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# Flavonoids in flowers of 16 *Kalanchoë blossfeldiana* varieties

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**Abstract**

*Kalanchoë blossfeldiana* varieties with orange, pink, red and magenta flowers were found to contain 3,5-*O*-β-D-diglucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Pink, red and magenta varieties contained relatively high amounts of quercetin based flavonols. Four distinct quercetin flavonols were identified, namely quercetin 3-*O*-β-D-glucoside and three that were quercetin 3-*O*-α-L-rhamnoside based, with either glucose, xylose or arabinose attached to position 2 of the rhamnose. In addition, the presence of at least three kaempferol based diglycosides was suggested from LC–MS analyses. Orange varieties contained very low amounts of flavonol co-pigments and of delphinidin derivatives. The flower extracts of the varieties 'Diva' (magenta) and 'Molly' (red) had identical anthocyanin ratios but differed significantly in flavonol content. The magenta variety contained four times as much quercetin relative to anthocyanidin as the red variety. This difference was mainly due to a larger content of quercetin 3-*O*-(2″-*O*-β-D-glucopyranosyl-α-L-rhamnopyranoside). Based on pigment and co-pigment analyses, approaches for molecular breeding towards blue flower colour are discussed.


*Keywords:* *Kalanchoë blossfeldiana*; Crassulaceae; Flavonoids; Pelargonidin; Cyanidin; Peonidin; Delphinidin; Petunidin; Malvidin; Quercetin; Kaempferol; Rhamnose; Glucose; Arabinose; Xylose

## 1. Introduction

Flavonoid biosynthesis has been the subject of many studies and especially the anthocyanin pathway is well documented (Cooper-Driver, 2001; Winkel-Shirley, 2001). A general model of the anthocyanidin biosynthetic pathway was devised more than 10 years ago (Holton and Cornish, 1995). Subsequently, most work has focussed on the late biosynthetic steps involving mainly glycosylation and acylation of anthocyanins to form more complex anthocyanin molecules, e.g., to provide blue flower colour. These secondary modifications offer interesting prospects for molecular breeding (Mol et al., 1998).

No reports on the flavonoid content of *Kalanchoë blossfeldiana* have appeared since a pioneering study in 1963 (Neyland et al., 1963). This study identified cyanidin 3-*O*-glucoside; cyanidin 3,5-*O*-diglucoside and peonidin 3,5-*O*-diglucoside in flowers and leaves of the variety 'Tom Thumb'. Since then, breeding efforts have expanded the flower colour spectrum immensely, from the reddish orange varieties available in the sixties to the bright yellow, orange, pink, red, magenta and white varieties available today. The natural variation in flower pH remains unknown and the potential to obtain new flower colours by traditional breeding supported by modern LC analysis and molecular breeding have not been exploited.

This research communication reports identification of the anthocyanins and flavonol–glycosides present in 16 of the *K. blossfeldiana* varieties currently available and

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discusses plausible modifications of the general biosynthesis pathway to generate a blue flower colour.

## 2. Results and discussion

Sixteen *K. blossfeldiana* varieties were obtained from two commercial growers on the island of Fyn, Denmark. The varieties are listed in Table 1 together with their colour, flower pH, anthocyanidin and quercetin content.

The anthocyanidin, flavonol and flavone content of acid hydrolysed flower extracts were determined by LC and comparison with authentic standards. This revealed presence of very low amounts of pelargonidin, relatively high amounts of cyanidin and varying amounts of peonidin, delphinidin, petunidin and malvidin as illustrated for the two cultivars 'Diva' and 'Molly' (Fig. 1). Hydrolysed extracts often contained relatively high amounts of quercetin and small amounts of kaempferol. No myricetin and no flavones (apigenin, eriodictyol and tricetin) were detected.

To determine the structure of the anthocyanins, non-hydrolysed extracts were analysed using LC–MS. This established the parent ions $[M + H^+]$ of six anthocyanins as $m/z$ 595, 611, 625, 627, 641 and 655. These masses correspond to the diglucosides of the anthocyanidins found. To establish the glycosylation pattern, retention times and UV-spectra of the components were compared with 3,5-*O*-β-D-diglucosides from *Crocus* 'Tricolor' (Norbaek and Kondo, 1998). The *Crocus* anthocyanins had identical chromatographical and UV-spectral characteristics to the

Table 1
Content of anthocyanidins and co-pigments in hydrolysed flower extracts from 16 varieties of *K. blossfeldiana*

| Variety | Flower color | Flower pH $LSD_{0.95}$=0.11 | Anthocyanidin µg/g fw | Quercetin µg/g fw | % relative to total anthocyanidin content $LSD_{0.95}$=8 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | % pel | % cyd | % peo | % del | % pet | % mal | % que |
| Titan | orange | 4.40 | 57 | 1 | 5 | 88 | 0 | 7 | 0 | 0 | 1 |
| Fame | red orange | 4.60 | 206 | 2 | 5 | 83 | 9 | 3 | 0 | 0 | 1 |
| Petero | red orange | 4.65 | 289 | 3 | 1 | 96 | 3 | 1 | 0 | 0 | 1 |
| Debbie | orange red | 4.50 | 254 | 3 | 4 | 70 | 23 | 3 | 0 | 0 | 1 |
| Altar | orange red | 4.35 | 392 | 88 | 0 | 78 | 19 | 3 | 0 | 0 | 22 |
| Sumaco | dark red | 4.50 | 360 | 49 | 0 | 42 | 12 | 23 | 17 | 6 | 14 |
| Molly | dark red | 4.50 | 248 | 30 | 0 | 31 | 26 | 10 | 12 | 21 | 12 |
| Diva [A] | magenta | 4.50 | 125 | 65 | 0 | 39 | 22 | 15 | 9 | 14 | 52 |
| Neon [a] | dark pink | 4.55 | 93 | 60 | 0 | 56 | 36 | 8 | 0 | 0 | 65 |
| Pinky [a] | pink | 4.55 | 50 | 54 | 0 | 59 | 27 | 14 | 0 | 0 | 108 |
| Cora [B] | pink | 4.40 | 21 | 17 | 0 | 62 | 5 | 27 | 6 | 0 | 82 |
| Dark Cora [b] | dark pink | 4.30 | 55 | 23 | 0 | 59 | 9 | 21 | 11 | 0 | 41 |
| Bromo [C] | magenta | 4.65 | 155 | 99 | 0 | 22 | 12 | 24 | 17 | 24 | 64 |
| Brava [c] | dark pink | 4.50 | 99 | 82 | 0 | 47 | 30 | 15 | 3 | 5 | 83 |
| Oriba | dark pink | 4.55 | 158 | 10 | 1 | 76 | 13 | 7 | 3 | 0 | 6 |
| Lican | magenta | 4.65 | 124 | 26 | 0 | 21 | 16 | 21 | 17 | 25 | 21 |

del, delphinidin; cyd, cyanidin; pet, petunidin; pel, pelargonidin; peo, peonidin; mal, malvidin; que, quercetin. [a–c] indicates that the varieties are spontaneous mutants from the ancestors [A–C], respectively.

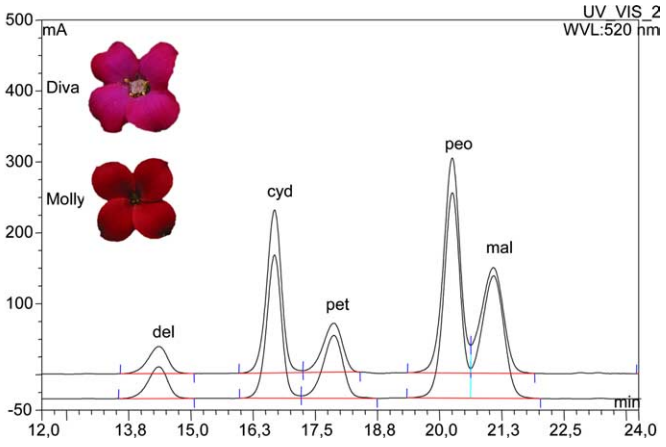


Fig. 1. Anthocyanin content of *K. blossfeldiana* 'Diva' (top) and 'Molly' (bottom) as determined by LC analyses and monitoring at 520 nm. Del = delphinidin 3,5-*O*-β-D-diglucopyranoside; cyd = cyanidin 3,5-*O*-β-D-diglucopyranoside; pet = petunidin 3,5-*O*-β-D-diglucopyranoside; peo = peonidin 3,5-*O*-β-D-diglucopyranoside; mal = malvidin 3,5-*O*-β-D-diglucopyranoside.

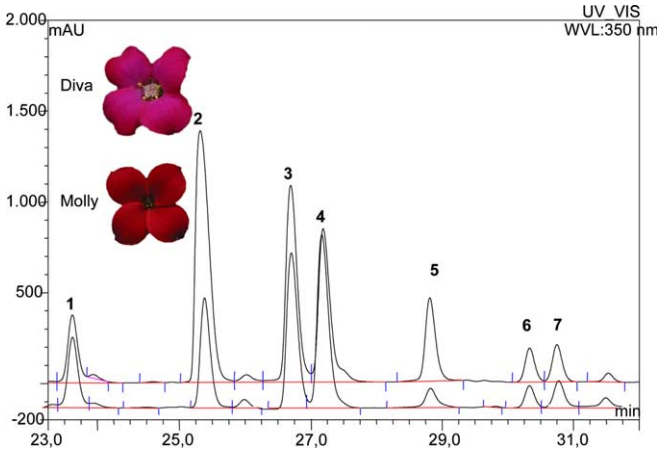


Fig. 2. Co-pigment content of *K. blossfeldiana* var. Diva (top) and Molly (bottom) as determined by HPLC analyses and monitoring at 350 nm. The structure of compound **1** was identified by comparison with an authentic standard, whereas compounds **2**, **3** and **4** were identified by NMR. The *m/z* of $[M + H]^+$ is shown in parenthesis. **5**, **6** and **7** are putative kaempferol analogs of **2**, **3** and **4**, respectively. **1** = quercetin-3-*O*-β-D-glucopyranoside (465); **2** = quercetin-3-*O*-(2″-*O*-β-D-glucopyranosyl)-α-L-rhamnopyranoside (611); **3** = quercetin-3-*O*-(2″-*O*-α-L-arabinopyranosyl)-α-L-rhamnopyranoside (581); **4** = quercetin-3-*O*-(2″-*O*-β-D-xylopyranosyl)-α-L-rhamnopyranoside (581).

anthocyanins found in *K. blossfeldiana*. We therefore conclude, that the anthocyanins found in *K. blossfeldiana* flowers are 3,5-*O*-β-D-diglucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (abbreviated anthocyanidin 3,5-diglucosides).

Flower colour of the two *K. blossfeldiana* varieties 'Diva' and 'Molly' is quite different (Table 1). Nevertheless, the ratio between the individual anthocyanins found in each variety did not differ (Fig. 1). LC analyses of the flavonol content (Table 1) showed that the quercetin content in 'Diva' was approx. four times as high as in 'Molly'. Since 'Diva' has the bluest flower colour, this indicated that the difference in flower colour was due to flavonol–glycoside co-pigmentation.

LC profiles of the flavonol–glycoside content of flowers of *K. blossfeldiana* 'Diva' and 'Molly' are presented in Fig. 2. Component **1** was identified as quercetin 3-*O*-β-D-glucopyranoside by comparison with retention times and UV-absorption spectrum of an authentic sample. A comparison of the flavonol–glycoside composition of the varieties 'Molly' and 'Diva' demonstrated that the differences in quercetin content were mainly due to the larger amount of component **2** in 'Diva', as component **2** yielded quercetin when isolated and subjected to acid hydrolysis. LC–MS analyses revealed $[M + H]^+$ and $[M + Na]^+$ at *m/z* 611 and 633, respectively. This corresponded to the mass of rutin (quercetin 3-*O*-rutinoside = quercetin 3-*O*-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside)), but the retention time was clearly different when compared with an authentic rutin sample. Component **2** was therefore isolated by preparative LC and identified as quercetin 3-*O*-(2″-*O*-β-D-glucopyranosyl-α-L-rhamnopyranoside) by NMR ($^1H$, $^{13}C$, DEPT, COSY, HSQC, HMBC, NOESY, TOCSY, JRES). The identity was confirmed by GC–MS of the glucose and rhamnose derived products obtained by acid hydrolysis followed by trimethylsilylation. $^1H$ and $^{13}C$ chemical shifts were in full agreement with values reported in literature (Hasler et al., 1992). Components **3** and **4** both afforded quercetin when subjected to acid hydrolysis and both exhibited $[M + H]^+$ at *m/z* 581 and $[M + Na]^+$ at *m/z* 603. $MS^2$ spectra revealed a fragment ion at *m/z* 303 likely to be the quercetin part of the molecule. NMR analyses (same techniques as above) resulted in identification of component **3** as quercetin 3-*O*-(2″-*O*-α-L-arabinopyranosyl-α-L-rhamnopyranoside) and component **4** as quercetin 3-*O*-(2″-*O*-β-D-xylopyranosyl-α-L-rhamnopyranoside) (see spectroscopic data in Tables 2 and 3). These structures explained the almost identical LC–MS properties and absorption spectra. The sugar content (rhamnose and arabinose/xylose) was verified by GC–MS of the silylated monomers.

Components **5**, **6** and **7** all afforded kaempferol when subjected to acid hydrolysis and analysis by LC. Component **5** gave a parent ion $[M + H^+]$ at *m/z* 595 and a fragmentation pattern similar to component **2**, but with an ion at 287 instead of 303, supporting that the co-pigment was kaempferol rather than quercetin based. Similarly, **6** and **7** gave parent ions $[M + H^+]$ at 565 and revealed substitution of the 303 ion with an ion at 287. This indicated that **5**, **6** and **7** were the kaempferol analogs of **2**, **3** and **4**, but it was not possible to purify enough of the compounds to conduct conclusive NMR analyses.

The flavonol–glycosides identified in *K. blossfeldiana* are not novel. **2** has previously been reported in *Ginkgo biloba* (Hasler et al., 1992), an ancient tree with many interesting compounds used in traditional medicine. **3** had been reported in a close relative to *K. blossfeldiana*, namely *Kalanchoë pinnata* (Rossi-Bergmann et al., 1997) as well as

Table 2
$^{13}$C NMR spectroscopic data for compounds **2**–**4** ($CD_3OD$)

| C | 2 | 3 | 4 |
|---|---|---|---|
| 2 | 159.4 | n.d | n.d |
| 3 | 136.8 | 137.0 | 137.1 |
| 4 | 179.7 | n.d | n.d |
| 5 | 163.2 | n.d. | n.d. |
| 6 | 100.0 | 99.9 | 100.1 |
| 7 | 166.0 | 165.9 | n.d |
| 8 | 94.8 | 94.8 | 94.9 |
| 9 | 158.6 | n.d. | n.d |
| 10 | 105.9 | n.d. | n.d. |
| 1′ | 122.9 | 123.0 | 122.8 |
| 2′ | 117.1 | 116.9 | 117.1 |
| 3′ | 146.7 | 146.6 | n.d. |
| 4′ | 149.6 | n.d. | n.d. |
| 5′ | 116.7 | 116.5 | 116.7 |
| 6′ | 123.0 | 122.7 | 123.0 |
| 1″ | 102.8 | 103.5 | 103.5 |
| 2″ | 83.0 | 82.8 | 82.9 |
| 3″ | 71.9 | 72.0 | 72.0 |
| 4″ | 73.4 | 73.8 | 73.7 |
| 5″ | 72.2 | 72.1 | 71.9 |
| 6″ | 17.7 | 17.8 | 17.8 |
| 1‴ | 107.3 | 108.1 | 108.1 |
| 2‴ | 75.6 | 73.1 | 75.5 |
| 3‴ | 77.9 | 74.2 | 78.0 |
| 4‴ | 71.1 | 70.0 | 71.1 |
| 5‴ | 77.9 | 67.6 | 67.2 |
| 6‴ | 62.3 | | |

Data were retrieved from the 1D ($^{13}$C and DEPT) or 2D spectra (HSQC, HMBC). **2**: $^{13}$C NMR data were in good agreement with those reported in 1992 in *Ginkgo biloba* (Hasler et al., 1992). **3**: No $^{13}$C NMR data were found in the literature. **4**: $^{13}$C NMR data were in good agreement with those reported in 1994, but the $^{1}$H shifts deviate slightly (Slowing et al., 1994).
n.d. = not detected for sensitivity reasons.

in *Alphitonia philippinensis* (Jou et al., 2004). **3** has also been reported present in *Aerva javanica* (El-Seedi and Sobaih, 1999), *Licania pyrifolia* (Bilia et al., 1996), *Moghania faginea* (Soicke et al., 1990) and in *A. philippinensis* (Jou et al., 2004). **4** had been reported as an anti-inflammatory agent from *Erythrospermum monticolum* (Recio et al., 1995) and also to co-occur with its myricetin counterpart in the leaves of *Eugenia jambos* (Slowing et al., 1994). **7** had been reported as the major flavonol–glycoside together with two coumaroylated complex delphinidin 3-*O*-glucosides to form the blue flower pigment of *Ceanothus papillosus* (Bloor, 1997). It is known that flavonol–glycosides may stack with anthocyanins and thereby contribute to a blue-shift in colour and a more pH stable molecular complex. Therefore, the "more bluish" flower petals of 'Diva' in comparison to 'Molly' may be explained primarily by its higher content of flavonol–glucosides.

Table 3
$^{1}$H NMR spectroscopic data for compounds **2**, **3** and **4** ($CD_3OD$)

| H | 2 | 3 | 4 |
|---|---|---|---|
| 6 | 6.21 (2.1) | 6.20 (2.1) | 6.20 (2.1) |
| 8 | 6.38 (2.1) | 6.37 (2.1) | 6.37 (2.1) |
| 2′ | 7.36 (2.1) | 7.36 (2.1) | 7.36 (2.1) |
| 5′ | 6.93 (8.3) | 6.92 (8.3) | 6.93 (8.3) |
| 6′ | 7.32 (8.3 2.1) | 7.31 (8.3 2.1) | 7.31 (8.3 2.2) |
| 1″ | 5.64 (1.6) | 5.37 (1.4) | 5.34 (1.3) |
| 2″ | 4.27(1.6 3.5) | 4.18 (n.d.) | 4.18 (1.6 3.7) |
| 3″ | 3.85 (3.5 9.8) | 3.88 (n.d.) | 3.86 (n.d.) |
| 4″ | 3.34 (n.d.) | 3.33 (2 × 9.8) | 3.33 (2 × 9.7) |
| 5″ | 3.60 (n.d.) | 3.88 (n.d.) | 3.86 (n.d.) |
| 6″ | 0.98 (6.2) | 1.02 (6.2) | 1.02 (6.2) |
| 1‴ | 4.37 (7.8) | 4.19 (7.3) | 4.24 (7.6) |
| 2‴ | 3.22 (7.8 9.3) | 3.54 (7.3, 9.3) | 3.17 (7.6 9.0) |
| 3‴ | 3.35 (n.d.) | 3.46 (9.3, 3.4) | 3.27 (2 × 9.0) |
| 4‴ | 3.35 (n.d.) | 3.72 (n.d.) | 3.38 (n.d.) |
| 5‴ | 3.16 (n.d.) | 3.64 3.37 (n.d.) | 3.63 3.04 (n.d.) |
| 6‴ | 3.66 (n.d.) | | |

Chemical shifts were retrieved from the 1D or 2D spectra (COSY, HSQC, HMBC). Coupling constants (given in Hz in parentheses) were retrieved from the 1D or 2D JRES spectra.
n.d. = not determined due to overlapping signals.

The pigment analyses showed that orange flowers contained a high amount of cyanidin 3,5-*O*-diglucoside and a relatively low content of flavonol–glycosides. Red varieties similarly had a low content of delphinidin-derived pigments but a more variable amount of flavonol–glycosides. Pink and magenta varieties had a relatively high content of delphinidin-derived anthocyanins and flavonol–glycosides (Table 1). These results are in good agreement with the general understanding of the interaction between anthocyanins and flavonol co-pigments stipulating that hydroxylation at the 3′ and 5′ positions of the B-ring in anthocyanidins cause a blue shift in colour, just like high amounts of co-pigments cause a blue shift relative to low amounts (Goto and Kondo, 1991; Mol et al., 1998). Variations in flower pH appear minute and to play a negligible role in the observed colour differences.

The flavonoid composition of *K. blossfeldiana* revealed a high content of cyanidin derived anthocyanins (cyanidin and peonidin based), inferring a high flavonoid 3′-hydroxylase (F3′H) activity in the varieties examined. The content of delphinidin derived anthocyanins (delphinidin, petunidin and malvidin) varied from very low to high, inferring a great variability in flavonoid 3′,5′-activity (F3′5′H) among the varieties (Holton and Cornish, 1995). The presence of delphinidin and high F3′5′H activity are often correlated to purple/blue flower colours. The presence of different amounts of flavonols inferred flavonol synthase (FLS) activity in some varieties, and the lack of myricetin indicated that the *K. blossfeldiana* FLS enzyme exhibited a similar substrate specificity as the FLS enzyme characterised from *Petunia* (Holton et al., 1993). The lack of flavones (apigenin, luteolin and tricetin) indicated that flavone synthase (FNSI or FNSII) was either not present or not active (Winkel-Shirley, 2001).

Spontaneous mutants in varieties of *K. blossfeldiana* identified during commercial production are named "sports" by breeders and commercial growers. Sports often exhibit very similar characteristics except for one trait – typically flower colour. This information combined with the flavonoid composition in each variety may be utilised to dissect and characterise the nature of the mutation and thus genetic differences between the varieties. Analysis of the mutational background for the varieties

(see footnotes for Table 1) showed that such mutations may result in: (1) a reduced content of delphinidin derived anthocyanins, indicating a mutation in the F3′5′H gene ('Bromo', 'Brava') and/or (2) an altered methylation pattern ('Diva', 'Neon', 'Pinky'), pointing to a mutation in the gene(s) important for the methylation of delphinidin. It is interesting, that delphinidin methylation may be down-regulated without simultaneous down-regulation of the methylation of cyanidin based anthocyanins. This points to the presence of at least two distinct types of anthocyanin methyltransferases (AMT's) (Frick et al., 2001), or a mutation in an AMT leading to an altered substrate specificity as demonstrated in *Mesembryanthemum crystallinum* (Vogt, 2004). The 'Diva' to 'Neon' or 'Pinky' mutation eliminated a major part of the F3′5′H activity indicating that B-ring hydroxylation would essentially be mediated solely by F3′H. The enzymes catalysing flavonoid biosynthesis are thought to be organised within metabolons (Winkel, 2004; Jørgensen et al., 2005). Accordingly, the F3′5′H containing metabolon, responsible for synthesis of delphinidin would be much less abundant in mutants and the B-ring hydroxylation pattern would reflect the activity of the cyanidin synthesising metabolon. With this in mind, the two distinct AMT activities may be hypothesised derived from a single AMT enzyme that exerts a different substrate specificity depending on the type of metabolon into which it is associated. The small amounts of delphinidin detected in many flower extracts may be a result of overlapping enzyme activity of the F3′H and F3′5′H enzymes (Kaltenbach et al., 1999).

Many investigations of the flavonoid content within a single or few varieties of different plant species have been carried out (Davies and Schwinn, 1997; Mitchell et al., 1998) whereas only few reports address analysis of the variation in flavonoid content within a single plant species. In one such study, thirteen pelargonium varieties ranging from orange, pink and red to magenta (purplish red) flower colour were subjected to extensive investigation of their anthocyanin and flavonol content (Mitchell et al., 1998). In several ways, the results obtained were comparable to those obtained in *K. blossfeldiana*: The same six anthocyanidins were found as 3,5-diglucosides and a relatively low content of flavonol mono- or di-glucosides were present. Measurements of the pH of flower macerates may provide an approximate estimation of vacuolar pH (Markham et al., 1997; Yamaguchi et al., 2001). The pH in pelargonium flowers was measured to be between 3.1 and 3.9 (Mitchell et al., 1998) – well below the range of 4.3–4.7 found in *K. blossfeldiana*. A low pH is generally in disfavour of blue colour formation (Goto and Kondo, 1991; Mol et al., 1998). A rise of vacuolar pH in flowers is possible without significant effects on anthocyanin content and apparent growth and may result in a more bluish flower colour (Spelt et al., 2002; van Houwelingen et al., 1998). It is concluded that flower colour in *K. blossfeldiana* and pelargonium is mainly dependent on the hydroxylation pattern of the anthocyanidins and on the relative amount of flavonols. In contrast, blue *Crocus* 'Tricolor' flowers were found to contain mainly delphinidin 3,5-diglucosides as well as flavonol- and flavone–glycosides that may serve as co-pigments. The literature does not provide data on co-pigmentation effects in *Crocus* and also does not report flower pH, rendering direct comparison difficult (Norbaek and Kondo, 1998, 1999a,b; Norbaek et al., 2002a). Our own analysis of *Crocus* 'Tricolor' flowers revealed a flavonol to anthocyanin ratio of approx. 2:1. The rose cultivar 'Rhapsody in Blue' is one of the most bluish roses available and contains mainly cyanidin 3,5-diglucoside and flavonol based co-pigments with a flavonol to anthocyanin ratio of 3:1 (Gonnet, 2003). Of the *Kalanchoë* varieties tested, *K. blossfeldiana* 'Diva' was found to have one of the highest relative flavonol contents with a flavonol:anthocyanin ratio about 1:2. Accordingly, the content of co-pigment in *K. blossfeldiana* is 4–6 times lower than in ''blue'' flowers identifying a higher co-pigment content in *Kalanchoë* as a key breeding target. In vitro experiments with anthocyanidin 3,5-diglucosides have demonstrated that the presence of aluminium ions may induce blue colour formation (Moncada et al., 2003). *K. blossfeldiana* shows great tolerance for acidic soil conditions and high $Al^{3+}$ content in the soil. However, *K. blossfeldiana* is a facultative Crassulacean Acid Metabolism (CAM) plant and accumulates large amounts of malate during flowering (Black and Osmond, 2003). Malate is known to form very stable complexes with aluminium ions. It is therefore unlikely that $Al^{3+}$ will be able to reach the floral epidermis cells in sufficient quantity to create blue anthocyanin complexes (Luttge, 2000; Schottelndreier et al., 2001; Venturini-Soriano and Berthon, 2001). This is supported by feeding experiments that showed that *K. blossfeldiana* did not alter flower colour upon administration of $Al^{3+}$ ions in solution and by ICP-MS analyses of 'Molly' and 'Diva' flowers that demonstrated a low content of metal ions such as $Al^{3+}$ in corallae.

The presence of fairly high amounts of cyanidin in all *K. blossfeldiana* varieties, serves to shift the flower colour towards reddish hues. A shift in anthocyanidin biosynthesis towards synthesis of more delphinidin and less cyanidin would therefore be an obvious approach to generate more bluish flowers. This approach could include over-expression of F3′5′H or down-regulation of F3′H activity. Anthocyanins accumulate as simple 3,5-diglucosides that are neither acylated with malonyl- nor with aromatic caffeoyl- or coumaroyl-groups. This disfavours blue colour formation because acylation with especially aromatic groups is a characteristic of many blue flowered plants (Saito et al., 1995; Norbaek and Kondo, 1998; Bloor and Falshaw, 2000; Suzuki et al., 2000; Bloor, 2001; Suzuki et al., 2001; Tanaka et al., 2001; Norbaek et al., 2002b; Williams et al., 2002;Kazuma et al., 2003). Introduction of relevant acyl transferases in *K. blossfeldiana* would therefore be an interesting approach towards *Kalanchoë* flowers with bluish colours. Several of these approaches are currently being pursued in our laboratory.

## 3. Experimental

### 3.1. Plant material

*K. blossfeldiana* plants were obtained from the growers Poul Erland Nielsen, Kerteminde and Mogens Madsen, Marslev, both producers located near Odense, Fyn, Denmark.

### 3.2. Extraction of flavonoids

Fresh flower corollae (0.5 g, 15–20 flowers depending on flower size) was ground in 2.5 ml of 3 N HCl for hydrolytic analysis or 2.5 ml of MAW (9 MeOH:1 AcOH:10 Water) for non-hydrolytic extraction. For hydrolysis, the extract was heated at 100 °C for 30 min. Samples were filtered through a 0.45-μm pore syringe filter before HPLC analysis.

For preparation of large amounts of flavonol co-pigment, flower corollae (50 g) were extracted with MeOH (500 ml, 2 h), filtered and MeOH evaporated. The dried extract was re-dissolved in $H_2O$ containing 0.5% TFA (20 ml), and extracted with *n*-pentane and EtOAc to remove lipophilic compounds. The aqueous phase was evaporated and the extract was redissolved in Milli-Q water with 0.5% (v/v) trifluoroacetic acid (TFA) (10 ml) for preparative HPLC purification using a reversed phase C-18 column as indicated below.

### 3.3. Identification of flavonoids

Extracts were analysed using a Dionex ASI-100 auto injector, P680 HPLC pump and UVD340U detector (all Dionex, CA, USA) fitted with an Agilent ODS reversed phase column (125 × 4 mm) (Agilent Technologies, Germany). Anthocyanins and anthocyanidins were detected at 520 nm, flavones and flavonols at 350 nm. Spectra from 200–595 nm were recorded. Flow rate was 0.8 ml/min. The elution gradient for anthocyanins was 5–20% $CH_3CN$ from 0 to 30 minutes. Anthocyanidins were separated using a gradient of 5–10% $CH_3CN$ from 0 to 3 min and 10% to 20% $CH_3CN$ from 3 to 33 min. Flavonols and flavones were separated on a 10–50% $CH_3CN$ gradient from 0 to 40 min, all with 0.5% (v/v) TFA added to sharpen peaks.

Identification and quantification was done by comparison with purified standards from Apin Chemicals, UK and from Extrasynthese, France.

Analytical LC–MS (electrospray ionization) was carried out using an Agilent 1100 Series LC (Agilent Technologies) coupled to a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The spectrometer was run in positive mode.

NMR spectra were recorded in methanol-$d_4$ on a Bruker Avance 400 NMR spectrometer.

### 3.4. pH in flower macerates

Corollae (5 pieces) were macerated in 100 μl of water. The macerate was loaded onto the electrode of an IFET pH meter KS723 (Shindengen, Japan). pH was measured twice for each variety, once 1–2 h after harvesting the flower stalks and again 20 h after harvest. Measurements were analysed as a two sided analysis of variance without replications. $F_{\text{variety},15,15} = 7.9^{***}$ and $F_{\text{time},1,15} = 18.8^{***}$. $s = 0.053$ with 15 DF, giving an $\text{LSD}_{0.95,\text{variety}} = 0.11$. $\text{pH}_{1\,\text{h}} = 4.55$, $\text{pH}_{20\,\text{h}} = 4.47$.

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## References

Bilia, A.R., Ciampi, L., Mendez, J., Morelli, I., 1996. Phytochemical investigations of *Licania* genus. Flavonoids from *Licania pyrifolia*. Pharm. Acta Helv. 71, 199–204.

Black, C.C., Osmond, C.B., 2003. Crassulacean acid metabolism photosynthesis: 'working the night shift'. Photosynth. Res. 76, 329–341.

Bloor, S.J., 1997. Blue flower colour derived from flavonol-anthocyanin co-pigmentation in *Ceanothus papillosus*. Phytochemistry 45, 1399–1405.

Bloor, S.J., 2001. Deep blue anthocyanins from blue *Dianella* berries. Phytochemistry 58, 923–927.

Bloor, S.J., Falshaw, R., 2000. Covalently linked anthocyanin-flavonol pigments from blue *Agapanthus* flowers. Phytochemistry 53, 575–579.

Cooper-Driver, G.A., 2001. Contributions of Jeffrey Harborne and coworkers to the study of anthocyanins. Phytochemistry 56, 229–236.

Davies, K.M., Schwinn, K.E., 1997. Flower colour. In: Geneve, R.L., Preece, J.E., Merkle, S.A. (Eds.), Biotechnology of Ornamental Plants. CAB International, Wallingford, pp. 259–294.

El-Seedi, H.R., Sobaih, S.A.-M., 1999. Triterpenes and flavonol glycosides from *Aerva javanica*. Rev. Latinoamer. Quím. 27, 17–21.

Frick, S., Ounaroon, A., Kutchan, T.M., 2001. Combinatorial biochemistry in plants: the case of *O*-methyltransferases. Phytochemistry 56, 1–4.

Gonnet, J.-F., 2003. Origin of the color of cv. Rhapsody in blue rose and some other so-called "blue" roses. J. Agric. Food Chem. 51, 4990–4994.

Goto, T., Kondo, T., 1991. Structure and molecular stacking of anthocyanins – Flower color variation. Angew. Chem. Int. Ed. 30, 17–33.

Hasler, A., Gross, G.-A., Meier, B., Sticher, O., 1992. Complex flavonol glycosides from the leaves of *Ginkgo biloba*. Phytochemistry 31, 1391–1394.

Holton, T.A., Brugliera, F., Tanaka, Y., 1993. Cloning and expression of flavonol synthase from *Petunia hybrida*. Plant J. 4, 1003–1010.

Holton, T.A., Cornish, E.C., 1995. Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7, 1071–1083.

Jou, S.-J., Chen, C.-H., Guh, J.-H., Lee, C.-N., Lee, S.-S., 2004. Flavonol glycosides and cytotoxic triterpenoids from *Alphitonia philippinensis*. J. Chin. Chem. Soc. 51, 827–834.

Jørgensen, K., Rasmussen, A.V., Morant, M., Nielsen, A.H., Bjarnholt, N., Zagrobelny, M., Bak, S., Møller, B.L., 2005. Metabolon formation and metabolic channeling in the biosynthesis of natural products. Curr. Opin. Plant Biol. 8, 280–291.

Kaltenbach, M., Schröder, G., Schmelzer, E., Lutz, V., Schröder, J., 1999. Flavonoid hydroxylase from *Catharanthus roseus*: cDNA, heterologous expression, enzyme properties and cell-type specific expression in plants. Plant J. 19, 183–193.

Kazuma, K., Noda, N., Suzuki, M., 2003. Flavonoid composition related to petal color in different lines of *Clitoria ternatea*. Phytochemistry 64, 1133–1139.

Luttge, U., 2000. The tonoplast functioning as the master switch for circadian regulation of crassulacean acid metabolism. Planta 211, 761–769.

Markham, K.R., Mitchell, K.A., Boase, M.R., 1997. Malvidin geraniums-3-*O*-glucoside-5-*O*-(6-acetylglucoside) and its colour manifestation in 'Johnson's Blue' and other 'blue'. Phytochemistry 45, 417–423.

Mitchell, K.A., Markham, K.R., Boase, M.R., 1998. Pigment chemistry and colour of *Pelargonium* flowers. Phytochemistry 47, 355–361.

Mol, J., Grotewold, E., Koes, R., 1998. How genes paint flowers and seeds. Trends Plant Sci. 3, 212–217.

Moncada, M.C., Moura, S., Melo, M.J., Roque, A., Lodeiro, C., Pina, F., 2003. Complexation of aluminum(III) by anthocyanins and synthetic flavylium salts – a source for blue and purple color. Inorg. Chim. Acta 356, 51–61.

Neyland, M., Ng, Y.L., Thimann, K.V., 1963. Formation of anthocyanin in leaves of *Kalanchoe blossfeldiana* – a photoperiodic response. Plant Physiol. 38, 447–451.

Norbaek, R., Brandt, K., Nielsen, J.K., Orgaard, M., Jacobsen, N., 2002a. Flower pigment composition of *Crocus* species and cultivars used for a chemotaxonomic investigation. Biochem. Syst. Ecol. 30, 763–791.

Norbaek, R., Kondo, T., 1998. Anthocyanins from flowers of *Crocus* (Iridaceae). Phytochemistry 47, 861–864.

Norbaek, R., Kondo, T., 1999a. Flavonol glycosides from flowers of *Crocus speciosus* and *C. antalyensis*. Phytochemistry 51, 1113–1119.

Norbaek, R., Kondo, T., 1999b. Further anthocyanins from flowers of *Crocus antalyensis* (Iridaceae). Phytochemistry 50, 325–328.

Norbaek, R., Nielsen, K., Kondo, T., 2002b. Anthocyanins from flowers of *Cichorium intybus*. Phytochemistry 60, 357–359.

Recio, M.D., Giner, R.M., Manez, S., Talens, A., Cubells, L., Gueho, J., Julien, H.R., Hostettmann, K., Rios, J.L., 1995. Anti-inflammatory activity of flavonol glycosides from *Erythrospermum monticolum* depending on single or repeated local TPA administration. Planta Med. 61, 502–504.

Rossi-Bergmann, B., Costa, S.S., de Morales, V.L.G., 1997. Brazilian medicinal planta: a rich source of immunomodulatory substances. Clência Cult. J. Brazil Ass. Adv. Sci. 49, 395–401.

Saito, N., Tatsuzawa, F., Yoda, K., Yokoi, M., Kasahara, K., Iida, S., Shigihara, A., Honda, T., 1995. Acylated cyanidin glycosides in the violet-blue flowers of *Ipomoea purpurea*. Phytochemistry 40, 1283–1289.

Schottelndreier, M., Norddahl, M.M., Strom, L., Falkengren-Grerup, U., 2001. Organic acid exudation by wild herbs in response to elevated Al concentrations. Ann. Bot. 87, 769–775.

Slowing, K., Söllhuber, M., Carretero, E., Vincieri, F.F., 1994. Flavonoid glycosides from *Eugenia jambos*. Phytochemistry 37, 255–258.

Soicke, H., Goerler, K., Waring, H., 1990. Flavonol glycosides from *Moghania figinea*. Plant Med. 56, 410–412.

Spelt, C., Quattrocchio, F., Mol, J., Koes, R., 2002. ANTHOCYANIN1 of *Petunia* controls pigment synthesis, vacuolar pH, and seed coat development by genetically distinct mechanisms. Plant Cell 14, 2121–2135.

Suzuki, H., Nakayama, T., Yonekura-Sakakibara, K., Fukui, Y., Nakamura, N., Nakao, M., Tanaka, Y., Yamaguchi, M., Kusumi, T., Nishino, T., 2001. Malonyl-CoA: anthocyanin 5-*O*-glucoside-6‴-*O*-malonyltransferase from scarlet sage (*Salvia splendens*) flowers – Enzyme purification, gene cloning, expression, and characterization. J. Biol. Chem. 276, 49013–49019.

Suzuki, K., Xue, H., Tanaka, Y., Fukui, Y., Fukuchi-Mizutani, M., Murakami, Y., Katsumoto, Y., Tsuda, S., Kusumi, T., 2000. Flower color modifications of *Torenia* hybrida by cosuppression of anthocyanin biosynthesis genes. Mol. Breeding 6, 239–246.

Tanaka, M., Fujimori, T., Uchida, I., Yamaguchi, S., Takeda, K., 2001. A malonylated anthocyanin and flavonols in blue *Meconopsis* flowers. Phytochemistry 56, 373–376.

van Houwelingen, A., Souer, E., Spelt, K., Kloos, D., Mol, J., Koes, R., 1998. Analysis of flower pigmentation mutants generated by random transposon mutagenesis in *Petunia hybrida*. Plant J. 13, 39–50.

Venturini-Soriano, M., Berthon, G., 2001. Aluminum speciation studies in biological fluids Part 7 A quantitative investigation of aluminum(III)-malate complex equilibria and their potential implications for aluminum metabolism and toxicity. J. Inorg. Biochem. 85, 143–154.

Vogt, T., 2004. Regiospecificity and kinetic properties of a plant natural product *O*-methyltransferase are determined by its N-terminal domain. FEBS Lett. 561, 159–162.

Williams, C.A., Greenham, J., Harborne, J.B., Kong, J.M., Chia, L.S., Goh, N.K., Saito, N., Toki, K., Tatsuzawa, F., 2002. Acylated anthocyanins and flavonols from purple flowers of *Dendrobium* cv. 'Pompadour'. Biochem. Syst. Ecol. 30, 667–675.

Winkel, B.S.J., 2004. Metabolic channeling in plants. Ann. Rev. Plant Biol. 55, 85–107.

Winkel-Shirley, B., 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 126, 485–493.

Yamaguchi, T., Fukada-Tanaka, S., Inagaki, Y., Saito, N., Yonekura-Sakakibara, K., Tanaka, Y., Kusumi, T., Iida, S., 2001. Genes encoding the cacuolar $Na^+/H^+$ exchanger and flower coloration. Plant Cell Physiol. 42, 451–461.