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Flavonoid metabolism in *Forsythia* flowers

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Abstract

The flavonoid pathway metabolism was studied in petals and sepals of *Forsythia* X *intermedia* cv. 'Spring Glory'. The activities of the flavonoid enzymes chalcone synthase (CHS), flavanone 3-hydroxylase (FHT) and flavonol synthase (FLS) were measured. The dihydroflavonol 4-reductase (DFR) role was also studied by comparing flavonoid accumulation in transgenic plants for a heterologous DFR gene and wild-type *F.* X *intermedia* cv. 'Spring Glory', already investigated for DFR gene expression and activity. HPLC analyses complemented enzymatic investigations, showing that: (i) rutin (quercetin 3-rutinoside) is the major flavonol accumulated in petals and sepals (ca. 90% of the flavonol pool) and; (ii) quercetin and cyanidin derivatives are the exclusive flavonols and anthocyanins in sepals, respectively. The overall data demonstrated that the flavonoid pathway in *F.* X *intermedia* flower organs leads to the major accumulation of 3′,4′-dihydroxylated compounds, and that 3′-hydroxylation of the B-ring occurs mainly at flavonoid intermediate stage(s). Comparative HPLC analyses of *F. X intermedia* cv. 'Spring Glory' and three other genotypes (*F. giraldiana*, *F.* X *intermedia* 'Korfor' Goldzauber® and *F. ovata* 'Robusta') confirmed the major production of flavonols by *Forsythia* flavonoid metabolism and suggested a method for screening *Forsythia* genotypes based on anthocyanin accumulation pattern in sepals.

Keywords: Anthocyanins; Flavonoid enzymes; Flowers; *Forsythia*; Transgenic plants; Woody ornamentals

1. Introduction

Flavonoids are important plant secondary metabolites that have been studied in genetic works for breeding and ornamental purposes. The complexity of the flavonoid pathway has been

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unraveled in the last decades by a great deal of studies, lately supported by molecular investigations [1,2]. In particular, biochemical studies first characterized the enzymatic steps involved in flavonoid production and the wide array of compounds produced by this pathway.

Forsythia is a widely cultivated yellow-flowering shrub. All known genotypes accumulate large amounts of carotenoids (xanthophylls) but lack anthocyanins in petals. Against such genetic background, biotechnologies provide effective tools to modify ornamental traits in valuable genotypes [3]. Our molecular breeding program at INRA Angers Station is aimed at changing flower color in *Forsythia* by inducing anthocyanin biosynthesis in petals. A prerequisite to success is the thorough knowledge of biochemistry of the pathway under study. Previous reports on secondary metabolism in *Forsythia* dealt with aspects not concerning ornamental breeding ([4,5] and references therein). Flavonols were found in all *Forsythia* genotypes, quercetin (Qu) derivatives and namely rutin (Qu 3-rutinoside) being the most abundant. Other flavonoids such as flavones were reported to be absent in *Forsythia* leaves [6] and no work has ever dealt with the characterization of anthocyanins, which proved to be useful markers in other ornamental species [7,8]. A previous work of ours [9] lead to the recovery of transgenic plants containing a dihydroflavonol 4-reductase (DFR) gene from *Antirrhinum majus* (DFR_{Ant}). Such DFR_{Ant} transformants had dramatically increased DFR gene expression and activity, but displayed a wild-type (wt) phenotype. Further biochemical investigations on both wt and transgenic plants would allow a better understanding of flavonoid pathway in *Forsythia* and possibly provide tools for genotype characterization and indications for our genetic engineering program.

This work deals with flavonoid pathway enzymatic and HPLC analyses on *Forsythia* genotypes and transformed plants, with a special emphasis on *F.* X *intermedia* cv. 'Spring Glory', the subject of our current transformation program for molecular breeding of flower color.

2. Materials and methods

2.1. Plant material

The four *Forsythia* wt genotypes studied in this work were grown in the INRA Angers collection. Open field-grown plants of *F.* X *intermedia* cv. 'Spring Glory' (collection reference: C6), *F. ovata* 'Robusta' (C8), *F. giraldiana* (C22) and *F.* X *intermedia* 'Korfor' Goldzauber® (C28) were used as source of material in 1996. Seven independent C6 DFR_{Ant} transgenic plants, among those obtained in a previous work [9], were also compared to wt C6 in 1997.

2.2. Chemicals and synthesis of substrates

Authentic standards for HPLC analyses were purchased from Extrasynthese (Lyon, France). [2-^{14}C]malonyl-CoA (spec. act. 0.67 MBq/mol) was from American Radiolabeled Chemicals (St. Louis, MO, USA). 4-coumaroyl-CoA and caffeoyl-CoA were a gift from W. Heller (Neuherberg, Germany). [4a,6,8-^{14}C]naringenin (NAR) was prepared as described [10,11] using partially purified chalcone synthase (CHS) and chalcone isomerase (CHI) from parsley suspension cell cultures. [4a,6,8–^{14}C]dihydrokaempferol (DHK) was prepared enzymatically using flavanone 3-hydroxylase (FHT) expression vector in *Escherichia coli* according to [12]. [4a,6,8-^{14}C]eriodictyol (ERI) and [4a,6,8–^{14}C]dihydroquercetin (DHQ) were prepared from their respective 4′-hydroxylated labeled precursors by incubation with microsome extracts of *Gerbera* hybrids (Asteraceae) or using caffeoyl-CoA as substrate for CHS instead of 4-coumaroyl-CoA. Radioactivity incorporated in labeled substrates was quantified by scanning sample aliquots after migration on cellulose plates (G 1440; Schleicher & Schüll, Göttingen, Germany) using a Bio-Imaging Analyzer Fuji BAS 1000.

2.3. Preparation of protein extracts and general enzyme assay procedures

Proteins were extracted from petals and sepals at different developmental stages according to

[13]. The resulting supernatant was directly used for CHS assays, or filtered through Sephadex G-50 columns equilibrated with 0.1 M Tris buffer (pH 7.5) for FHT and flavonol synthase (FLS) assays. Protein concentration was determined according to [14], using BSA as a standard. After incubation for 20 (FHT and FLS) or 30 min (CHS) at 30°C, reactions were stopped by 2 × 100 μl EtOAc extractions. The pooled EtOAc phase was chromatographed on cellulose plates with either CAW (chloroform:acetic acid:water) 50:45:5 for CHS assays or 15% HOAc for FHT and FLS assays. Labeled products were localized and quantified by scanning the plates as above mentioned.

2.4. Enzyme assays

Standard CHS enzyme assay (100 μl) contained: 75 μl 0.1 M K–Pi buffer, 5 μl 4-coumaroyl-CoA (1 nmol), 5 μl [2-^{14}C]malonyl-CoA (2.5 nmol; spec. act. 0.67 MBq/μmol) and 15 μl enzyme extract (ca. 10 μg protein). For competition experiments, the volume of K–Pi buffer was reduced to 70 μl and both 5 μl 4-coumaroyl-CoA (1 nmol) and 5 μl caffeoyl-CoA (1 nmol) were added.

FHT and FLS enzyme system (200 μl) contained: 155 μl 0.1 M Tris buffer (pH 7.5), either [4a,6,8-^{14}C]NAR or [4a,6,8-^{14}C]ERI or both of them (83 Bq each) as substrate, 30 μl cofactors (50 nmol 2-oxoglutarate, 10 nmol ferrous sulphate and 1 μM sodium ascorbate) and 15 μl enzyme extract (ca. 10 μg protein).

2.5. Extraction, separation and quantitation of polyphenolic compounds

Petals and sepals were harvested at full bloom and from flower buds 2 weeks before the start of anthesis, respectively, from all genotypes. Organ samples (150 mg fresh weight) were extracted in 1 ml MeOH–HCl 0.1% for 3 h at room temperature. Extracts were filtered through Millipore membranes (0.22 μm pore size) and stored at −80°C until analysis. Samples were analyzed by reverse-phase HPLC (Varian 5500). The polyphenolic fraction was determined at 280 nm using an Alltech 150 × 4.6 mm Adsorbosphere C18 3 μm column (0.8 ml/min flow rate). Separation of phenolics was performed with H_2O (solvent A) and MeOH:H_2O:MeCN (3:1:1) (solvent B). The gradient (in % solvent B) was: 0 min, 5; 5 min, 12; 10 min, 12; 16 min, 15; 26 min, 15; 60 min, 50; 75 min, 50. The anthocyanin composition was determined at 520 nm using an Alltech 250 × 4.6 mm Lichrosorb RP-18 5 m column (0.8 ml/min flow rate). Separation of anthocyanins was performed with MeCN (solvent A), 10% HCOOH (solvent B) and H_2O:HCOOH:MeCN (6:1:3) (solvent C). The gradient (in % each solvent) was: 0 min, 0:80:20; 50 min, 0:15:85; 55 min, 100:0:0. The presence of each compound was confirmed by comparison of retention times and spectra (photodiode array detector Waters 990) and co-chromatography with authentic standards. Flavonols and anthocyanins were quantified by external calibration with rutin and cyanidin 3-glucoside respectively as external standards. Measurements were carried out in triplicate in 1996 and as a single reading in 1997, due to the reduced standard deviation ($< 5\%$) from 1996 data treatment.

3. Results

3.1. Chromatographic studies

Chromatographic studies were performed to analyze flavonoid composition in flower organs (petals and sepals) of various *Forsythia* genotypes, including DFR_{Ant} transformants. Anthocyanins were absent in petals of wt genotypes. In sepals, the three detected anthocyanins were all cyanidin (Cy) glycosides, that is, Cy 3-galactoside (Cy 3-gal), Cy 3-glucoside (Cy 3-glu) and Cy 3-rutinoside (Cy 3-rut). The four studied *Forsythia* genotypes were characterized by different accumulation patterns of such pigments (Table 1). Cy 3-gal and Cy 3-glu were present in sepals of all genotypes. Only C22 sepals lacked Cy 3-rut and had the highest concentration of Cy 3-gal, while the highest amounts of Cy 3-glu and Cy 3-rut were found in C8 sepals. *F.* X *intermedia* genotypes C6 and C28 displayed similar anthocyanin accumulation ratios (*a*:*b*:*c* ratio, Table 1), which

Table 1
HPLC data on flavonol and anthocyanin concentration in petals and sepals of the four *Forsythia* genotypes analyzed in 1996[a]

	Petals		Sepals					
	Flavonols		Flavonols		Anthocyanins			
Genotype	Total	Rutin	Total	Rutin	Cy 3-gal (a)	Cy 3-glu (b)	Cyt 3-rut (c)	Ratio *a*:*b*:*c*
C6	2830	2511	3946	3627	25	39	33	1:1.6:1.4
C8	2293	2235	4844	4210	44	123	209	1:2.8:4.7
C22	956	824	1993	1814	198	76	0	1:0.4:0.0
C28	2055	1914	4319	4083	68	99	72	1:1.5:1.5

[a] Values are expressed in μg/g tissue FW and are means of three independent measurements.

were markedly different from those of the two other genotypes.

Flavonols, namely Qu derivatives, accounted for the majority of flavonoid compounds in studied organs (Table 1). Their concentration in sepals was one to two orders of magnitude higher than that of anthocyanins, and more than 2-fold higher than in petals in all genotypes except C6 (1.4-fold). C22 had by far the lowest petal and sepal flavonol concentration values. Rutin was consistently the major flavonol compound, accounting for ca. 90% of the total flavonol pool in petals and sepals of all *Forsythia* genotypes (Table 1). Kaempferol (Km) derivatives turned out to be absent in C6 sepals, while they were detected in petals, albeit in very low amounts (2% of the flavonol pool) with respect to the Qu derivatives (98%). Finally, no flavones were detected in any genotype, confirming preliminary TLC analyses.

In order to modify flower pigment composition and thus assess DFR role in *Forsythia* flavonoid pathway, C6 DFR_{Ant} transformants were recovered [9] and seven independent clones further investigated in this study for anthocyanin and flavonol composition in petals and sepals. Petals of all DFR_{Ant} transformants lacked anthocyanins, as those of wt C6. In addition, compared to wt C6, DFR_{Ant} clones displayed no variation for anthocyanin accumulation in sepals (data not shown). On the other side, differences were observed in flavonol accumulation patterns (Table 2). In sepals, accumulation of Qu derivatives with respect to wt C6 was slightly reduced in the clone DFR_{Ant} 3 (-11%) and strongly reduced in clones DFR_{Ant} 6 and DFR_{Ant} 7 to 48 and 50%, respectively. Surprisingly, the flavonol content in petals augmented in all DFR_{Ant} clones. The increase percentage in total flavonols varied between 17 (DFR_{Ant} 6) and 75% (DFR_{Ant} 1).

3.2. Enzymatic studies

Following chromatographic analyses, we investigated different flavonoid enzymes to demonstrate the formation of the main 3′,4′-hydroxylated compounds (Qu- and Cy-derivatives) found in *Forsythia* flower organs. The activity of the key enzyme CHS (Fig. 1) was extremely low in sepals of C6 throughout anthesis ($<1\%$ radioactivity incorporated in [4a,6,8–

Table 2
HPLC data on flavonol concentration in petals and sepals of wt C6 and seven C6 DFR_{Ant} transgenic genotypes analyzed in 1997[a]

Genotype	Total (% wt)	
	Petals	Sepals
wt C6	2893	6174
DFR_{Ant} 1	5072 (175)	6339 (103)
DFR_{Ant} 2	4029 (139)	6339 (103)
DFR_{Ant} 3	3920 (135)	6075 (98)
DFR_{Ant} 4	4123 (143)	6229 (101)
DFR_{Ant} 5	4380 (151)	6316 (102)
DFR_{Ant} 6	3381 (117)	2965 (48)
DFR_{Ant} 7	4170 (144)	3069 (50)

[a] Values are expressed in μg/g tissue FW and represent data from a single experiment. Flavonol content percentage ratios of transformants vs. wt C6 are shown in brackets.

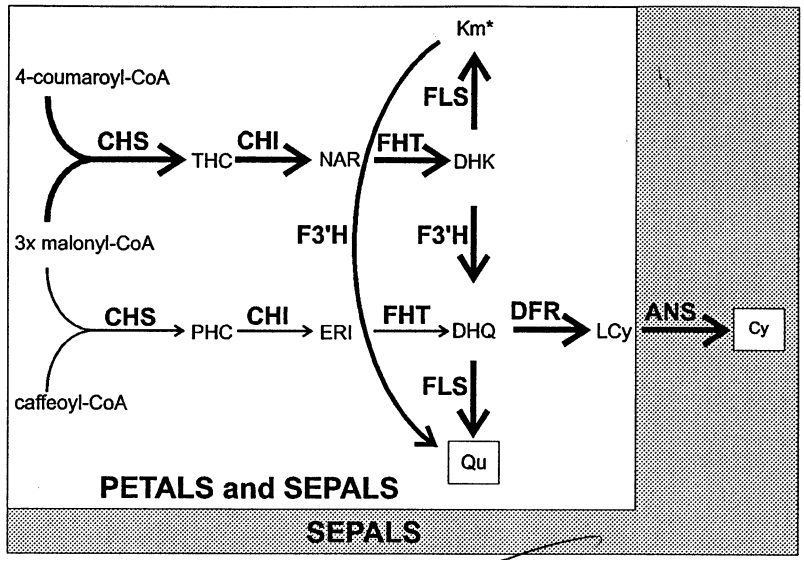

Fig. 1. Proposed flavonoid pathway in *F.* X *intermedia* cv. 'Spring Glory' petals and sepals, according to the results described in this paper. Thick and thin lines represent major and minor pathway steps, respectively. Major end-products are boxed. *, accumulation of Km derivatives occurs in petals only. THC, 2′,4′,6′,4-tetrahydroxychalcone. PHC, 2′,4′,6′,3,4-pentahydroxychalcone. Lcy, leucocyanidin.

^{14}C]NAR). By contrast, CHS activity was high in petals and peaked 3 days after the beginning of anthesis (petal length 2–3 mm out of flower buds). Standard tests were established and carried out with cell-free extracts (10 μg protein per reaction) from such maximum activity stage. The petal CHS showed increasing activity with regard to NAR formation in the pH range from 6 to 8 (Fig. 2). When 4-coumaroyl-CoA was substituted by caffeoyl-CoA, the pH optimum for ERI formation was found around 7. [4a,6,8-^{14}C]NAR formation was always higher than that of [4a,6,8–^{14}C]ERI, irrespective of pH value (Fig. 2). In competition experiments within the previous pH range, [4a,6,8–^{14}C]NAR formation (represented as relative amount of label incorporated in the C15 fraction) was as high as 82.1% at pH 6 and reached its maximum at pH 8 (94.9%) (data not shown).

The next investigated step concerned the conversion of flavanones to dihydroflavonols by the 2-oxoglutarate-dependent dioxygenase FHT (Fig. 1). Following incubation of [4a,6,8–^{14}C]NAR with cell-free extracts in the presence of required cofactors, two new products were found which could be identified as DHK and Km. This demonstrated the combined activity of FHT and a second dioxygenase responsible for flavonol formation, known as FLS (Fig. 1). To test substrate specificity of FHT and FLS, [4a,6,8–^{14}C]NAR and [4a,6,8–^{14}C]ERI were used as substrate. With [4a,6,8–^{14}C]NAR as substrate, the produced labeled DHK and Km accounted for 77% of the total radioactivity ([DHK + Km]/NAR ratio 3.3). On the other hand, the remnant [4a,6,8–^{14}C]ERI fraction was as high as 57%, while 43% of the radioactivity was incorporated in DHQ and Qu ([DHQ + Qu]/ERI ratio 0.75). The [4a,6,8–^{14}C]Km fraction was 45% of the total radioactivity, 8-fold higher than that of the [4a,6,8–^{14}C]Qu fraction (6%), indicating DHK as the preferred substrate for flavonol formation. In competition experiments, where equal amounts of both labeled NAR and ERI were used, the amount of produced DHK + Km was almost 2-fold that of DHQ + Qu.

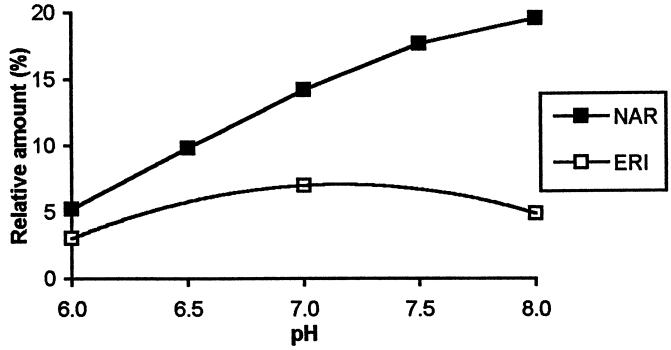

Fig. 2. CHS activity and pH dependence in *Forsythia* X *intermedia* cv. 'Spring Glory' petals. The percentage amounts of radioactivity incorporated into [4a,6,8-^{14}C]NAR and [4a,6,8-^{14}C]ERI after incubation of [2-^{14}C]malonyl-CoA with either 4-coumaroyl-CoA or caffeoyl-CoA, respectively, are shown.

4. Discussion

The flavonoid pathway in *Forsythia* flower organs was investigated by means of chromatographic and enzymatic analyses. This study was aimed at complementing molecular and transformation work which has been carried out on *Forsythia* X *intermedia* cv. 'Spring Glory' [9], investigated more thoroughly than other genotypes. Nevertheless, due to similarities in flavonol composition, it is likely that a number of results from enzymatic analyses of C6 could be extrapolated to the other studied *Forsythia* genotypes. Finally, anthocyanins were qualitatively and quantitatively characterized for the first time to our knowledge in *Forsythia*, and turned out to be possible markers for genotype identification.

3′,4′-hydroxylated compounds, namely flavonol rutin, were the major class of flavonoids found in all *Forsythia* genotypes, in agreement with previous quantitative data in *Forsythia* [6,15]. The absence of flavones in flowers of all genotypes confirmed previous data on leaf flavonoid composition [6]. Compared to 1996 data (Table 1), total flavonol content values scored in 1997 for C6 were consistent for petals, while significantly varied in sepals (Table 2). The expression and regulation of flavonoid enzymes are known to be subjected to environmental factors, such as light conditions and temperature ([16], and references therein). The observed difference could be accounted for by year-to-year variation in this flavonol-rich organ, and stress the importance of collecting samples in the same year when comparative studies are to be carried out. Actually, the four wt varieties were studied in 1996, while wt C6 was compared to DFR_{Ant} transformants in 1997. Rutin concentration was constantly high in all genotypes, not allowing genotype discrimination. On the other hand, anthocyanin accumulation patterns were genotype-specific: the absence of Cy 3-rut discriminated C22 genotype, and the anthocyanin accumulation ratios allowed to distinguish qualitatively cultivars belonging to different *Forsythia* species (Table 1). *F.* X *intermedia* genotypes were discriminated on the basis of their quantitative anthocyanin accumulation, more than 2-fold higher for each compound in C28 than in C6. Therefore, anthocyanins seem to be promising markers for the identification of *Forsythia* genotypes, unlike the flavonoid fraction [this study, 6], encouraging further investigations to validate this assumption.

The exclusive accumulation of Cy-derivative anthocyanins in *Forsythia* sepals can be due to either substrate-specificity of flavonoid enzymes (CHS, FHT and DFR [1]) or to the aforementioned strong F3′H activity (Fig. 1). The absence of Km derivatives in such organs suggests that the latter hypothesis cannot be ruled out, or that in sepals F3′H is more active and/or efficient than in petals, due to possible regulatory factors modulating the organ-specific expression of flavonoid genes [2,16]. In any case, Cy derivatives are likely

to be the exclusive pigments that would be produced following the induction of anthocyanin biosynthesis in petals by molecular breeding.

To proof the formation of 3′,4′-hydroxylated compounds, different flavonoid enzymes were investigated for their activity and substrate specificity. CHS activity data in petals and sepals were in agreement with previous molecular data, showing a strong induction of CHS gene in petals at the beginning of anthesis and very low transcript levels in sepals [9]. In the latter organ, already pigmented at the time of sampling, early flavonoid enzymes as CHS are likely to have already finished their action. Earlier (autumn) samplings did not lead to the detection of higher activity, indicating that CHS activity in sepals could be constantly low throughout the period before dormancy. CHS tests were performed to determine whether the formation of 3′,4′-hydroxylated flavonoids in *Forsythia* organs derives either from incorporation of caffeoyl-CoA instead of 4-coumaroyl-CoA or by 3′-hydroxylation of flavonoid compounds (Fig. 1). The first case was found in a few plants and seems to be strongly dependent on pH [1]. CHS assays showed that 4-coumaroyl-CoA is the preferred substrate for the synthesis of flavonoid skeleton in *Forsythia*, a finding common to most plant species [1]. FHT/FLS assays led to the main formation of 4′-hydroxylated products: thus, a strong flavonoid 3′ hydroxylase (F3′H) acting preferably at the dihydroflavonol and flavonol level is implied by the major accumulation of 3′,4′-hydroxylated compounds in petals and sepals and the absence of Km derivatives in sepals of both wt C6 and C6 DFR_{Ant} transformants. However, the incorporation of caffeoyl-CoA could represent a minor pathway for the synthesis of 3′,4′-hydroxylated flavonoids (Fig. 1). FLS activity was demonstrated in petals and inferred in sepals, due to their similar flavonol accumulation pattern.

The overexpression of the DFR_{Ant} gene in transformants neither altered the anthocyanin accumulation in sepals nor induced pigment synthesis in petals, confirming that DFR activity is not the limiting factor for anthocyanin biosynthesis in *Forsythia* [9]. The effect of the DFR_{Ant} gene on flavonol accumulation in transformant petals and sepals are contrasting. In sepals, the reduced flavonol accumulation observed in the clone DFR_{Ant} 3 and especially in clones DFR_{Ant} 6 and DFR_{Ant} 7 could be easily explained by a competition for dihydroflavonol substrate(s) of the overexpressed DFR against FLS. In petals, it can be hypothesized that overproduced leucoanthocyanidins, not processed because of the lack of ANS activity in these organs (C. Rosati et al., submitted for publication), inhibit DFR metabolism, leading to higher flavonol accumulation. Nevertheless, further investigations on the composition of other leucoanthocyanidin derivatives such as flavan-3-ols and proanthocyanidins are needed to confirm such speculative hypotheses.

The whole body of results from biochemical analyses of C6 are summarized in the proposed flavonoid pathway for this cultivar (Fig. 1), which leads to the major synthesis of 3′4′-hydroxylated compounds. Petals and sepals are likely to share a common pathway (except for accumulation of Km derivatives) as far as DFR. Instead, anthocyanins accumulate only in sepals. Lack of ANS gene expression in *Forsythia* petals (C. Rosati et al., submitted for publication) would be the cause of the absence of anthocyanins in these organs.

Future work is planned to characterize other flavonoid enzymes and compounds and to extend chemotaxonomical studies to other *Forsythia* genotypes.

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