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Formation of UV-honey guides in *Rudbeckia hirta*

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ABSTRACT

The UV-honey guides of *Rudbeckia hirta* were investigated by UV-photography, reflectance spectroscopy, LC–MS analysis and studies of the enzymes involved in the formation of the UV-absorbing flavonols present in the petals. It was shown for the first time that the typical bull's eye pattern is already established at the early stages of flower anthesis on the front side of the petal surface, but is hidden to pollinators until the buds are open and the petals are unfolded. The rear side of the petals remains UV-reflecting during the whole flower anthesis. Studies on the local distribution of 19 flavonols across the petals confirmed that the majority are concentrated in the basal part of the ray flower. However, in contrast to the earlier studies, eupatolitin 3-*O*-glucoside (6,7-dimethoxyquercetin 3-*O*-glucoside) was present in both the basal and apical parts of the petals, whereas eupatolin (6,7-dimethoxyquercetin 3-*O*-rhamnoside) was exclusively found in the apical parts. The enzymes involved in the formation of the flavonols in *R. hirta* were demonstrated for the first time. These include a rare flavonol 6-hydroxylase, which was identified as cytochrome P450-dependent monooxygenase and did not accept any methylated flavonol as substrate. All enzymes were present in the basal and apical parts of the petals, although some of them clearly showed higher activities in the basal part. This indicates that the local accumulation of flavonols in *R. hirta* is not achieved by a locally restricted presence of the enzymes involved in flavonol formation.

1. Introduction

Honey guides are part of the pigmentation pattern, which occurs particularly frequently in bee-pollinated flowers. Their physiological function is to guide pollinators to the reproduction organs and to the nectar in the flower centre (Daumer, 1956, 1958; Kevan and Mulligan, 1973; Harborne, 1993; Kevan et al., 2001). A broad variety of forms are known. They may appear as color contrast – as in snapdragons (*Antirrhinum majus*) where a yellow spot is located on the lip of an otherwise red or orange flower – or may take the form of colored dots or lines in particular areas of the petals. Often, honey guides are formed by local accumulation of UV-absorbing substances and are therefore invisible to the human eye. Such UV-patterns on flower petals may be perceived by many pollinating insects because of their specific color sense, which makes them blind to scarlet red coloration but sensitive to the UV-range of the spectrum (Eisner et al., 1969; Silberglied, 1979; McCrea and Levy, 1983; Dyer, 1996; Indsto et al., 2006, 2007).

The prime example for the formation of UV-honey guides is *Rudbeckia hirta* (Fig. 1). In 1972, Thompson et al. were able to show that the flowers, which are uniformly yellow to the human eye, have a characteristic UV-absorbing centre, whilst the outer parts of the ray petals are UV-reflecting. For UV-sensitive insects, the *Rudbeckia* flower appears as a bull's eye with two concentric dichromatic circles against the green meadow background (Abrahamson and McCrea, 1977). The UV-reflecting apical part is responsible for the long-distance orientation and acts as a landing site for the pollinators, whereas the UV-absorbing centre of the flower acts as a 'honey guide', helping the pollinators to orient themselves within the flower after landing (Daumer, 1956, 1958; Jokl and Fürnkranz, 1989; Burr et al., 1995). In contrast to the fully developed *Rudbeckia* flower, small buds were described as being

Abbreviations: CHI, chalcone isomerase; CHS, chalcone synthase; DHK, dihydrokaempferol; EGME, ethylene glycol mono methyl ether; EtOAC, ethyl acetate; FHT, flavanone 3-hydroxylase; FLS, flavonol synthase; F3′H, flavonoid 3′-hydroxylase; F6H, flavonol 6-hydroxylase; F7GT, flavonol 7-*O*-glucosyltransferase; F3GT, flavonol 3-*O*-glucosyltransferase; Glu, glucose; Rha, rhamnose; SAM, S-Adenosylmethionine; UDPG, UDP-glucose.

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uniformly colored to the insect's eyes. This was explained as part of the ontogenetic development, because only fertile flowers rely on pollinator attraction (Jokl and Fürnkranz, 1989; Burr et al., 1995). First chemical analysis revealed that local accumulation of three flavonols at the petal base is responsible for the basal UV-absorbing zones of the mature ray flower (dark in Fig. 1): quercetagetin (6-hydroxyquercetin), patulitrin (6-methoxyquercetin 7-*O*-glucoside) and 6,7-dimethoxyquercetin 3-*O*-glucoside (Thompson et al., 1972) (Fig. 2). It is remarkable that all the three flavonols show an extra hydroxyl or methoxyl group in position 6 in addition to the basic 5,7-hydroxylation pattern of the common flavonols, which considerably influences the light absorption. Apart from their UV-absorbance, they also contribute to the yellow color of the flower. These so-called 'yellow flavonols' are particularly formed in members of Asteraceae and Leguminosae (Harborne, 1967, 1976).

Interestingly, carotenoids are also present in *R. hirta*. In contrast to the flavonols, they are uniformly distributed in the flowers (Thompson et al., 1972). The co-occurrence of two yellow pigment types in flowers has frequently been observed, particularly in highly evolved plant species (Harborne, 1988). At the first sight, this seems to be a waste of biochemical resources, which could be justified only by their different physiological functions. Actually, the co-occurrence of different yellow pigment types is frequently connected to the formation of UV-honey guides (Harborne, 1988). In contrast to the UV-absorbing flavonols in the inner part, UV-reflecting carotenoids are responsible for the formation of the bright circle of the ray disk of *R. hirta*.

In addition to the flavonols identified by Thompson et al. (1972), further flavonols were isolated later from *R. hirta* petals (Cisowski et al., 1993): quercimeritrin (quercetin 7-*O*-glucoside), quercetagitrin (quercetagetin 7-*O*-glucoside), 6,7-dimethoxyquercetin and 6,7-dimethoxyquercetin 3-*O*-rhamnoside (Fig. 2). However, their contribution to the formation of UV-honey guides has not been studied so far. We investigated the formation of UV-honey guides in *R. hirta* by UV-photography, reflectance spectroscopy, LC–MS analysis and biochemical studies. We provide an in-depth insight into the local distribution of different flavonols in *R. hirta* petals and show for the first time the biochemical pathway leading to the pigments involved in the formation of UV-honey guides.

2. Results and discussion

2.1. UV-photography and UV-reflectance spectroscopy

UV-photography is a helpful and well-established tool for visualizing floral patterns that are invisible to the human eye (Kevan et al., 1973; Jokl et al., 1999; Dyer et al., 2004; Chittka and Kevan, 2005). In contrast to the earlier studies, which commonly show UV-photos of whole plants or flowers (Jokl and Fürnkranz, 1989; Burr et al., 1995), we photographed the front- and rear sides of single petals of four stages of flower anthesis with UV-transmittable filters. Even the youngest petals exhibited the typical bull's eye pattern at their surface (Fig. 3A). In contrast, the rear sides of the petals remain UV-reflecting in all developmental stages (Fig. 3B). Thus, the honey guides are already formed at early stages but are hidden to the pollinators, because juvenile *Rudbeckia* petals are two times folded and only the rear side is visible at the early stages of flower anthesis. Apparently, the bull's eye is formed only on the front side of the flower as the relevant side for pollen and nectar collection.

Fig. 1. UV-photography of *R.hirta* cv. 'Indian Summer', showing the reflectance at around 360 nm. The circular dark honey guide is known as 'bull's eye-effect'.

UV-photography (Williams and Williams, 1993) gives a qualitative picture of the reflectance distribution across the petal surface for a selected, rather narrow wavelength range (in this case around 360 nm ± 20 nm) and also runs the risk of interpretational difficulties (Kevan, 1979). In contrast, reflectance spectroscopy can provide a quantitative value of spectral reflectance for selected areas of the petal over a wide spectral range from UV to near infrared (Galsterer et al., 1999; Langanger et al., 2000). In the present study, reflectance curves (Fig. 4, Table 1) of both petal sides were also obtained. Each reflectance curve is the average value obtained from five different petals of the same flower, measured either at the apical or at the basal area of the petal. Additionally shown in Fig. 4 is the standard deviation of the mean value, given as 95% confidence interval.

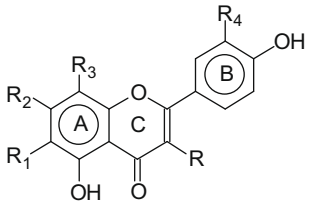
Whereas the rear side of the flower petal does not show any significant difference between the reflectance of the apical and the basal part of the petal during anthesis over the whole spectral range, for the front side this difference is already perceptible in the ultraviolet spectral range in anthesis stages ST1 and ST2, and becomes quite significant during the anthesis stages ST3 and ST4.

2.2. Local distribution of flavonols across the Rudbeckia petals

We investigated the local distribution of 19 flavonols in basal and apical parts of the petals in four different anthesis stages. The studies comprised the compounds previously described in *R. hirta* (Thompson et al., 1972; Cisowski et al., 1993) and the related flavonols, which could be potential intermediates or end-products in the flavonol formation in *R. hirta*. This included kaempferol (Km), kaempferol 3-*O*-glucoside (Km3glu), kaempferol 7-*O*-glucoside (Km7glu), quercetin (Qu), quercetin 3-*O*-glucoside (Qu3glu), quercetin7-*O*-glucoside (Qu7glu), 6-hydroxykaempferol (6-OHKm), quercetagetin (Quag), quercetagetin 3-*O*-glucoside (Quag3glu), quercetagetin 7-*O*-glucoside (Quag7glu), 6-methoxykaempferol (6-MeOKm), 6,7-dimethoxykaempferol 3-*O*-rhamnoside (6-MeOKm3rha), 7,8-dimethoxykaempferol 3-*O*-glucoside (7,8-DiMeOKm3glu), patuletin (6-MeOQu), patulitrin (6-MeOQu7glu), eupatolitin (6,7-DiMeOQu), eupatolitin 3-*O*-glucoside (6,7-DiMeOQu3glu), eupatolin (6,7-DiMeOQu3rha), and eupatolitin 3-*O*-rhamnoglucoside (6,7-DiMeOQu3glu-rha) (Fig. 2).

Qu7glu, Quag7glu, 6-MeOQu7glu, 6,7-DiMeOQu3glu and 6,7-DiMeOQu3rha were the main flavonols present in the *R. hirta* petals. Km3glu, Qu3glu, 6-MeOQu, and 6,7-DiMeOKm3rha were present in medium amounts and only small amounts of Qu, Quag and 6,7-DiMeOQu3glu-rha could be detected. Km, Km7glu, 6-OHKm, 6-MeOKm, and 7,8-DiMeOKm3glu could not be detected in the petals. The LC–MS spectra indicated the presence of further unidentified flavonols (data not shown), which were not included in the current study.

During the flower development, flavonol concentration increased continuously. The majority of the flavonols present in the petals were concentrated at the basal parts of the petals. Fig. 5 shows the local distribution of the main flavonols across the *R. hir-*



Name	Abbreviation	R	R_1	R_2	R_3	R_4
Kaempferol	Km	OH	H	OH	H	H
Kaempferol 3-*O*-glucoside	Km3glu	OGlu	H	OH	H	H
Kaempferol 7-*O*-glucoside	Km7glu	OH	H	OGlu	H	H
7-Methoxykaempferol	7-MeOKm	OH	H	OCH_3	H	H
Quercetin	Qu	OH	H	OH	H	OH
Quercetin 3-*O*-glucoside	Qu3glu	OGlu	H	OH	H	OH
Quercetin 7-*O*-glucoside	Qu7glu	OH	H	OGlu	H	OH
7-Methoxyquercetin	7-MeOQu	OH	H	OCH_3	H	OH
6-Hydroxykaempferol	6-OHKm	OH	OH	OH	H	H
Quercetagetin	Quag	OH	OH	OH	H	OH
Quercetagetin 3-*O*-glucoside	Quag3glu	OGlu	OH	OH	H	OH
Quercetagetin 7-*O*-glucoside	Quag7glu	OH	OH	OGlu	H	OH
6-Methoxykaempferol	6-MeOKm	OH	OCH_3	OH	H	H
Patuletin (6-methoxyquercetin)	6-MeOQu	OH	OCH_3	OH	H	OH
Eupatolitin (6,7-dimethoxyquercetin)	6,7-DiMeOQu	OH	OCH_3	OCH_3	H	OH
Patulitrin (patuletin 7-*O*-glucoside)	6-MeO Qu7glu	OH	OCH_3	OGlu	H	OH
7,8-Dimethoxykaempferol 3-*O*-glucoside	7,8-DiMeOKm3glu	OGlu	OCH_3	OH	OCH_3	H
6,7-Dimethoxykaempferol 3-*O*-rhamnoside	6,7-DiMeOKm3rha	ORha	OCH_3	OCH_3	H	H
Eupatolitin 3-*O*-glucoside	6,7-DiMeOQu3glu	OGlu	OCH_3	OCH_3	H	OH
Eupatolin (Eupatolitin 3-*O*-rhamnoside)	6,7-DiMeOQu3rha	ORha	OCH_3	OCH_3	H	OH

Fig. 2. Chemical structures of flavonols included in the studies.

ta petal (black columns: basal parts, white columns: apical parts). However, in contrast to the earlier studies, considerable amounts of 6,7-DiMeOQu3glu could be detected in both petal parts and 6,7-DiMeOQu3rha and 6,7-DiMeOKm3rha were present exclusively in the apical parts (Fig. 5).

2.3. Biochemical pathway leading to the flavonols present in R. hirta

Flavonol biosynthesis is well established and has been studied in many different plants (Forkmann and Heller, 1999). The key step in the flavonol formation is the introduction of a double bond between C-2 and C-3 of dihydroflavonols, which is catalyzed by flavonol synthase (FLS), a 2-oxoglutarate-dependent dioxygenase. The dihydroflavonols are formed by the consecutive action of chalcone synthase (CHS), chalcone isomerase (CHI) and flavanone 3-hydroxylase (FHT) (Forkmann and Heller, 1999). The presence of all enzymes involved in flavonol formation could be demonstrated in preparations from *R. hirta* (Fig. 6). In addition, a high flavonoid 3′-hydroxylase (F3′H) activity could be detected which correlates well to the fact that the majority of flavonols in *R. hirta* have a 3′,4′-hydroxylation pattern in ring B.

The spectrum of flavonols present in *R. hirta* indicated the presence of a number of modifying enzymes which catalyzed the (i) introduction of a hydroxyl group in position 6, (ii) methylation of hydroxyl groups in positions 6 and 7 and (iii) glucosylation of hydroxyl groups in positions 3 and 7.

Up to now, hydroxylation in position 6 has been observed only in a few plant species (Anzellotti and Ibrahim, 2000, 2004; Latun-

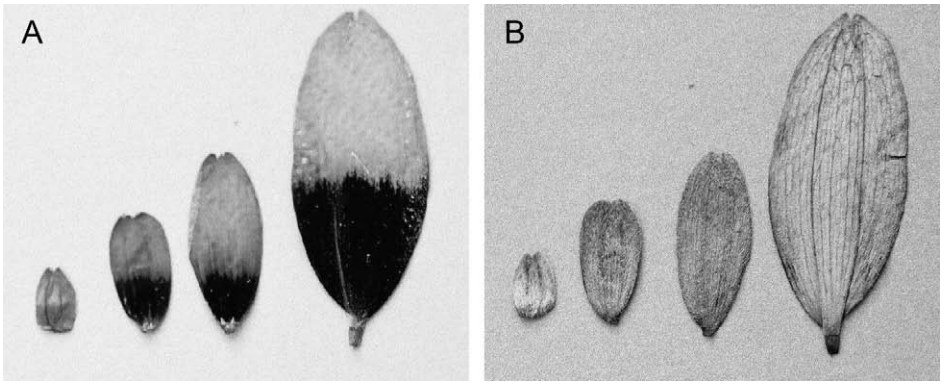


Fig. 3. UV-photography of four developmental stages (from left to right: ST1, ST2, ST3, and ST4) of *Rudbeckia* petals, showing the reflectance at around 360 nm. A: front side; B: rear side.

de-Dada et al., 2001; Halbwirth et al., 2004). Interestingly, two different enzyme systems were shown to catalyze this reaction in the flavonoid pathway. *Chrysosplenium americanum*, a semiaquatic weed, accumulates a variety of partially methylated flavonol glucosides in the leaves. In this plant, hydroxylation in position 6 is catalyzed by a 2-oxoglutarate-dependent dioxygenase, which requires methylated flavonols as substrates (Anzellotti and Ibrahim, 2000, 2004). Five distinct *O*-methyltransferases, which have high specificity for a definite position, sequentially methylate the hydroxyl groups of the flavonol substrates. It was shown that methylation in positions 3 and 7, but not necessarily in position 4′, precedes the hydroxylation in position 6 in *C. americanum* (Ibrahim et al., 1987).

In contrast, a cytochrome P450-dependent monooxygenase catalyzes the formation of Quag from Qu in *Tagetes patula*, but does not convert any partially methylated flavonols (Halbwirth et al., 2004). In *T. patula,* glycosylated 6-hydroxyflavonols contribute to the yellow color of the flower (Tarpo, 1969).

As *C. americanum*, *R. hirta* contains partially methylated flavonols, but the pigments described so far show methoxy groups only in positions 6 and 7. We investigated whether there was a defined sequence for the hydroxylation, methylation and glycosylation of the flavonols in *R. hirta* and which enzyme class is responsible for the introduction of the additional group in position 6. From the substrate specificities and conversion rates observed, the potential pathway leading to the flavonols identified from *R. hirta* was suggested (Fig. 7).

2.3.1. Hydroxylation in position 6

Incubation of [^{14}C]Qu with crude enzyme preparations from petals of *R. hirta* in the presence of NADPH led to the formation of [^{14}C]Quag, which was identified as previously described by HPLC co-chromatography with the authentic reference substance using a photodiode array detector coupled with a radioactivity detector (Halbwirth et al., 2004, 2006). No product formation was observed in the presence of typical dioxygenase cofactors (2-oxoglutarate, ascorbate, and Fe^{2+}). Preparations of the microsomal protein fraction showed a 3.5-fold enrichment of the enzyme activity compared to the crude extract, and only 8% of the activity remained in the supernatant after the final centrifugation step, thus indicating that the enzyme is membrane bound. Therefore all further enzymatic studies were performed with the microsomal fraction.

When Km was used as a substrate, the formation of Quag, but not of 6-OHKm, was observed, which is a result of the high F3′H activity present in the microsomal fraction. No reaction products were detected, when Quag3glu, Quag7glu or methylated quercetin derivatives were incubated under the same conditions. Flavanones, flavones, dihydroflavonols and 6′-deoxychalcones were not accepted as substrates either.

The enzymatic formation of Quag was studied in more detail (Table 2). The reaction was strictly dependent on NADPH and oxygen, and the activity was significantly reduced when NADPH was substituted by NADH (Table 2). Highest reaction rates were observed in the presence of 1.45 mM NADPH, at higher concentrations, the F6H activity decreased continuously. Addition of FAD resulted in slightly increased enzyme activities (Table 2). Highest reaction rates were measured at pH 7.0 and at 30 °C, where the formation of Quag was linear with time up to 15 min and with protein concentration up to 8 μg protein in the standard assay. At 10 °C, the rate was about 50% of the maximum; at temperatures higher than 30 °C the reaction rate decreased strongly. The highest specific activities were measured in the developmental stage 2, where the specific activity was 222 nkat/mg protein under standard conditions. The cytochrome P450-specific inhibitors ketoconazole and tetcyclacis clearly reduced enzyme activity, whereas ancymidol, had only a weak influence (Table 2). The addition of a crude fraction of cytochrome P450 reductase-specific antibodies led to a total loss of enzyme activity, whereas heat-inactivated antibodies did not have any effect (Table 2).

The localization in the microsomal fraction, the dependence on NADPH and oxygen and the specific inhibition by cytochrome P450 reductase antibodies (Benveniste et al., 1989; Nielsen and Møller, 1999; Halbwirth et al., 2004) clearly indicate that the F6H of *R. hirta* is a cytochrome P450-dependent enzyme. The introduction of an additional hydroxyl group in ring A is the first modification step in the biosynthetic pathway to the flavonols formed in *R. hirta,* because methylated and/or glucosylated flavonols are not accepted as substrates. In addition, the formation of flavonols has to precede A-ring hydroxylation because flavanones and dihydroflavonols are not hydroxylated in position 6. Thus, it is obvious that the methoxylated flavonols present in *R. hirta* are formed in a different way than those in *C. americanum.*

2.3.2. Methylation of hydroxyl groups of flavonols

Incubation of Qu with enzyme preparations from petals of *R. hirta* in the presence of [^{14}C]S-Adenosylmethionine (SAM) led to the formation of [^{14}C]7-MeOQu, which was identified by HPLC co-chromatography with the authentic reference substance using a photodiode array detector coupled with a radioactivity detector. When Km was used as a substrate, 7-MeOKm was formed accordingly. Incubation of Quag with crude enzyme preparations from petals of *R. hirta* in the presence of [^{14}C]SAM primarily resulted in the formation of 6-MeOQu. In addition, a by-product was formed in very low amounts which was identified as 6,7-DiM-

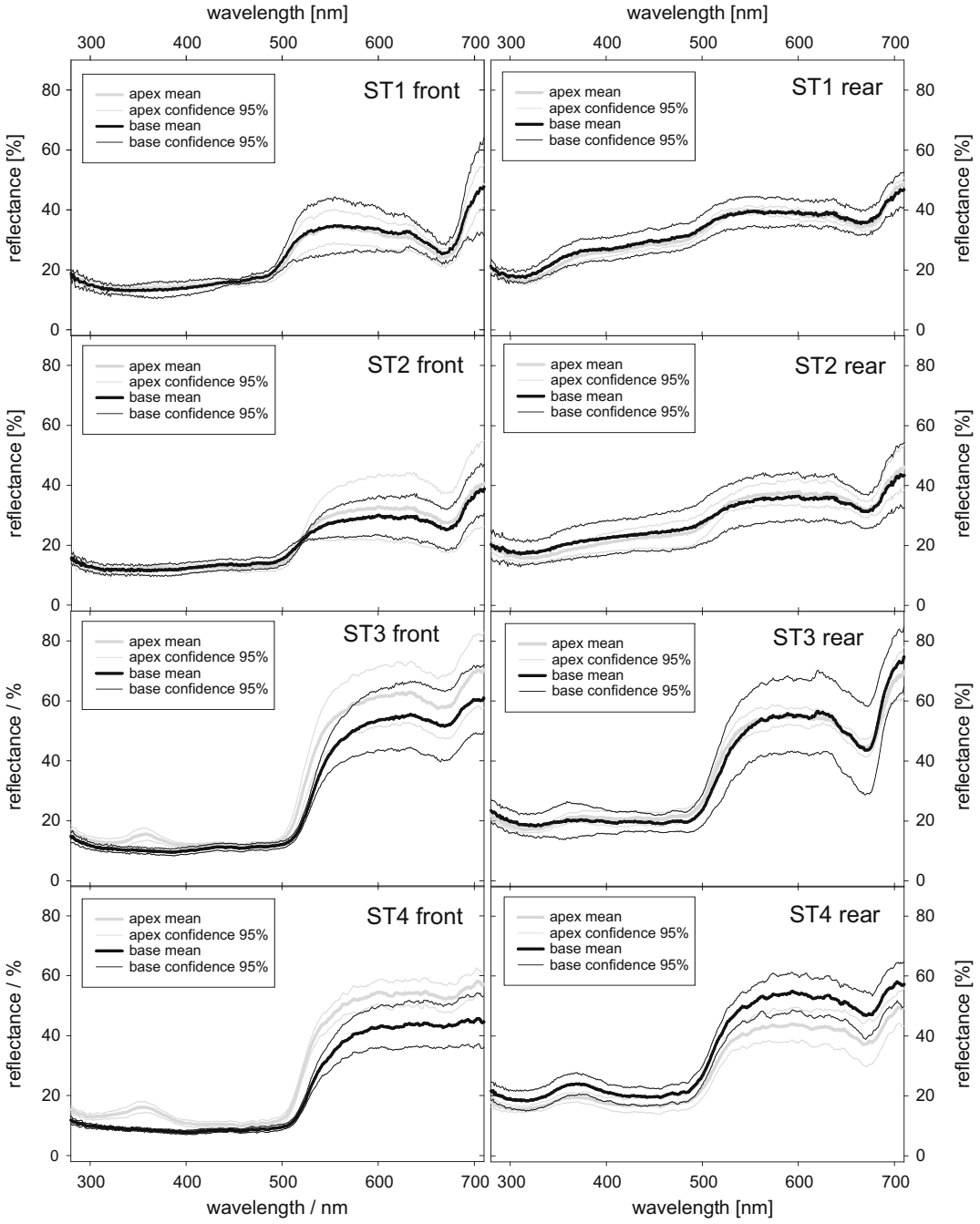


Fig. 4. Reflectance spectra obtained from the apical part and the basal part of the front side and the rear side of *R. hirta* petals during flower anthesis (compare Fig. 3 with the UV-photography of the four developmental stages ST1, ST2, ST3, ST4). Each reflectance curve is the average value obtained from five different petals of the same flower; additionally shown is the standard deviation of the mean value, given as 95% confidence interval.

eOQu. Glucosylated flavonols were accepted as substrates only to a very low extent.

The formation of 7-MeOQu showed a maximum at pH 7.5, whilst formation of 6-MeOQu and 6,7-DiMeOQu slightly increased at higher pH values (with a maximum at pH 8.5). Using Quag as a substrate, highest reaction rates were measured at 30 °C, where the formation of 6-MeOQu was linear with time up to 15 min and with protein concentration up to 50 μg protein in the standard assay. However, at 25 and 40 °C, 75% of the maximum activity at 30 °C was still observed, whereas at higher temperatures, the conversion rates decreased drastically. The highest specific activities were measured in the developmental stage 4, where the activity in the basal part of the petals was two times higher than that in the apical part. However, the other three anthesis stages did not show these pronounced differences of the methyltransferase (MT) activities.

2.3.3. Glucosylation of hydroxyl groups of flavonols

Incubation of Quag with enzyme preparations from petals of *R. hirta* in the presence of [^{14}C]UDP-glucose (UDPG) led to the formation of [^{14}C]Quag7glu, which was identified by HPLC co-chromatography with the authentic reference substance using a photodiode array detector coupled with a radioactivity detector. When Qu was used as a substrate, the formation of [^{14}C]Qu7glu and [^{14}C]Qu3glu was observed. We assume that two different glucosyltransferases, flavonol 7-*O*-glucosyltransferase (F7GT)

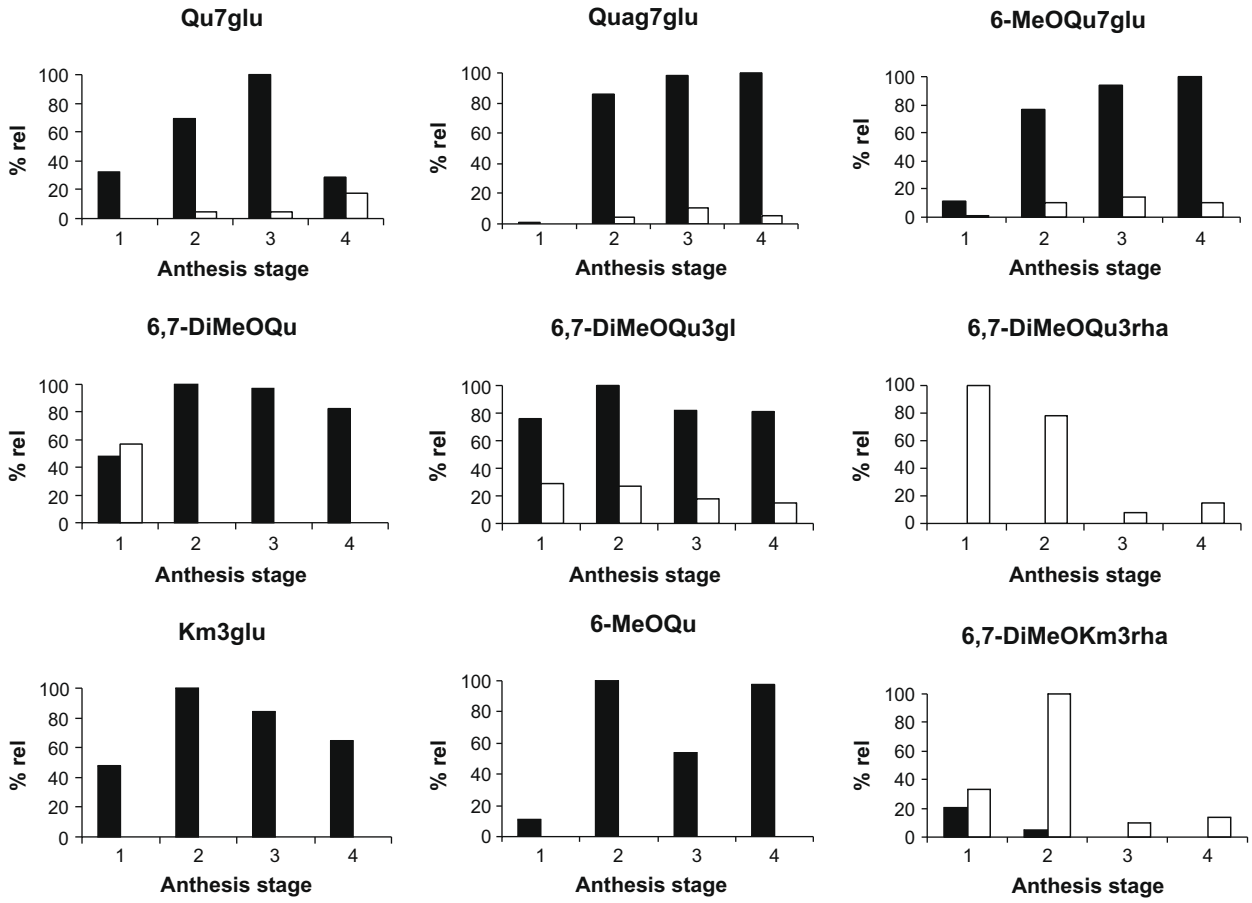


Fig. 5. Relative distribution (% rel.) of selected flavonols in basal (black columns) and apical (white columns) parts of *R. hirta* petals in four anthesis stages. Values were calculated in relation to the highest amount of each compound present.

and flavonol 3-*O*-glucosyltransferase (F3GT), are present in the petals of *R. hirta*, because glucosyltransferases frequently act specifically on a distinct position (Stich et al., 1997; Forkmann and Heller, 1999).

Apart from Qu and Quag, Km, 6-OHKm, 6-MeOKm and 6-MeOQu were also glucosylated in position 7. However, the highest reaction rates were observed with Quag and 6-OHKm as substrates followed by 6-MeOQu and 6-MeOKm. With Qu and Km as substrates, 10-fold lower activities were observed. This indicates that in the biochemical pathway, glucosylation tends to precede the methylation reaction. For all substrates tested, the glucosylation in position 7 showed pH optima between pH 7.5 and 8.25 (Quag, 6-MeOKm: 7.75; Qu: 7.5; Km, 6-OHKm: 8.0; 6-MeOQu: 8.25).

Using Quag as a substrate, the highest reaction rates were measured at 30 °C, where the formation of Quag7glu was linear with time up to 25 min and with protein concentration up to 15 μg protein in the standard assay. The highest specific activities were measured in anthesis stage 4. Interestingly, F7GT activity was much higher in the basal part than that in the apical part of the petals (Fig. 6), which correlates well with the fact that only traces of flavonol 7-*O*-glucosides could be detected in the apical parts (Fig. 2).

Compared to the F7GT activity, glucosylation in position 3 was distinctly lower. The highest reaction rates were observed using 6,7-DiMeOQu as a substrate. With Km and Qu as substrates, the reaction rates were 10 times lower. No flavonol 3-*O*-glucosides were formed, when Quag, 6-MeOQu, 6-OHKm or 6-MeOKm were incubated with enzyme preparations of *R. hirta* in the presence of UDPG. Using 6,7-DiMeOQu as a substrate, the highest reaction rates were measured at pH 7.5 and at 30 °C, where the formation of 6,7-DiMeOQu3glu was linear with time up to 40 min and with protein concentration up to 20 μg protein. The highest specific activities were measured in the developmental stage 3. F3GT did not show these pronounced activity differences between basal and apical parts of the petals as observed for F7GT (Fig. 6).

2.4. Formation of UV-honey guides

UV-photographs of *Rudbeckia* flowers suggest the absence of flavonols in the apical part of the petals and actually, several flavonols could only be found in the basal parts (Thompson et al., 1972). Thus we assumed a local restriction of the flavonol biosynthesis and the absence of the involved enzyme in the apical parts. Our investigations have confirmed that many flavonols are concentrated in the basal parts of the flowers (Table 1), whereas carotenoids are present in the basal and apical parts of the petals in the four developmental stages. However, in contrast to earlier studies, 6,7-DiMeOQu3glu was surprisingly found in the basal and apical parts and 6,7-DiMeOQu3rha was found exclusively in the apical parts.

The presence of two flavonol pigments in the apical part of the ray flower raises the question of why this does not lead to UV-absorption in the outer part. It could be speculated that the concentration gradient of flavonols in the different parts of the petals is high enough to result in the differences in the UV-absorption observed. In addition, the flavonol 7-*O*-glucosides are exclusively accumulated in the basal parts. In contrast to the 3-*O*-glucosides, they show yellow fluorescence in the UV-light and this could also

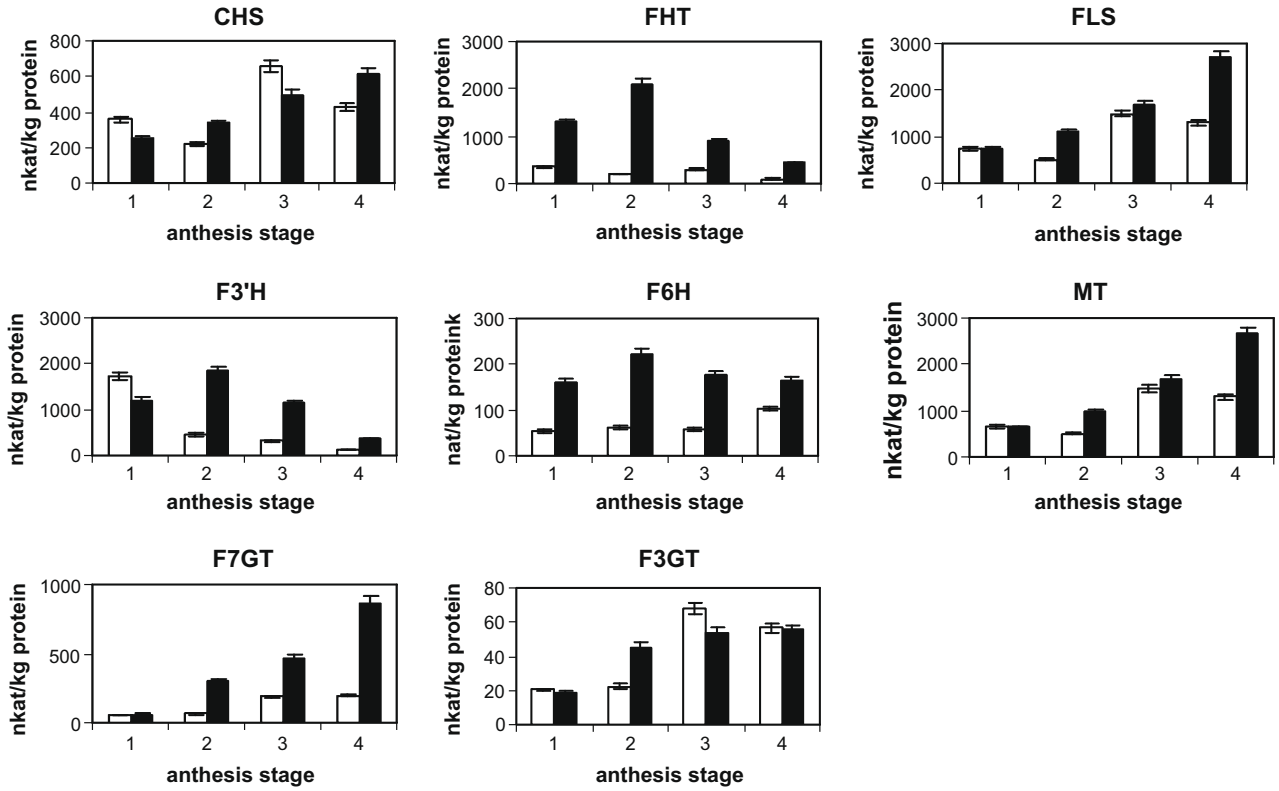


Fig. 6. Specific activities (nkat/kg protein) of selected flavonoid enzymes in basal (black) and apical parts (white) of the petals in four anthesis stages. F7GT and MT assays were performed with quercetagetin as substrate, F3GT with quercetin.

contribute to the pollinator's perception of the flower as two concentric dichromatic circles. However, 3-*O*-glucosides and 7-*O*-glucosides of flavonols may not be differentiated by UV-photography because both show UV-absorption at 360 nm and therefore may appear dark.

In accordance with the presence of 6,7-DiMeOQu3glu, the activities of all enzymes involved in its formation could be demonstrated in both parts of the petals (Fig. 6). For FHT, FLS, F6H and F7GT, the activities in the basal parts were considerably higher than that in the apical parts. However, a strict ± system could not be observed. The methylating and glucosylating enzymes showed highest activities in the late developmental stages, whereas most other enzymes had a climax in stage 2. There was no indication that flavonol biosynthesis increases at the end of the flower life-cycle. The absence of 6,7-DiMeOQu3rha in the basal parts of the petals could be explained by a local lack of flavonol 3-*O*-rhamnosyltransferase activity. However, up to now, we have not been able to demonstrate the presence of this enzyme in any part of the petal, despite continued efforts.

The previous work on anthesis in Asteraceae reported that the bull's eye pattern is present only in fertile flowers whilst juvenile and senescent flowers appear uniformly dark UV-absorbing (Jokl and Fürnkranz, 1989). Our investigations have shown that in *R. hirta* the bull's eye pattern is established already in the earliest developmental stages. However, as long as the buds are closed and the petals are folded, the honey guides remain invisible to pollinators, because the rear side is uniformly UV-reflecting. This implicates that the honey guide forming pigments are accumulated only in the surface cell layers of the petals. In this case, a potential ± system would not be detected with enzyme preparations from the whole basal and apical parts. Further work will concentrate on the identification of the corresponding genes involved and the detection of their local expression by *in situ* hybridization techniques. Work on the identification of further flavonols present in *R. hirta* is also in progress.

3. Experimental

3.1. General experimental procedures

UV photography was carried out with a Nikon FM2 camera with a UV-Nikkor lens (f = 105 mm, 1:4.5, Nikon, Vienna, Austria) and a T-Max 100 black & white film 135-36 (Kodak, Vienna, Austria) (Williams and Williams, 1993). The system used for LC-UV-ESI-MSn analysis was a Bruker esquire 3000 plus mass spectrometer, equipped with an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) composed of an Agilent 1100 quaternary pump and an Agilent 1100 variable wavelength detector. HPLC analysis was performed on a Perkin Elmer Series 200 system (Perkin Elmer, Vienna, Austria) equipped with a photodiode array detector coupled with a 500TR Flow Scintillation Analyzer for the detection of radiolabelled substances. Radiolabelled substances were also detected with a TLC Linear Analyzer (Berthold LB 2842, Wildbad, Germany) or with a Winspectral scintillation counter (Wallac, Vienna, Austria).

3.2. Plant material

The investigations were performed on *R. hirta* cv. 'Indian Summer' (Austrosaat, Vienna, Austria). The plant material was cultivated in the municipal parks of Vienna, collected during the summer periods 2005 and 2006, shock frozen in liquid nitrogen and stored at −80 °C. Morphological criteria were used for subdividing the developmental process into four anthesis stages (Fig. 3). ST1 and 2 refer to closed buds (5 and 10 mm petal length, respectively), ST3 refers to buds just opening (petal length 15 mm) and ST4 refers to open flowers (petal length 20 mm).

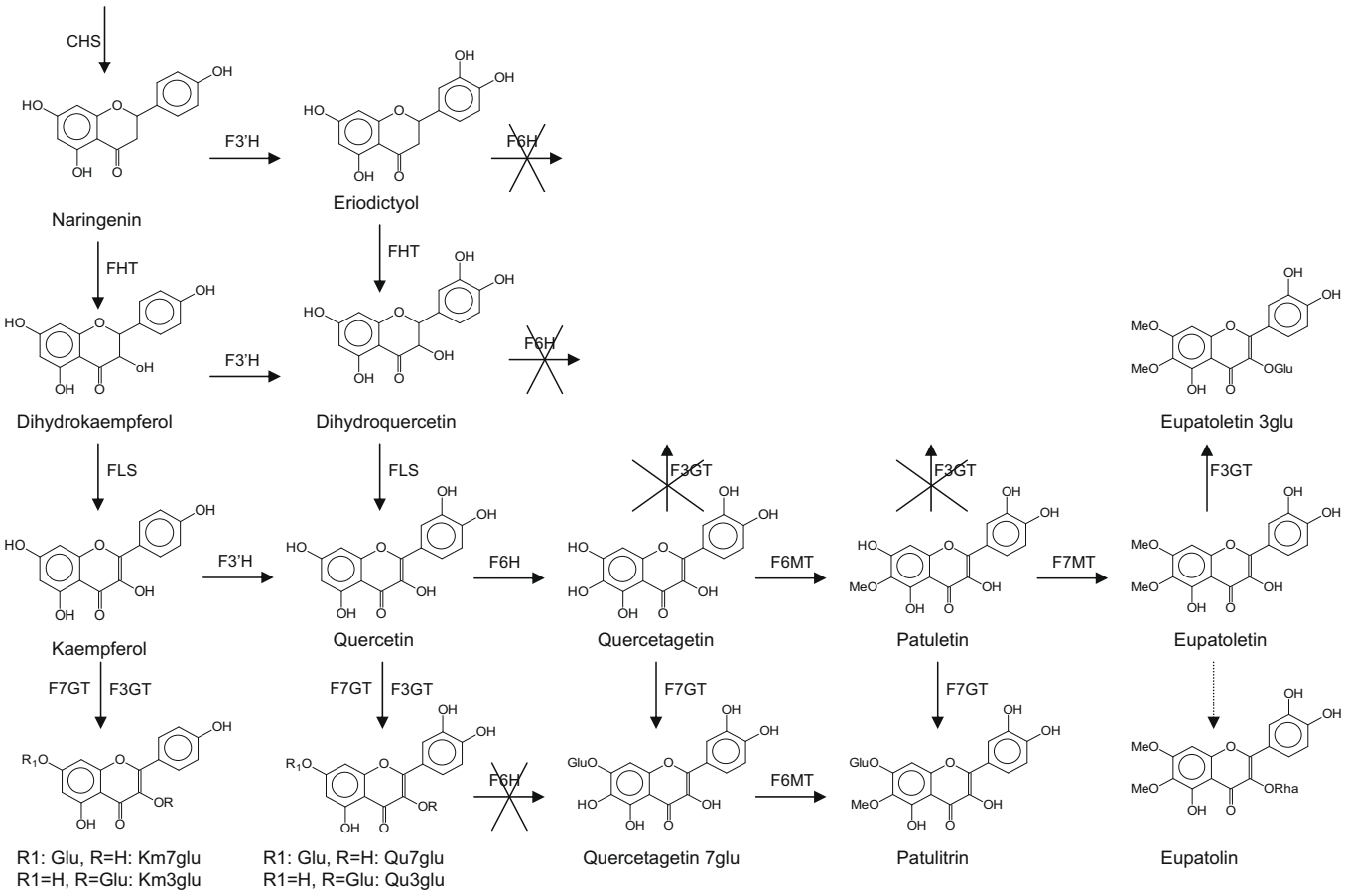


Fig. 7. Biochemical pathway leading to the flavonols in *R. hirta*.

3.3. Chemicals

UDP-D-[U-^{14}C]glucose (12.0 GBq/mmol), S-adenosyl-L-[methyl-^{14}C]methionine (2.15 GBq/mmol) and [2-^{14}C]malonyl-coenzyme A (2 GBq/mmol) were purchased from GE Healthcare (Freiburg, Germany). Non-labelled UDPG, SAM and malonyl-CoA (all Sigma-Aldrich, Vienna, Austria), respectively, were added to their corresponding [^{14}C]compounds to achieve the specific activities indicated in the enzyme assay section. [^{14}C]Flavonoids were prepared as previously described (Halbwirth et al., 2004, 2006). Km, Km3glu, Km7glu, Qu, Qu3glu, Quag and Quag7glu, were purchased from Extrasynthese (Genay, France), 7-MeQu and 3-MeQu from Fluka (Vienna, Austria) and Qu7glu, 3,7-DiMeQu, 6-MeOQu, and 6-MeOQu 7glu from Apin Chemicals (Abingdon, UK). 6,7-DiMeOQu, 6,7-DiMeOQu3rha, and 6,7-DiMeOKm3rha were from the laboratory collection of M. Luczkiewicz. 6-OHKm, 6-MeOKm and Quag3glu came from E. Wollenweber (TU Darmstadt, Germany). The cytochrome P450 reductase-specific antiserum from Jerusalem artichoke was a gift from F. Durst and I. Benveniste (CNRS, Strasbourg, France). NADPH, cytochrome c, glucose oxidase and catalase were obtained from Sigma (Vienna, Austria). Cytochrome P450-specific inhibitors were a gift from W. Rademacher (BASF, Limburgerhof, Germany).

3.4. Reflectance spectroscopy

The reflectance curves of the flower petals were obtained in the spectral region between 300 and 700 nm as the fraction of the light spectrum reflected by the sample in relation to the light spectrum reflected by an ideally white, diffuse reflecting surface. The illumi-

Table 1
(Addition to Fig. 4). Maximal UV reflectance (%) of apical and basal petal parts and the corresponding wavelength positions for the front side and the rear side in four different anthesis stages.

Anthesis stage		1	2	3	4
Frontal side					
Apex	Max. UV-reflectance (%)	14.3 ± 1.0	13.0 ± 1.0	15.5 ± 2.1	16.2 ± 1.8
	wavelength number	367.3	367.3	356.6	356.6
Base	Max. UV-reflectance (%)	13.5 ± 2.6	11.8 ± 1.8	10.0 ± 1.1	8.5 ± 0.7
	wavelength number	367.3	367.3	356.6	356.6
Rear side					
Apex	Max. UV-reflectance (%)	24.3 ± 2.2	19.1 ± 2.3	21.5 ± 2.0	19.5 ± 1.5
	Wavelength number	367.3	367.3	374.4	372.0
Base	Max. UV-reflectance (%)	25.7 ± 4.0	21.1 ± 5.5	20.3 ± 5.3	24.0 ± 3.4
	Wavelength number	367.3	367.3	374.4	372.0

Table 2
Dependence of the F6H on cofactors and influence of potential enzyme inhibitors.

Additions	Relative activity (%)
None	0
NADPH, 1.45 mM	100
NADH, 1.45 mM	20
NADPH,1.45 mM + FAD, 1 μM	113
NADH, 1.45 mM + FAD, 1 μM	28
Oxygen consuming system	15
Oxygen consuming system, heat inactivated	104
Ketoconazol, 50 mM	18
Tetcyclacis, 50 mM	11
Ancymidol, 50 mM	70
EDTA, 5 mM	100
Cyt. P450 red. specific antibodies, 10 μg	0
Cyt. P450 red. specific antibodies, 10 μg inactivated	98

All activities were expressed per kg of total protein and relative activities were calculated compared to the activity of the microsomal fraction obtained in the presence of 1.45 mM NADPH. 100% correspond to 0.2 μkat/kg protein.

nation of the samples was accomplished using a 75 W ozone-free Xenon arc lamp (Oriel 6251) as white light source (color temperature 5500 K). An F/1.0 quartz condenser (Oriel 68806) was used to obtain a collimated light beam, which passed an infrared blocking filter (Oriel 6123) for avoidance of thermal stress. The petal samples were fixed on an opaque black holder (non-reflecting background) with the help of an opaque black mask having a 3×3 mm hole, the latter allowing the illumination of a selected area of the petal. The light diffusely reflected by the petal was focused through a quartz lens ($f = 140$ mm) into the entrance slit of a grating spectrometer (Jobin Yvon Triax 190), spectrally dispersed by a diffraction grating with 300 lines mm^{-1}, and detected by a Peltier cooled CCD camera (Jobin Yvon) at the exit aperture of the spectrometer. As reference sample a white, diffuse reflecting standard (Spectralon TM SrS-99-010, 99% reflectance from 300 to 1200 nm) was used. Determining the ratio of the sample spectrum divided by the reference automatically eliminates the necessity for grating efficiency correction and spectral sensitivity correction of the CCD.

3.5. Chromatography

Substrates and products were separated by TLC on Merck precoated cellulose (without fluorescence indicator, 1.0571.001, VWR International, Vienna, Austria) using the following solvent: (1) *n*-butanol/acetic acid/water (6:1:2, v/v/v); (2) chloroform/acetic acid/water (10:9:1, v/v/v) (3) 30% (v/v) acetic acid. Product identification was performed by HPLC analysis according to Kim et al. (2005) using a Perkin Elmer Series 200 system equipped with a photo diode array detector coupled with a 500TR Flow Scintillation Analyzer for the detection of radiolabelled substances (R_t 6-MeO Qu7glu: 4.40; R_t Qu3glu: 4.80; R_t Qu7glu: 5.30; R_t Quag7glu: 6.90; R_t 7-MeQu: 8.53; R_t 7-MeQu: 9.40; R_t Quag: 9.74; R_t 6-MeQu: 11.08; R_t 6,7-DiMeOQu: 12.5; R_t Qu: 13.58). 6-MeO Qu7glu, Quag7-glu, Qu7glu and Qu3glu were purified *via* paper chromatography in solvent 3 before they were subjected to HPLC analysis. LC-UV-ESI-MSn analysis was performed according to Lunkenbein et al. (2006).

3.6. Buffers used

The following buffers were used for the enzyme assays: buffer 1 (CHS, F3′H and GT assays): 0.1 M KH_2PO_4/K_2HPO_4 (containing 0.4% Na-ascorbate), pH 7.5; buffer 2: (FHT, FLS and MT assays): 0.1 M Tris/HCl (containing 0.4% Na-ascorbate), pH 7.6; buffer 3 (F6H assays): 0.1 M KH_2PO_4/K_2HPO_4 (containing 0.4% Na-ascorbate), pH 7.0.

3.7. Enzyme preparations and protein determination

Crude enzyme preparations and microsomal preparations from petals of *R. hirta* were obtained as described earlier (Halbwirth et al., 2006), using buffer 2. To remove low molecular compounds, crude enzyme preparations were passed through a gel chromatography column (Sephadex G25, GE Healthcare, Freiburg, Austria). Protein content was determined by a modified Lowry procedure (Sandermann and Strominger, 1972) using crystalline bovine serum albumin as a standard.

3.8. Enzyme characterization

All data represent an average of at least three independent experiments. Subcellular determination was carried out according to Halbwirth et al. (2004). Determination of the pH optimum was carried out as described for the standard assay, but using 0.2 M buffers with pH values between 6.0 and 8.5. Exclusion of oxygen from the enzyme assay was carried out using an oxygen consuming system consisting of glucose, glucose oxidase and catalase according to Wimmer et al. (1998).

3.9. Enzyme assays

In a final volume of 100 μl: the CHS/CHI assay contained 40 μl enzyme preparation (29–151 μg total protein), 5 μl [^{14}C]malonyl-CoA (1.5 nmol, 1300 Bq), 5 μl *p*-coumaroyl-CoA (1.0 nmol), and 50 μl buffer 1; the FHT assay contained 0.046 nmol [^{14}C]naringenin (108 Bq), 30 μl enzyme preparation (9–56 μg total protein), 5 μl 3.48 mM 2-oxoglutarate (aqueous), 5 μl 2.01 mM $FeSO_4 \times 7H_2O$ (aqueous), and 80 μl buffer 2; the FLS assay contained 0.046 nmol [^{14}C]DHK (108 Bq), 40 μl enzyme preparation (29-151 μg total protein), 5 μl 3.48 mM 2-oxoglutarate (aqueous), 5 μl 2.01 mM $FeSO_4 \times 7H_2O$ (aqueous), and 50 μl buffer 2; the F3′H assay contained 0.046 nmol [^{14}C]naringenin (108 Bq), 20 μl enzyme preparation (6.5–44 μg total protein), 5 μl 1.30 mM NADPH (aqueous), and 75 μl buffer 1; and the F6H assay contained 0.046 nmol [^{14}C]Qu (108 Bq), 40 μl microsomal preparation (27–112 μg total protein), 5 μl 1.45 mM NADPH (aqueous) and 55 μl buffer 3. In a final volume of 50 μl: the MT assay contained 20 μl enzyme preparation (29–151 μg protein), 30 nmol flavonoid substrate (dissolved in 5 μl EGME), 5 μl 1.45 mM $MgCl_2$ (aqueous), 5 μl [^{14}C]SAM (0,43 nmol, 925 Bq, aqueous), and 20 μl buffer 2 and the GT assay contained 20 μl enzyme preparation (15–75 μg total protein), 2.5 μl [^{14}C]UDPG (0.2 nmol, 1500 Bq), 15 nmol flavonoid substrate (dissolved in 2.5 μl ethylene glycol mono methyl ether), and 25 μl buffer 1.

The assays were incubated for 30 min at 30 °C. PAL, CHS/CHI and MT assays were stopped with 200 μl ethyl acetate and 10 μl acetic acid and the amounts of product formed were determined on a scintillation counter. For MT assays, the remaining organic phase was applied to a cellulose plate and chromatographed with solvent system 2 to separate the different products formed. FHT, FLS, F3′H and F6H assays were terminated by the addition of 70 μl ethyl acetate and 10 μl acetic acid. To FLS assays 10 μl of 0.1 mM EDTA was also added before the extraction. The organic phases were transferred to a precoated cellulose plate (Merck, Germany). After developing the TLC plates in solvent system 2 (FHT, FLS, and F3′H) or solvent 1 (F6H) conversion rates were determined with a TLC linear analyzer. GT assays were terminated by the addition of 10 μl acetic acid and 25 μl methanol. The mixture was chromatographed on Schleicher and Schüll 2043b paper in solvent 3. The zones containing the labelled products were cut out and radioactivity was quantified on a scintillation counter.

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References

Abrahamson, W.G., McCrea, K.D., 1977. Ultraviolet light reflection and absorption patterns in populations of *Rudbeckia*. Rhodora 72, 269–277.

Anzellotti, D., Ibrahim, R.K., 2000. Novel flavonol 2-oxoglutarate dependent dioxygenase: affinity purification, characterization, and kinetic properties. Arch. Biochem. Biophys. 382, 161–172.

Anzellotti, D., Ibrahim, R.K., 2004. Molecular characterization and functional expression of flavonol 6-hydroxylase. BMC Plant Biol. 4, 20–26.

Benveniste, I., Lesot, A., Hasenfratz, M.P., Durst, F., 1989. Immunochemical characterization of NADPH-cytochrome P-450 reductase from Jerusalem artichoke and other higher plants. Biochem. J. 259, 847–853.

Burr, B., Rosen, D., Barthlott, W., 1995. Untersuchungen zur Ultraviolettreflexion von Angiospermenblüten III. Dilleniidae und Asteridae. Trop. u. Subtrop. Pflanzenwelt 93, 1–185.

Cisowski, W., Dembinska-Migas, W., Luczkiewicz, M., 1993. Flavonoids from the *Rudbeckia hirta* L. herb. Pol. J. Chem. 67, 829–836.

Chittka, L., Kevan, P.G., 2005. Flower colour as advertisement. In: Dafni, A., Kevan, P.G., Husband, B.C. (Eds.), Practical Pollination Biology. Enviroquest Ltd., Cambridge, ON, Canada, pp. 157–196.

Daumer, K., 1956. Reizmetrische Untersuchungen des Farbsehens der Biene. Z. Vergl. Physiol. 38, 413–478.

Daumer, K., 1958. Blumenfarben, wie sie die Bienen sehen. Z. Vergl. Physiol. 41, 49–110.

Dyer, A.G., 1996. The reflection of near ultraviolet radiation from flowers of Australian native plants. Aust. J. Bot. 44, 473–488.

Dyer, A.G., Muir, L.L., Muntz, W.R.A., 2004. A calibrated gray scale for forensic ultraviolet photography. J. Forensic Sci. 49, 1056.

Eisner, T., Silberglied, R.E., Aneshansley, D., Carrel, J.F., Howlans, H.C., 1969. Ultraviolet video-viewing: the television camera as an insect eye. Science 166, 1172–1174.

Forkmann, G., Heller, W., 1999. Biosynthesis of flavonoids. In: Barton, D., Nakanishi, K., Meth-Cohn, O. (Eds.), Comprehensive Natural Products Chemistry, vol. 1. Elsevier, Oxford, pp. 713–748.

Galsterer, S., Musso, M., Asenbaum, A., Fürnkranz, D., 1999. Reflectance measurements of glossy petals of *Ranunculus lingua* (Ranunculaceae) and of non-glossy petals of *Heliopsis helianthoides* (Asteraceae). Plant Biol. 1, 70–78.

Halbwirth, H., Forkmann, G., 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 Sci. 167, 129–135.

Halbwirth, H., Puhl, I., Haas, U., Jezik, K., Treutter, D., Stich, K., 2006. Two-phase flavonoid formation in developing strawberry (*Fragaria* × *ananassa*) fruit. J. Agric. Food Chem. 54, 1479–1485.

Harborne, J.B., 1967. Comparative Biochemistry of the Flavonoids. Academic Press, London.

Harborne, J.B., 1976. Functions of flavonoids in plants. In: Goodwin, T.W. (Ed.), Chemistry and Biochemistry of Plant Pigments, vol. 1. Academic Press, London, pp. 736–778.

Harborne, J.B., 1988. The flavonoids: recent advances. In: Goodwin, T.H. (Ed.), Plant Pigments. Academic Press, London, pp. 299–344.

Harborne, J.B., 1993. Introduction to Ecological Biochemistry. Academic Press, London.

Ibrahim, R.K., De Luca, V., Khouri, H., Latchinian, L., Brisson, L., Charest, P.M., 1987. Enzymology and compartmentation of polymethylated flavonol glucosides in *Chrysosplenium americanum*. Phytochemistry 26, 1237–1245.

Indsto, J.O., Weston, P.H., Clements, M.A., Dyer, A.G., Batley, M., Whelan, R.J., 2006. Pollination of *Diuris maculate* (Orchidaceae) by male *Trichocolletes venustus* bees. Aust. J. Bot. 54, 669–679.

Indtso, J.O., Weston, P.H., Clements, M.A., Dyer, A.G., Batley, M., Whelan, R.J., 2007. *Diuris alba* (Orchidaceae) is pollinated by small bees and wasps and shows evidence of weak pea flower mimicry. Aust. J. Bot. 55, 628–634.

Jokl, S., Fürnkranz, D., 1989. Antheseabhängige UV-Muster in Blütenständen von Asteraceen. Plant Syst. Evol. 165, 91–94.

Jokl, S., Galsterer, S., Musso, M., Fürnkranz, D., Asenbaum, A., 1999. Ray flowers of *Heliopsis helianthoides* (Asteraceae) and their UV-reflectance during anthesis. Bot. Jahrb. Syst. 121, 279–287.

Kevan, P.G., Mulligan, G.A., 1973. Floral characteristics attracting insects. Can. J. Bot. 51, 1939–1952.

Kevan, P.G., Grainger, N.D., Mulligan, G.A., Robertson, A.R., 1973. A gray-scale for measuring reflectance and colour in the insect and human visual spectra. Ecology 54, 924–926.

Kevan, P.G., 1979. Vegetation and floral colors revealed by ultraviolet light: interpretational difficulties for functional significance. Am. J. Bot. 66, 749–751.

Kevan, P.G., Chittka, L., Dyer, A.G., 2001. Limits to the salience of ultraviolet: lessons from colour vision in bees and birds. J. Exp. Biol. 204, 2571–2580.

Kim, D.H., Kim, B.G., Lee, Y., Ryu, J.Y., Lim, Y., Hur, H.G., Ahn, J.H., 2005. Regiospecific methylation of naringenin to ponciretin by soybean *O*-methyltransferase expressed in *Escherichia coli*. J. Biotechnol. 119, 155–162.

Langanger, M., Jokl, S., Musso, M., 2000. UV-reflectance in flowers of *Nymphea alba* L. and *Nuphar lutea* (L.) Sm. (Nymphaeaceae). Aquat. Bot. 67, 13–21.

Latunde-Dada, A.O., Cabello-Hurtado, F., Czittrich, N., Didierjean, L., Schopfer, C., Hertkorn, N., Werck-Reichhart, D., Ebel, J., 2001. Flavonoid 6-hydroxylase from soybean (*Glycine max* L.), a novel plant P-450 monooxygenase. J. Biol. Chem. 276, 1688–1695.

Lunkenbein, S., Bellido, M., Aharoni, A., Salentijn, E., Kaldenhoff, R., Coiner, H., Munoz-Blanco, J., Schwab, W., 2006. Cinnamate metabolism in ripening fruit. Characterization of a UDP-glucose:cinnamate glucosyltransferase from strawberry. Plant Physiol. 140, 1047–1058.

McCrea, K.D., Levy, M., 1983. Photographic visualization of floral colors as perceived by honeybee pollinators. Am. J. Bot. 70, 369–375.

Nielsen, J.S., Møller, B.L., 1999. Biosynthesis of cyanogenic glucosides in *Triglochin maritima* and the involvement of cytochrome P450 enzymes. Arch. Biochem. Biophys. 368, 121–130.

Silberglied, R.E., 1979. Communication in the ultraviolet. Annu. Rev. Ecol. Syst. 10, 373–398.

Stich, K., Halbwirth, H., Wurst, F., Forkmann, G., 1997. UDP-glucose: flavonol 7-*O*-glucosyltransferase activity in flower extracts of *Chrysanthemum segetum*. Z. Naturforsch. C 52, 153–158.

Tarpo, E., 1969. Flavones of *Tagetes patula*. Farmacia 93, 98.

Thompson, W.R., Meinwald, J., Aneshansley, D., Eisner, T., 1972. Flavonols: responsible for ultraviolet absorption in nectar guide of flower. Science 177, 528–530.

Williams, A.R., Williams, G.F., 1993. The invisible image – a tutorial on photography with invisible radiation. Part 1. Introduction and reflected ultraviolet techniques. J. Biol. Photogr. 61, 115–132.

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, 1013–1016.