucaciu, Christian Molitor, Silvija Marinovic, Daria Agata Nitarska, Didem Aktas, Thomas Rattei, Ioannis Kampatsikas, Karl Stich, Christian Haselmair-Gosch, Heidi Halbwirth

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Research article

Dahlia variabilis cultivar 'Seattle' as a model plant for anthochlor biosynthesis

Benjamin Walliser [a], Calin Rares Lucaciu [b], Christian Molitor [a], Silvija Marinovic [a], Daria Agata Nitarska [a], Didem Aktaş [a], Thomas Rattei [b], Ioannis Kampatsikas [a,1], Karl Stich [a], Christian Haselmair-Gosch [a], Heidi Halbwirth [a,*]

[a] Institute of Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, 1060, Vienna, Austria
[b] Department of Microbiology and Ecosystem Science, University of Vienna, 1090, Vienna, Austria

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Chalcone reductase

ABSTRACT

We investigated the bi-colored dahlia cultivar 'Seattle', which exhibits bright yellow petals with white tips, for its potential use as a model system for studies of the anthochlor biosynthesis. The yellow base contained high amounts of the 6′-deoxychalcones and the structurally related 4-deoxyaurones, as well as flavones. In contrast, only traces of anthochlors and flavones were detected in the white tips. No anthocyanins, flavonols, flavanones or dihydroflavonols were found in the petals. Gene expression studies indicated that the absence of anthocyanins in the petals is caused by a lack of *flavanone 3-hydroxylase* (*FHT*) expression, which is accompanied by a lack of expression of the bHLH transcription factor *IVS*. Expression of other genes involved in anthocyanidin biosynthesis such as dihydroflavonol 4-reductase (*DFR*) and anthocyanidin synthase *(ANS)* was not affected. The yellow and white petal parts showed significant differences in the expression of *chalcone synthase 2* (*CHS2*), which is sufficient to explain the absence of yellow pigments in the white tips. Transcriptomes of both petal parts were *de novo* assembled and three candidate genes for chalcone reductase *(CHR)* were identified. None of them showed a significantly higher expression in the yellow base compared to the white tips. In summary, it was shown that the bicolouration is most likely caused by a bottleneck in chalcone formation in the white tip. The relative prevalence of flavones compared to the anthochlors in the white tips could be an indication for the presence of a so far unknown differentially expressed *CHR*.

1. Introduction

Yellow flower coloration occurred as a result of adaption to the color sense of insects, in order to attract them as pollinators (Harborne, 1993). Among several types of yellow pigments such as carotenoids, especially species of the Asteraceae family additionally accumulate the so-called anthochlor pigments (chalcones and aurones). Due to the different UV absorbance of anthochlor pigments and carotenoids, those plants can create UV nectar guides to attract UV sensitive insects as pollinators (Harborne and Smith, 1978; Scogin and Zakar, 1976). Accumulation of chalcones alone provides the main pigmentation of only a few ornamental species (Brouillard and Dangles, 1994).

Chalcones can be distinguished into two types, 6′-hydroxychalcones and 6′-deoxychalcones, defined by the presence or absence of a hydroxyl group in the 6′-position of the A-ring (Fig. 1). Chalcones are the initial intermediates of flavonoid biosynthesis and produced by the action of chalcone synthase (CHS), which merges *p*-coumaroyl-CoA and three molecules malonyl-CoA to the first C15 structure in the pathway. CHS acting alone leads to the formation of 6′-hydroxychalcones, which quickly isomerize either chemically or enzymatically, the latter being catalyzed by chalcone isomerase (CHI), to the corresponding flavanone. 6′-Hydroxychalcone accumulation is generally caused by a lack of activities of CHI and an enzyme further downstream, to prevent anthocyanin formation from chemically isomerized flavanone, and additionally requires the presence of a glucosyltransferase to stabilize and process the chalcone (Ogata et al., 2004). 6′-Hydroxychalcones as

Abbreviations: ANS, anthocyanidin synthase; AUS, aurone synthase; CH3H, chalcone 3-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; CHR, chalcone reductase; DFR, dihydroflavonol 4-reductase; FHT, flavanone 3-hydroxylase; FNS II, flavone synthase II.

* Corresponding author.
E-mail address: heidrun.halbwirth@tuwien.ac.at (H. Halbwirth).
[1] Present address: Universität Wien, Fakultät für Chemie, Institut für Biophysikalische Chemie, Vienna, Austria.

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main pigment for yellow flower coloration are therefore only rarely found e.g. in *Dianthus caryophyllus* (Forkmann and Dangelmayr, 1980), *Cyclamen persicum* (Miyajima et al., 1991) and *Antirrhinum majus* (Harborne, 1967). The 6′-deoxychalcones are formed when CHS acts mutually with the enzyme chalcone reductase (CHR), a rarely occurring enzyme belonging to the aldo-keto reductase (AKR) superfamily, so far described only in the Leguminosae family (Welle and Griesebach, 1988). The suggested mechanism of CHR is the reduction of the polyketide intermediate of CHS, producing the 6′-deoxychalcone (Bomati et al., 2005). Enzymatic isomerization of 6′-deoxychalcones requires a CHI with broad substrate specificity, which was so far only found in the Fabaceae family (Kimura et al., 2001), and chemical isomerization occurs at a much lower rate compared to 6′-hydroxychalcones (Miles and Main, 1989). Therefore, 6 ′-deoxychalcones, if formed at all, have a higher chance for accumulation in the petals than the 6′-hydroxychalcones. Accumulation of 6′-deoxychalcone pigments has been described for species of the Asteraceae family, e.g *Cosmos sulphureus, Bidens ferulifolia, Coreopsis grandiflora* or *Dahlia variabilis* (Bohm, 1988, 1993; Halbwirth et al., 2008).

Aurones are formed from chalcones as immediate precursors and can be therefore also distinguished in hydroxy and deoxy types (Fig. 1).

Aurone formation was primarily studied in *Antirrhinum majus*, which accumulates 4-hydroxyaurones, catalyzed by the polyphenol oxidase homologue aureusidin synthase (AUS (Nakayama T. et al., 2000; Nakayama T. et al., 2001; Sato et al., 2001; Ono et al., 2006; Ono et al., 2006)). More recently, the formation of 4-deoxyaurones in plants of the Asteracaea species such as *B. ferulifolia* (Miosic et al., 2013) or *C. grandiflora* (Kaintz et al., 2014) was studied. AUS accepts 6′-deoxychalcones as substrates and converts them into the corresponding 4-deoxyaurones (Molitor et al., 2015). The introduction of a second hydroxyl group in the B-ring seems to be catalyzed by an Asteraceae specific chalcone 3-hydroxylase (CH3H) (Wimmer et al. 1998; Schlangen et al., 2010a, 2010b), but may also occur during the formation of the 4-deoxyaurones (Molitor et al., 2016). Pigments carrying three hydroxy groups on the B-ring are not present in dahlia, neither in flavonoids nor in anthochlors (Halbwirth et al., 2008).

Dahlia (*Dahlia variabilis* hort.), a member of the Asteraceae family, is with more than 20,000 cultivars one of the most popular garden plants world-wide (McClaren, 2009). The flower color ranges from nearly black to any imaginable hue of magenta, red, orange, white and yellow, based on the accumulation of flavonoids and anthochlors (Giannasi, 1975; Nordström and Swain, 1956; Halbwirth et al., 2008). Therein, three basic color types can be distinguished: red hues (including black) are based on the presence of anthocyanins. Yellow color is a result of the formation of anthochlors. Acyanic varieties typically show ivory hues and are characterized by the presence of flavones (Lawrence, 1931b). Real white varieties, which show complete absence of flavonoids and anthochlors, occur rarely in dahlia (Lawrence, 1932; Ohno et al., 2011b). The presence of flavonols has also been shown, but does not seem to be related to a specific color type (Thill et al., 2012). Due to the octaploidy of dahlia, variegation in dahlia flowers is frequently observed (Lawrence, 1931b; Ohno et al., 2011b).

Whereas the anthocyanin pathway leading to red hues is well studied, the formation of 6′-deoxychalcones and the corresponding 4-deoxyaurones remains unclear. In this study, we investigated a yellow-white bicolored dahlia (Fig. 2), which accumulates anthochlor pigments in its inner petal parts, whereas only low amounts of pigments without traces of anthochlor pigments can be found in the outer petal parts. We used the cultivar as a potential model plant for studying anthochlor biosynthesis and identified the biosynthetic bottleneck in the formation of anthochlors. We observed a lack of CHS expression correlating with the absence of anthochlors in the white tips. The relative prevalence of flavones compared to the anthochlors in the white tips could additionally indicate the presence of a so far unknown differentially expressed

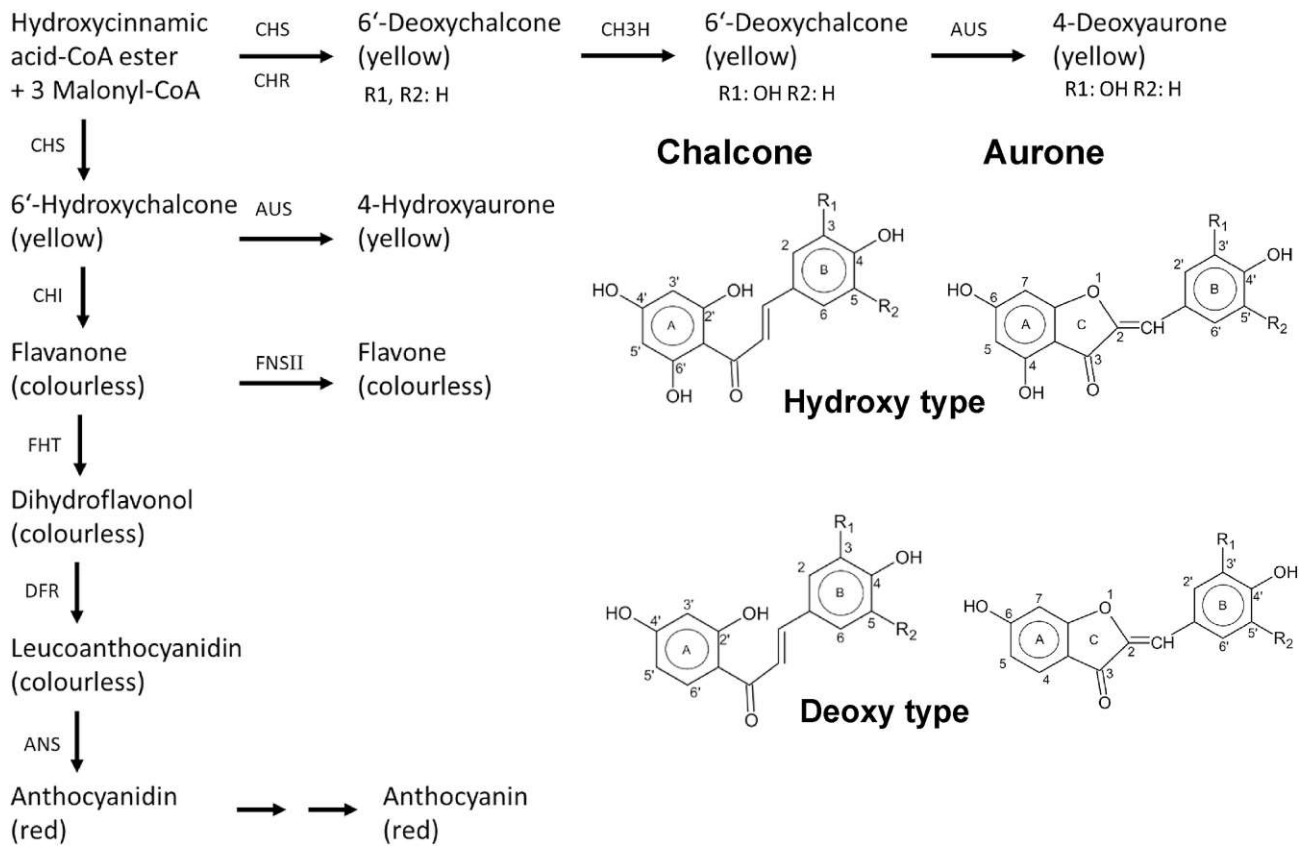

Fig. 1. Overview of the flavonoid pathway in *Dahlia variabilis* and the general structure of chalcones and aurones; please note the divergent ring numbering in the chalcone and aurone structure. In dahlia, anthochlor pigments do not carry hydroxy groups in position R_2.

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Fig. 2. *Dahlia variabilis* cultivar 'Seattle'.

CHR. Finally, three candidates were isolated and subjected to gene expression studies and phylogenetic analysis, but do not seem to be involved in the anthochlor pathway.

2. Material & methods

2.1. Plant material

Dahlia variabilis hort. cv. 'Seattle' (Landgard Blumen und Pflanzen GmbH, Germany) were cultivated in a private garden near Graz (Austria) during the summers of 2017–2018. Petals from at least three plants were harvested and separated in the yellow base and the white tips. The intermediate area was discarded to avoid cross contamination. The separated parts were shock frozen in liquid nitrogen and stored at −80 °C. All investigations were carried out on at least three biological replications. Material for the preparation of the 2 × 150 bp *paired-end* cDNA libraries and the 1 × 75 bp *single-end* 3′ cDNA libraries was harvested in different years and was sent for processing and Illumina sequencing on dry ice to Vertis Biotechnologie AG (Freising, Germany).

2.2. Chemicals

Isoliquiritgenin, butein, sulfuretin, apigenin, luteolin, naringenin, eriodictyol, dihydrokaempferol, dihydroquercetin, marein, maritimein, pelargonidin, cyanidin were purchased from Extrasynthese (Genay, France). Okanin and maritimetin were obtained from marein and maritimein by enzymatic hydrolysis. Briefly, 20 mg of the glycoside dissolved in 20 mL ethanol were incubated for 30 min at 40 °C with 10 mg naringinase (Sigma-Aldrich, Vienna, Austria) in 80 mL 0.1 M McIlvaine buffer (pH 5.5). Thereafter, the aglycones were extracted with ethyl acetate.

2.3. Pigment analysis

For the determination of the pigment composition and content, 0.5 g of shock frozen petal parts were pulverized and extracted with 1 mL methanol. The homogenate was centrifuged for 1 min at 4 °C and 10.000 g. For enzymatic hydrolysis of flavones, flavonols and anthochlors, 20 μL of the methanolic extract was mixed with 100 μL 0.1 M McIlvaine Buffer (pH 4.0) containing 10 U naringinase (Sigma-Aldrich, Austria) and incubated for 10 min at 40 °C. The reaction was stopped by addition of 30 μL methanol and used in HPLC analysis according to Thill et al. (2012). A Thermo Scientific Dionex UltiMate(R) 3000 RSLC System with DAD-3000RS Photodiode Array Detector (Thermo Scientific, Germany) using an Acclaim™ column RSLC 120 C18, 2.2 μm, 120 Å, 2.1 × 150 mm (Dionex bonded Silica Products: No. 071399) was operated at 25 °C. For analysis of flavones, flavonols and anthochlor pigments, 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). The compounds were detected at 290 nm and identified by retention times and comparison of the 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.

2.4. RNA extraction

mRNA was isolated from the separated petal parts using the μMACS mRNA Isolation Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). cDNA was prepared using the RevertAid H Minus MuLV reverse transcriptase (Fermentas Life Science, St. Leon-Rot, Germany) with an oligo (-dT) anchor primer. Total RNA was isolated using the *mir*Vana miRNA Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA).

2.5. PCR and qPCR

Quantitative expression of genes involved in anthochlor biosynthesis was analyzed with a StepOnePlus system (Applied Biosystems, CA, USA) and Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, UK). At least three biological replications with technical triplicates each, were done. A list of primers used for qPCR can be found in Suppl. Table S1. All primers were derived from Thill et al. (2012), except for the *AUS* and *CHR*. *AUS* primers, *CHRl1* primers and *CHRl2* primers were derived from the cloned *AUS* and *CHRl* sequences (section 2.8). The expression was normalized to the housekeeping gene and the relative expression was calculated as decribed (Pfaffl, 2001). The efficiency of the PCR primers was determined based on 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). 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).

2.6. 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, 2007). The Shapiro-Wilk test was used for testing 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. For all statistical significance tests, the significance level was set to 0.05 (5%).

2.7. Transcriptome studies

All steps were performed by a commercial supplier (Vertis Biotechnologie AG, Freising, Germany). Two strategies were used. First, 2 × 150 bp paired-end cDNA libraries were constructed from the yellow base and the white tip. This included preparation of total RNA using the mirPremier™ miRNA isolation kit (Sigma-Aldrich, St. Louis, USA), depletion of ribosomal RNA via the Ribo-Zero rRNA Removal Kit for plants (Illumina, San Diego, USA), synthesis of first-strand cDNA using an N6 randomized primer, preparation of the paired end libraries and Illumina PE sequencing on an Illumina NextSeq 500 system. In addition,

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1 × 75 bp *single-end* 3′ cDNA libraries were constructed from the yellow base and the white tips (each in two replicates). For this, poly(A)+ RNA was isolated from the total RNA using Dynabeads® Oligo (dT)25–61002 (Thermo Fisher Scientific, Waltham, USA). The first-strand cDNA was synthesized using an oligo (dT)-adapter primer and M-MLV reverse transcriptase. After fragmentation, the first-strand cDNA was purified and the 5′ Illumina TruSeq sequencing adapter was ligated to the 3′ end of the antisense cDNA. cDNA was amplified by PCR and the *single-end* libraries were sequenced at Vertis Biotechnologie AG using an Illumina NextSeq500 system.

From the total RNA-seq, reads representing rRNA were depleted with SortMeRna tool (v 4.1) (Kopylova et al., 2012), with all the included databases. Low-quality reads (below an average of 20 quality score) were filtered out and trimmed using Trimmomatic v 0.39 (Bolger et al., 2014) with the parameters TRAILING:20 AVGQUAL:20 SLIDINGWINDOW:5:20 MINLEN:75 for the *paired end* data. Trimmed paired end reads from both parts of the petal were further merged together and assembled with Trinity v 2.8.4 (Haas et al., 2013). The transcriptome was functionally annotated against the *Arabidopsis thaliana* reference genome (TAIR10 (https://doi.org/10.1002/dvg.22877)) and the Swiss-Prot (https://doi.org/10.1093/nar/gkw1099) databases with blast 2.8.1 (https://doi.org/10.1186/1471-2105-10-421) with an *e*-value < 1E-20 (Bairoch and Apweiler, 2000; Falcon and Gentleman, 2007).

For transcript abundance quantification, both type of Illumina reads (paired end and single end) were aligned to the transcriptome or to a specific list of genes of interest with RSEM 1.3.1 (https://doi.org/10.1186/1471-2105-12-323) tool with bowtie2 (Langmead and Salzberg, 2012) selected as alignment method.

To estimate relative gene expression of the anthochlor pathway in white and yellow parts, illumina data from both parts of the petal were mapped against selected genes (*DvCHS1-4* AB576660, AB591825, LC223139, LC223141; *DvCHI* AB591827; *DvCH3H* GQ479804, *DvF3′H* GQ281058 and GQ281059; *DvFNSII* GQ489011; *DvAUS* MT181750) and the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values were calculated using the method for quantification of RNA expression RSEM (v 1.3.0) (Li and Dewey, 2011). Transcriptome data are available at the Short Read Archive of the International Nucleotide Sequence Database Collaboration (BioProject PRJNA627342, SAMN14668017, SAMN14668018, SAMN14840801, SAMN14840802).

2.8. Cloning of cDNA clones from AUS and CHR candidates

A partial sequence of *AUS* was PCR amplified from *D. variabilis* cv. Rubens cDNA by using the Go*Taq* DNA Polymerase Kit (Promega, Germany) with the primers CuA forward and CuB reverse as described earlier (Kaintz et al., 2014). 3′- and 5′-ends of the *AUS* were identified by application of 3′- and 5′-RACE (rapid amplification of cDNA ends) techniques, using the SMARTer RACE cDNA Amplification Kit (Clontech, Takara Bio Europe, France). Based on the obtained sequence, full-length AUS was amplified with the PCR primers *DvAUS*F and *DvAUS*R (Suppl. Table S1) by using the *Taq/Pwo* Expand High Fidelity PCR System (Roche, Germany). The *AUS* cDNA of *D. variabilis* cv. Rubens consisted of 1839 bp and the sequence showed an open reading frame encoding 612 amino acids. The sequence was deposited in the Genbank database (accession No. MT181750). The *DvAUS* gene showed 79.5% (accession No. KC878307) and 70% (accession No. KC972611) sequence identity in comparison to the *AUS* genes reported from *Coreopsis grandiflora* (Kaintz et al., 2014).

Three full size *CHR*-like cDNA sequences (*CHRl1*, *CHRl2* and *CHRl3*) were obtained from the assembled transcriptome by using the amino acid sequence of *CHR* from *Medicago sativa* (Q40309_MEDSA) as a template in a TBLASTN search.

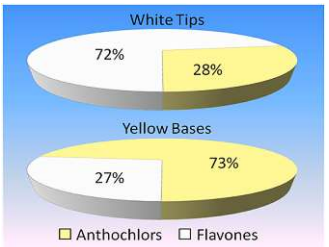
Based on these sequence information, full size cDNAs were amplified with primers *DvCHRl1&2*F in combination with *DvCHR1*R and *DvCHR2*R, respectively (Suppl. Table S1) using Q5® High-fidelity DNA Polymerase (New England Biolabs, Vienna, Austria). *CHRl1, CHRl2* and *CHRl3* consisted of 1081 bp and the sequences showed an open reading frame encoding 322 (CHRl1 and CHRl2) and 320 (CHRl3) amino acids. The sequences were deposited in the Genbank database (accession No. MW269536-38). *CHRl1* and *CHRl2* cDNA clones showed 92% sequence identity to each other and 54–58% with *CHR*s from the Leguminosae family.

2.9. Phylogenetic analysis

Amino acid sequences of the AKR4 family were obtained from the AKR superfamily homepage https://hosting.med.upenn.edu/akr/(Hyndman et al., 2003). A list with the accession numbers of the used sequences can be found in Table S2. Sequences were aligned using the Muscle algorithm at the EMBL-EBI webserver (Madeira et al., 2019). The phylogenetic tree was prepared from the obtained data with TreeGraph 2 (Stöver and Müller, 2010).

Table 1
Content of anthochlors and flavonoids (quantified as aglycones after acidic hydrolysis) in differently colored parts of *Dahlia variabilis* cv. 'Seattle'. Pie charts show the relative distribution of anthochlors and flavones in the white tips and yellow bases. Absolute values are provided as mg/g fresh weight. Mean ± standard error are presented (n = 3).

	White Tip [mg/g]	Yellow Base [mg/g]	White Tip %	Yellow Base %
Total	**0.54 ± 0.05**	**9.06 ± 0.07**	**6.0**	**94.0**
Anthochlors	0.15 ± 0.02	6.63 ± 0.04	2.3	97.7
Isoliquiritigenin	0.04 ± 0.00	2.93 ± 0.01	1.2	98.8
Butein	0.04 ± 0.01	1.67 ± 0.01	2.5	97.5
Sulfuretin	0.07 ± 0.01	2.03 ± 0.02	3.5	96.5
Flavones	0.39 ± 0.03	2.43 ± 0.03	13.8	86.2
Apigenin	0.19 ± 0.01	1.80 ± 0.01	9.4	90.6
Luteolin	0.20 ± 0.02	0.64 ± 0.01	24.1	75.9
Flavonols	n.d.	n.d.		
Kaempferol	n.d.	n.d.		
Quercetin	n.d.	n.d.		
Flavanones	n.d.	n.d.		
Naringenin	n.d.	n.d.		
Eriodictyol	n.d.	n.d.		



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3. Results

3.1. Pigment analysis

The pigment composition of the two differently colored petal parts was analyzed by HPLC after acidic hydrolysis (Table 1). Anthochlors were prevalently present (70.5% of analyzed pigments), followed by flavones (29.5% of analyzed pigments). Flavonols and dihydroflavonols could not be detected in the petals.

The two petal parts differed significantly in pigment composition and concentration. Whereas high amounts of anthochlors and flavones were found in the yellow base, the white tips were generally characterized by very low pigment concentrations (Table 1) and contained only 6% of the pigments detected in the whole petals. In the white tips, flavones dominated over the anthochlors with a percentage of 72%, whereas in the yellow bases anthochlors were prevalently present (73%). With respect to the B-ring hydroxylation pattern, mono- and dihydroxylated types of flavones and anthochlors were found (Table 1).

In the case of flavones, the monohydroxylated apigenin dominated with 70% over the dihydroxylated luteolin, however there was an unequal distribution between the petal parts, as the white tips contained apigenin and luteolin based pigments to almost the same extent, whereas in the yellow parts apigenin based pigments were predominant with 73% of the total flavone content. In the case of anthochlors, the ratio of mono- and dihydroxylated types was comparable in both petal parts (Table 1). The larger part of anthochlor pigments was present as 6′-deoxychalcone glycosides with 69% in the yellow parts and 48% in the white tips. In total, the white tips contained only 0.15 mg anthochlor pigments/g fresh weight and 0.39 mg flavones/g fresh weight, which was below the detection level of the naked eye.

3.2. Expression of genes (qPCR)

Yellow and white parts of the petals were separated, and relative gene expression was measured independently in order to examine the differences in gene expression between the two differently colored parts. Beside the genes involved in anthochlor biosynthesis (*CHS*, *CH3H* and *AUS*), the expression of genes and transcription factors which are involved in flavonoid pathway (Suppl. Table S1) was measured. Expression data were normalized to the expression of the housekeeping gene *actin* (Fig. 3).

Chalcone synthase is the first key enzyme in the flavonoid biosynthesis and more than one copy is present (Lehnert and Walbot, 2014; Ohno et al., 2018). For *DvCHS1* we observed a relatively low expression in both petal parts. In contrast, *DvCHS2* showed a significantly higher expression in the yellow base compared to the white tip (23 times higher). The ratio between *DvCHS1* and *DvCHS2* in the white tip was comparably low.

Further downstream of the anthochlor pathway, *CH3H* is a gene responsible for the conversion of the 6′-deoxychalcone isoliquiritigenin to butein by introducing a hydroxyl-group at position 3 of the A-ring. qPCR results showed slightly higher relative expression of *DvCH3H* in the yellow base, however without significant differences compared to the white tip (Fig. 3). Ring closure, and thus conversion of chalcones into aurones is catalyzed by aurone synthase (AUS) which can also alternatively introduce the hydroxyl group. The relative expression ratios for

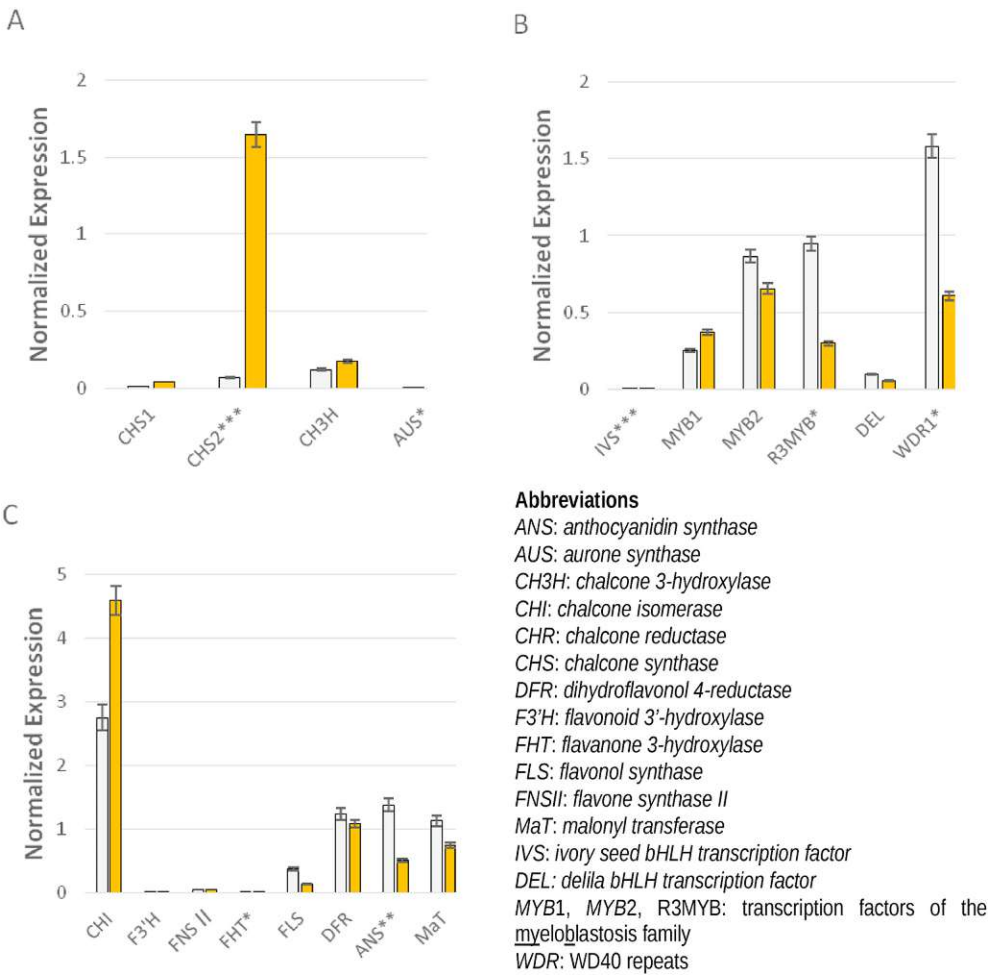

Fig. 3. Expression of structural genes (A: anthochlor pathway, C: flavonoid pathway) and regulatory genes (B) in differently colored petal parts of *Dahlia variabilis* cv. 'Seattle': Expression was normalized to the housekeeping gene *actin* in the white tip (white bars) and the yellow base (yellow bars); stars represent the significance of differences between the relative gene expression *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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DvAUS in dahlia showed significantly higher expression in the yellow base compared to the white tip.

3.3. Transcriptome studies

Total RNA was isolated from the yellow base and the white tips of bicolored petals and used for cDNA library generation. A total of 34,281,650 and 63,908,282 Illumina 150 bp RNA reads were generated for the white tip and for the yellow base of the petal, respectively. After removing reads representing rRNA data and filtering by quality, 25,748,951 and 49,513,941 number of Illumina reads remained for transcriptome assembly from white and yellow petal parts (Suppl. Fig.1). The assembled Trinity transcriptome contains 315,419 isoforms that are clustered into 134,666 genes with an N50 of 1287. A total of 91,709 transcripts corresponding to 27,297 genes were annotated against the Swiss-Prot database and 108,803 transcripts corresponding to 31,430 genes were functionally annotated against TAIR10 list of proteins.

Out of 108,803 annotated transcripts 1521 were homologues to *Arabidopsis thaliana* genes belonging to flavonoid metabolic pathway (Carbon et al., 2008).

From single end samples, 24,180,610 plus 18,767,796 Illumina 3′end data of 75 bp were sequenced from the white tip and 20,276,902 plus 25,260,797 Illumina 3′end data of 75 bp were sequenced from the yellow base.

However, when FPKM values were normalized to the highest expression for each gene and average values were calculated for the yellow base and the white tips, the RNA seq data were largely in

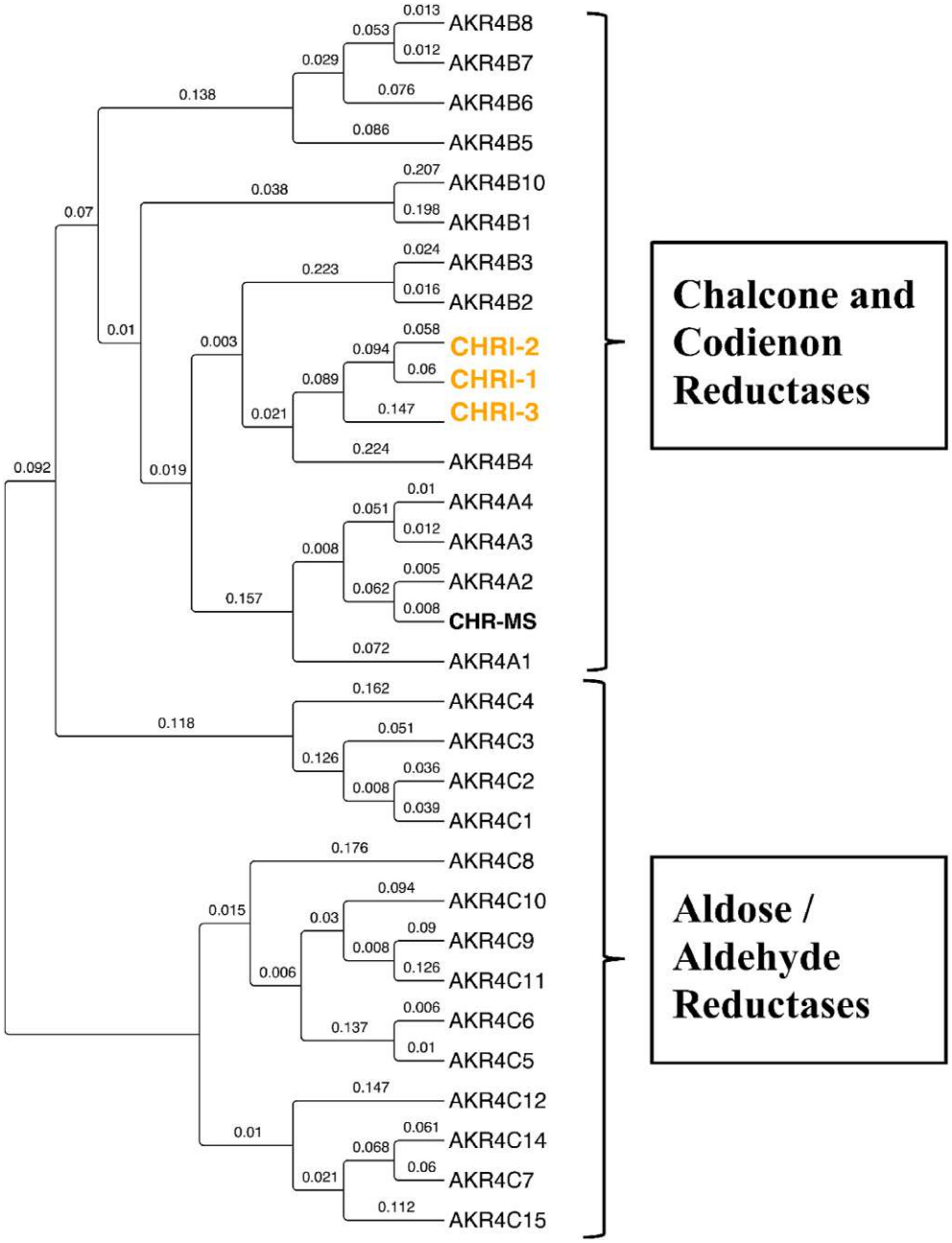

Fig. 4. Phylogenetic tree showing the AKR4 superfamily (Hyndman et al., 2003) including the dahlia CHR-like proteins (CHRI; orange) and the used template for homology search CHR-MS (bold; Q40309). The CHR-like proteins (orange) cluster within the family of chalcone (AKR4A) and codein reductases (AKR4B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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agreement with the qPCR results (Suppl. Fig. S3). Particularly, a lower gene expression for *CHS1-4* in the white tips was observed, pointing at a decisive bottleneck for pigment formation in the white tips.

3.4. Phylogenetic and expression analysis of CHR-like genes (CHRl)

A TBLASTN search for homologues to the known CHR from *Medicago sativa* through the assembled transcriptome resulted in the identification of three full size sequences. The three *CHRls* showed high similarity to loci identified in the first dahlia transcriptome (Lehnert and Walbot, 2014), *CHRl1* (96% sequence identity to locus 44502), *CHRl2* (93% sequence identity to locus 44502) and *CHRl3* (84% sequence identity to locus 24145). These homologues belong to the AKR4 family and cluster in the subfamily of CHR and codienone reductases (Fig. 4). The AKR4B4 (D-galacturonate reductase from *Fragaria x ananassa*) represents the closest homologue of the potential chalcone reductases.

The RNA seq data showed elevated FPKM values for the three CHR candidates in the white tips rather than in the yellow base. Additionally, we analyzed the gene expression of the *CHRls* by quantitative real-time PCR.

Based on the close sequence similarity of *CHRl1* and *CHRl2*, their expression was checked simultaneously, whereas a second primer pair specifically detected *CHRl3*. No statistically significant differences in the *CHRl1* and *CHRl2* expressions could be observed between white tips and the yellow base (Fig. 5). In comparison to the housekeeping gene as well as to *DvCHS* and *DvCHI*, gene expression was relatively low, but was comparable to other genes from the pathway such as *DvF3′H* and *DvAUS*.

4. Discussion

Color mutations in the petals of *D. variabilis* are relatively common and typically occur as mericlinal chimeras (Lawrence, 1931a). The mutations may involve any of the three pigment classes present in dahlia flowers, anthocyanins, anthochlors and flavones (Lawrence, 1931b). Such bicolored varieties can be powerful models to identify the key enzymes of the pathways leading to the formation of the different pigment types. There is still no good model for the anthochlor pathway available, which particularly lacks the identification of the key enzyme for 6′-deoxychalcone formation in the Asteraceae species, a presumable analogue to the CHR as described for the Leguminosae family (Boucherle et al., 2017). We therefore investigated the yellow-white *D. variabilis* cultivar 'Seattle' for its potential to take this role.

4.1. Absence of anthocyanins in the petals of cv. 'Seattle'

The bicolored cultivar 'Seattle' accumulated chalcones and aurones in its yellow base, but only trace amounts of other flavonoids in its white tips. In contrast to common white varieties, which typically show an ivory hue, the white areas appear to be pure-white as described previously for red-white bicolored dahlias (Ohno et al., 2011b). The cultivar does not accumulate any anthocyanins in the petals. Our studies show that this is primarily caused by the absence of FHT activity as indicated by gene expression (Fig. 3) and confirmed by the accumulation of flavones and the absence of flavonoids that are formed downstream of the FHT step (Table 1). Anthocyanin formation in dahlia was previously shown to be associated with the bHLH transcription factor *DvIVS*, which was reported to simultaneously activate *DvCHS1, DvFHT, DvDFR, DvANS* and *DvMaT* (Ohno et al., 2011a). In cv. 'Seattle', both petal parts showed low expression only for *DvIVS, DvCHS1* and *DvFHT,* but not for *DvDFR, DvANS* and *DvMaT*.

Previous studies showed that neither single colored white dahlias nor purely yellow colored dahlias showed high expression ratios for the genes of pathway leading to anthocyanins (Thill et al., 2012). Despite the absence of anthocyanin formation in both petal parts of cv. 'Seattle', the genes involved in the formation of anthocyanidins from chalcones (*DvFHT*, *DvDFR*, *DvANS, DvMaT*) were higher expressed in the white tips, besides *DvCHI*, which mediates the enzymatic conversion of chalcones to the corresponding flavanones. Statistically significant differences in the gene expression between the white and yellow areas were, however, only observed in case of *DvANS, DvFHT* and *DvIVS,* as well in the WD repeat-containing protein 1 (*WDR1*), which is known to be part of the transcriptional complex for the regulation of the anthocyanin biosynthesis (Matus et al., 2010).

4.2. Absence of anthochlors in the white tips of cv. 'Seattle'

The first dahlia transcriptome (Lehnert and Walbot, 2014) indicated the presence of at least six genes putatively encoding CHS with an amino acid sequence identity of 39–82% between each other, some of them showing tissue specificity. Thereof, loci 7595 and 18623 are most closely related and could be allelic variants. Of the six CHS variants, locus 49578 (*DvCHS1*; GBDN01034951) and locus 27 (*DvCHS2*; GBDN01000027) are closer related to CHS proteins, compared to the other putative CHS loci (GBDN01007304, GBDN01016765, GBDN01008961, GBDN01003095), which seem to be closer related to polyketide synthases when the translated amino acid sequence is compared (Ohno et al., 2018). In the qPCR studies, we therefore focused

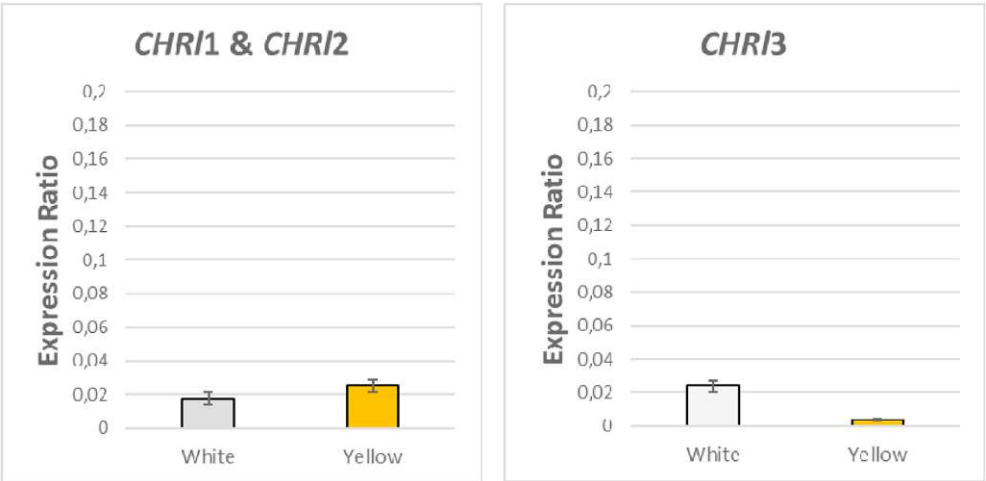

Fig. 5. Expression of *CHRl* genes in comparison to the housekeeping gene *actin* in the white tip and the yellow base of *Dahlia variabilis* cv. 'Seattle'. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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on *DvCHS1* and *DvCHS2*, which show 68% nucleotide sequence identity and 82% amino acid sequence identity and are particularly involved in the formation of pigments in dahlia flowers (Ohno et al., 2011b). Whereas *DvCHS1* was reported to be correlated with anthocyanidin biosynthesis, *DvCHS2* seems to be expressed independently of the flower coloration (Ohno et al., 2018). In agreement with this, we observed almost no expression of *DvCHS1* in both acyanic petal parts, but a high relative expression of *DvCHS2* in the yellow base that correlated with the high concentrations of anthochlors and flavones. The low level of *CHS* expression in the white tips provides a good and per se sufficient explanation for the low concentrations of flavones and anthochlors and the lack of color in the white tips. Expression of other genes of the anthochlor pathway such as *CH3H* and *AUS* was comparable to expression of *FNS II*, which opens the side branch to flavone formation, but was drastically lower than gene expression of the anthocyanin pathway (Fig. 3). Whereas no significant differences in *DvCH3H* expression could be observed between the white tip and the yellow base, *DvAUS* showed significant higher expression ratios in the yellow base than in the white tip. The expression level, however, was quite low compared to the housekeeping genes.

AUS are plant polyphenol oxidases, which are supposed to enable the synthesis of aurones in chloroplasts, indicated by the presence of an N-terminal chloroplast transit peptide and a thylakoid transfer domain (Molitor et al., 2016). In the Asteraceae family, *AUS* genes (KC972611, KC878307) have so far only been identified in *Coreopsis grandiflora* (Kaintz et al., 2014). Based on sequence homology we isolated the first *AUS* cDNA clone from *Dahlia variabilis* (*DvAUS*). The significantly higher expression in the yellow base compared to the white tip could reflect an active role in the anthochlor pathway. However, at this stage the presence of a multigene family cannot be excluded.

To our knowledge, no CHR candidate genes are known in Asteraceae species so far. Transcriptomic data were therefore screened for chalcone reductase like genes (*CHRl*) and their relationship within the AKR4 family was examined by phylogenetic analysis. Homology studies using CHR from Leguminosae species delivered four candidate sequences (GBDN01031364, GBDN01033202, GBDN01009250, GBDN01020960) in the published dahlia transcriptome from *Dahlia variabilis* plants (Lehnert and Walbot, 2014), of which three (MW269536-38) were also present in the novel transcriptome from *Dahlia variabilis* cv. 'Seattle' flowers. Phylogenetic analysis revealed that these proteins cluster within the chalcone and codienone reductase subfamily (AKR4A and AKR4B) with D-galacturonate reductase from *Fragaria x ananassa* (AKR4B4) being the closest homologue. Even though the known Leguminosae CHRs cluster within the AKR4A clade, the absence of an AKR4A homologue in dahlia is not suspicious, as also other known flavonoid enzymes of the Asteraceae family frequently show phylogenetic inconstancy. The Asteraceae F3′5′H, for example, belongs to the CYP75B (usually F3′H) subfamily instead to the ordinarily CYP75A (usually F3′5′H) subfamily (Seitz et al., 2006; Yonekura-Sakakibara et al., 2019). Furthermore, aurone synthase from *Coreopsis grandiflora* showed low sequence identity to other polyphenol oxidases and revealed several unique features (Molitor et al., 2015). Therefore, a slightly further distance to Leguminosae CHRs is not surprising.

Primers derived from those sequences resulted in the isolation of three *CHR-like* genes (*CHRl1*, *CHRl2, CHRl3*), which were tested for gene expression differences in the yellow base and the white tips. Both RNA seq and real time PCR data indicated a higher or similar expression in the white tips compared to the yellow base, which does not demonstrate their physiological relevance for the anthochlor biosynthesis. On the other hand, involvement can also be not completely excluded as the relative gene expression in comparison to the housekeeping gene was comparable with *DvAUS*, *DvCH3H* and *DvF3′H*, which are part of the pathway. Furthermore, the expression of the three *CHR* candidates was relatively low in comparison to *CHS*, which coacts with CHR to form 6′-deoxychalcones (Welle and Griesebach, 1988; Bomati et al., 2005). From the Leguminosae family, in which 6′-deoxychalcone formation has been exclusively studied so far, there is no clear hint available about the optimal ratio of CHS/CHR, as earlier studies used a broad range from excess and balanced to shortfall amounts in their studies (Welle and Griesebach, 1988; Ayabe et al., 1988; Bomati et al., 2005; Boucherle et al., 2017). Further studies will concentrate on the identification of further candidate *CHR*s.

5. Conclusion

In summary, it was shown that the bicoloration of cv. 'Seattle' is most likely caused by a bottleneck of CHS in the white tip rather than caused by a differentially expressed *CHR*. However, a difference in expression of an enzyme catalyzing a chalcone reductase-like reaction could not be excluded, in addition to the different *CHS* expression. The relative prevalence of flavones compared to the anthochlors in the white tips could be an indication that the presumable key enzyme for 6′-deoxychalcone formation is less expressed in white tips than in the yellow base. Cloning and heterologous expression of these candidate genes with subsequent activity assays are ongoing and remain to be identified in further studies.

Author contribution statement

HH, KS and TR conceived the research. BW, HH and TR designed the experiments. BW, CRL, CM, SM, DN, DA, CHG, IK, performed the experiments and analyzed the data. CL and TR performed the bioinformatical analysis. BW, CM and HH wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.12.016.

References

Ayabe, S.-I., Udagawa, A., Furuya, T., 1988. NAD(P)H-dependent 6′-deoxychalcone synthase activity in Glycyrrhiza echinata cells induced by yeast extract. Arch. Biochem. Biophys. 261 (2), 458–462. https://doi.org/10.1016/0003-9861(88)90362-1.

Bairoch, A., Apweiler, R., 2000. The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. Nucleic Acids Res. 1, 45–48. https://doi.org/10.1093/nar/28.1.45.

Bohm, B.A., 1988. The minor flavonoids. In: Harborne, J.B. (Ed.), The Flavonoids: Advances in Research since 1980. Chapman & Hall, London.

Bohm, B.A., 1993. The minor flavonoids. In: Harborne, J.B. (Ed.), The Flavonoids: Advances in Research since 1986. Chapman & Hall, London.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30 (15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Bomati, A.K., Austin, M.B., Bowman, M.E., Dixon, R.A., Noel, J.P., 2005. Structural elucidation of chalcone reductase and implications for deoxychalcone biosynthesis. J. Biol. Chem. 34, 30496–30503.

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Boucherle, B., Peuchmaur, M., Boumendjel, A., Haudecoeur, R., 2017. Occurrence, Biosynthesis and properties of aurones as high-end evolutionary products. Phytochemistry 142, 92–111. https://doi.org/10.1016/j.phytochem.2017.06.017.
Brouillard, R., Dangles, O., 1994. Flavonoids and flower colour. In: Harborne, J.B. (Ed.), The Flavonoids - Advances in Research. Chapman & Hall, London, pp. 565–586.
Carbon, S., Ireland, A., Mungall, C.J., Shu, S., Marshall, B., Lewis, S., 2008. AmiGO: online access to ontology and annotation data. Bioinformatics 25 (2), 288–289. https://doi.org/10.1093/bioinformatics/btn615.
de Mendiburu, F., 2007. Agricolae: statistical procedures for agricultural research. Available online at: https://CRAN.R-project.org/package=agricolae.
Falcon, S., Gentleman, R., 2007. Using GOstats to test gene lists for GO term association. Bioinformatics 23 (2), 257–258. https://doi.org/10.1093/bioinformatics/btl567.
Forkmann, G., Dangelmayr, B., 1980. Genetic control of chalcone isomerase activity in flower of Dianthus caryophyllus. Biochem. Genet. 18, 519–527.
Giannasi, D.E., 1975. The flavonoid systematics of the genus Dahlia (Compositae). Memoir. N. Y. Bot. Gard. 2 (26), 1–125.
Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., et al., 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat. Protoc. 8 (8), 1494–1512. https://doi.org/10.1038/nprot.2013.084.
Halbwirth, H., Muster, G., Stich, K., 2008. Unraveling the biochemical base of dahlia flower coloration. Natural Product Commun. 3 (8), 1259–1266.
Harborne, J.B., 1967. Comparative Biochemistry of Flavonoids. Academic Press, London.
Harborne, J.B., 1993. Introduction to the Ecological Biochemistry. Academic Press.
Harborne, J.B., Smith, D.M., 1978. Anthochlors and other flavonoids as honey guides in the Compositae. Biochem. Systemat. Ecol. (6), 287–291.
Hyndman, D., Bauman, D.R., Heredia, V.V., Penning, T.M., 2003. The aldo-keto reductase superfamily homepage. Chem. Biol. Interact. 621–631. https://doi.org/10.1016/S0009-2797(02)00193-X.
Ihaka, R., Gentleman, R., 1996. R: a language for data analysis and graphics. J. Comput. Graphical Studies (3), 299–314.
Kaintz, C., Molitor, C., Thill, J., Kampatsikas, I., Michael, C., Halbwirth, H., Rompel, A., 2014. Cloning and functional expression in E. coli of a polyphenol oxidase transcript from C. grandiflora involved in aurone formation. FEBS Lett. 588, 3417–3426. https://doi.org/10.1016/j.febslet.2014.07.034.
Kimura, Y., Aoki, T., Ayabe, S.-i., 2001. Chalcone isomerase isozymes with different substrate specificities towards 6′-hydroxy-and 6′-deoxychalcones in cultured cells of Glycyrrhiza echinata, a leguminous plant producing 5-deoxyflavonoids. Plant Cell Physiol. 10, 1169–1173.
Kopylova, E., Noé, L., Touzet, H., 2012. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. Bioinformatics 28 (24), 3211–3217. https://doi.org/10.1093/bioinformatics/bts611.
Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359. https://doi.org/10.1038/nmeth.1923.
Lawrence, W.J., 1931a. Mutation or segregation in the Octopoild dahlia variabilis. J. Genet. (24), 307–324.
Lawrence, W.J., 1931b. The Genetics and Cytology of dahlia variabilis. J. Genet. (3), 257–305.
Lawrence, W.J., 1932. Interaction of flavones and anthocyanins. Nature 834. https://doi.org/10.1038/129834b0.
Lehnert, E.M., Walbot, V., 2014. Sequencing and de novo assembly of a Dahlia hybrid cultivar transcriptome. Front. Plant Sci. 340 https://doi.org/10.3389/fpls.2014.00340.
Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinf. 12 (323) https://doi.org/10.1186/1471-2105-12-323.
Madeira, F., Park, Y.m., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., et al., 2019. The EMBL-EBI Search and Sequence Analysis Tools APIs in 2019, W1 ed., vol. 47. Nucleic Acids Research. https://doi.org/10.1093/nar/gkz268.
Matus, J.T., Poupin, M.J., Canón, P., Bordeu, E., Alcalde, J.A., Arce-Johnson, P., 2010. Isolation of WDR and bHLH genes related to flavonoid synthesis in grapevine (Vitis vinifera L.). Plant Mol. Biol. 607–620. https://doi.org/10.1007/s11103-010-9597-4, 72.
McClaren, M., 2009. Dahlia: history and species. In: Encyclopedia of Dahlias. Timber Press, Portland, Oregon, USA, pp. 161–166.
Miles, C.O., Main, L., 1989. Kinetics and mechanism of the cyclisation of 2',6'-dihydroxychalcone and derivatives. J. Chem. Soc. Perkin Trans. I 1623–1632.
Miosic, S., Knop, K., Holscher, D., Greiner, J., Gosch, C., Thill, J., Halbwirth, H., 2013. 4-Deoxyaurone formation in Bidens ferulifolia (Jacq.) DC. PloS One 5 (8).
Miyajima, I., Maehara, T., Kage, T., Fujieda, K., 1991. Identification of the main agent causing yellow color of yellow-flowered cyclamen mutant. J. Jpn. Soc. Hortic. Sci. 60, 409–414.
Molitor, C., Mauracher, S.G., Rompel, A., 2016. Aurone synthase is a catechol oxidase with hydroxylase activity and provides insights into the mechanism of plant polyphenol oxidases. Proc. Natl. Acad. Sci. U.S.A. 113, 1806–1815.
Molitor, C., Mauracher, S.G., Pargan, S., Mayer, R.L., Halbwirth, H., Rompel, A., 2015. Latent and active aurone synthase from petals of C. grandiflora: a polyphenol oxidase with unique characteristics. Planta 242, 519–537. https://doi.org/10.1007/s00425-015-2261-0.
Nakayama, T., Sato, M., Fukui, Y., Yonekura-Sakakibara, K., Hayashi, H., Tanaka, Y., et al., 2001. Specificity analysis and mechanism of aurone synthesis catalyzed by aureusidin synthase, a polyphenol oxidase homolog responsible for flower coloration. FEBS Lett. 499, 107–111.
Nakayama, T., Sato, T., Fukui, Y., Yonekura-Sakakibara, K., Hayashi, K., Tanaka, Y., et al., 2000. Aureusidin synthase: a polyphenol oxidase homolog responsible for flower coloration. Science 290, 1163–1165. https://doi.org/10.1126/science.290.5494.1163.
Nordström, C.G., Swain, T., 1956. The flavonoid glycosides of Dahlia variabilis. II. Glycosides of yellow varieties. Arch. Biochem. Biophys. 60, 329–344.
Ogata, J., Ito, Y., Ishida, M., Yoshida, H., Ozeki, Y., 2004. Cloning and heterologous expression of cDNAs encoding flavonoid glucosyltransferases from Dianthus caryophyllus. Plant Biotechnol. (5), 367–375.
Ohno, S., Hori, W., Hosokawa, M., Tatsuzawa, F., Doi, M., 2018. Post-transcriptional silencing of chalcone synthase is involved in phenotypic lability in petals and leaves of bicolor dahlia (Dahlia variabilis) 'Yuino. Planta 247 (2), 413–428. https://doi.org/10.1007/s00425-017-2796-3.
Ohno, S., Hosokawa, M., Hoshino, A., Kitamura, Y., Morita, Y., Park, K.I., et al., 2011a. A bHLH Transcription Factor, DvIVS, Is Involved in Regulation of Anthocyanin Synthesis in Dahlia (Dahlia Variabilis), vol. 62, pp. 5105–5116. https://doi.org/10.1093/jxb/err216, 14.
Ohno, S., Hosokawa, M., Kojima, M., Kitamura, Y., Hoshina, A., Tatsuzawa, F., et al., 2011b. Simultaneous post-transcriptional gene silencing of two different chalcone synthase genes resulting in pure white flowers in the octoploid dahlia. Planta 234 (5), 945–958. https://doi.org/10.1007/s00425-011-1456-2.
Ono, E., Fukuchi-Mizutani, M., Nakamura, N., Fukui, Y., Yonekura-Sakakibara, K., Yamaguchi, M., et al., 2006a. Yellow flower generated by expression of the aurone biosynthetic pathway. Proc. Natl. Acad. Sci. Unit. States Am. 103, 11075–11080.
Ono, E., Hatayama, M., Isono, Y., Sato, T., R, W., Yonekura-Sakakibara, K., et al., 2006b. Localization of a flavonoid biosynthetic polyphenol oxidase in vacuoles. Plant J. 45, 133–143. https://doi.org/10.1111/j.1365-313X.2005.02625.x.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29 (9) https://doi.org/10.1093/nar/29.9.e45.
Razali, N.M., Wah, Y.B., 2011. Power comparisons of shapiro-wilk, Kolmogorov-smirnov, lilliefors and anderson-darling tests. J. Stat. Modeling Anal. (1), 21–33.
Sato, T., Nakayama, T., Kikuchi, S., Fukui, Y., Yonekura-Sakakibara, K., Ueda, T., et al., 2001. Enzymatic formation of aurones in the extracts of yellow snapdragon flowers. Plant Sci. 160, 229–236.
Schlangen, K., Miosic, S., Halbwirth, H., 2010a. Allelic variants from Dahlia variabilis encode flavonoid 3'hydroxylases with functional differences in chalcone 3-hydroxylase activity. Arch. Biochem. Biophys. 494, 40–45.
Schlangen, K., Miosic, S., Thill, J., Halbwirth, H., 2010b. Cloning, functional expression, and characterization of a chalcone-3-hydroxylase from Cosmos sulphureus. J. Exp. Bot. 61, 3451–3459. https://doi.org/10.1093/jxb/erq169.
Scogin, R., Zakar, K., 1976. Anthochlor pigments and floral UV patterns in the genus Bidens. Biochem. Systemat. Ecol. (4), 165–167.
Seitz, C., Eder, C., Deiml, B., Kellner, S., Martens, S., Forkmann, G., 2006. 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 Mol. Biol. 365–381. https://doi.org/10.1007/s11103-006-0012-0.
Stöver, B.C., Müller, K.F., 2010. TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. BMC Bioinf. 11.
Thill, J., Miocis, S., Romel, A., Schlangen, K., Muster, G., Stich, K., Halbwirth, H., 2012. A decline in flavone formation correlates with the rare color of black dahlia (Dahlia variabilis hort.) flowers. BMC Plant Biol. (12).
Welle, R., Griesebach, H., 1988. Isolation of a novel NAPDH-depended reductase which coacts with chalcone synthase in the biosynthesis of 6'-deoxychalcones. FEBS Lett. 221–225, 236.
Wimmer, G., Halbwirth, H., Wurst, F., Forkmann, G., Stich, K., 1998. Enzymatic hydroxylation of 6'-deoxychalcones with protein preparations from petals of Dahlia variabilis. Phytochemistry 47 (6), 1013–1016. https://doi.org/10.1016/S0031-9422(98)80063-0.
Yonekura-Sakakibara, K., Higashi, Y., Nakabayashi, R., 2019. The origin and evolution of plant flavonoid metabolism. Front. Plant Sci. https://doi.org/10.3389/fpls.2019.00943.

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4.3. Publication 3

Introduction of Genes involved in anthochlor biosynthesis into plants using the GoldenBraid system

Benjamin Walliser, Marika Takacs, Robert Boehm, Silvija Marinovic, Renate Peltram, Christian Haselmair-Gosch, Christopher Schlosser, Karl Stich and Heidi Halbwirth

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Introduction of Genes involved in anthochlor biosynthesis into plants using the GoldenBraid system

Abstract

Alteration of flower coloration in plants to hues which they do not naturally exhibit, offers great monetary potential to ornamental plant breeders. *Pelargonium* does not produce bright yellow flowers. Anthochlors, a pigment class related to flavonoids, contribute to the yellow coloration of many members of the Asteraceae family, for instance *Helianthus*, *Dahlia* or *Bidens*. The proposed biosynthetic pathway involves three to five enzymes and the precursor molecules can be found in nearly all plants. The GoldenBraid cloning system provides a method to introduce multiple transcription cassettes in a single construct into plants. In this study, twelve GoldenBraid constructs, harboring all or groups of genes involved in anthochlor biosynthesis, were designed and introduced transiently or stable in *Dianthus caryophyllus*, *Pelargonium zonale* and *Petunia hybrida*. Independent of the used plant species, activity of the reporter genes *dsRED* and *gfp* was observable in transient transformations. Additionally, transcripts of the introduced genes can be found in the transformed areas. A visible color change from white to pale yellow in transiently transformed pelargonia could not be linked to the presence of anthochlors. During stable transformation, the regeneration of transgenic *Pelargonium* failed due to heavy contamination with endogenous fungi and bacteria. The regeneration of transgenic *Petunia hybrida* produced a number of regenerants. However, though *nptII* integration could be verified, no alteration of flower color was observable. Although, no color change could be achieved, this study demonstrates i) the potential of the GoldenBraid cloning system and ii) highlights potential obstacles and ways how to overcome them when introducing the anthochlor pathway into plants.

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Introduction

Yellow flower color is often related to carotenoid accumulation. Besides carotenoids, especially species of the Asteraceae family additionally accumulate the so-called anthochlor pigments (chalcones and aurones). Due to different UV absorbance of anthochlor pigments and carotenoids, those plants can create UV nectar guides to attract UV sensitive insects as pollinators. Only a few ornamental species accumulate chalcones alone as the main pigment for yellow coloration (Brouillard and Dangles 1994/1988).

Anthochlors can be distinguished into two types, the hydroxy-type and deoxy-type, defined by the presence or absence of a hydroxyl group in the 6'-position of the A-ring for chalcones or position 4 at the B-ring of aurones, respectively (Fig. 1). Chalcones are the initial intermediates of flavonoid biosynthesis. One molecule of *p*-coumaroyl-CoA and three molecules malonyl-CoA are merged to a C_{15} structure by the action of the chalcone synthase (CHS). CHS acting alone leads to the formation of 6'-hydroxychalcones, which quickly isomerize either chemically or are isomerized enzymatically by chalcone isomerase (CHI), to the corresponding flavanone. 6'-Hydroxychalcone as main pigments are only rarely found for example in *Dianthus caryophyllus* (Forkmann and Dangelmayr 1980), *Cyclamen persicum* (Miyajima et al. 1991) and *Antirrhinum majus* (Harborne 1967). The accumulation usually requires a lack of CHI activity and additionally the presence of a glycosyltransferase to stabilize the chalcone (Ogata et al. 2004).

The 6'-deoxychalcones are formed when CHS acts mutually with the enzyme chalcone reductase (CHR), a rarely occurring enzyme so far described only in the Leguminosae family (Welle and Grisebach 1988). Bomati et al. (2005) suggest that CHR reduces the polyketide intermediate of CHS, resulting in 6’-deoxychalcones. 6'-Deoxychalcones have a higher chance for accumulation in the petals than the 6'-hydroxychalcones due to the requirement of a CHI with broad substrate specificity for isomerization of 6'-deoxychalcones. Such a CHI was so far only found in the Fabaceae family (Kimura et al. 2001), and chemical isomerization occurs at a much lower rate compared to 6'-hydroxychalcones (Miles and Main 1985, 1989). Accumulation of 6'-deoxychalcone based pigments has been described for species of the Asteraceae family, e.g. *Cosmos sulphureus, Bidens ferulifolia, Coreopsis grandiflora* or *Dahlia variabilis* (Bohm 1988; Halbwirth et al. 2008).

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Aurone formation was primarily studied in *Antirrhinum majus*, which accumulates 4-hydroxyaurones, catalyzed by the polyphenol oxidase homolog aureusidin synthase (AUS) (Nakayama et al. 2000; Nakayama et al. 2001; Sato et al. 2001; Ono et al. 2006a; Ono et al. 2006b). More recently, Miosic et al. (2013) studied the formation of 4-deoxyaurones in *Bidens ferulifolia* and Kaintz et al. (2014) published findings on *Coreopsis grandiflora*. 6'-Deoxychalcones are accepted as substrates and converted into the corresponding 4-deoxyaurones by AUS (Molitor et al. 2015). The introduction of a second hydroxyl group in the B-ring seems to be catalyzed by an Asteraceae specific chalcone 3-hydroxylase (CH3H) (Schlangen et al. 2009; Schlangen et al. 2010a; Schlangen et al. 2010b)). Molitor et al. (2016) proposed that the hydroxylation may also occur during the formation of the 4-deoxyaurones. Pigments carrying three hydroxy groups in the B-ring are not present in dahlia, neither in the flavonoids nor in anthochlors (Halbwirth et al. 2008).

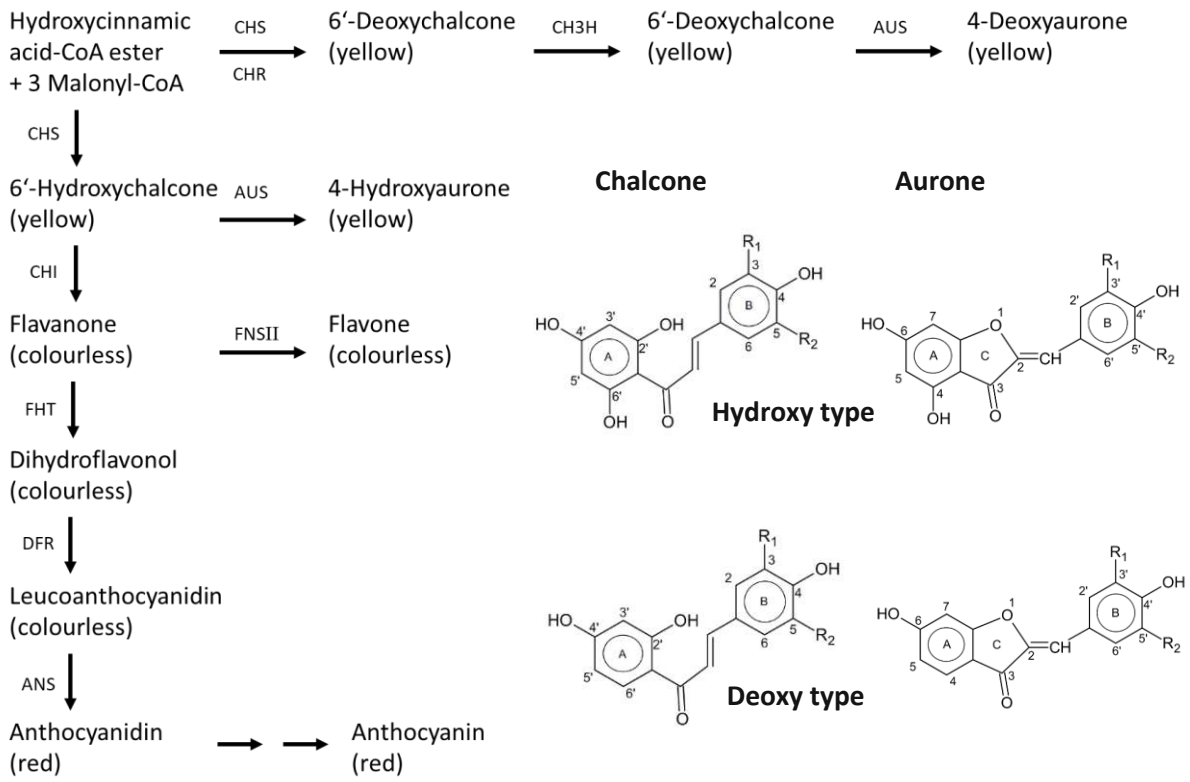

Fig. 1. Overview of the biosynthetic pathway of flavonoids in Dahlia variabilis and the general structure of chalcones and aurones; please note the divergent ring numbering in the chalcone and aurone structure level expression of CHS.

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Introduction of single genes involved in anthochlor biosynthesis such as a legume CHR in *Petunia hybrida* (Shimada et al. 2006; Davies et al. 2002) or an AUS and a glycosyltransferase in *Torenia hybrida* (Ono et al. 2006a) was already achieved in the past, resulting in pale yellow petals. However, there is no study that combines all genes involved in anthochlor biosynthesis. Introduction of multiple genes is often achieved by co-transformation using multiple expression cassettes or sequential crossing between already transgenic lines. Disadvantages of these strategies involve varying expression levels depending on the integration loci (Felipe et al. 2006; Lechtenberg et al. 2003). A potential approach would be the introduction of a single vector containing multiple expression cassettes under the control of different regulatory elements to avoid co-suppression effects (Daniell and Dhingra 2002; Fagard and Vaucheret 2000). The GoldenBraid (GB) system provides a tool for overcoming co-suppression effects by enabling the user to combine different regulatory elements, based on the Golden Gate cloning system, one of the most effective methods for vector construction (Engler et al. 2008; Weber et al. 2011).

The GoldenBraid cloning system was designed for modularity (Sarrion-Perdigones et al. 2011; Sarrion-Perdigones et al. 2013; Kundert et al. 2020a). This can be achieved by the usage of modules called “GoldenBraid parts” or GBparts. These GBparts are prefabricated modules using a standardized “grammar” and involve a variety of different promotors, terminators, coding sequences, etc. It is possible for the user to introduce own desired sequences into GBparts using the so-called “domestication process”. Domestication of sequences involves elimination of internal restriction sites, addition of 4 bp flanking restriction sites produced after cleavage with a Type IIS restriction enzyme, either BsaI or BtgZI, and introduction into a suitable domestication plasmid called pUPD2. GBparts then can be combined in different combinations to form “transcriptional units”, comprising in its simplest pattern a promotor, the coding sequence of a gene of interest and a terminator. This reaction is a one-tube multipartite reaction utilizing type IIs restriction enzymes and special destination plasmids, called pDGBα1, pDGBα2, pDGBΩ1and pDGBΩ2 (Fig. 2A). Each of those TUs is the minimal instruction which can be introduced into an organism. However, it is possible to combine several TUs into larger constructs in Multipartite GB assembly reactions. During these reactions, pDGBα1 and pDGBα2 are linked together into an Ω-level destination plasmid and vice versa (Fig. 2B). Upon ligation, the recognition sites of the enzymes used in the assembly will disappear, therefore the composite part cannot be

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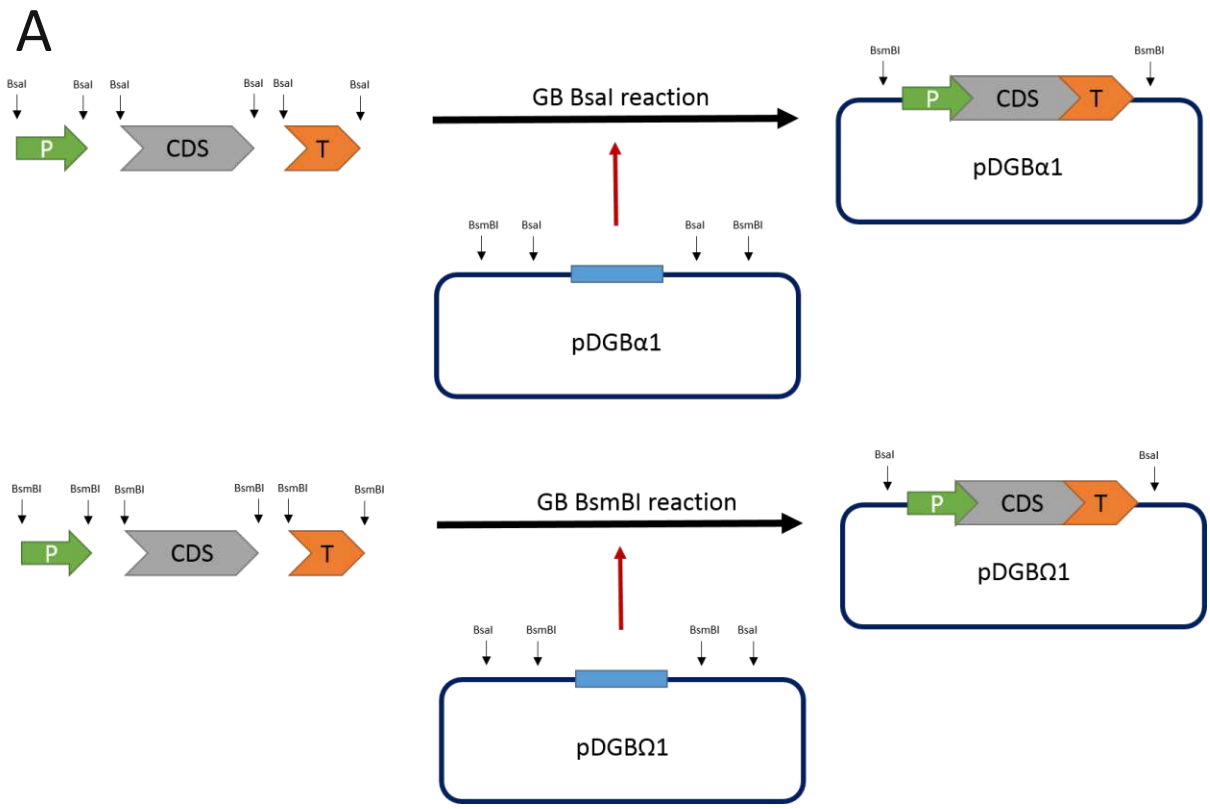
broken apart anymore. pDGBs are themselves binary plasmids, and therefore the resulting composite parts are ready for plant transformation.

In this study, we designed and cloned multigene constructs harboring structural genes to induce accumulation of deoxy-type anthochlors using the GB system. These constructs enable the user to introduce single or groups of genes involved in anthochlor biosynthesis in order to elucidate the individual contribution of genes alone or their combination. Furthermore, the resulting GB constructs were introduced into model and target plants using transient and stable transformation.



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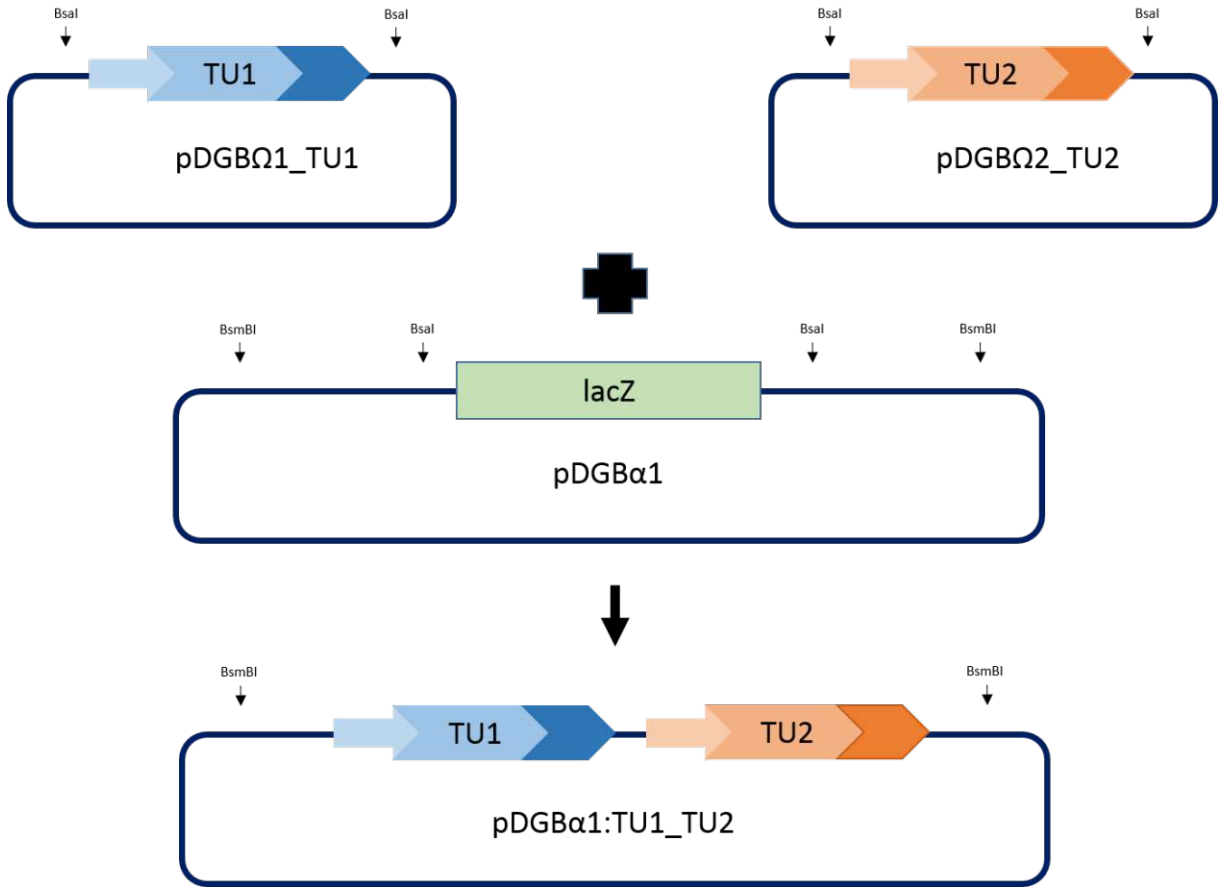

Fig. 2: Schematic illustration of GB cloning reactions; A) Multipartite GB BsaI reaction, using pDGBα plasmid as destination plasmid B) Multipartite GB BsmBI reaction, using a pDGBΩ plasmid as destination plasmid C) Binary assembly of a TU in a pDGBΩ1 plasmid is combined with a TU in a complementary pDGBΩ2 plasmid into an α-level (either 1 or 2) pDGB plasmid

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Material and Methods

Strains and growth conditions

Escherichia coli TOP10 (Thermo Scientific) was used for cloning. *Agrobacterium tumefaciens* strain GV3101 was used for transient expression and transformation experiments. Both strains were grown in Luria-Bertani (LB) medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract; pH = 7.0) under agitation (200 rpm) at 37°C and 28°C, respectively. Ampicillin (50 µg/mL), kanamycin (50 µg/mL) and spectinomycin (100 µg/mL) were used for *E. coli* selection. Rifampicin, the construct specific selection antibiotic, was also used for *A. tumefaciens* selection at 50 µg/mL. 5-Bromo-4-chloro-3-indolyl-β-d-galactopyranoside acid (40 µg/mL) and isopropylthio-β-galactoside (0.5 mM were used on LB agar plates for the white/blue selection of clones.

Plant material

Target plants for stable and transient transformation are five *Pelargonium* cultivars and one *Petunia hybrida* cultivar from the company Selecta One (Stuttgart, Germany). The petunia cultivar 'Lilac Glo' was cultivated *in vitro,* whereas the pelargonia were cultivated in a green house. The *Pelargonium* cultivars 'Sailing '12' (white), 'Sunrise' (white), 'Goesta' (pink), 'Michelle' (salmon) were obtained from Selecta One (Stuttgart, Germany) and 'French Vanilla' (yellow-creamish) was obtained from PAC-Elsner, respectively.

GoldenBraid Domestication

All cloning steps were guided and planned to use the "Domestication", "GB TU Assembler" and "GB Binary Assembler" tools from the GoldenBraid homepage (https://gbcloning.upv.es/). All primers used during domestication can be found in table 1 and were obtained from LGC Genomics (Berlin, Germany). DNA patches were obtained by PCR amplification using suitable templates. The Phusion High-Fidelity DNA Polymerase (ThermoScientific) was used for amplification following the manufacturer's protocols. All PCR amplification products were analyzed on an 1% agarose gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). Quantification was done using the Nano Drop Spectrophotometer 2000.

For domestication, 40 – 75 ng of each amplicon and 75 ng of the domestication vector (pUPD) were mixed with *Bsm*BI (New England Biolabs) and T4 DNA ligase in 1x T4 DNA ligase Buffer (Promega) in a 25-cycle restriction-ligation reaction (2 min at 37 °C,

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5 min at 16 °C). One microliter of the reaction was used for *E. coli* transformation and positive clones were selected through blue/white selection and PCR. Plasmid DNA was extracted using the GenElute™ HP Plasmid Miniprep Kit (Sigma-Aldrich). Correct assemblies were confirmed by sequencing (Microsynth, Vienna, Austria).

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Gene	Source		Sequence 5' → 3'
Lj_CHR	*Lotus japonica*	F	GCGCCGTCTCGCTCGAATGGCTGCTATTATTGAAATCCC
		R	GCGCCGTCTCGCTCAAAGCTTATGCTTGGTCATCAAAGAGATC
Bf_CHR	*Bidens ferulifolia*	F	GCGCCGTCTCGCTCGAATGGTATCCATTCCAGTAACAAC
		R	GCGCCGTCTCGCTCAAAGCTTACTGAAATGCCAGACCAGC
Cs_CH3H_1	*Cosmos sulphureus*	F	GCGCCGTCTCGCTCGAATGACTATTCTACCCCTACTAC
		R	GCGCCGTCTCGGAAGACGGTTAGGGTGACGG
Cs_CH3H_2		F	GCGCCGTCTCGCTTCCGCCGGGACCAAGCCC
		R	GCGCCGTCTCGCGATCTCGACCAACTACATTG
Cs_CH3H_3		F	GCGCCGTCTCGATCGGCTTGTAACTGAATTAGAC
		R	GCGCCGTCTCGATGTCTCTTTTACAATGGCTTGTA
Cs_CH3H_4		F	GCGCCGTCTCGACATTTAGGCTTCACCCTTCA
		R	GCGCCGTCTCGCTCAAAGCTTAATGACTTCCATACACGTGTG
Cg_AUS1_1	*Coreopsis grandiflora*	F	GCGCCGTCTCGCTCGAATGGCTCCCATAACAGCTCC
		R	GCGCCGTCTCGGGATCTCCATTCGCAACGGG
Cg_AUS1_2		F	GCGCCGTCTCGATCCGTCAGTCGGGTCCGTG
		R	GCGCCGTCTCGCTCAAAGCCTAGGCTTTAGGAATAGGAAC
Cg_AUS2	*Coreopsis grandiflora*	F	GCGCCGTCTCGCTCGAATGGCGCCCGTAACAACTCC
		R	GCGCCGTCTCGCTCAAAGCTTAAAGCCGAGGAACCAACTT
Md_GT_1	*Malus domestica*	F	GCGCCGTCTCGCTCGAATGAAGAGGCCAGCACAACT

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		R	GCGCCGTCTCGCATCTCTGACGTGAGATTTGT
Md_GT_2		F	GCGCCGTCTCGGATGCCGTTATCAACGTACTC
		R	GCGCCGTCTCGCTCAAAGCTTAAATTTTATCAATAAAACGCCCTAATG
Mutation_CH3H		F	aGAAACCTTTAGGCTTCAC
		R	TTACAATGGCTTGTAGAAAATTTAAC

Table 1: List of Primers used for Domestication of GB parts

Name	Sequence		
Promotor		**Terminator**	
Ubq3-fw	GGTGTTGTTTTGATTTCTCTTACGGCTTT	Thsp18.2-rv	AGCACACAAGCTTTTTATTTGACACACC
Ubq10-fw	GATCTGGTGTTAGTTTCTAGTTTGTGCG	Tact2-rv	CCAGAGAGTTTGTCACACACAAGTG
Pnos-fw	ATGCTCCACTGACGTTCCATAAATTCC	Tnos-rv	CAAGACCGGCAACAGGATTCAATCT
35s_new-fw	GATGTGATATCTCCACTGACGTAAGG	T35s-rv	TGCTCAACACATGAGCGAAACCC
pUPD2-fw	GCTTTCGCTAAGGATGATTTCTGG	pUPD2-rv	CAGGGTGGTGACACCTTGCC
Bf_CHR_fw	ATGGTATCCATTCCAGTAACAACG	Bf_CHR_rv	TTACTGAAATGCCAGACCAGCATC
Cs_CH3H_fw	ATGACTATTCTACCCCTACTACTC	Cs_CH3H_rv	TTAATGACTTCCATACACGTGTGGA
Cg_AUS1_fw	ATGGCTCCCATAACAGCTCCTG	Cg_AUS1_rv	CTAGGCTTTAGGAATAGGAACCAACTC
Cg_AUS2_fw	ATGGCGCCCGTAACAACTCCTGATA	Cg_AUS2_rv	TTAAAGCCGAGGAACCAACTTAATTTTGATC
Md_GT_fw	ATGAAGAGACCAGCACAACTAGTG	Md-GT-rv	GCCCTAATGAAGAGTAAGAAGAACC

Table 2: List of Primers used for validation of correct assemblies

Transcriptional Unit and Binary Assembly

Transcriptional Unit (TU) and Binary Assemblies (BA) were carried out similar to the domestication reactions, unless otherwise noted. For assembling the initial or subsequent transcriptional units in α-level vectors, 75 ng each of the individual GBparts (promotor, gene of interest (GOI) and terminator) or TU in Ω-level vectors were mixed with 75 ng of the desired α-level vector, *Bsa*I (New England Biolabs) and T4 DNA ligase in 1x T4 DNA ligase Buffer (Promega) in a 25-cycle restriction-ligation reaction (2 min at 37 °C, 5 min at 16 °C), followed by *E. coli* transformation. For assembling the TUs in Ω-level vectors, 75 ng each of the individual GBparts or TU in α-level vectors were mixed with 75 ng of the desired Ω-level vector, *Bsm*BI (New England Biolabs) and T4 DNA ligase in 1x T4 DNA ligase Buffer (Promega) in a 25-cycle restriction-ligation reaction (2 min at 37 °C, 5 min at 16 °C), followed by *E. coli* transformation. Initial and process cloning were carried out using pDGB1 vectors, the final assembly of the finished constructs were cloned in pDGB3 α-level vectors or Ω-level vectors.

Site-directed mutagenesis

Mutations were generated by use of the Q5 Site-Directed Mutagenesis Kit (NewEngland Biolabs, Vienna, Austria). Primers were designed using the NEBase ChangerTM provided at http://nebasechanger.neb.com. The sequences are given in Table 1. The integrity of the constructs was confirmed by commercial sequencing (Microsynth, Vienna, Austria).

Agrobacterium preparation

All chemicals, if not otherwise stated, were purchased from Duchefa (Haarlem, Netherlands). Transformation of *Agrobacterium tumefaciens* strain GV3101 was performed according to xxx. 5 mL of Luria-Bertani (LB) medium were inoculated with *A. tumefaciens* clones and incubated over night at 28 °C and 200 rpm agitation. 500 µL of overnight culture were transferred into cooled 1.5 mL Eppendorf reaction tubes containing 5 – 10 µg plasmids and carefully mixed by inverting. After chilling on ice for 5 min, the tubes were frozen for 5 minutes in liquid nitrogen, followed by immediate incubation in a 37 °C water bath for 5 minutes. After addition of 1 mL LB medium, the

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agrobacteria were incubated for 4 h at room temperature. Selection for positive clones was achieved by plating 50 and 130 µL of the *A. tumefaciens* culture onto LB plates containing 50 µL/mL rifampicin and the construct specific selection antibiotic.

For transformation, an overnight culture of *A. tumefaciens* was pelleted at 3,000 rpm for 15 minutes and washed twice with infiltration medium (10 mM MES-NaOH pH 5.5, 10 mM $MgSO_4$) before the pellet was resuspended in infiltration medium. For stable transformation, the OD of the infiltration culture was adjusted to 0.4 – 0.6 and for transient transformation, the OD was adjusted to 0.1 – 0.15. 200 µM acetosyringon was added to both infiltration cultures and incubated for 2 – 3 h at room temperature and 200 rpm agitation.

Transformation Protocol

The stable transformation was conducted according to (Winkelmann et al. 2005). Leaves and petiols were incubated in 500 mL sodium hypochloride with 1.5 % chlorine with one drop of Tween20 for 10 min, followed by three washing steps in sterile water for 10 minutes each. Petiols were cut into segments of 4 – 6 mm length and were placed horizontally on the medium in 6 cm petri dishes containing 10 mL basal medium (CaCl: 320 mg/L; KH_2PO_4: 170 mg/L; $MgSO_4$: 180 mg/L; NH_4NO_3: 1,650 mg/L; KNO_3: 1,900 mg/L; 2 mL/L of 500x Microelement Stock Solution ($CoCl_2$: 12.5 mg; $CuSO_4$: 12.5 mg; H_3BO_3: 3,100 mg; KI: 415 mg; $MnSO_4$: 8,450 mg; Na_2MoO_4: 125 mg; $ZnSO_3$: 4,300 mg; FeNaEDTA: 18,750 mg); 100 µL/L of 10,000x Vitamine Stock Solution (Glycine: 20 g; Niacin: 500 mg; Pyridoxine: 5 g; Thiamine: 1 g); Sucrose: 20 g/L; µ-inositol: 10 mL/L from 10 g/L stock solution; PVP-20: 5 g/L; phytohormones: IAA: 0.5 mg/L; TDZ: 1 mg/L; Zeatin: 1 mg/L); 1 mL/L Plant Preservative Mixture (Plant Cell Technology, Washington D.C. USA); Agar: 7.6 g/L; pH=5.8).

Leaf blade explants were cut into 5 x 5 mm discs and were incubated with the adaxial side up. The petri dishes were sealed with parafilm and all cultures were kept at 23 – 25 °C under 16 h of light (20-40 µmol m^{-2} s^{-1}) for three days.

Petiole and leaf disc explants were incubated for 10 min in *A. tumefaciens* infiltration culture, then blotted dry on sterile filter paper and placed horizontally (leaf discs with the adaxial facing up, respectively) on the medium in 6 cm petri dishes containing 10 mL basal medium. After two and four days of co-cultivation, the explants were washed

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three times in 150 mg/L timentin, followed by three washing steps in sterile water for 10 minutes and transferred to basal medium containing 500 mg/L cefotaxim, 150 mg/L timentin and 50 mg/L kanamycin. The Petri dishes were sealed with Parafilm and cultures were kept at 23 – 25 °C under 16 h of light (20-40 µmol m^{-2} s^{-1}) for four weeks. Every four weeks or when visible contamination occurred, the non-contaminated explants were transferred onto new plates.

Small plantlets were excised and placed into boxes containing basal medium as described above. Leafs were harvested, shock-frozen in liquid nitrogen, and stored at -80 °C.

For transient transformation, two strategies were used. One was the introduction of *A. tumefaciens* using a needleless syringe and the other was vacuum infiltration. For the syringe method, ~ 1 mL of the infiltration culture of *A. tumefaciens* is injected into petals using a needleless syringe after carefully scratching the abaxial side of a petal using a scalpel. The plants are either kept under greenhouse conditions or flower heads were harvested and placed in plastic containers containing 10 g/mL water agar. Color changes and/or fluorescence was checked optically.

Vacuum infiltration was accomblished according to (Leuzinger et al. 2013) unless otherwise stated. The flower heads were submerged upside down into the *A. fumefaciens* solution and placed inside an exsiccator. A pressure of 100 mbar was applied and hold for two to five minutes to allow the bacteria to infiltrate the intercellular space. Afterwards, pressure compensation was done over the course of additional five minutes with only minor changes in air pressure at a time to exclude additional damaging of the petals. Infiltrated plants were kept in the dark overnight before placing them again under light.

Infiltrated petals showing alterations were harvested 7 to 13 days post infiltration (dpi), shock-frozen in liquid nitrogen and stored and -80 °C.

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DNA and RNA extraction

Genomic DNA was isolated from the leafs using the Invisorb Spin Plant Mini Kit from Invitek (Berlin, Germany).

mRNA was isolated from the separated petal parts using the µMACS mRNA Isolation Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). cDNA was prepared using the RevertAid H Minus MuLV Reverse Transcriptase (Fermentas Life Science, St. Leon-Rot, Germany) with an oligo(dT)-SMART anchor primer (AAGCAGTGGTATCAACGCAGAGTAC(T23)VN) .

Pigment Analysis

For the validation of the presence and contents of anthochlor pigments, 0.5 g of shock frozen petals from transformed and wildtype plants were pulverized and extracted with 1 mL methanol. The homogenate was centrifuged for 1 min at 4 °C and 10.000 x g. For enzymatic hydrolysis of flavones, flavonols and anthochlors, 20 µL of the methanolic extract were mixed with 100 µL 0.1 M McIlvaine Buffer (pH 4.0) containing 10 U naringinase (Sigma-Aldrich, Austria) and incubated for 10 min at 40 °C. The reaction was stopped by addition of 30 µL methanol and used in HPLC analysis according to Thill et al. (36). A Thermo Scientific Dionex UltiMate(R) 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 x 150 mm (Dionex bonded Silica Products: No. 071399) was operated at 25 °C. For analysis of flavones, flavonols and anthochlor pigments, 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). The compounds were detected at 290 nm and identified by retention times and comparison of the 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.

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Results

Designing und cloning of GB vectors

Aim of this study is the induction of the biosynthesis of anthochlor pigments in plants, which do not naturally accumulate those compounds. We used the bipartite multigene construction system GoldenBraid, comprising the steps domestication of GOI, combining those GOI with promotors and terminators to transcriptional units (TU) and finally combining those TU to binary assemblies (BA). The utilization of the GoldenBraid vector system enabled us to design constructs carrying multiple genes involved in anthochlor biosynthesis in one vector under different regulatory elements. The basis structural genes chalcone reductase (CHR), chalcone 3-hydroxylase (CH3H) and aurone synthase (AUS) should all origin from members of the Asteraceae family. The sequence of CHR was obtained from previous studies in *Bidens ferulifolia* (unpublished) and, as a secondary approach, from the legume *Lotus japonica* (GenBank: AB263016.1). *Cosmos sulphureus* CH3H was obtained from petals based on the findings of (Kaintz et al. 2014) (GenBank: FJ216429.1). Two slightly different AUS, named AUS1 (GenBank: KC878307.1) and AUS2 (GenBank: KC972611.1) were isolated from *Coreopsis grandiflora* (Kaintz et al. 2014). An additional flavonoid glycosyltransferase from *Malus* x *domestica* (GenBank: DQ103712.1) (Gosch et al. 2010) was cloned for glycosylation and storing of anthochlors in the vacuole. The genes were either available as plasmid or were cloned from cDNA using a PCR-based cloning strategy and were domesticated in pUPD2 vectors (Addgene plasmid #68161) from the GoldenBraid library in a restriction-ligation reaction using *Bsm*BI as restriction enzyme. Domestication describes the process of removing internal restriction sites used by the GoldenBraid system from the genes of interests, addition of *Bsm*BI restriction sites to the N- and C-terminus and embedding those genes into domestication vectors named pUPD2.

After domestication, the DNA parts were combined in individual conjunction with promotors and terminators and embedded in pDGB1-vectors depending on the further combination strategy, resulting in specific and combinable TU. Depending on the later transformation strategy, two different selection marker TU were chosen from the GoldenBraid database. For transient transformation, the green fluorescent protein GFP or the red fluorescent protein dsRED, both under the control of the cauliflower mosaic virus CaMV 35S promoter were cloned in the aforementioned manner and the

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neomycin phosphotransferase gene *nptII* under the control of the nopaline synthase promotor (Addgene plasmid #68212) was used for selecting stable transformations. In total, we created nine basic TU (Fig. 3A). These TU were further combined in several combination steps, resulting in twelve transformation vectors (Fig. 3B). Common to all is the kanamycin resistance gene for selection of transformed plants. Based on the proposed biosynthetic pathway of anthochlor pigments, the first gene in all BA is CHR, opening the branch for 6'-deoxy chalcone synthesis (Fig. 1). Most of the constructs, including those containing additional genes of the anthochlor pathway, carry the Asteraceae *Bidens ferulifolia* CHR. In parallel, a legume CHR from *Lotus japonica* was used to create secondary vectors for co-transformation, using the same promotor-terminator combination as Bf_CHR and cloned together with the red fluorescent protein dsRED.

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Publication 3

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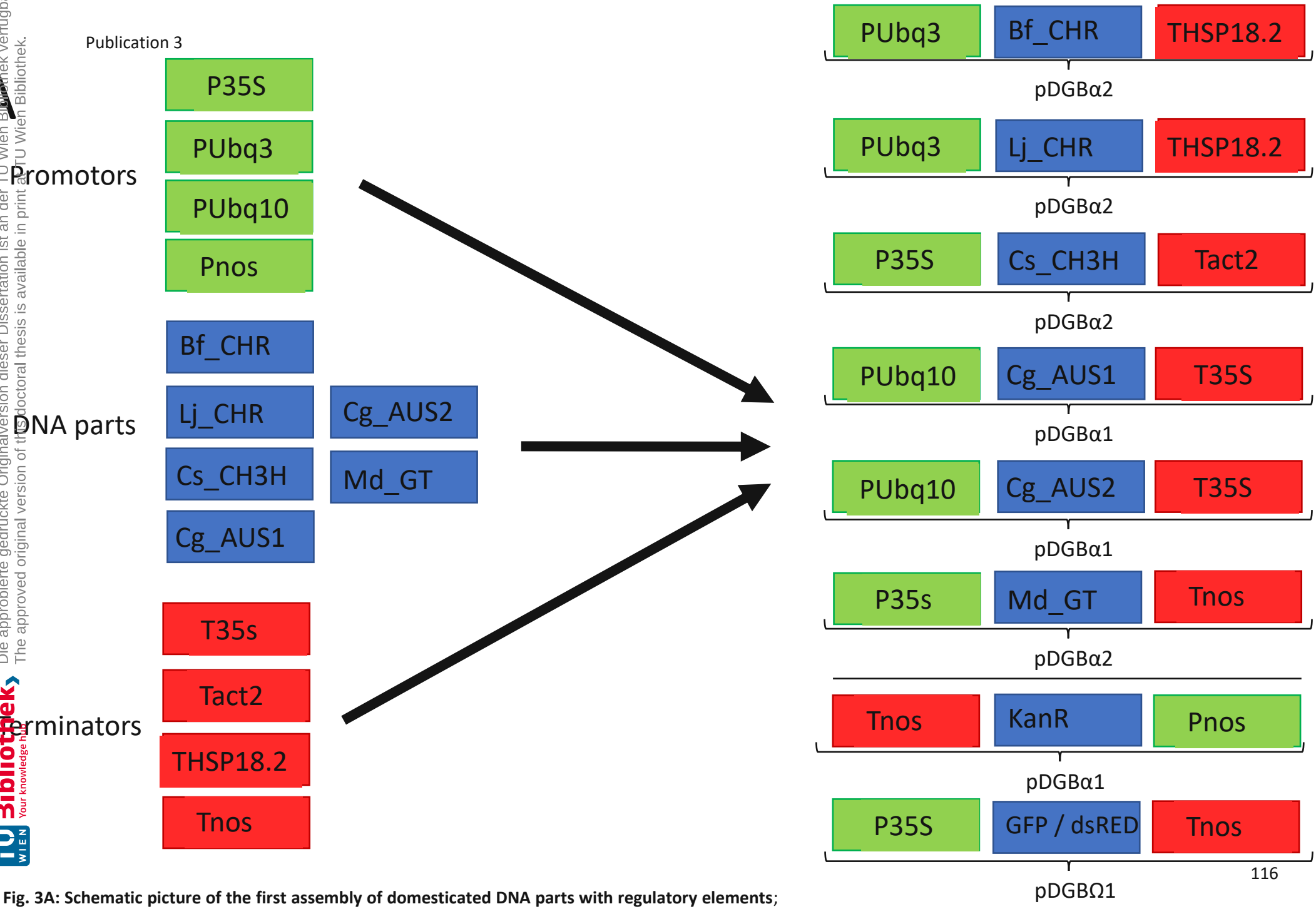

Fig. 3A: Schematic picture of the first assembly of domesticated DNA parts with regulatory elements; note the different destination vector numeration

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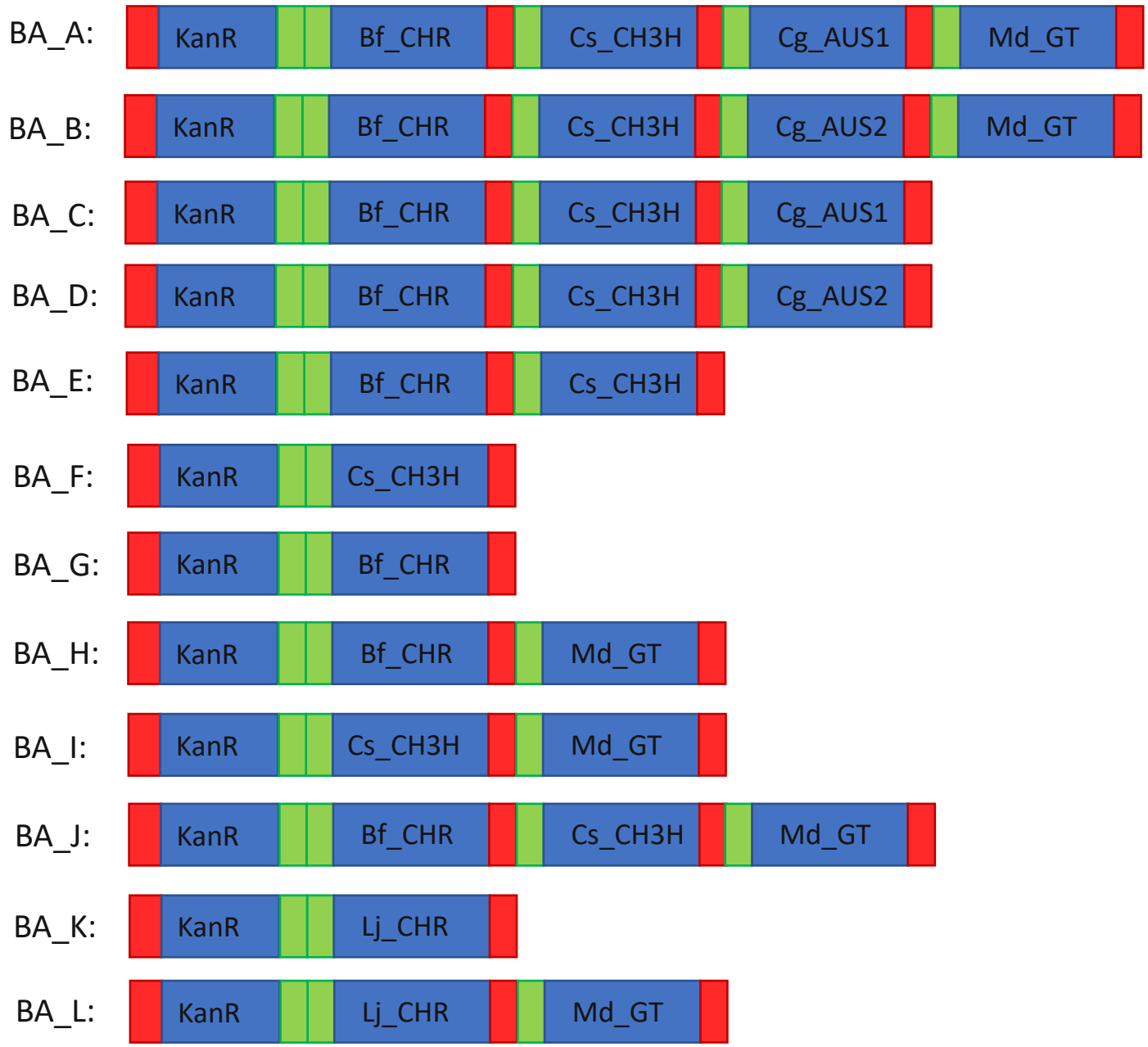

Fig. 3B: Schematic picture of the finished GoldenBraid Binary Assemblies; green boxes mark promotors, green boxes mark terminators. The exakt promotor-/terminator composition can be found in Figure 3A

During vector construction, we found that a mutation of Cs_CH3H must have occurred already during domestication. The mutation at position 1193, a deletion of an adenine, lead to a frameshift, resulting in a truncated and nonfunctional protein (Fig. 4). Therefore, site-directed mutagenesis was carried out on the BA_F vector and delivered successfully a repaired gene which was then used for further cloning of the final constructs.

```
             1171  1180      1190      1200      1210      1220      1230
             |--------+---------+---------+---------+---------+---------+
Cos_CH3H     ATTTTCTACAAGCCATTGTAAAAGAAACCTTTAGGCTTCACCCTTCAACACCACTCTCGT
BA_F1_Mut    ATTTTCTACAAGCCATTGTAAAAGAAACCTTTAGGCTTCACCCTTCAACACCACTCTCGT
BA_F1        ATTTTCTACAAGCCATTGTAAA-GAAACCTTTAGGCTTCACCCTTCAACACCACTCTCGT
Consensus    ATTTTCTACAAGCCATTGTAAAaGAAACCTTTAGGCTTCACCCTTCAACACCACTCTCGT
```

Fig. 4: Alignment of Cs_CH3H before and after site-directed mutagenesis; Cos_CH3H marks the GenBank sequence FJ216429.1, BA_F1 is the domesticated gene showing the missing adenine at position 1193 and BA_F1_Mut is the mutated and recovered gene

Transient transformation

In parallel to stable transformations, the constructs were transiently transformed using *Agrobacterium*-mediated gene transfer. Due to time and workload reasons, only a collection of constructs was used for transformation: BA_GFP, BA_dsRED, BA_A, BA_F, BA_J and BA_L.

We used white and yellow flowering *Dianthus caryophyllus* with just opened buds (cut flowers and potted flowers), the yellow-white bicolored *Dahlia variabilis* 'Seattle', the red-white colored unstable *Dahlia variabilis* 'Franzosendahlie' and potted or cuttings from different colored *Pelargonium zonale*. Modus operandi was *Agrobacterium*-mediated transformation with a needleless syringe or vacuum infiltration.

Only the petals of *Dianthus caryophyllus* were robust enough withstand the physical stress of vacuum infiltration. For transient transformation, infiltration cultures of agrobacteria carrying selection vectors (GFP or dsRED) were mixed with infiltration cultures carrying the anthochlor pathway genes, resulting in co-transformation with two constructs. Four events were created with three plants (cut flowers) or one pot per constructs. Two days post infiltration (dpi), the white carnations began to show necrotic

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areas at the petal border areas. Neither the white nor the yellow varieties showed a visible color change from white to yellow or a more intense yellow. The successful transformation was checked by the presence of dsRED activity under UV light. All white varieties showed dsRED activity in the injected areas (Fig. 5A). A surprising side effect was a color change from white to pale pink in the injected region of the petals, caused by the production of pelargonidin (Fig. 5B). Yellow varieties did not exhibit dsRED activity. Potted plants showed the same patterns, except for one yellow flower which showed a bolder yellow coloration (Fig. 5C). Petals showing dsRED were harvested and analyzed for transcripts. All of the tested tissue showed positive signals for the inserted genes (Fig. 7). However, when subjected to HPLC, in none of the samples, accumulation of isoliquiritigenin, butein or sulfuretin could be measured.

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Fig. 5: Phenotypes of transiently transformed *Dianthus caryophyllus*; A) Left picture half shows potted carnations after vacuum infiltration with BA_dsRED and BA_L, the right picture half shows cut carnations after infiltration using the syringe method and the same constructs. Both pictures were taken 7 days post infiltration; B+C) Light rose coloration occurred in the same region where dsRED activity was observable. Photos were taken 7 days post infiltration; D) Comparison between a wildtype yellow potted carnation and a flower of a transiently transformed yellow potted carnation; the used construct was BA_I and the photo was taken 13 days post infiltration

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We used the white flowering ‘Paloma’ and the salmon colored ‘Goesta’ for transient transformation in pelargonia. *Pelargonium* petals were only possible to transform by injecting the infiltration medium using a needleless syringe. Additionally, transformation had to be done in the greenhouse since the plants were grown in the greenhouse and were highly susceptible to endogenous pathogens. Fungi and bacteria would rapidly overgrow the excised cuttings when they were placed in moist boxes. On the other hand, when leaving the flowers attached to the mother plant, the petals endured the transformation process and no alteration in petal fitness could be observed compared to the wildtype controls. Similar to the transformation process in *Dianthus*, co-transformation of fluorescence marker and anthochlor genes was the modus operandi. Regardless of the used variety, infiltrated plants exhibited fluorescence after three to five dpi due to the expression of dsRED and/or GFP. Although most of the fluorescent petals did not show alterations in coloration, around 5 % of the fluorescent petals exhibited a slight color change towards pale yellow (Fig 6). However, HPLC results showed no measurable accumulation of anthochlors. The presence of transcripts of the inserted genes was verified through PCR (Fig. 7).

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Fig. 6: Phenotypes of transiently transformed *Pelargonium zonale*; Upper two pictures show fluorescence induced by expression of dsRED (left) and GFP (right), the injection sites are visible as dark spots inside the glowing area; The lower two pictures show color changes from white to pale yellow in 'Paloma' (left) and 'Goesta' (right). 'Paloma' was transformed with BA_L + BA_A and 'Goesta' was transformed with BA_L

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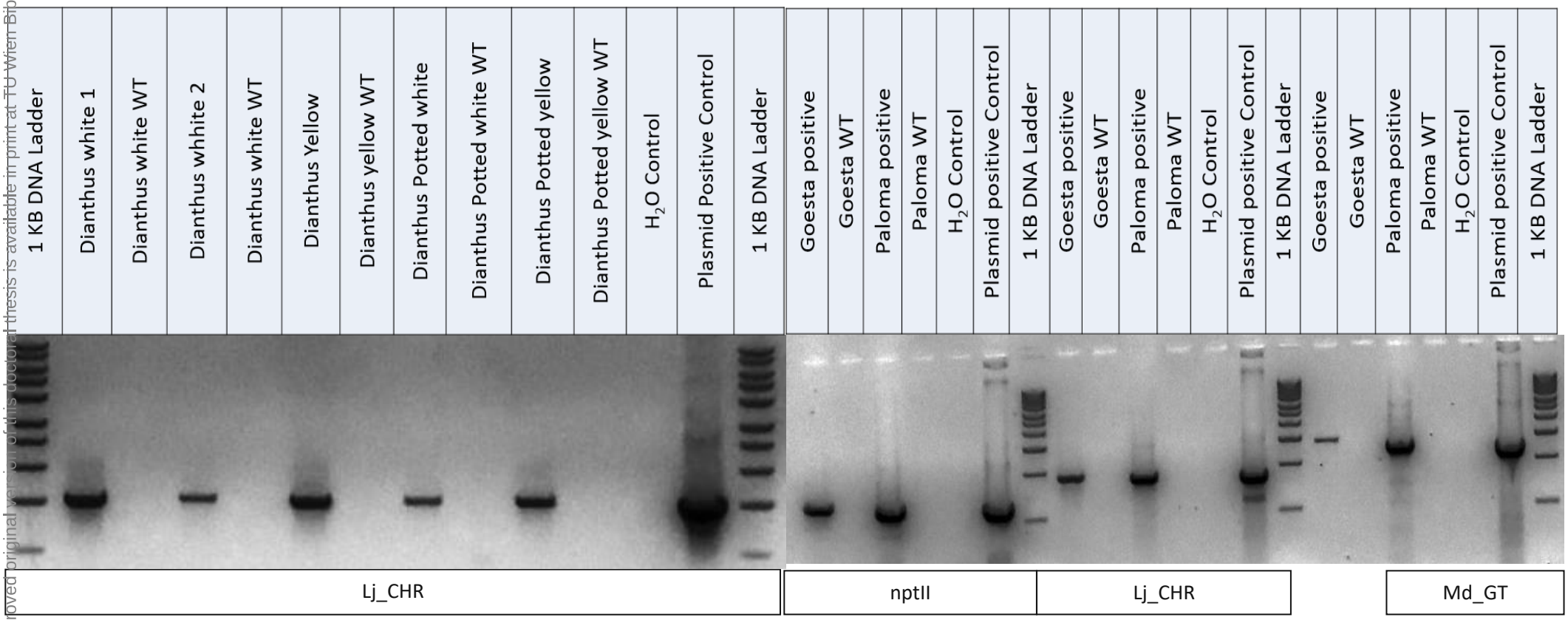

Fig. 7: PCR evaluation of cDNA from transiently transformed *Dianthus caryophyllus* and *Pelargonium zonale*; the upper picture shows the presence of bands specific for CHR from *Lotus japonica;* the lower picture shows the presence of *nptII*, CHR from *Lotus japonica* and a glycosyltransferase from *Malus domestica*

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Stable transformation

Pelargonia do not naturally produce anthochlor pigments nor express a bold yellow flower coloration by accumulation of other bright yellow pigments. Stable transformation with vectors harboring genes involved in anthochlor biosynthesis provides a powerful tool for anthochlor research.

Plant	Variety	Construct	No. of explants
Pelargonium zonale	Moonlight Sailing'12	BA_L-1	372
		BA_L-2	298
		BA_L-3	488
		BA_L + BA_D	487
	Paloma	BA_L + BA_GFP-1	260
		BA_L + BA_GFP-2	315
		BA_L + BA_GFP-3	367
		BA_L + BA_B	277
		BA_L-1	611
		BA_L-2	503
		BA_L-3	421
		BA_L-4	488
	French Vanilla	BA_L	564
		BA_L + BA_B	628
Petunia hybrida	Lilac Glo	BA_L	402
		BA_L + BA_GFP	315
		BA_L + BA_F-1	442
		BA_L + BA_F-2	352
		BA_L + BA_D	370

Table. 1: List of constructs used for stable transformation; explanation of the detailed construct composition can be found in figure 3B;

Transformation was done according to (Winkelmann et al. 2006) with minor changes which can be found in material and methods. We used the white flowering varieties 'Moonlight Sailing '12' and 'Sunrise Paloma' and the pale-yellow flowering variety 'French Vanilla'. Due to contamination problems during regeneration, the *Petunia hybrida* variety 'Lilac Glo', flowering with a pale pink color, was chosen as a second transformation platform since this variety was already established *in vitro* and was

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already used in stable transformation experiments in the lab by Nitarska et. al (personal communication).

From in total 6079 explants excised from pelargonium (excluding wildtype controls) divided in 14 events, no explants survived the transformation process. Most explants in the first approaches were lost due to heavy infection of endogenous bacteria and fungi in the initial days of regeneration. Since the original surface sterilization process (incubation for 10 minutes in 1.5 % hypochloride, followed by three washing steps) did not produce satisfying results, the sterilization process was optimized two times. The first optimization changed the sterilization protocol to washing with sterile water for 20 minutes, followed by incubation in 4% PPM for three hours with continuous agitation. The explants were then rinsed with sterile water, dried on sterile paper blots and placed on basal medium containing 1 mL/L PPM and 150 mg/L timentin. During pre-culture, the explants would start browning from the ends. The middle of the explants stayed green and alive. This method led to a loss of 40 – 60 % of explants, but the surviving cells showed normal callus growth and did not show infection with endogenous pathogens (Fig. 8). However, after treated with this PPM-intensive sterilization, neither wildtype control nor *A. tumefaciens* incubated explants did produce plantlets.

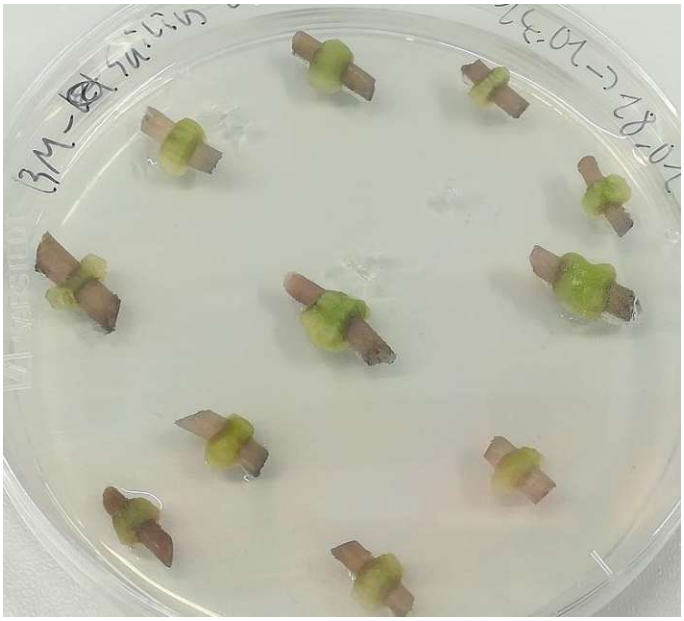

Fig. 8: Calli of explants treated with the PPM-intensive sterilization method; explants were incubated in 4 % PPM for three hours and produced calli in the surviving middle part

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A second optimization combined both approaches. The explants were first washed for 10 minutes in 1.5 % hypochloride, rinsed with sterile water, followed by incubation in 2 % PPM for 1 h under continuous agitation, subsequent rinsing with sterile water and blotted dry on sterile paper sheets. With this method, the explants showed a higher survivability compared to the PPM-intensive treatment (70% compared to 40 – 60 %) and the infection with endogenous pathogens was on an acceptable level. Additionally, the explants produced plantlets which could be excised from the callus and placed in medium for rooting.

Incubation of *Pelargonium* explants with *A. tumefaciens* lead to heavy losses of explants due to overgrowth with agrobacteria. We tried several strategies to overcome this problem. Washing of the explants with antibiotics with varying concentrations and/ or PPM did not produce satisfying results, but worsened the spread of bacteria, leading to higher losses than not washing the explants. Increasing the concentration of timentin and cefotaxime in the basal medium stepwise from 50 µg/L (100 µg/L for cefotaxime) up to 150 µg/L (250 µg/L for cefotaxime) only restricted the growth of the bacteria to the explants and stopped the growth onto the medium and thus the spreading. However, losses of 50% or more of the incubated explants per event were still occurring. Additionally, the stress of transformation seemed to trigger the growth of endogenous pathogens once more, leading to additional losses. Surviving explants without bacterial or fungal contamination kept growing calli. The concentration of kanamycin as selection marker had to be lowered stepwise from 100 mg/L down to 10 mg/L to prevent total losses of explants. After the course of eight to ten weeks, 2 to 5 % of transformed explants kept growing and produced small plantlets (5 to 10 per callus) (Fig. 9A). These plantlets were excised and placed into growth medium. However, these plantlets failed to root and stopped growth. Changes in medium composition, different phytohormones, lighting scheme and removal of kanamycin did not enhance the rooting, thus leading to not a single surviving transformed pelargonium.

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Fig. 9: Regenerants of transformed pelargonia and petunia; A) explants on calli of *Pelargonium zonale* explants, 8 weeks after transformation (upper and right picture) and excised regenerants placed in medium (lower picture); B) explants on calli of *Petunia hybrida,* note the hyperhydrocity

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Transformation of *Petunia* explants did not produce the same problems with endogenous pathogens as the transformation of *Pelargonium* did. However, the strong contamination of *A. tumefaciens* in combination with the need for lower kanamycin concentrations was observable in the petunia transformations as well. Only the transformation events “BA_L”, “BA_L + BA_F-2” and “BA_L + BA_D” lead to a total of 39 regenerates which were transferred from the calli to medium (9 for “BA_L”, 27 for “BA_L + BA_F-2” and 3 for BA_L + BA_D). Many of the regenerates had glossy and fragile tissue, a disorder commonly known as hyperhydricity (Fig. 9B). Screening for the integration of *nptII* showed the presence in three plants of the event “BA_L + BA_F-2” and one plant of the event “BA_L + BA_D”. No integration of *nptII* could be detected in plants of the event “BA_L”. This corresponds to a transformation efficiency of 0% for the event “BA_L”, 0.85 % for the event “BA_L + BA_F-2” and 0.27 % for the event “BA_L + BA_D”, respectively. The amount of false positive plants was 2.2 % for “BA_L”, 7.7 % for “BA_L + BA_F-2” and 0.81 % for “BA_L + BA_D” (Table 2).

Plant	Construct	No. of explants	No. of rooted plants	*nptII* presence	Positive / False positive
Lilac Glo	BA_L	402	9	0	0 % / 2.2 %
Lilac Glo	BA_L + BA_F-2	352	27	3	0.85 % / 6.8 %
Lilac Glo	BA_ + BA_D	370	3	1	0.27 % / 0.5 %

Table. 2: List of transformation efficiency of *Petunia hybrida* ‘Lilac Glo’; in three events, the transformation efficieny ranges between 0 % and 0.85 % with false positives ranging between 0.5 and 6.8 % of tested plants

When verifying the presence of transcripts via RT-PCR, no transcripts of the genes involved in anthochlor biosynthesis (Lj_CHR, Cs_CH3H and Md_GT for “BA_L+BA_F”; Lj_CHR, Cs_CH3H, Cg_AUS2 and Md_GT for “BA_L + BA_D”) could be found (Fig. 10).

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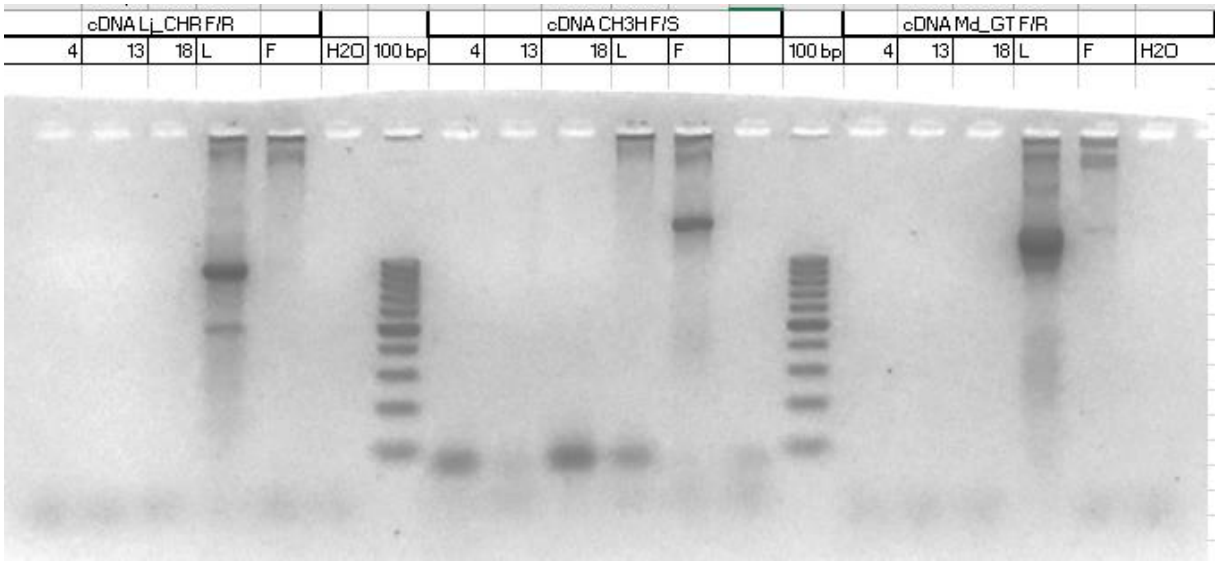

Fig. 10: Exemplary RT-PCR on the presence of genes of the anthochlor biosynthesis; cDNA from the three plants tested *nptII*-positive from the event "BA_L + BA_F" were tested on the presence of the transcripts Lj_CHR, Cs_CH3H and Md_GT, with the plasmids BA_L and BA_F as positive controls. No transcripts were found

Nonetheless, all *nptII*-positive plants were transferred to the greenhouse and brought to flowering in order to observe a potential color change. However, the flowers showed the same coloration as the wild type 'Lilac Glo' controls.

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Discussion

Pelargonium zonale is one of the plants which naturally do not exhibit a bright yellow flower coloration, nor produce pigments belonging to the class of anthochlors. Anthochlors are a pigment class closely related to flavonoids. In contrast to other flavonoids, the exact pathway of biosynthesis of anthochlor pigments is not fully understood. This study consists of three parts. 1) Designing and cloning of plant transformation vectors combining genes involved in anthochlor biosynthesis from different plants of the Asteraceae family. These multigene constructs were designed using the GoldenBraid cloning system. The constructs were used for 2) transient transformation of *Dianthus caryophyllus* and *Pelargonium zonale* and 3) stable transformation of *Pelargonium zonale* and *Petunia hybrida*.

Previous studies showed the functional activity of the used proteins. Enzyme activity assays performed with purified, codon optimized recombinant CHR from *Bidens ferulifolia* in yeast showed a clear conversion of naringenin chalcone to isoliquiritigenin. When using petal raw extract disrupted with lysozyme, no conversion was observable (Barbara Roth, personal communication). Schlangen et al. (2010b) examined in their study the activity of CH3H from *Cosmos sulphureus*. The activity of both aurone synthases from *Coreopsis grandiflora* was studied by Kaintz et al. (2014). Similarly, the glycosyltransferase used in this study was chosen based on the positive enzyme activity assays done by Gosch et al. (2010).

The domestication, the combination of transcriptional units and assembling of binary constructs proved to be quick and easy. Domestication and most of the combination to transcriptional units was done in parallel within three days, including transformation and screening of *E. coli* (Sarrion-Perdigones et al. 2011; Sarrion-Perdigones et al. 2013). The utilization of type IIS restriction sites was reported by various other studies (Engler et al. 2008; Weber et al. 2011; Da Lin and O'Callaghan 2018; Lee et al. 2020; Damalas et al. 2020), but only a few rely on the GoldenBraid system (Kundert et al. 2020a; Vazquez-Vilar et al. 2016).

Transient expression of genes in plants is a rapid and efficient alternative to the costly and time-consuming stable transformation of plants for e.g. production of pharmaceuticals (Leuzinger et al. 2013; Giritch et al. 2006; Nausch et al. 2012a; Nausch et al. 2012b) or the research of the physiological roles of gene products (Tyurin

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et al. 2020). The transient transformation of model plants was planned to A) verify the general functionality of GoldenBraid constructs and B) to verify the functionality of the genes involved in anthochlor biosynthesis. To reach the first objective, GoldenBraid constructs carrying the reporter genes GFP and dsRED were designed and introduced *Agrobacterium*-mediated either using vacuum infiltration (Leuzinger et al. 2013) or syringe infiltration. Whereas the syringe infiltration method is a reliable small-scale infiltration method, vacuum infiltration of whole plants delivers the possibility for larger-scale and cost-efficient plant transformation.

Target plants for transient transformation were the yellow-white bicolored *Dahlia variabilis 'Seattle'* and the red-white colored unstable *Dahlia variabilis* 'Franzosendahlie' due to their well-studied metabolism (Halbwirth et al. 2008; Walliser et al. 2021). Especially 'Seattle' was already in the focus of anthochlor research. For similar reasons, white and yellow flowering *Dianthus caryophyllus*, either potted or as cut flowers, were obtained (Forkmann and Dangelmayr 1980; Ogata et al. 2004; Mato et al. 2000).

Independent of the used transformation method, dahlia was very susceptible to infiltration in general. After vacuum infiltration, with or without agrobacteria, petal necrosis occurred two days post infiltration, leading to the loss of the whole flower without any signs of successful transformation, seemingly due to the physical stress during the infiltration process. Transformation with the gentler syringe method of single petals, either still attached to the flower or separated and placed on petri dishes filled with water agar, lead to a slightly higher survival rate. However, the separated explants still spoiled before any activity of the inserted genes could be observed. The soft petals of *Dahlia variabilis* were too fragile for the physical stress during infiltration with the used methods.

The petals of carnations, on the other hand, were very robust and survived the stress of transformation very well regardless of the performed method. Syringe infiltration showed the best results due to the big and robust petals and the relative gentle physical stress. Necrotic areas were only found at the border regions of infiltrated petal, regardless if agrobacteria were used or if it was the agrobacterium-free infiltration solution. All infiltrated petals exhibited visible dsRED or GFP activity and the presence of transcripts were verified by PCR, proving the successful transient transformation. Vacuum infiltration using whole potted carnations did produce a number of infiltrated

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petals, but the ratio of glowing petals to infiltrated petals did not match the syringe infiltration method. The presence of genes of the anthochlor biosynthesis introduced by co-transformation was confirmed by PCR as well. However, no visible color change was observable, in neither white nor yellow carnations except for a slight shift from white to red in white carnations transformed with dsRED and one flower of yellow potted carnations co-transformed with dsRED and chalcone reductase from *Lotus japonica*. The red coloration can be ascribed to the surprising induction of anthocyanidin synthesis according to the presence of the single-hydroxylated anthocyanidin pelargonidin. The used dsRED, first described in 1999, is derived from the coral *Discosoma* sp. “red” and is excited with yellow-green light and shows and emission maximum of 583 nm (Matz et al. 1999). This is within the range of red light which was shown, together with UV(B) light, to induce anthocyanidin synthesis (Takeda and Abe 1992) since anthocyanidins and flavonols in general act as UV and light protection (Halbwirth 2010). HPCL measurements of the dark yellow *Dianthus* flower showed slightly elevated flavonol contents, but no indication of the presence of anthochlor pigments.

The same result can be observed when *Pelargonium zonale* petals were used for transformation. Transformation of pelargonia was performed by syringe infiltration in petals still attached to the mother plant inside the greenhouse. Removing single petals and flowers, followed by transformation by vacuum infiltration and storing in containers failed due to strong endogenous contamination with bacteria and fungi. Necrosis of the infiltrated petals, independent of the presence of agrobacteria in the infiltration medium, occurred in roughly 75 % of infiltrated petals. The ratio could be lowered by infiltrating only up to a half of the total petals on one flower head. The additional advantage of this approach lies in the wildtype control directly attached to the test object. Surviving petals transformed in that manner began to show fluorescence after three to five days post infiltration. A small number of plants showing GFP or dsRED activity exhibited a color change from white to pale yellow. Corresponding to the transformation of *Dianthus*, transcripts of all inserted genes could be found in the transformed petals, but no traces of the desired compounds were detected by HPLC. It remains unclear whether the genes were eventually translated into proteins after the transformation and the fate of those proteins remains unknown. Transcription of the inserted constructs is functional, as the activity of marker proteins and the presence of transcripts on the mRNA level state. Ohno et al. (2022) showed in their study that only

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the mutual expression of CHR, a chalcone glycosyltransferase and a MYBA transcription factor lead to successful accumulation of 6'-deoxychalcones. They report from the same findings we observed, so introducing the transcription factor MYBA may be a feasible approach.

Due to the low yield of each transformation, no enzyme activity assays of petal raw extracts were performed to investigate the activity of potentially translated genes of the anthochlor pathway. Enhancement of transformation capacities and optimization of the transformation process for improving survivability of the transformants could deliver more plant material for subsequent evaluations. The most suitable approach is syringe-assisted transformation of *Dianthus caryophyllus* due to the relative low effort in combination with the observed transformation success rate, tissue fitness and high amounts of resulting tissue.

Whilst the transient transformations of carnations and dahlias were performed beforehand, the transient transformation of *Pelargonium* was done in parallel to the stable transformations. Though the GoldenBraid system is planned to introduce multiple transcriptional units in one transformation step (Sarrion-Perdigones et al. 2011; Sarrion-Perdigones et al. 2013; Kundert et al. 2020a), we decided to perform co-transformations of BA_L (carrying a chalcone reductase from *Lotus japonica* and a flavonoid glycosyltransferase from *Malus domestica*) with other constructs carrying other combinations of genes downstream in the pathway of anthochlor biosynthesis. Those GB constructs were **BA_F**, carrying a chalcone 3-hydroxylase from *Coreopsis grandiflora*, **BA_B**, carrying the full set of the anthochlor pathway including the Asteracaea chalcone reductase from *Bidens ferulifolia*, a chalcone 3-hydroxylase from *Coreopsis grandiflora*, the aurone synthase 2 from *Cosmos sulphureus* and a flavonoid glycosyltransferase from *Malus domestica* and **BA_D**, constructed like BA_B but lacking the glycosyltransferase. BA_GFP, carrying the green fluorescent protein GFP was co-transformed with BA_L as well for optical control of successful transformation.

Since the lab was not prepared for the amounts of sterile grown *Pelargonium* plants needed for the planned transformations, we had to use greenhouse grown *Pelargonium* plants. Though available in sufficient quantities, the explants from greenhouse grown plants proofed to be hard to use. In the beginning, most to all explants prepared according to the original sterilization protocol from Winkelmann et al. were lost due to heavy infection of endogenous bacteria and fungi. The first

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alteration of the sterilization protocol involved washing the explants in diluted Plant Preservative Medium© (PPM), according to manuals and reviews (Lata et al. 2009; Miyazaki et al. 2010; Orlikowska et al. 2012). Though the intensive washed explants were cleaned from endogenous bacteria and fungi, the successfully formed calli failed to produce plantlets for further cultivation, irrespective of wildtype or agrobacterium mediated transformation. Reduction of washing time and/or PPM concentration did not produce satisfying results. Either the explants still did not produce plantlets, or the endogenous pathogens survived the sterilization and overgrew the explants (Orlikowska et al. 2012). The latest optimization, combining the strong surface sterilization involving chlorine and deep-tissue cleaning using PPM with moderate incubation times lead to a tolerable amount of surviving healthy explants and occurring contaminations. Wildtype plants, without incubation with the agrobacterium infiltration solution, produced numbers of healthy and subsequent rooting plantlets, verifying the general suitability of the sterilization and regeneration protocol. The utilization of *in vitro* grown *Petunia* for agrobacterium-mediated transformation did not result in the same problems as the explants of *Pelargonium*, showing the importance of clean source plant material.

Now with the problems from endogenous bacteria and fungi removed, marauding agrobacteria possessed a threat to the explants. After just a few days post transformation of the explants with the agrobacteria transformation solution, most of the explants were overgrown by *A. tumefaciens,* regardless of the origin of the plant material. After increasing the concentration of antibiotics in the media, the agrobacteria seemed to grow only on the explants. No bacterial growth could be observed when the bacteria reached the plant growth medium, indicating that the bacteria were then susceptible enough to the antibiotics contained in the media. Antibiotic resistance assays performed after the first occurrence of this phenomenon showed already high resistances in the non-transformed *A. tumefaciens* GV3101 strain used in the lab during this project (data not shown), seemingly indicating a contamination of the initial wildtype strain. Since agrobacterium-mediated transformations weren't performed for some time in the lab, this contamination remained unnoticed.

Whilst five to ten percent of the transformed pelargonia calli produced plantlets, these failed to root when excised and placed in appropriate medium. Alteration of phytohormone concertation (Reuther 1983) or composition (Rao 1994) did not results

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in enhanced rooting. Wojtania (2011) showed that the addition of the plant growth regulator meta-topolin enhances not only the organogenesis but also showed a better rooting for *Pelargonium* cultivars. Additionally, other studies implied that brassinosteroides used as a plant growth regulated resulted in improved root growth in *Pelargonium* (Swamy and Rao 2006). Furthermore, the used cytokinin Thidiazuron (TDZ) seemed to play a vital role in the occurred abnormalities. (Dewir et al. 2018) published a review article, addressing several problems which emerged during the regeneration and growing of the explants when TDZ was incorporated in the growth media. One of the most common effects of TDZ is hyperhydricity, resulting in the observed phenotype of the *Petunia* regenerants in our study. This hyperhydricity is also known for other plant species when treated with TDZ (Kadota and Niimi 2003; Ivanova and van Staden 2011, 2008; Dewir et al. 2006; Hutchinson and Saxena 1996).

Although PPM was essential to hold endogenous bacteria in check, several studies showed that PPM may have a negative impact on growth and development of shoots and roots. Compton and Koch (2001) analyzed the potential effect of PPM on different plant species and explant types with varying PPM concentrations. They concluded that 0.3 % PPM resulted in decreased bud numbers in *Petunia* explants, whereas even high amounts of PPM did not influence the regeneration of tobacco. Sensitivity of *Petunia* to PPM was also confirmed by Miyazaki et al. (2010). Furthermore, *Arabidopsis thaliana* (Paul et al. 2001) and *Chrysanthemum* (Orlikowska et al. 2012; George and Tripepi 2001) were proven to be highly susceptible to PPM in the growth media, resulting in diminished rooting and/or development of microshoots. These studies agree with our findings of reduced microshooting and rooting in *Pelargonium* and *Petunia*.

Transformation and regeneration of *Pelargonium* (Chabannes et al. 2009; García-Sogo et al. 2012; Gehl et al. 2018; Hassanein et al. 2005; Winkelmann et al. 2006; Boase et al. 1998) and *Petunia* (Avila and Day 2021; Broderick and Jones 2014; Conner et al. 2009; Meyer et al. 1987; Kasai et al. 2013; Haselmair-Gosch et al. 2020; Haselmair-Gosch et al. 2018; Fraley et al. 1984; Thirukkumaran et al. 2009) has been successfully achieved in multiple studies. However, in contrast to this study, most of the aforementioned authors used the *A. tumefaciens* strain LBA4404 for their stable transformations. GV3101, the agrobacterium strain we used in this study for both transient and stable transformation, is more commonly used for transient

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transformation (Sheikh et al. 2014). But there are also studies which used GV3101 for successful stable transformations (Chabannes et al. 2009; Li et al. 2014; Chetty et al. 2013), indicating that the *A. tumefaciens* strain alone may not be responsible for a successful transformation. Nonetheless, all the aforementioned problems resulted in very low numbers of regenerants. And even though the presence of the *nptII* gene was detected in four regenerated plants on the genomic DNA level, none of them showed transgene expression. Since the expression of other transcriptional units did work when transiently transformed, we can assume that the system should work when integrated correctly into the genome.

Another approach for elucidation the individual role of the genes involved in anthochlor biosynthesis was the utilization of two different silencing methods. One was the “stable” silencing of single genes in plants which do naturally produce anthochlor pigments using the CRISPR/Cas9 system based on *Streptococcus pyogenes* (Steinert et al. 2015). Silencing vectors were constructed, harboring single-guide RNA (sgRNA) from *Bidens ferulifola* CHR, *Cosmos sulphureus* CH3H and *Coreopsis grandiflora* AUS. High homology of the 20 bp sequences between the plant species when selecting the sdRNA was one of the main aims, so the constructs can be used for several species. Another approach was utilizing virus-induced gene silencing (VIGS) for transient silencing of target genes. A modified strain of the *Tobacco rattle virus* (TRV) with fused GFP for optical observation of the infection tracking was used for vector construction (Tian et al. 2014). Though the silencing approach seemed to be promising, they had to be postponed due to time reasons and data are therefore not shown in this thesis. However, these two approaches may give highly interesting insights into anthochlor biosynthesis.

Generally, this work gained valuable insights in potential obstacles in introducing new biosynthetic pathways in plants. I have to admit that the goals of this work may were too ambitious for the given time frame. Nonetheless, the results gained lay an excellent foundation for further investigations. Beginning with the construction and testing of the genes embed in GB constructs, transient pre-experiments of different promoter-terminator combinations may elevate the expression profiles of the single genes. Additionally, using viral vectors for high expression, maybe generating tagged proteins and testing the purified recombinant proteins, may be rewarding to shed light on the functionality of the enzymes. Another approach may be fusing the genes of interest

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with a stabilizing fusion partner like GFP to examine the stability of the recombinant protein in model and target plants (Walliser 2015). GFP as a fusion partner offers the additional effect of visible observation of the presence in planta. The results we obtained for regeneration of plant cells regarding media composition and *A. tumefaciens* strains are highly valuable for repeating the stable transformation experiments.

References

Avila, E. M., and Day, A. (2021), 'Stable Plastid Transformation of Petunia for Studies in Basic Research', *Methods in molecular biology (Clifton, N.J.)*, 2317: 229–245.

Boase, M. R., Bradley, J. M., and Borst, N. K. (1998), 'An improved method for transformation of regal pelargonium (Pelargonium Xdomesticum Dubonnet) by Agrobacterium tumefaciens', *Plant Science*, 139/1: 59–69.

Bohm, AB (1988), *The minor flavonoids the flavonoids advances in research since 1980 (p. 352)*.

Bomati, E. K., Austin, M. B., Bowman, M. E. et al. (2005), 'Structural elucidation of chalcone reductase and implications for deoxychalcone biosynthesis', *The Journal of biological chemistry*, 280/34: 30496–30503.

Broderick, S. R., and Jones, M. L. (2014), 'An Optimized Protocol to Increase Virus-Induced Gene Silencing Efficiency and Minimize Viral Symptoms in Petunia', *Plant molecular biology reporter*, 32: 219–233.

Brouillard, R., and Dangles, O. (1988), 'Flavonoids and flower color', in J. B. Harborne (ed.), *The Flavonoids - Advances in Research*, 565–86.

Chabannes, M., Marazuela, E., and Borja, M. (2009), 'EXPRESSION OF AN ARABIDOPSIS ASPARTIC PROTEASE IN PELARGONIUM', *Acta Hortic.*, 2009: 271–275.

Chetty, V. J., Ceballos, N., Garcia, D. et al. (2013), 'Evaluation of four Agrobacterium tumefaciens strains for the genetic transformation of tomato (Solanum lycopersicum L.) cultivar Micro-Tom', *Plant cell reports*, 32/2: 239–247.

Compton, M. E., and Koch, J. M. (2001), 'Influence of plant preservative mixture (PPM)TM on adventitious organogenesis in melon, petunia, and tobacco', *In Vitro Cell.Dev.Biol.-Plant*, 37/2: 259–261.

Conner, A. J., Albert, N. W., and Deroles, S. C. (2009), 'Transformation and Regeneration of Petunia', in , *Petunia* (Springer, New York, NY), 395–409 <https://link.springer.com/chapter/10.1007/978-0-387-84796-2_19>.

Da Lin, and O'Callaghan, C. A. (2018), 'MetClo: methylase-assisted hierarchical DNA assembly using a single type IIS restriction enzyme', *Nucleic acids research*, 46/19: e113.

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Damalas, S. G., Batianis, C., Martin-Pascual, M. et al. (2020), 'SEVA 3.1: enabling interoperability of DNA assembly among the SEVA, BioBricks and Type IIS restriction enzyme standards', *Microbial Biotechnology*, 13/6: 1793–1806.

Daniell, H., and Dhingra, A. (2002), 'Multigene engineering: dawn of an exciting new era in biotechnology', *Current Opinion in Biotechnology*, 13/2: 136–141 <https://www.sciencedirect.com/science/article/pii/s0958166902002975>.

Davies, K. M., Bloor, S. J., Spiller, G. B. et al. (2002), 'Production of yellow colour in flowers. Redirection of flavonoid biosynthesis in Petunia', *The Plant Journal*, 13/2: 259–266.

Dewir, Y. H., Chakrabarty, D., Ali, M. B. et al. (2006), 'Lipid peroxidation and antioxidant enzyme activities of Euphorbia millii hyperhydric shoots', *Environmental and Experimental Botany*, 58/1-3: 93–99.

Dewir, Y. H., Nurmansyah, Naidoo, Y. et al. (2018), 'Thidiazuron-induced abnormalities in plant tissue cultures', *Plant cell reports*, 37/11: 1451–1470.

Engler, C., Kandzia, R., and Marillonnet, S. (2008), 'A one pot, one step, precision cloning method with high throughput capability', *PLOS ONE*, 3/11: e3647 <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0003647>.

Fagard, M., and Vaucheret, H. (2000), '(TRANS)GENE SILENCING IN PLANTS: How Many Mechanisms?', *Annual review of plant physiology and plant molecular biology*, 51: 167–194.

Felipe, P. de, Luke, G. A., Hughes, L. E. et al. (2006), 'E unum pluribus: multiple proteins from a self-processing polyprotein', *Trends in Biotechnology*, 24/2: 68–75 <https://www.sciencedirect.com/science/article/pii/s0167779905003288>.

Forkmann, G., and Dangelmayr, B. (1980), 'Genetic control of chalcone isomerase activity in flowers of Dianthus caryophyllus', *Biochem Genet*, 18/5-6: 519–527 <https://link.springer.com/article/10.1007/bf00484399>.

Fraley, R. T., Horsch, R. B., Matzke, A. et al. (1984), 'In vitro transformation of petunia cells by an improved method of co-cultivation with A. tumefaciens strains', *Plant molecular biology*, 3/6: 371–378 <https://link.springer.com/article/10.1007/bf00033384>.

García-Sogo, B., Pineda, B., Roque, E. et al. (2012), 'Production of engineered long-life and male sterile Pelargonium plants', *BMC plant biology*, 12: 156.

Gehl, C., Wamhoff, D., Schaarschmidt, F. et al. (2018), 'Improved leaf and flower longevity by expressing the etr1-1 allele in Pelargonium zonale under control of FBP1 and SAG12 promoters', *Plant Growth Regul*, 86/3: 351–363.

George, M. W., and Tripepi, R. R. (2001), 'Plant Preservative Mixture™ Can Affect Shoot Regeneration from Leaf Explants of Chrysanthemum, European Birch, and Rhododendron', *HortSci*, 36/4: 768–769.

Giritch, A., Marillonnet, S., Engler, C. et al. (2006), 'Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors', *Proceedings of the National Academy of Sciences of the United States of America*, 103/40: 14701–14706 <https://www.pnas.org/content/103/40/14701.short>.

Gosch, C., Halbwirth, H., Schneider, B. et al. (2010), 'Cloning and heterologous expression of glycosyltransferases from Malus x domestica and Pyrus communis, which convert phloretin to phloretin 2′-O-glucoside (phloridzin)', *Plant Science*, 178/3: 299–306 <https://www.sciencedirect.com/science/article/pii/s016894521000004x>.

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TU WIEN Bibliothek Your knowledge hub

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., Muster, G., and Stich, K. (2008), 'Unraveling the Biochemical Base of Dahlia Flower Coloration', *Natural Product Communications*, 3/8: 1934578X0800300.

Harborne, J. B. (1967), 'Comparative biochemistry of the flavonoids-VI', *Phytochemistry*, 6/12: 1643–1651.

Haselmair-Gosch, C., Miosic, S., Nitarska, D. et al. (2018), 'Great Cause-Small Effect: Undeclared Genetically Engineered Orange Petunias Harbor an Inefficient Dihydroflavonol 4-Reductase', *Frontiers in plant science*, 9: 149.

Haselmair-Gosch, C., Nitarska, D., Walliser, B. et al. (2020), 'Event-specific qualitative polymerase chain reaction analysis for two T-DNA copies in genetically modified orange Petunia', 2020.

Hassanein, A., Chevreau, E., and Dorion, N. (2005), 'Highly efficient transformation of zonal (Pelargonium x hortorum) and scented (P. capitatum) geraniums via Agrobacterium tumefaciens using leaf discs', *Plant Science*, 169/3: 532–541.

Hutchinson, M. J., and Saxena, P. K. (1996), 'Acetylsalicylic acid enhances and synchronizes thidiazuron-induced somatic embryogenesis in geranium (Pelargonium x hortorum Bailey) tissue cultures', *Plant cell reports*, 15/7: 512–515.

Ivanova, M., and van Staden, J. (2008), 'Effect of ammonium ions and cytokinins on hyperhydricity and multiplication rate of in vitro regenerated shoots of Aloe polyphylla', *Plant Cell Tiss Organ Cult*, 92/2: 227–231.

—— (2011), 'Influence of gelling agent and cytokinins on the control of hyperhydricity in Aloe polyphylla', *Plant Cell Tiss Organ Cult*, 104/1: 13–21.

Kadota, M., and Niimi, Y. (2003), 'Effects of cytokinin types and their concentrations on Effects of cytokinin types and their concentrations on shoot proliferation and hyperhydricity in in vitro pear cultivar shoots', *Plant Cell Tiss Organ Cult*, 72/3: 261–265.

Kaintz, C., Molitor, C., Thill, J. et al. (2014), 'Cloning and functional expression in E. coli of a polyphenol oxidase transcript from Coreopsis grandiflora involved in aurone formation', *FEBS letters*, 588/18: 3417–3426.

Kasai, M., Matsumura, H., Yoshida, K. et al. (2013), 'Deep sequencing uncovers commonality in small RNA profiles between transgene-induced and naturally occurring RNA silencing of chalcone synthase-A gene in petunia', *BMC genomics*, 14: 63.

Kimura, Y., Aoki, T., and Ayabe, S. (2001), 'Chalcone isomerase isozymes with different substrate specificities towards 6'-hydroxy- and 6'-deoxychalcones in cultured cells of Glycyrrhiza echinata, a leguminous plant producing 5-deoxyflavonoids', *Plant & cell physiology*, 42/10: 1169–1173.

Kundert, P., Sarrion-Perdigones, A., Gonzalez, Y. et al. (2020), 'A GoldenBraid cloning system for synthetic biology in social amoebae', *Nucleic acids research*, 48/8: 4139–4146.

Lata, H., Chandra, S., Khan, I. A. et al. (2009), 'Propagation through alginate encapsulation of axillary buds of Cannabis sativa L. - an important medicinal plant', *Physiology and molecular biology of plants : an international journal of functional plant biology*, 15/1: 79–86.

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Lechtenberg, B., Schubert, D., Forsbach, A. et al. (2003), 'Neither inverted repeat T-DNA configurations nor arrangements of tandemly repeated transgenes are sufficient to trigger transgene silencing', *The Plant Journal*, 34/4: 507–517.

Lee, J. H., Won, H. J., Oh, E.-S. et al. (2020), 'Golden Gate Cloning-Compatible DNA Replicon/2A-Mediated Polycistronic Vectors for Plants', *Frontiers in plant science*, 11: 559365.

Leuzinger, K., Dent, M., Hurtado, J. et al. (2013), 'Efficient agroinfiltration of plants for high-level transient expression of recombinant proteins', *JoVE (Journal of Visualized Experiments)*, 2013: e50521 <https://www.jove.com/t/50521/efficient-agroinfiltration-plants-for-high-level-transient-expression>.

Li, Y., Gao, Z., Piao, C. et al. (2014), 'A stable and efficient Agrobacterium tumefaciens-mediated genetic transformation of the medicinal plant Digitalis purpurea L', *Applied biochemistry and biotechnology*, 172/4: 1807–1817.

Mato, M., Onozaki, T., Ozeki, Y. et al. (2000), 'Flavonoid biosynthesis in white-flowered Sim carnations (Dianthus caryophyllus)', *Scientia Horticulturae*, 84/3-4: 333–347.

Matz, M. V., Fradkov, A. F., Labas, Y. A. et al. (1999), 'Fluorescent proteins from nonbioluminescent Anthozoa species', *Nature biotechnology*, 17/10: 969–973.

Meyer, P., Heidmann, I., Forkmann, G. et al. (1987), 'A new petunia flower colour generated by transformation of a mutant with a maize gene', *Nature*, 330/6149: 677–678 <https://www.nature.com/articles/330677a0>.

Miles, C. O., and Main, L. (1985), 'Kinetics and mechanism of the cyclisation of 2′,6′-dihydroxy-4,4′-dimethoxy-chalcone; influence of the 6′-hydroxy group on the rate of cyclisation under neutral conditions', *J. Chem. Soc., Perkin Trans. 2*, 1985: 1639–1642.

—— (1989), 'Kinetics and mechanism of the cyclisation of 2′,6′-dihydroxychalcone and derivatives', *J. Chem. Soc., Perkin Trans. 2*, 1989: 1623–1632.

Miosic, S., Knop, K., Hölscher, D. et al. (2013), '4-Deoxyaurone formation in Bidens ferulifolia (Jacq.) DC', *PloS one*, 8/5: e61766.

Miyajima, I., Maehara, T., Kage, T. et al. (1991), 'Identification of the Main Agent Causing Yellow Color of Yellow-Flowered Cyclamen Muatnt', *J. Japan. Soc. Hort. Sci.*, 60/2: 409–414 <https://www.jstage.jst.go.jp/article/jjshs1925/60/2/60_2_409/_article/-char/ja/>.

Miyazaki, J., Tan, B. H., and Errington, S. G. (2010), 'Eradication of endophytic bacteria via treatment for axillary buds of Petunia hybrida using Plant Preservative Mixture (PPMTM)', *Plant Cell Tiss Organ Cult*, 102/3: 365–372.

Molitor, C., Mauracher, S. G., Pargan, S. et al. (2015), 'Latent and active aurone synthase from petals of C. grandiflora. A polyphenol oxidase with unique characteristics', *Planta*, 242/3: 519–537.

Molitor, C., Mauracher, S. G., and Rompel, A. (2016), 'Aurone synthase is a catechol oxidase with hydroxylase activity and provides insights into the mechanism of plant polyphenol oxidases', *PNAS*, 113/13: E1806-15 <https://www.pnas.org/content/113/13/e1806.short>.

Nakayama, T., Sato, T., Fukui, Y. et al. (2001), 'Specificity analysis and mechanism of aurone synthesis catalyzed by aureusidin synthase, a polyphenol oxidase homolog responsible for flower coloration', *FEBS letters*, 499/1-2: 107–111.

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Nakayama, T., Yonekura-Sakakibara, K., Sato, T. et al. (2000), 'Aureusidin synthase: a polyphenol oxidase homolog responsible for flower coloration', *Science (New York, N.Y.)*, 290/5494: 1163–1166.

Nausch, H., Mikschofsky, H., Koslowski, R. et al. (2012a), 'High-level transient expression of ER-targeted human interleukin 6 in Nicotiana benthamiana', *PLOS ONE*, 7/11: e48938.

Nausch, H., Mikschofsky, H., Mischofsky, H. et al. (2012b), 'Expression and subcellular targeting of human complement factor C5a in Nicotiana species', *PLOS ONE*, 7/12: e53023.

Ogata, J., Itoh, Y., Ishida, M. et al. (2004), 'Cloning and heterologous expression of cDNAs encoding flavonoid glucosyltransferases from Dianthus caryophyllus', *Plant Biotechnology*, 21/5: 367–375 <https://www.jstage.jst.go.jp/article/plantbiotechnology/21/5/21_5_367/_article/-char/ja/>.

Ohno, S., Yamada, H., Maruyama, K. et al. (2022), *Identification of a novel chalcone reductase gene for isoliquiritigenin biosynthesis in dahlia (Dahlia variabilis)*.

Ono, E., Fukuchi-Mizutani, M., Nakamura, N. et al. (2006a), 'Yellow flowers generated by expression of the aurone biosynthetic pathway', *Proceedings of the National Academy of Sciences of the United States of America*, 103/29: 11075–11080.

Ono, E., Hatayama, M., Isono, Y. et al. (2006b), 'Localization of a flavonoid biosynthetic polyphenol oxidase in vacuoles', *The Plant Journal*, 45/2: 133–143.

Orlikowska, T., Zawadzka, M., Zenkteler, E. et al. (2012), 'Influence of the biocides PPM tm and Vitrofural on bacteria isolated from contaminated plant tissue cultures and on plant microshoots grown on various media', *The Journal of Horticultural Science and Biotechnology*, 87/3: 223–230.

Paul, A. L., Semer, C., Kucharek, T. et al. (2001), 'The fungicidal and phytotoxic properties of benomyl and PPM in supplemented agar media supporting transgenic arabidopsis plants for a Space Shuttle flight experiment', *Applied microbiology and biotechnology*, 55/4: 480–485.

Rao, P. (1994), 'In vitro plant regeneration of scented-leaved geranium Pelargonium graveolens', *Plant Science*, 98/2: 193–198.

Reuther, G. (1983), 'PROPAGATION OF DISEASE-FREE PELARGONIUM CULTIVARS BY TISSUE CULTURE', *Acta Hortic.*, 1983: 311–320.

Sarrion-Perdigones, A., Falconi, E. E., Zandalinas, S. I. et al. (2011), 'GoldenBraid. An iterative cloning system for standardized assembly of reusable genetic modules', *PloS one*, 6/7: e21622.

Sarrion-Perdigones, A., Vazquez-Vilar, M., Palací, J. et al. (2013), 'GoldenBraid 2.0. A comprehensive DNA assembly framework for plant synthetic biology', *Plant physiology*, 162/3: 1618–1631.

Sato, T., Nakayama, T., Kikuchi, S. et al. (2001), 'Enzymatic formation of aurones in the extracts of yellow snapdragon flowers', *Plant Science*, 160/2: 229–236 <https://www.sciencedirect.com/science/article/pii/s016894520000385x>.

Schlangen, K., Miosic, S., Castro, A. et al. (2009), 'Formation of UV-honey guides in Rudbeckia hirta', *Phytochemistry*, 70/7: 889–898.

Schlangen, K., Miosic, S., and Halbwirth, H. (2010a), 'Allelic variants from Dahlia variabilis encode flavonoid 3'-hydroxylases with functional differences in chalcone 3-hydroxylase activity', *Archives of Biochemistry and Biophysics*, 494/1: 40–45.

Die approbierte gedruckte Originalversion dieser Dissertation ist an der TU Wien Bibliothek verfügbar.
The approved original version of this doctoral thesis is available in print at TU Wien Bibliothek.
TU Bibliothek Your knowledge hub WIEN

Schlangen, K., Miosic, S., Thill, J. et al. (2010b), ‘Cloning, functional expression, and characterization of a chalcone 3-hydroxylase from Cosmos sulphureus’, *Journal of experimental botany*, 61/12: 3451–3459.

Sheikh, A. H., Raghuram, B., Eschen-Lippold, L. et al. (2014), ‘Agroinfiltration by cytokinin-producing Agrobacterium sp. strain GV3101 primes defense responses in Nicotiana tabacum’, *Molecular plant-microbe interactions : MPMI*, 27/11: 1175–1185.

Shimada, N., Nakatsuka, T., Nishihara, M. et al. (2006), ‘Isolation and characterization of a cDNA encoding polyketide reductase in Lotus japonicus’, *Plant Biotechnology*, 23/5: 509–513 <https://www.jstage.jst.go.jp/article/plantbiotechnology/23/5/23_5_509/_article/-char/ja/>.

Steinert, J., Schiml, S., Fauser, F. et al. (2015), ‘Highly efficient heritable plant genome engineering using Cas9 orthologues from Streptococcus thermophilus and Staphylococcus aureus’, *The Plant journal : for cell and molecular biology*, 84/6: 1295–1305.

Swamy, K. N., and Rao, S. S. R. (2006), ‘Influence of Brassinosteroids on Rooting and Growth of Geranium (Pelargonium sp.) Stem Cuttings’, *Asian J. of Plant Sciences*, 5/4: 619–622.

Takeda, J., and Abe, S. (1992), ‘LIGHT-INDUCED SYNTHESIS OF ANTHOCYANIN IN CARROT CELLS IN SUSPENSION—IV. THE ACTION SPECTRUM’, *Photochem Photobiol*, 56/1: 69–74.

Thirukkumaran, G., Ntui, V. O., Khan, R. S. et al. (2009), ‘Thidiazuron: an efficient plant growth regulator for enhancing Agrobacterium-mediated transformation in Petunia hybrida’, *Plant Cell Tiss Organ Cult*, 99/1: 109–115 <https://link.springer.com/article/10.1007/s11240-009-9581-3>.

Tian, J., Pei, H., Zhang, S. et al. (2014), ‘TRV-GFP. A modified Tobacco rattle virus vector for efficient and visualizable analysis of gene function’, *Journal of experimental botany*, 65/1: 311–322.

Tyurin, A. A., Suhorukova, A. V., Kabardaeva, K. V. et al. (2020), ‘Transient Gene Expression is an Effective Experimental Tool for the Research into the Fine Mechanisms of Plant Gene Function. Advantages, Limitations, and Solutions’, *Plants (Basel, Switzerland)*, 9/9.

Vazquez-Vilar, M., Bernabé-Orts, J. M., Fernandez-del-Carmen, A. et al. (2016), ‘A modular toolbox for gRNA-Cas9 genome engineering in plants based on the GoldenBraid standard’, *Plant Methods*, 12/1: 10 <https://plantmethods.biomedcentral.com/articles/10.1186/s13007-016-0101-2>.

Walliser, B. (2015), ‘Characterization of the expression of the fusionprotein GFP:CphB in Nicotiana benthamiana’, Masterarbeit (Rostock, Universität Rostock).

Walliser, B., Lucaciu, C. R., Molitor, C. et al. (2021), ‘Dahlia variabilis cultivar 'Seattle' as a model plant for anthochlor biosynthesis’, *Plant physiology and biochemistry : PPB*, 159: 193–201.

Weber, E., Engler, C., Gruetzner, R. et al. (2011), ‘A Modular Cloning System for Standardized Assembly of Multigene Constructs’, *PLOS ONE*, 6/2: e16765 <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0016765>.

Welle, R., and Grisebach, H. (1988), ‘Isolation of a novel NADPH-dependent reductase which coacts with chalcone synthase in the biosynthesis of 6′-

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deoxychalcone', *FEBS letters*, 236/1: 221–225 <https://www.sciencedirect.com/science/article/pii/0014579388803181>.

Winkelmann, T., Kaviani, K., and Serek, M. (2005), 'Development of a shoot regeneration protocol for genetic transformation in Pelargonium zonale and Pelargonium peltatum hybrids', *Plant Cell Tiss Organ Cult*, 80/1: 33–42.

Winkelmann, T., Kayiani, K., and Serek, M. (2006), 'AGROBACTERIUM-MEDIATED TRANSFORMATION OF PELARGONIUM (PELARGONIUM ZONALE HYBRIDS AND PELARGONIUM PELTATUM HYBRIDS)', *Acta Hortic.*, 2006: 737–746.

Wojtania, A. (2011), 'Effect of Meta-topolin on in vitro propagation of Pelargonium × hortorum and Pelargonium × hederaefolium cultivars', *Acta Soc Bot Pol*, 79/2: 101–106.

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