Accumulation of Anthocyanins and Flavones during Bud and Flower Development in *Campanula isophylla* Moretti

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In the blue flowers of Italian bellflower (*Campanula isophylla* Moretti), the formation of anthocyanins progresses from simple unacylated anthocyanins, delphinidin 3–glucoside and bisdeacylplatyconin, through a series of progressively-acylated and glycosylated compounds, including diacylated violodelin and monodeacylcampanin, to the triacylated campanin. In this study, anthocyanin and flavone contents were very low in buds until a few days before anthesis, after which they increased rapidly. Bisdeacylplatyconin and luteolin 7-O-glucoside peaked 2 d before anthesis. The more complicated luteolin glucosides peaked 2 d after anthesis, slightly preceding monodeacylcampanin and campain. Total anthocyanin content peaked approx. 3 d after anthesis followed by a slow decline. The highest total flavone content was reached at anthesis, after which it remained almost constant, but with some changes in the proportion of individual compounds. In the investigation two phenotypes were used, types B and C. Acylation of monodeacylcampanin to campanin is blocked in type B, but not in type C plants. Conversion of bisdeacylplatyconin into acylated anthocyanins was shown to be slower in type C than in type B plants.

Key words: *Campanula isophylla*, Campanulaceae, Italian bellflower, anthocyanin, flavone, biosynthesis, flower development

INTRODUCTION

Flower colour is an important factor in the breeding of pot plants for the introduction of new flower colours and the improvement of quality of existing colours. Development of pale flowers when plants are kept under unfavourable conditions can reduce quality. To support the breeding of cultivars with improved flower colour, it is important to understand the accumulation of the compounds responsible for colour formation. In many plants these are the anthocyanins together with phenolic co-pigments, commonly other flavonoids (Brouillard, 1988).

The anthocyanins in petals of the blue-flowering *Campanula isophylla* and *C. carpatica* have been investigated (Brandt et al., 1993). The main anthocyanins are bisdeacylplatyconin, violodelin, monodeacylcampanin and campanin, synthesized in the named order, with each step representing one or more acylations and/or glucosylations. Three phenotypes have been distinguished on the basis of their anthocyanin composition. Type A has a block in the biosynthetic pathway to campanin immediately after bisdeacylplatyconin; in type B, the block occurs after monodeacylcampanin, whereas type C is able to synthesize all four compounds (Fig. 1). Since flowers of type B and C are both blue–purple, these phenotypes can not be distinguished visually. In contrast, despite comparable levels of total anthocyanin, type A flowers containing only the unacylated bisdeacylplatyconin are light blue or almost colourless, showing that acylation is a prerequisite for the blue flower colour in these species (Brandt, 1990).

Apart from anthocyanins, glucosides of the flavone luteolin constitute the majority of floral flavonoids in *C. isophylla*. Both flavones and anthocyanins are formed from a flavanone precursor, but flavones are on a separate branch and can not be converted into anthocyanins or vice versa (Fig. 1). The occurrence of luteolin glucosides is in agreement with investigations of the epigeal part of *Campanula rotundifolia*, *C. patula* and *C. persicifolia*, where the major flavonoids were derivatives of the flavone luteolin: five different luteolin-7-O-glucosides, one luteolin-4′-O-glucoside, three luteolin-4′-O-glucoside-7-O-glucosides and one luteolin-3′-glucoside-0-7-glucoside (Teslov 1980a, b, 1981, 1984, 1987, 1989; Teslov and Koretskaya, 1983; Teslov, Koretskaya and Tsareva, 1985). Flavones are common in flowers, where, they can function as co-pigments, enhancing the colour of the anthocyanins (Brouillard, 1988). However, co-pigmentation is mostly known from species with relatively simple anthocyanins, whereas polyacetylated anthocyanins such as those occurring in *Campanula* are not known to interact with other flavonoids (Goto and Kondo, 1991).

The existence of a multistep pathway from the biosynthesis of acylated anthocyanins in flowers of *C. isophylla* makes this species a possible source for the demonstration of, as yet, unknown enzymes involved in the final steps of anthocyanin biosynthesis. Knowledge of the time-course of flavone and
Fig. 1. Biosynthetic pathway to campanin in *Campanula isophylla*. Only the structures shown have been unequivocally identified. The sequence was deduced from genetic data and comparison of the structures. The general phenylpropanoid pathway is well established, up to the formation of each class of aglycons. In contrast, little is known of the enzymes that modify the anthocyanins by glycosylation or acylation, nor are the donors of acyl or glycosyl groups known in *Campanula*. Redrawn from Brandt *et al.* (1993).
anthocyanin accumulation in relation to floral development is critical for the isolation of biosynthetic enzymes, by indicating the stage of flower development at which the enzymes exhibit their maximum activity.

Previous work has concentrated mainly on elucidating the relationship between the activity of known enzymes in the early stages of flavonoid biosynthesis and the total concentration of anthocyanin or other flavonoids (e.g. Dangelmayr et al., 1983; Stich, Eidenberger and Wurst, 1992); few authors have investigated the time-course of accumulation of individual pigments (Pecket, 1965; Hagen, 1966; Kim et al. 1989; Toki and Kawanishi, 1992).

The overall aim of the present investigation was to examine the time-course of accumulation of anthocyanins and other flavonoids in *C. isophylla* during bud and flower formation.

**MATERIALS AND METHODS**

The plants used were unnamed genotypes of blue coloured *Campanula isophylla* Moretti, generated by a breeding program at L. Dæhnfeldt Inc., Odense, Denmark. They were siblings from a cross, segregating type B and C, and ten plants of type B and four plants of type C were used. Voucher specimens of the parent genotypes are retained at the Department of Ornamentals in Arslev and are available upon request. Established plants were cut back and placed in a glasshouse at a minimum temperature of 18 °C and 12 h daylength, provided by natural light, supplemented with metal halide lamps (approx. 30 W m⁻²) for 1 month to support the growth of new shoots. Flower formation was initiated by extending the photoperiod to 16 h. Nine different developmental stages of buds were defined: in stages 1–7, the petals were between 0–14 mm long, at intervals of 2 mm for each stage. Both stage 8 and 9 had petals longer than 14 mm but, while stage 8 buds were whitish–green, the blue colour was predominant in buds at stage 9.

The experiment started when plants were in full bloom, 2 weeks after anthesis of the first flower and 3 months after the start of long day treatments. At least 30 buds of each stage were labelled according to stage, and left to develop on the plants so that the date of anthesis could be recorded. The rest of the buds were harvested and frozen at −20 °C. Sixteen days later, all open flowers from the labelled buds were harvested. The number and fresh weight of buds and flowers of each age from each plant were recorded.

Chromotypes were determined by HPLC analysis of anthocyanins as described by Brandt et al. (1993). The materials for chemical analysis were either paired samples of buds from each plant, extracted and analysed separately, or individual flowers, one or two of each age from each plant. Missing values were estimated by interpolation within the data from a single plant. Entire flower buds or flowers, including ovaries, were extracted by homogenizing with 4 ml cold MeOH–H₂O–HCO₂H (10:3:3) per g frozen plant material. If a sample had less than 1 g plant material, 2 ml of MeOH–H₂O–HCO₂H (10:7:3) were added to the mixture to facilitate homogenization. The extract was cleared by centrifugation and 10 µl injected into the chromatograph without further purification. The analysis was carried out using a RP-18 column with solvent A [H₂O–HCO₂H (19:1)], and B [MeOH–H₂O–HOAc (10:7:3)]. The column was equilibrated with 0% B (100% A) for 10 min before injection, and then linear gradients were used, with 10% B at 3 min, 25% B at 8 min, 60% B at 22 min, and 100% B at 25 min. Anthocyanins were detected at 535 nm, flavones at 360 nm.

To determine the concentrations from absorption values,
it was necessary to know whether there were differences in the molar extinction coefficients (ε) of the pigments. The following values have been reported for bisdeacylplatyconin, violodelphin, monodeacylcampanin and campanin, respectively: log ε = 4.59 at 540 nm (Goto et al., 1983), log ε = 4.27 at 547 nm (Kondo et al., 1990), log ε = 4.42 at 550 nm (Brandt et al., 1993) and log ε = 4.75 at 550 nm (Terahara et al., 1990). These differences in molar extinction coefficients are surprisingly large for compounds with the same basic structure. For this reason, the relative extinction coefficients of bisdeacylplatyconin, violodelphin, monodeacylcampanin and campanin were compared by preparing HPLC chromatographs of solutions of each anthocyanin in 10% aqueous HCl:MeOH 1:1 before and after 20 min hydrolysis at 95 °C.

The molar extinction coefficients of luteolin 7-O-β-apiofuranosyl-β-d-glucopyranosid (log ε = 4.51 at 355 nm) and an acylated luteolin 7-apioxylosid (log ε = 4.42 at 352 nm) have also been determined (Teslov, 1977) but since most of the flavones were not definitively identified, the mean of the extinction coefficients of these two compounds was used to calculate the concentration of flavones.

Statistics

Before the average time to flowering of buds from each stage was calculated, the lifetest procedure (SAS Institute, 1989), was used to test whether there were differences in time to flowering between the two types. Concentrations of anthocyanins and flavones were logarithmically-transformed before analysis. For each plant the rate of disappearance of bisdeacylplatyconin was calculated using data from day −1 to day 15 and the difference between type B and C was analysed by the general linear models procedure (GLM) (SAS Institute, 1989). Significant differences among clones within each phenotype were considered to be caused by random variation. Missing values for individual plants were interpolated before calculation of average values shown in Figs 2 and 3.

RESULTS

There were no significant differences in time to flowering of the buds in type B and C plants. The morphological bud stages were found to correspond to approx. 24, 12, 9, 6, 5, 3, 2 and 1 d before anthesis, respectively.

All anthocyanins were completely hydrolysed to delphinidin after 20 min at 95 °C. No significant difference was found among the relative extinction coefficients, measured as the change in ε during hydrolysis to delphinidin, and none of the observed differences exceeded 15%. For that reason, the logarithmic mean of the published molar extinction coefficients (log ε = 4.51), was used to calculate the molar concentrations of all four anthocyanins.

The contents of bisdeacylplatyconin, violodelphin, monodeacylcampanin, campanin and total anthocyanin in buds and flowers were determined (Fig. 2). Since delphinidin 3-glucoside had the same retention time as monodeacylcampanin, these two compounds were not separated. In any case, it is very unlikely that monodeacylcampanin is present in the early bud stages, since no other polyacetylated anthocyanins appear before stage 5, 6 d before anthesis. Thus it can be assumed that, from approx. day −24 to day −9, this peak represented delphinidin 3-glucoside whereas, from day −6 onward it was monodeacylcampanin. The total anthocyanin content per bud remained at a constant level from 24 to 12 d before anthesis, but since the fresh weight of the buds increased in that period, the anthocyanin concentration actually decreased. In the early bud stages, the proportion of the anthocyanins changed: delphinidin 3-glucoside decreased and bisdeacylplatyconin increased, but the other anthocyanins were present only at trace levels until a few days before anthesis, after which the accumulation of these pigments increased rapidly. In both genotypes, bisdeacylplatyconin reached its maximum level 2 d after anthesis. However, the subsequent decline was significantly slower (P < 0.001) in type C than in type B plants. Violodelphin, monodeacylcampanin and campanin reached their maximum levels within 4 d after anthesis. Type B plants accumulated higher levels of monodeacylcampanin and violodelphin than type C (P < 0.001 for both), but no significant difference in total anthocyanin content was found between the two types. The difference in monodeacylcampanin content was 68%, but only 26% for violodelphin. The total anthocyanin content increased until about 4 d after anthesis, when a decline became established. The decline affected both anthocyanin concentration and anthocyanin content per flower, since the fresh weight per flower did not change significantly.
stabilized around 1 anthesis, then falling rapidly until anthesis, where it changed during development, rising to 350 times 9 d before was 70 times higher than that of anthocyanins, but this ratio stage 1, 24 d before anthesis, the concentration of flavones

compounds no. 1a and 1b were poorly separated, but had clearly different kinetics of accumulation, as was also observed with delphinidin-3-glucoside and monodeacyl-

In view of the fact that the total amount of flavones remained constant, it is likely that this change occurred through the glycosylation of early flavone compounds. One of these flavones, compound no. 1a, peaked early (between 10 and 5 d before anthesis), but the kinetics of the other (no. 7) were similar to bisdeacylplatyconin. The contents of three compounds [nos 4 (luteolin 7-O-glucoside), 5 and 6] rose much more slowly, giving a constant concentration as long as the bud was green. A few days before anthesis these early compounds were replaced by three newly-appearing compounds (nos 1b, 2 and 3), probably polyglucosylated luteolins (Brandt, 1990). In view of the fact that the total amount of flavones remaining constant, it is likely that this change occurred through the glycosylation of early flavone compounds. Compounds no. 1a and 1b were poorly separated, but had clearly different kinetics of accumulation, as was also observed with delphinidin-3-glucoside and monodeacylcampanin.

No differences were observed in the ability of the two phenotypes to synthesize the individual flavones. At bud stage 1, 24 d before anthesis, the concentration of flavones was 70 times higher than that of anthocyanins, but this ratio changed during development, rising to 350 times 9 d before anthesis, then falling rapidly until anthesis, where it stabilized around 1-3 times, with a small increase at the end of the lifetime of the flowers (Fig. 4).

DISCUSSION

Rapid accumulation of anthocyanins in petals has commonly been observed in the later stages of flower development (Hess, 1963; Miller, Miller and Deal, 1967; Davies et al., 1993), and it has been shown that this rapid increase in anthocyanins is caused by de novo synthesis, e.g. in Hibiscus mutabilis (Amrhein and Frank, 1989). The decline in anthocyanin content observed here after the flowers had matured was unexpected, as no fading was detectable to the naked eye. Degradation of anthocyanins as flowers age has also been observed in Chrysanthemum (Stickland, 1972), in contrast to Matthiola incana whose anthocyanins are quite stable (Dangelmayr et al., 1983).

Stick et al. (1992) found that flavonol synthase and dihydroflavonol 4-reductase, the key enzymes of the flavonol and anthocyanin pathways, respectively, compete for dihydroflavonols (the branch point for flavonol and anthocyanin formation) as substrates in Dianthus caryophyllus. Similar observations have been made in Matthiola incana (Sprille and Forkman, 1984) but there is ample evidence that flavone and flavonol synthesis is regulated independently of anthocyanin synthesis (Hess, 1963; Jackson, Roberts and Martin, 1992; Davies et al., 1993). In the present study, the continuous changes in the proportions of individual compounds within each group make it difficult to demonstrate any direct competition for common precursor flavanones between the enzymes of the two pathways although, overall, accumulation of flavones occurred somewhat earlier than that of anthocyanins. Stich et al. (1992) found that the flavonol content of Dianthus increased until a few days after anthesis and remained at a constant level in the later stages. However, they determined the total flavonol content only, by spectrophotometry, without distinguishing among individual flavonoid glycosides. Thus their results do not contradict the present findings.

It is noticeable that the more complicated luteolin glucosides are accumulated after anthesis, precisely when the most complicated anthocyanins appear, and the contents of simpler flavone glycosides begin to decline. Campanin is almost completely absent from type B (Fig. 2) as are all polyacylated anthocyanins from type A (Brandt et al., 1993), even though the flavonoid profiles are identical for the chemotypes. This shows that at least the benzoyl transferases are highly specific. Since the most complicated luteolin glucosides are synthesized almost in parallel with anthocyanins, this concerted regulation indicates that flavones may play a role in colour formation in C. isophylla. The relations between flavones and flower colour will be investigated in a subsequent study.

In type C plants, less than 70% of monodeacylcampanin is converted into campanin, the content of violodelphin (which can be converted to monodeacylcampanin without further acylation) is lower, and the decline in bisdeacylplatyconin is slower than in type B. Both of these features indicate that the capacity for accumulation of acylated anthocyanins in type C plants may be inadequate, possibly owing to competition for a common substrate donating the benzoyl group to the different acylation enzymes. Experiments are in progress to investigate this hypothesis.

Fig. 4. Changes in flavone:anthocyanin ratio during bud and flower development in Campanula isophylla. The data shown are weighted means from 14 plants.

Other flavonoids, mainly flavones (Brandt, 1990) were already present in the small buds (Fig. 3). The total flavone content per bud increased up to its maximum level around anthesis, after which it remained almost constant (Fig. 3). This accumulation closely paralleled bud growth such that when total flavone content was expressed on a fresh weight basis, it remained approximately constant (data not shown). Two flavones showed relatively rapid accumulation followed by a decline, indicating that enzymes catalyzing their biosynthesis and metabolism probably appear at specific stages of bud development. One of these flavones, compound no. 1a, peaked early (between 10 and 5 d before anthesis), but the kinetics of the other (no. 7) were similar to bisdeacylplatyconin. The contents of three compounds [nos 4 (luteolin 7-O-glucoside), 5 and 6] rose much more slowly, giving a constant concentration as long as the bud was green. A few days before anthesis these early compounds were replaced by three newly-appearing compounds (nos 1b, 2 and 3), probably polyglucosylated luteolins (Brandt, 1990). In view of the fact that the total amount of flavones remaining constant, it is likely that this change occurred through the glycosylation of early flavone compounds. Compounds no. 1a and 1b were poorly separated, but had clearly different kinetics of accumulation, as was also observed with delphinidin-3-glucoside and monodeacylcampanin.
The present results indicate that, in any attempt to demonstrate the unknown enzymes that catalyse the interconversion of the four main anthocyanins in C. isophylla, work should be done at bud stage 8 or 9 when the rate of synthesis, and thus presumably the enzyme activity, is greatest. This also applies to the preparation of flavonoid-specific CDNA clones for studying gene action and regulation at the molecular level.

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LITERATURE CITED


