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Investigation of the biosynthesis of 3-deoxyanthocyanins in *Sinningia cardinalis*

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3-Deoxyanthocyanins provide bright orange-red colours to flowers of some members of the Gesneriaceae, including sinningia (*Sinningia cardinalis*). We examined 3-deoxyanthocyanin biosynthesis in sinningia, in particular, the expression of key flavonoid biosynthetic genes and the activities of the encoded proteins. Two abundant 3-deoxyanthocyanins, luteolinidin 5-*O*-glucoside and apigeninidin 5-*O*-glucoside, three flavone glycosides, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide and apigenin 7-*O*-glucuronide, and the cinnamic acid verbascoside were identified in sinningia petal tissue. Small amounts of a 3-hydroxyanthocyanin were also detected in a limited region of the petal. cDNA clones for three flavonoid enzymes, flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase/flavanone 4-reductase (DFR/FNR) and anthocyanidin synthase (ANS), were isolated from a sinningia cDNA library made from petal RNA and used to measure transcript abundance during petal development. Only very low levels of F3H transcript were detected, while DFR/FNR transcript was highly abundant. ANS transcript levels were intermediate between these two. The F3H cDNA was shown to encode a functional F3H protein by complementation of the phenotype of an *Antirrhinum majus* F3H mutant. The recombinant DFR/FNR had activity against both flavanone and dihydroflavonol substrates to a comparable extent. The results suggest a mechanism of 3-deoxyflavonoid biosynthesis in sinningia similar to that reported for *Zea mays*, in which lack of F3H activity allows action of the DFR/FNR on flavanone substrates and production of flavan-4-ols. These are then likely converted to 3-deoxyanthocyanins through the action of the ANS and subsequent glucosylation.

Introduction

Flavonoids are one of the most abundant groups of plant secondary metabolites. The most obvious flavonoids are the anthocyanin pigments, which provide red to blue colours to the majority of flowers and other plant tissues (Harborne and Grayer 1994). Most anthocyanins are derived from 3-hydroxylated base anthocyanidins, e.g. pelargonidin (Fig. 1). However, there are rare, variant anthocyanidins that are the basis of important anthocyanin pigments in

Abbreviations – ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; FNR, flavanone 4-reductase; F3H, flavanone 3-hydroxylase; HPLC, high performance liquid chromatography.

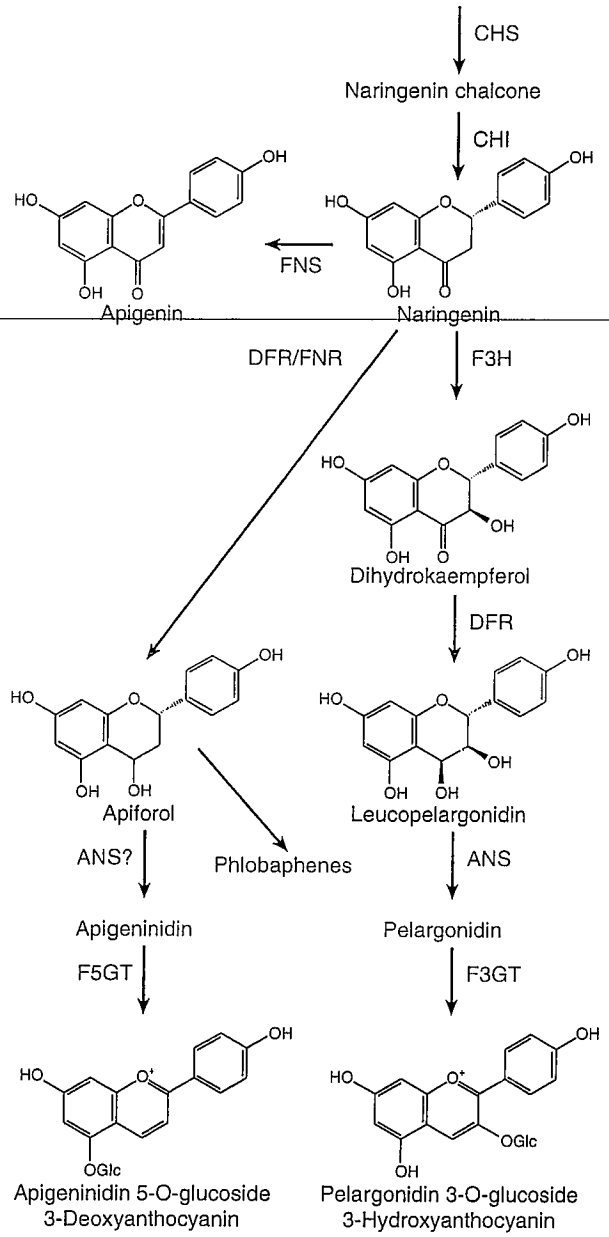


Fig. 1. Representation of a section of the flavonoid biosynthetic pathway leading to formation of flavones, 3-hydroxyanthocyanins, 3-deoxyanthocyanins and phlobaphenes. This diagrammatic representation is simplified by not showing the likely in vivo route to anthocyanins via an anthocyanidin pseudobase (a 3-flaven-2,3-diol), and by showing the proposed pathway for apigeninidin and pelargonidin monoglucosides only. Furthermore, some compounds, in particular apiforol, may be unstable in vivo, existing only as ephemeral intermediates. Enzyme abbreviations are CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FNS, flavone synthase; DFR, dihydroflavonol 4-reductase; FNR, flavanone 4-reductase; ANS, anthocyanidin synthase; F3GT, UDP-glucose: flavonoid 3-*O*-glucosyltransferase; F5GT, UDP-glucose: flavonoid 5-*O*-glucosyltransferase.

some species. Of particular interest are the 3-deoxyanthocyanins.

3-Deoxyflavonoids have been reported principally from ferns, mosses, the Gesneriaceae and some of the Poaceae, such as maize (*Zea mays*), sorghum (*Sorghum bicolor*) and sugarcane (*Saccharum* interspecific hybrids). There are also reports of their occurrence in a few other plant species (Bohm 1998) including *Camellia sinensis* (tea) (Roberts and Williams 1958). In maize, 3-deoxyanthocyanins can occur in various tissues, most obviously in the flower silks, and the related tannin-like 3-deoxyflavonoid phlobaphenes are also produced (Styles and Ceska 1975, 1977). The production by sorghum and sugarcane of 3-deoxyanthocyanidins and 3-deoxyanthocyanins as phytoalexins in response to fungal pathogens has received much interest (Chopra et al. 1999, 2002, Godshall and Lonergan 1987, Hipskind et al. 1990, 1996, Lo and Nicholson 1998, Lo et al. 1999, 2002, Viswanathan et al. 1996a, b). In the Gesneriaceae, the 3-deoxyanthocyanins provide bright red or orange-red colours to flowers of some of the Gesnerioideae subfamily, which occurs principally in the New World (Bohm 1998, Harborne 1966, 1967). It has been suggested that their biosynthesis may have arisen in response to selection pressure for bright orange-red flower colours to attract bird pollinators (Harborne 1966), although this hypothesis is not unequivocally supported by subsequent studies on bird pollination with regard to flower colour (Strack and Wray 1994).

The biosynthesis of the common 3-hydroxyanthocyanin pigments is one of the best characterized secondary metabolic pathways of plants. The synthesis of these anthocyanins proceeds via the sequential enzymatic activities of chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyaninidin synthase (ANS) and, most commonly, uridine diphosphate glucose: flavonoid 3-*O*-glucosyltransferase (Fig. 1). However, there have been relatively few studies on how the biosynthetic pathway produces 3-deoxyanthocyanins. Most information is available for the related phlobaphene pigments of maize. A MYB transcription factor, P, activates the biosynthetic genes encoding CHS, CHI and DFR but not F3H (Grotewold et al. 1994), so that flavanones are formed but conversion to dihydroflavonols does not occur. A similar regulatory system may operate in sorghum (Chopra et al. 1999, 2002). From genetic studies of maize (Styles and Ceska 1977) and later enzymatic studies of maize DFR (Halbwirth et al. 2003), it was concluded that the DFR of maize is able to catalyse reduction of the flavanones, in addition to its usual dihydroflavonol substrates, thus forming the

phlobaphene precursors flavan-4-ols rather than the flavan-3,4-diol anthocyanin precursors. A DFR-like enzyme showing the same activity on flavanones has also been assayed from flowers of the 3-deoxyanthocyanin accumulating Gesneriads, sinningia (*Sinningia cardinalis*) (Fig. 2) and *Columnea hybrida*, and termed flavanone 4-reductase (FNR) (Stich and Forkman 1988a, b). It is not known whether the FNR of sinningia represents a distinct enzyme or is due to FNR activity of the DFR enzyme, however, all enzymes characterized to date as having FNR activity have also been found to have DFR activity. The phlobaphenes are thought to be polymerization products from the flavan-4-ols, whereas 3-deoxyanthocyanins are presumably formed from the flavan-4-ols by ANS action and subsequent glycosylation. Furthermore, it is not known whether ANS and flavonoid glycosyltransferases specific to 3-deoxyanthocyanin biosynthesis have evolved. No molecular studies have been reported from a dicotyledonous plant or a species that produces only anthocyanins of the 3-deoxy type.

Enzymology studies suggest that a similar overall biosynthetic system as that for formation of flavan-4-ols in maize phlobaphene production may apply for 3-deoxyflavonoids in general, i.e. lack of F3H activity and acquisition of a FNR activity by DFR. Recombinant DFR proteins from apple (*Malus domestica*) and pear (*Pyrus communis*), which produce 3-deoxyflavonoids when F3H is chemically inhibited by the 2-oxoglutarate analogue prohexadione-Ca, exhibit both DFR and FNR activity (Fischer et al. 2003). DFR preparations from species that produce only 3-hydroxyanthocyanins generally lack FNR activity (Heller and Forkmann 1994, Martens et al. 2002). The recombinant DFR/FNR proteins studied to date demonstrate a preference for dihydroflavonol substrates compared with flavanones (Fischer et al. 2003, Gosch et al. 2003, Halbwirth et al. 2003, Schlangen et al. 2003), supporting the need for a mechanism promoting flavanones as the available substrate, such as occurs for maize phlobaphene production. There is further evidence for this from 3-deoxyanthocyanin producing flower silks of maize, as they have only low levels of F3H activity but show DFR/FNR activity (Halbwirth et al. 2003).

In this study, we have examined 3-deoxyanthocyanin biosynthesis in a species, sinningia, in which these compounds are the predominant flower pigments. In contrast, maize and sorghum produce 3-hydroxyanthocyanins in abundance in addition to the 3-deoxyflavonoids. We have characterized anthocyanin biosynthesis in sinningia in detail, confirming the production of 3-deoxyanthocyanins but also finding small amounts of 3-hydroxyanthocyanins produced in a restricted region of the petals. The expression of the key flavonoid biosynthetic genes has been examined, and the activities of

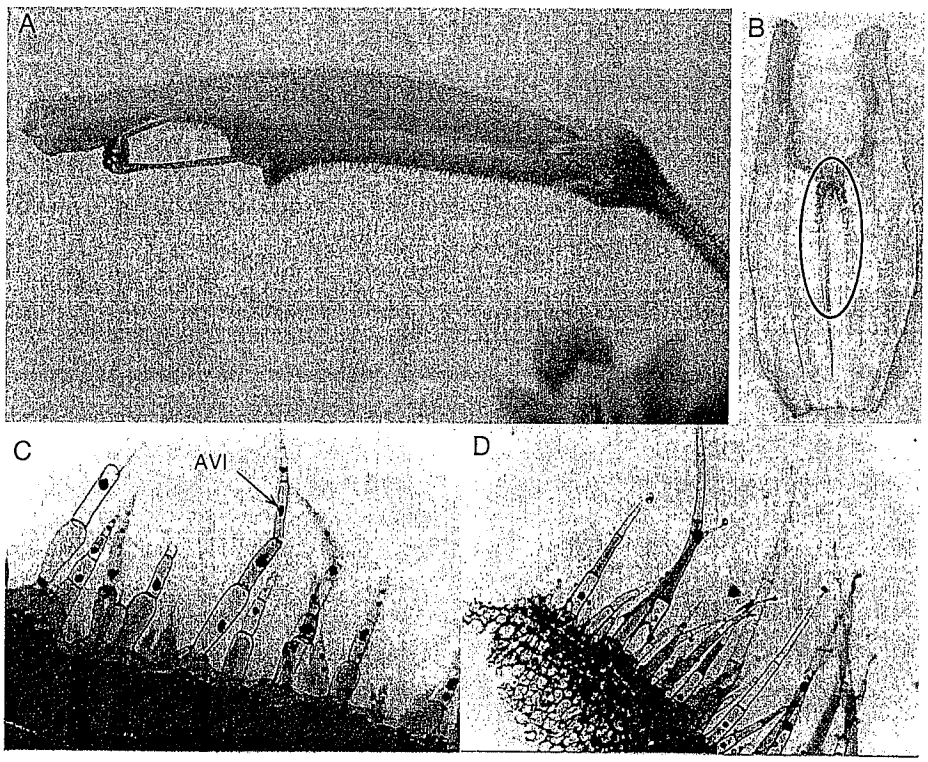


Fig. 2. A flower of sinningia (A) and a dissection of the flower (B) to show the irregular stripes on the ventral inner epidermis of the petal (ringed) that contain cyanidin-based anthocyanins. The lower panels are close-up images of the surface of the petal (C) and sepal (D) showing the trichomes and densely pigmented cellular bodies (labelled as anthocyanic vacuolar inclusion in C).

the encoded proteins characterized. The mechanism of 3-deoxyanthocyanin formation in the petals of the dicotyledon sinningia is found to have many commonalities with that of phlobaphene production in the monocotyledon maize.

Methods and materials

Plant material

Plants and seed for a cyanic sinningia line were obtained from Pukeiti Botanical Gardens (New Plymouth, New Zealand). Seed of the antirrhinum (*Antirrhinum majus*) *incolorata* line JI19 (Martin et al. 1991) was supplied by Dr Cathie Martin of The John Innes Centre (Norwich, UK).

Plants were grown in pots at 18–25°C under standard glasshouse conditions, in New Zealand, throughout the year. All tissue samples for RNA extraction were frozen in liquid N_2 immediately after harvest, and stored at −80°C until used. Tissue samples for chemical analysis were freeze-dried before analysis. Six stages of flower development of sinningia were defined as follows: stage 1 – closed bud; stage 2 – sepals separated and red corolla visible; stage 3 – corolla extended to the top of the sepals; stage 4 – corolla up to 10 mm above the sepals; stage 5 – corolla 10–20 mm above the sepals; stage 6 – open flower.

Chemical analysis

Flavonoid identification and quantification were by preparative chromatography, high performance liquid chromatography (HPLC) and NMR spectroscopy following the methods reported in Swinny et al. (2000) and Swinny (2001). The HPLC gradient for cyanidin 3-*O*-glucoside identification was modified slightly from Swinny et al. (2000) by increasing the starting amount of gradient solution A to 35% (v/v). The full ^{1}H and ^{13}C NMR assignments for the 3-deoxyanthocyanins have been reported previously (Swinny et al. 2000). Total anthocyanin concentration was determined as apigeninidin equivalents (ϵ = 38 000, MW = 255) from visible absorption maxima in 0.1 *N* HCL (Sweeny and Iacobucci 1977). Flavone and 2- (3′,4′-dihydroxyphenyl) ethanol 1-*o*-α-L-rhamnopyranosyl (1→3)-β-D (4-caffeoyl)-glucopyranoside (verbascoside, previously known as acteoside) concentration was calculated from HPLC peak integration data at 340 and 320 nm as apigenin 7-*O*-neohesperidoride and caffeic acid equivalents, respectively. Identification of cyanidin 3-*O*-glucoside was by HPLC retention time and by comparing on-line spectral data with an authentic standard.

RNA isolation and analysis

Total RNA was extracted using the TriZol reagent and method according to the manufacturer's instructions (Roche, Auckland, New Zealand). Transcript abundance was determined by northern RNA hybridization analysis as described by Winefield et al. (1999). Equivalent RNA loadings were determined by UV-spectroscopic measurements of the RNA, supported by ethidium bromide staining of the RNA gels and probing of northern membranes with a cDNA corresponding to a 25/26S rRNA from *Asparagus officinalis* (King and Davies 1992).

Isolation of cDNA clones

A cDNA library was constructed from poly(A)+ RNA prepared from total RNA isolated from a mix of stage 1–5 sinningia petal tissue. The cDNA library was constructed in λZAPII following the manufacturer's instructions (Stratagene, La Jolla, CA). The cDNA clones for F3H, DFR/FNR and ANS were isolated by heterologous screening using probes from cDNAs for apple F3H and ANS (Davies 1993a, b) and antirrhinum DFR (Beld et al. 1989). Library filters were prepared as in King and Davies (1992) and hybridized as in Winefield et al. (1999).

Nucleotide sequence determination of the cDNA clones was by a commercial service from University of Waikato (New Zealand). Sequence comparisons were carried out using CLUSTAL W within the MegAlign programme of the DNASTAR package (DNASTAR Inc, Madison, WI).

Biolistic transformation of antirrhinum

Particle bombardment experiments were performed with a helium particle inflow gun based on Finer et al. (1992), but modified with a high-speed direct current solenoid valve that allows more accurate opening times. Antirrhinum petal tissue was used from Stage 7 flowers (refer to Martin et al. (1991) for definition of antirrhinum stages). The tissue was sterilized and bombarded as in Deroles et al. (2002). Gold, 1 μ*M* in diameter (Bio-Rad, Auckland, New Zealand), was used as the standard microparticle DNA carrier. The process of Vain et al. (1993), with modifications as in Deroles et al. (2002), was used to prepare the gold and to precipitate the DNA on to the gold. Activity of the construct was measured as restoration of pigmentation to the F3H null mutant line JI19 (*incolorata*). Negative controls were gold particles alone (no DNA) and *CaMV 35S:GUS* or *CaMV 35S:GFP* constructs for β-galactosidase activity or green fluorescent protein, respectively. Several petals were shot for each construct, and all biolistic experiments were replicated at least twice.

For the biolistic experiments, a construct for the sinningia F3H was made by introducing the full-length F3H coding region into pART7 (Gleave 1992). The F3H cDNA was removed as an EcoRI-Asp718 fragment, the Asp718 site end filled with Klenow fragment enzyme and the fragment ligated into the EcoRI-SmaI site of pART7, so that the cDNA sequence was under the control of the *CaMV 35S* promoter and followed by the *ocs 3'* transcript termination region.

Heterologous expression of the sinningia DFR/FNR cDNA in yeast

The production of recombinant sinningia DFR/FNR in yeast (*Saccharomyces cerevisiae*) followed the method of Fischer et al. (2003). The cDNA was polymerase chain reaction (PCR) amplified from the plasmid clone with the Expand High Fidelity PCR System (Roche Diagnostics, Vienna, Austria) using the primers 5'-AATATGGAAACTGTTGCCCCAC-3' and 5'-GTTTC-ACTTTGTCTTTATCATGGG-3'. The sinningia translation initiation consensus sequence (ANNATGG) was introduced as part of the PCR primer. The dA-tailed PCR product was cloned into the expression vector pYES2.1 (TOPO TA Cloning Kit, Invitrogen, Karlsruhe, Germany). Presence and orientation of the insert were confirmed using PCR analysis.

Assay of DFR/FNR activity

DFR/FNR assays with enzyme preparations from yeast expressing the sinningia cDNA clone used the method of Stich and Forkmann (1988a) as updated in Halbwirth et al. (2003). [2-^{14}C]-Malonyl-CoenzymeA (55 mCi mmol^{-1}) was obtained from Amersham International (Amersham International, Little Chalfont, UK). [^{14}C]-Naringenin, [^{14}C]-eriodictyol, [^{14}C]-dihydrokaempferol and [^{14}C]-dihydroquercetin were prepared as described by Halbwirth et al. (2003).

Results

Flavonoid chemistry of sinningia

Using HPLC (Fig. 3) and NMR analysis (data not shown), two anthocyanins and three flavone glycosides were identified in extracts from whole petals of sinningia. They were luteolinidin 5-*O*-glucoside, apigeninidin 5-*O*-glucoside, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide and apigenin 7-*O*-glucuronide (Fig. 3). The predominant anthocyanin was apigeninidin 5-*O*-glucoside, and the most abundant flavone apigenin 7-*O*-glucuronide (Table 1). Minor flavone peaks on the HPLC at RT 30.1°C and

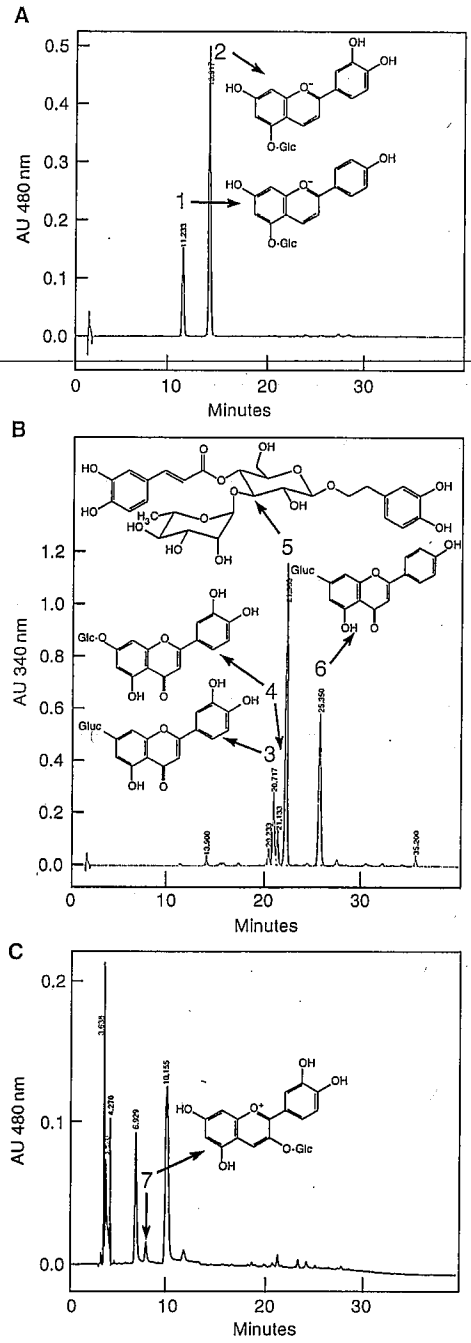


Fig. 3. Analysis of sinningia petal flavonoids. Panels A and B are high performance liquid chromatography (HPLC) chromatograms of anthocyanins (panel A) and general flavonoids (panel B) in extracts from whole petals of sinningia. The major peaks 1–6 represent luteolinidin 5-*O*-glucoside, apigeninidin 5-*O*-glucoside, luteolin 7-*O*-glucuronide, luteolin 7-*O*-glucoside, verbascoside and apigenin 7-*O*-glucuronide, respectively. Panel C is an HPLC chromatogram of flavonoids extracted from a small region of the petal throat, and peak 7 represents cyanidin 3-*O*-glucoside. Note that the left-hand scale is adjusted for the maximum peak value of each sample and is labelled in absorbance units (AU). Retention time is shown in minutes following sample loading.

Table 1. Estimated amounts of flavonoids in petals of sinningia. Estimates were calculated using a closely related pure compound as an equivalent.

Compound	Equivalent	mg g DW^{-1}
Luteolinidin 5-*O*-glucoside	Apigeninidin	1.61
Apigeninidin 5-*O*-glucoside	Apigeninidin	4.86
Luteolin 7-*O*-glucuronide	Apigenin 7-*O*-neohesperidoside	8.09
Luteolin 7-*O*-glucoside	Apigenin 7-*O*-neohesperidoside	3.41
Apigenin 7-*O*-glucuronide	Apigenin 7-*O*-neohesperidoside	19.71
Verbascoside	Caffeic acid	12.90

35.2 min may be the 4′-*O*-methyl ether derivatives of luteolin 7-*O*-glucuronide and apigenin 7-*O*-glucuronide, respectively, but their abundance was too low for unequivocal NMR determination. A major cinnamic acid peak was also seen during HPLC analysis (Fig. 3B, peak 5), and identified by NMR as 2- (3′,4′-dihydroxyphenyl) ethanol 1-*O*-α-L-rhamnopyranosyl (1→3)-β-D-(4-caffeoyl)-glucopyranoside, commonly known as verbascoside. Low levels of a cyanidin derivative appeared to be also present. It was thought that this might be derived from two irregular sized stripes with a distinct colour to the rest of the petal that are present on the ventral inner epidermis of the petal (Fig. 2B). Analysis of these tissue regions dissected from the rest of the petal identified cyanidin 3-*O*-glucoside as the major pigment (Fig. 3C).

Microscopy studies sugested that the anthocyanins were located in the petal and sepal epidermal cells and trichomes (Fig. 2). Most trichomes showed densely pigmented bodies. Chemical analysis of petal epidermal layer peels confirmed that both the anthocyanins and flavones were present in the epidermal cells (data not shown).

Sinningia flavonoid biosynthetic cDNA clones

A cDNA library was constructed from sinningia petal mRNA, and candidate cDNA clones isolated for F3H, DFR and ANS. The sinningia *F3H* cDNA (accession AAR01566) encodes a mRNA of 1329 bp, with the longest open reading frame (ORF) encoding a predicted polypeptide of 372 amino acids. Using a Blast search to the GenBank database, the most similar related amino acid sequences were all putative F3H sequences, with the closest being from a *Nierembergia sp.* cDNA with amino acid sequence identity of 79%. The sinningia *DFR/FNR* cDNA (accession AAR01565) encodes a mRNA of 1350 bp, with the longest ORF encoding a predicted polypeptide of 358 amino acids. The most similar sequences were all putative DFR sequences, with the closest being from a *Perilla frutescens* cDNA, with amino acid sequence identity of 70%. The sinningia *ANS* cDNA (accession AAR01567) encoded a mRNA of 1293 bp, with the longest ORF encoding a predicted polypeptide of 367 amino acids. The most similar sequences were all putative ANS sequences, with the closest being from a *P. frutescens* cDNA, with amino acid sequence identity of 73%.

As changes in F3H and DFR/FNR activity may be key to 3-deoxyanthocyanin biosynthesis, the deduced amino acid sequences of the sinningia cDNAs were compared with the sequences for F3H and DFR from other species (data not shown). The sinningia F3H sequence showed no major differences, such as deletions or additions, when compared with sequences that are known from mutant studies to be for functional F3H proteins. The sinningia DFR/FNR sequence was compared to a range of DFR sequences, including sequences for proteins having only DFR activity and sequences for proteins having both FNR and DFR activity (Fischer et al. 2003, Halbwirth et al. 2003, Martens et al. 2002). No consistent change in the amino acid sequence was found between proteins with FNR activity compared with those thought to lack FNR activity.

Transcript abundance for flavonoid genes of sinningia

F3H, *DFR/FNR* and *ANS* all had different patterns of transcript abundance in developing petals of sinningia (Fig. 4). *F3H* transcript abundance was very low, whereas that for DFR/FNR was highly abundant. The peak in expression was at stage 3. ANS also had high transcript levels, but less than for DFR/FNR. Expression was consistent over stages 1–4 and dropped markedly by stage 5. Some signal variation may be expected from variation in radiolabelling and hybridization efficiency of the cDNA probes. However, the cDNA probes were prepared using the same methodology and were of similar activity, based on total counts at

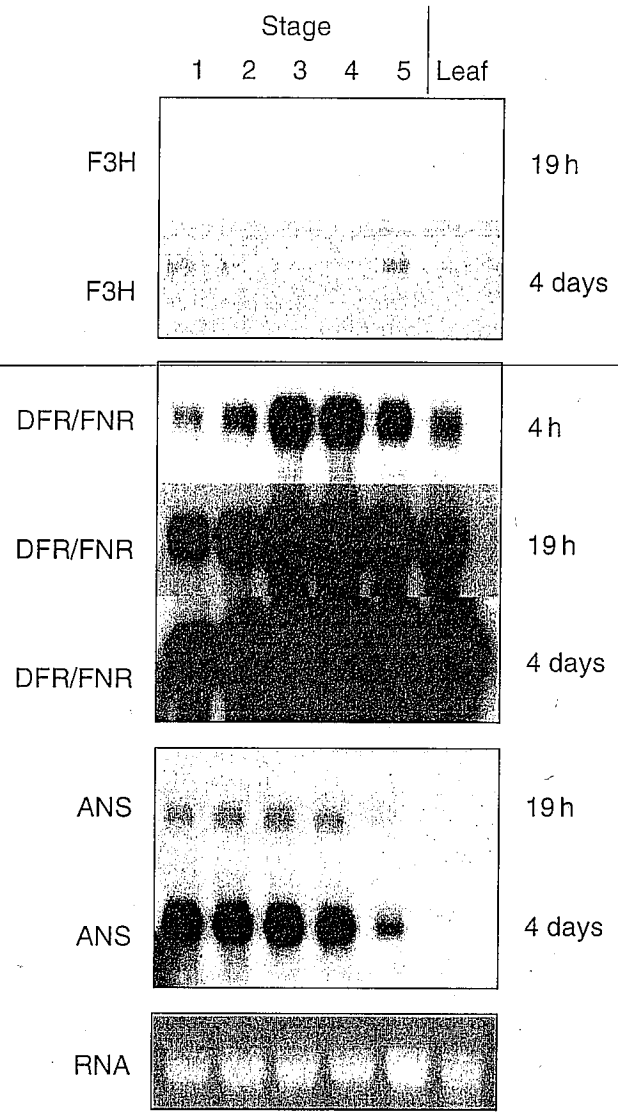


Fig. 4. Transcript abundance for flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase/flavanone 4-reductase (*DFR/FNR*) and anthocyanidin synthase (ANS) in petal tissue from five developmental stages of sinningia flowers and in leaf tissue. Transcript abundance in 20 μg of total RNA for each stage was measured by northern analysis using radiolabelled cDNA probes. The duration of autoradiography exposure for each image is shown on the right. A section of an ethidium bromide-stained agarose gel of the RNA samples prior to transfer to membranes for hybridization is shown to indicate RNA loadings for each sample.

a set distance from a GM tube detector, and autoradiography times and conditions were the same. The differences in signal intensity are of a magnitude to suggest a major difference in transcript abundance in the petals from the *F3H*, *DFR/FNR* and *ANS* gene(s). Transcript for all three cDNAs was also detected in RNA from leaf tissue, but in notable amounts only for DFR/FNR (Fig. 4).

Functional analysis of the sinningia F3H

A *CaMV 35S:sinningiaF3H* construct produced pigmented cell clusters when introduced by particle bombardment into petal tissue of the antirrhinum *F3H* mutant (Fig. 5). Gold particles alone produced neither pigmented sectors (Fig. 5) nor did control constructs such as *CaMV 35S:GUS* or *CaMV 35S:GFP* (data not shown).

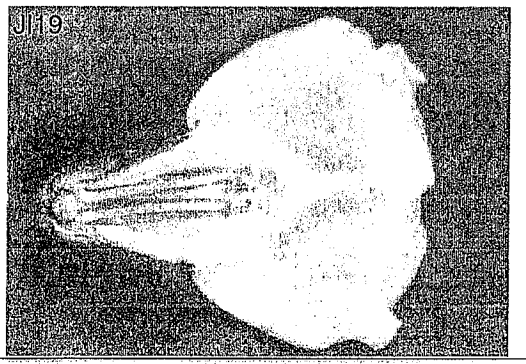
Assay of DFR/FNR activity

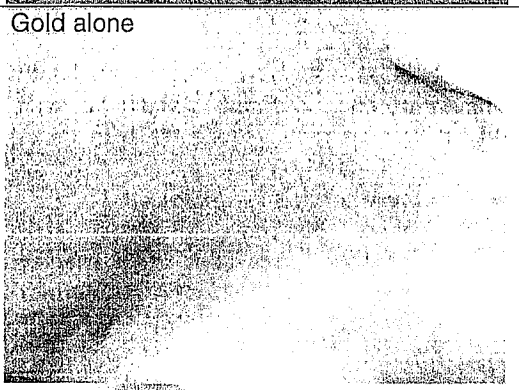
Enzyme preparations from yeast expressing the sinningia DFR/FNR cDNA showed both DFR and FNR activity (Fig. 6). Flavanones and dihydroflavonols were converted to a comparable extent. As with the DFR of maize, the substrate conversion rates of the sinningia enzyme in the yeast expression system were generally lower than those observed with various other DFRs (Fischer et al. 2003, Gosch et al. 2003, Martens et al. 2002, Schlangen et al. 2003). The pH optimum was pH 6.0 for the DFR reaction and pH 5.75 for the FNR reaction (data not shown).

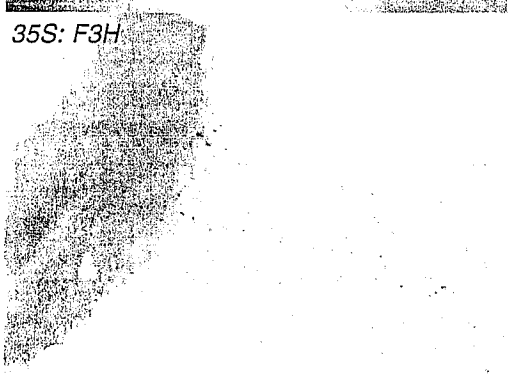
Discussion

There is an abundance of information on the biosynthesis of the common 3-hydroxyanthocyanin pigments. However, there have been few studies on how the anthocyanin biosynthetic pathway produces 3-deoxyanthocyanins. While gene expression studies have been conducted in relation to 3-deoxyflavonoid biosynthesis in maize, none have been reported for dicotyledonous species or for petal tissue. We report here molecular studies on the biosynthesis of the 3-deoxyanthocyanins in petals of the Gesneriaceae species *S. cardinalis*, in which they provide the main floral pigments.

We confirmed the occurrence of the two previously described anthocyanins in the petals of sinningia, luteolinidin 5-*O*-glucoside and apigeninidin 5-*O*-glucoside (Harborne 1966, Stich and Forkmann 1988a, Swinny et al. 2000). While small amounts of a cyanidin derivative were also found in whole petal extracts, based on relative abundances, it is obvious that the 3-deoxyanthocyanins are responsible for the bright orange-red colour of the flowers, with apigeninidin 5-*O*-glucoside the most abundant anthocyanin. Indeed, the low levels of cyanidin detected in whole petal extracts are likely to be derived from the two small stripes that have a distinct colour on the inner epidermis. Flavones were also present, and two flavone glycosides were identified that have not been previously reported for sinningia,







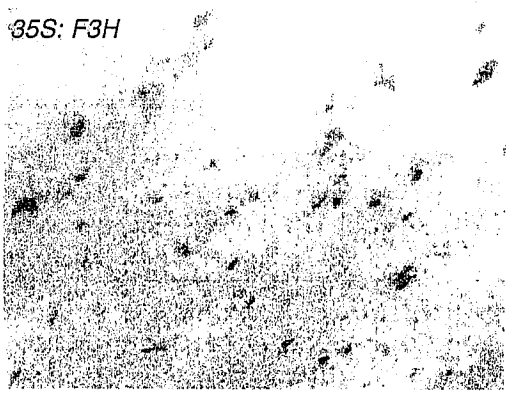


Fig. 5. Transient expression of a sinningia *CaMV 35S:F3H* construct in petal tissue of the *Antirrhinum majus* line JI19, which carries the *incolorata* mutation for the *F3H* gene, rendering the petals acyanic. The control was bombarded with gold particles alone. Two different magnifications of the *CaMV 35S:F3H* bombarded tissue are shown.

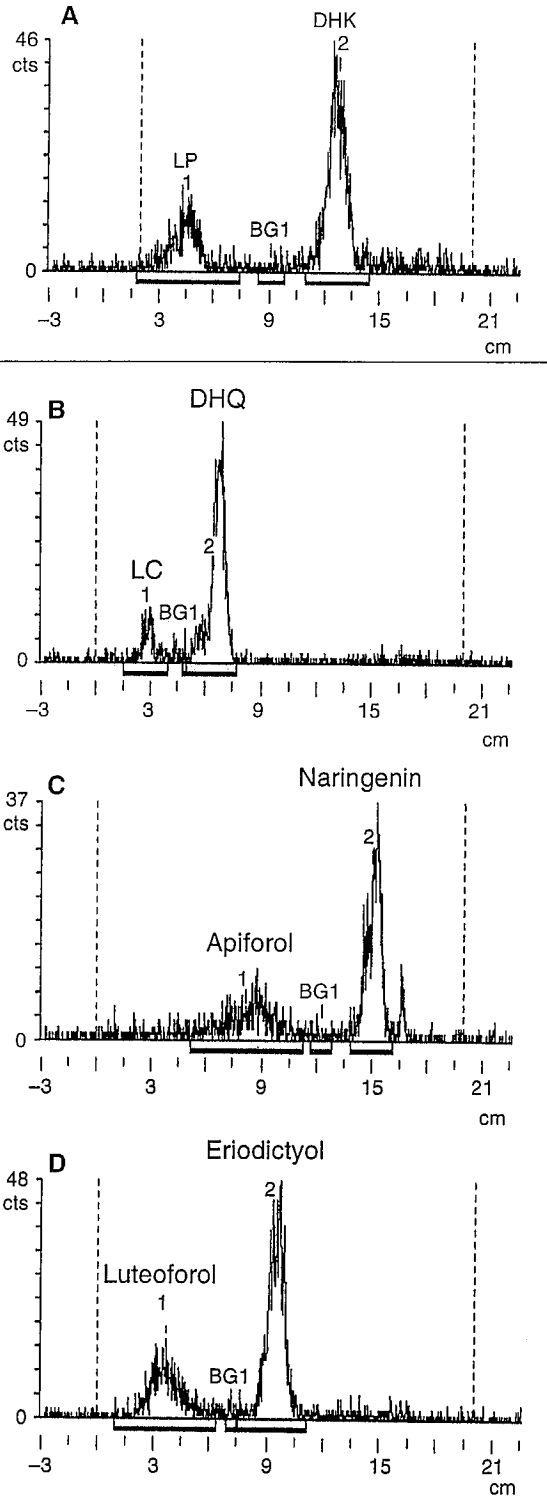


Fig. 6. Radioscans of thin layer chromatographs from incubation of extracts of yeast expressing the sinningia DFR/FNR cDNA, incubated with NADPH and (A) dihydrokaempferol (DHK) (B) dihydroquercetin (DHQ) (C) naringenin and (D) eriodictyol substrate. Products are leucopelargonidin (LP), leucocyanidin (LC), apiforol and luteoforol.

luteolin 7-*O*-glucuronide and apigenin 7-*O*-glucuronide. The cinnamic acid-derivative verbascoside was also found in abundance. Although its presence is a new report for sinningia, it is a widespread compound in plants and has been reported in another Gesneriad, *Conandron ramoidioides* (Nonaka and Nishioka 1977).

The 3-deoxyanthocyanins of sinningia can occur as inclusion bodies in the trichomes of the petal. Pigmented inclusion bodies have been reported for a range of species, although not in trichomes, including in carnation (*Dianthus caryophyllus*) (Markham et al. 2000), lisianthus (*Eustoma grandiflorum*) (Markham et al. 2000) and sweet potato (*Ipomoea batatas*) (Nozue et al. 1995, Xu et al. 2000, 2001) for 3-hydroxyanthocyanins and in sorghum for 3-deoxyflavonoids (Snyder and Nicholson 1990). Those of sinningia visually resemble the irregularly shaped, 'granular' anthocyanic vacuolar inclusions described for carnation and lisianthus, rather than the spherical, mobile inclusions of sweet potato and sorghum. The lisianthus and sweet potato inclusions have been shown to be associated with specific proteins, but the function, if any, of the bodies has yet to be determined.

The biosynthetic pathway for 3-hydroxyanthocyanins and 3-deoxyanthocyanins is thought to be common to the production of flavanones (Fischer et al. 2003, Halbwirth et al. 2003). Thus, we studied the genes encoding the enzymes that potentially use flavanones and their immediate derivatives as substrates, namely F3H, DFR/FNR and ANS. Putative full-length cDNA clones for all three of these enzymes, with high percentage sequence identities to proven F3H, DFR and ANS sequences of other species, were isolated from a sinningia cDNA library made from petal RNA. When the sinningia DFR/FNR-deduced amino acid sequence was compared with a range of DFR sequences, including proteins with only DFR activity and those with both FNR and DFR activity, no consistent change in the amino acid sequence was found that might account for acquisition of FNR substrate specificity. Furthermore, on a phylogenetic comparison, no grouping of proteins with FNR activity is seen (Fig. 7). Rather, general separation by evolutionary distance is observed, with the sequences from monocotyledonous species grouping distinct to those from dicotyledonous ones.

Recombinant enzyme from the sinningia DFR/FNR cDNA had both DFR and FNR activity, using flavanone and dihydroflavonol substrates to a comparable extent. Thus, the FNR activity previously reported in enzyme preparations of sinningia (Stich and Forkmann 1988a) was confirmed as being the result of the DFR enzyme and not a separate enzyme. The pH optima for

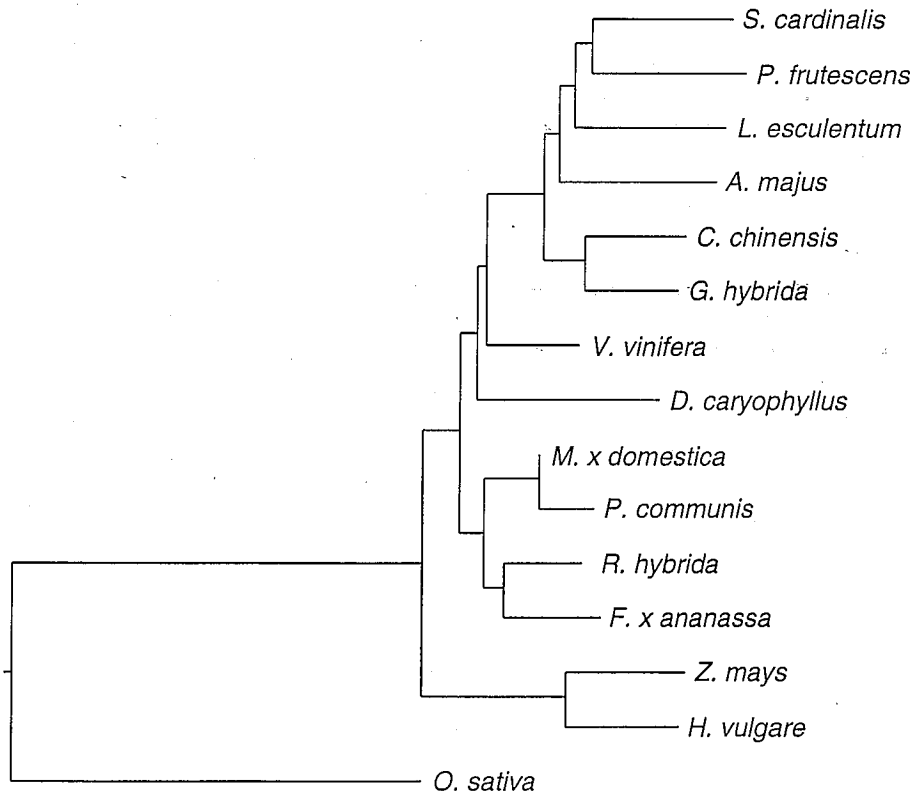


Fig. 7. Phylogenetic tree showing the relationship of various dihydroflavonol 4-reductase (DFR) proteins determined by CLUSTAL W analysis. DFR sequences *Sinningia cardinalis* (this study), *Perilla frutescens* (GenBank accession BAA19658), *Lycopersicon esculentum* (P51107), *Antirrhinum majus* (P14721), *Callistephus chinensis* (Z67981), *Gerbera hybrida* (Z17221), *Vitis vinifera* (P51110), *Dianthus caryophyllus* (Z67983), *Malus x domestica* (BAB92999), *Pyrus communis* (AY227730), *Rosa hybrida* (D85102), *Fragaria x ananassa* (AAS89833), *Zea mays* (P51108), *Hordeum vulgare* (S18595) and *Oryza sativa* (AAD24584).

flavanones and dihydroflavonols correspond with those described in the literature from studies on enzyme preparations from plant tissue. Substrate specificity studies with the recombinant enzymes have shown that the DFRs of apple, pear, grapevine (*Vitis vinifera*), strawberry (*Fragaria x ananasa*), cranberry (*Vaccinium macrocarpon*) and rose (*Rosa hybrida*) possess FNR activity (Fischer et al. 2003, Gosch et al. 2003, Halbwirth et al. 2003, Schlangen et al. 2003), although 3-deoxyanthocyanins or correlated precursors are not naturally formed in these plants. Moreover, the presence of FNR activity was also shown in preparations from grapevine, strawberry, peach (*Prunus persica*) and kiwifruit (*Actinidia deliciosa, Actinidia arguta*). For these plants, the accumulation of 3-deoxycatechins has been demonstrated after F3H inhibition by prohexadione-Ca treatment (Gosch et al. 2003). This common occurrence of the FNR activity supports the hypothesis that a key factor for the formation of 3-deoxyanthocyanins is the absence of F3H activity.

F3H transcript abundance was very low in sinningia petals during development of the flower, with only a weak signal detected by northern analysis. In contrast, transcript for DFR/FNR was highly abundant. An alternative that might result in lack of F3H enzyme activity, besides lack of F3H gene expression, is the presence of changes in the F3H coding sequence that result in production of no F3H protein or a non-functional F3H protein. However, the F3H transcript does correspond to a functional F3H protein, based on the deduced amino acid sequence and complementation of the antirrhinum *F3H* mutant. It is possible that the low level of F3H transcript is insufficient to produce sufficient F3H enzyme activity for 3-hydroxyanthocyanin production, or that the F3H transcript detected is associated with the small cyanidin-producing region inside the throat of the sinningia flower.

Given the common occurrence of FNR activity from recombinant DFR proteins, the rarity of 3-deoxyanthocyanin production in plants suggests that other biosynthetic factors may also be important besides the presence of FNR activity and the loss of F3H activity. These may include the need for a specific ANS or glucosyltransferase. The production of 3-deoxyanthocyanins in sinningia is accompanied by ANS transcript production. As 3-deoxyanthocyanins are the only abundant anthocyanins in the petals, it is likely that this ANS production is for conversion of the flavan-4-ols to the 3-deoxyanthocyanidins, which would then be glucosylated to the 3-deoxyanthocyanins. It is not known whether a specific ANS activity is required for action on the flavan-4-ols. The 3-hydroxyl has been proposed as a key component of the ANS reaction, with the initial step of the reaction being a stereospecific hydroxylation of the leucoanthocyanidin (flavan-3,4-*cis*-diol) at the C-3, producing a flavan-3,3,4-triol intermediate (Nakajima et al. 2001, Turnbull et al. 2001, 2004). The isolation of the sinningia ANS cDNA will enable comparison of recombinant sinningia ANS activity with that from species that produce only 3-hydroxyanthocyanins. From comparison of the deduced sequence of the sinningia cDNA with other available ANS sequences, no major amino acid differences were apparent that would indicate a coevolution of a distinct ANS and DFR/FNR for 3-deoxyanthocyanin biosynthesis.

In maize and sorghum, production of 3-deoxyflavonoids involves MYB transcription factors (Grotewold et al. 1994, Chopra et al. 1999). In maize, for the production of phlobaphenes, the MYB protein P activates the biosynthetic genes required for flavanone formation and DFR/FNR, but not F3H. Thus, flavanones are the only substrates available to the DFR/FNR, and the phlobaphene flavan-4-ols precursors are formed. In sinningia, the lack of F3H transcript, although a functional F3H gene(s) is present, and the very abundant DFR/FNR transcript suggest that 3-deoxyanthocyanin production may be achieved by a similar gene regulatory system to that in maize. However, additional regulatory changes may have occurred in sinningia to those described for maize. The *DFR/FNR* gene(s) are massively up-regulated compared with the other flavonoid biosynthetic genes examined in sinningia. Tight spatial regulation is also seen, with cyanidin produced only in one small region of the petal, perhaps to provide a nectar guide.

In this report, we present data on the transcript abundance and encoded activity of flavonoid genes accompanying 3-deoxyanthocyanin biosynthesis in flowers of the dicotyledon sinningia. The results suggest a biosynthesis mechanism similar to that reported for reproductive tissues of the monocotyledon maize, in which the key change from 3-hydroxyanthocyanin biosynthesis is lack of F3H activity allowing action of the DFR/FNR on flavanone substrates and resultant production of flavan-4-ols.

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