The strawberry transcription factor FaMYB1 inhibits the biosynthesis of proanthocyanidins in Lotus corniculatus leaves

Francesco Paolocci1,*, Mark P. Robbins2, Valentina Passeri1, Barbara Hauck2, Phil Morris2, Andrea Rubini1, Sergio Arcioni1 and Francesco Damiani1

1 National Research Council, Plant Genetics Institute-Perugia, Perugia Division, Via Madonna Alta 130, 06128 Perugia, Italy
2 Bioenergy and Biorenewables Programme, Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Gogerddan Campus, Aberystwyth, Ceredigion SY23 3EB, UK

* To whom correspondence should be addressed. E-mail: francesco.paolocci@igv.cnr.it

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Abstract
Proanthocyanidins (PAs) are agronomically important biopolymers in higher plants composed primarily of catechin and epicatechin units. The biosynthesis of these natural products is regulated by transcription factors including proteins of the R2R3MYB class. To gain insight into the genetic control of the catechin and epicatechin branches of the PA pathway in forage legumes, here the effects of the expression of FaMYB1, a flavonoid R2R3MYB repressor from strawberry, in Lotus corniculatus (birdsfoot trefoil), were tested. It was found that in leaves of T0 transgenic lines the degree of PA inhibition correlated with the level of FaMYB1 expression. These effects were heritable in the transgene-positive plant T1 generation and were tissue specific as the suppression of proanthocyanidin biosynthesis was most pronounced in mesophyll cells within the leaf, whereas other flavonoid and phenolic compounds were substantially unaltered. The data suggest that FaMYB1 may counter-balance the activity of the endogenous transcriptional MYB–bHLH–WD40 (MBW) complex promoting proanthocyanidin biosynthesis via the catechin and epicatechin branches and that FaMYB1 does not interfere with the expression levels of a resident R2R3MYB activator of PAs. It is proposed that in forage legumes leaf cell commitment to synthesize proanthocyanidins relies on the balance between the activity of activator and repressor MYBs operating within the MBW complex.

Key words: HPLC-DAD-ESI/MSn, Lotus, metabolite profiling, proanthocyanidin, R2R3MYB, transcriptional regulation.

Introduction
Flavonoids are plant secondary metabolites comprising a wide variety of compounds that are synthesized from flavanone precursors. Chemical end-products belonging to the flavonoid class include flavonols (colourless to pale pigments), anthocyanins (red to purple pigments), and colourless polymerized compounds which are classified as proanthocyanidins (PAs), also known as condensed tannins (Winkel-Shirley, 2001).

PAs play important roles in various plant functions such as pigmentation, protection against damage by ultraviolet light and phytopathogens, dormancy, and fertility (Winkel-Shirley, 2001; Lepiniec et al., 2006). In addition, benefits have been reported for human health, spanning from protection against free radical-induced oxidative damage to the inhibition of growth of human cancer cells in vitro (Santos-Buelga and Scalbert, 2000; Marles et al., 2003). PAs are potentially relevant for industrial purposes and, additionally, are important with reference to ruminant nutrition; tanniferous crops not only increase the efficiency of protein usage in ruminant livestock, but can also reduce methane
production from sheep, cattle, and other ruminant animals (Beauchemin et al., 2007). Alfalfa and clovers, the most important legume species worldwide, and the model legume species Medicago truncatula and Lotus japonicus are, however, unable to accumulate PAs in leaves. In contrast, some other forage species accumulate high levels of PAs in leaves and stems, but a high concentration of PA substantially depresses voluntary free intake by animals (Barry and McNabb, 1999). Thus, the modulation of the levels of PAs in leaves of forage legumes is of paramount relevance for forage breeders worldwide.

PAs are oligomers generated by the sequential addition of leucoanthocyanidin, catechin, or epicatechin extension units to starter epicatechin or catechin units (Dixon et al., 2005). Catechin and epicatechin units are biosynthesized by two different pathways, branching from common leucoanthocyanidin (flavan 3, 4-diol) intermediates (Fig. 1). While catechins are derived from the direct reduction of leucoanthocyanidin via the activity of leucoanthocyanidin reductase (LAR) (Tanner et al., 2003), epicatechin formation occurs via anthocyanidin synthesis and reduction. The two biosynthetic steps leading to epicatechins are catalysed by anthocyanidin synthase and anthocyanidin reductase, encoded by LDOX/ANS and BAN/ANR genes, respectively (Abrahams et al., 2003; Xie et al., 2003).

The PA biosynthetic pathway has been best characterized in Arabidopsis thaliana which synthesizes these metabolites in the seed coat. The MYB–bHLH–WD40 (MBW) complex regulates the expression of flavonoid biosynthetic genes at the transcription level (Lepinec et al., 2006). The combinatorial interactions between MYB and bHLH transcription factors are central to the regulation of gene expression in order to control various stages of flavonoid pathway(s). It would appear that this MBW complex is involved in flavonoid biosynthesis in all plant species. Three regulators, namely TT2 (R2R3MYB), TT8 (bHLH), and TTG1 (WD40 protein), play a central role in the regulation of PA-specific genes in Arabidopsis. Within this complex, TT2 is the key component to specify proanthocyanidin biosynthesis (Nesi et al., 2000, 2001). Conversely, the bHLH and WD40 components are also involved in mucilage production and epidermal cell patterning processes (Zhang et al., 2003). Lotus japonicus and other species have genomes including TT2 orthologues that regulate the tissue-specific expression of PA pathways in these crops (Matus-Cadiz et al., 2008; Yoshida et al., 2008). However, negative modulation of flavonoid biosynthesis by R2R3 and R3MYBs has also been reported in different plant species (Aharoni et al., 2001; Dubos et al., 2008; Matsui et al., 2008).

Here tests were carried out to determine whether the expression of a negative regulator gene of the flavonoid pathway is sufficient to down-regulate the PA pathway in leaves of a high PA genotype of Lotus corniculatus. For this purpose, the FaMYB1 gene from strawberry was used, which has been reported to interact with bHLH partners and repress the anthocyanin pathway in tobacco (Aharoni et al., 2001). Here it was found that the ectopic expression of FaMYB1 repressed the expression of the structural genes of both epicatechin and catechin branches of PA biosynthesis. This action was dose dependent and specific in that it reduced the accumulation of PA polymers only in leaf tissues. Such depletion was heritable, and the analysis of other flavonoid end-products indicated that FaMYB1-mediated interventions were restricted to proanthocyanidin biosynthesis.

### Materials and methods

**Plant material**

The binary vector containing the FaMYB1 cDNA inserted between a double 35S cauliflower mosaic virus (CaMV) promoter and a nopaline synthase terminator was kindly provided by Dr A. Aharoni (Aharoni et al., 2001). An Agrobacterium rhizogenes strain harbouring the wild-type Ri plasmid 1855 and the binary vector described above were used to transform L. corniculatus genotype S41, which is characterized by a high level of foliar PA accumulation (Carron et al., 1994). Transgenic hairy roots were cultured in kanamycin selective media as described in Damiani et al. (1993) and independent transgenic lines were regenerated to plants. The resulting T0 plants were screened for leaf PA accumulation in leaves using 4-dimethylaminocinnamaldehyde (DMACA) (Li et al., 1996), a histological stain which gives a qualitative estimate of PA content. Seeds from pollination under controlled conditions among FaMYB1 transgenic lines (T1) as well as among S41 control lines (S1) were recovered and sown in Petri dishes. The resulting seedlings were transferred to a glasshouse and screened for leaf PA accumulation at monthly intervals.

**Isolation and analysis of nucleic acids**

Isolation, blotting, and Southern hybridization of genomic DNA from primary (T0) transformants harbouring FaMYB1 as well as from T1 lines were performed as in Damiani et al. (1999). An aliquot of ~8 µg (3.5×10⁶ genomes) of genomic DNA was restricted with XbaI (New England Biolabs) which cuts once outside the FaMYB1 fragment in the T-DNA, and filters were hybridized as reported in Aharoni et al. (2001). PCR analysis for a rapid screening of transgenic lines was performed using the 35S-specific primer (5’-GACCTTTCTTCTATATAAGG-3’) and a primer (5’-TTAAGCAACTTGAGGATCGC-3’) which anneals at the extreme 3’ end of the FaMYB1 coding sequence.

### Fig. 1. Schematic version of flavonoid metabolism in Lotus. Key genetic steps are indicated; major flavonoid end-products are underlined. A question mark indicates the putative activity of the protein coded by LAR2.
RNA was isolated from leaves of FaMYB1 T0 and T1 transgenic lines and from control untransformed and β-glucuronidase (GUS)-transformed S41 lines; plants were grown contemporaneously in a glasshouse under identical conditions, and healthy young trifoliate leaves were harvested. RNA was isolated using the Nucleo Spin RNA Plant Isolation kit (Macherey Nagel) according to the supplier’s instructions, after which a further DNase treatment was added. The quality and quantity of RNA were verified by agarose gel electrophoresis and spectrophotometric analysis. The absence of DNA contaminating the RNA preparations was tested by the null PCR amplification of DNase-treated RNAs in the presence of the universal rDNA primer pair ITS1/ITS4 as described in Paolocci et al. (2005).

For real-time PCR (RT-PCR) analysis, cDNA from transgenic and control lines was synthesized from ~5 µg of total RNA using SuperScriptIII H-Reverse Transcriptase (Invitrogen) and 100 pmol of random hexamers (Pharmacia Biotech) according to the enzyme’s supplier. Primer pairs used to amplify fragments corresponding to CHS, DFR, ANS, ANR, LARI, LAR2, and 1xEF were as described by Paolocci et al. (2005, 2007). Primers for the transgene were qFaMYB1 Fw (5'-GGGCTGTTCTGATCCAGA-3') and qFaMYB1 Rev (5'-GCAACCCTCAGCCTTGTITT-3'). The primers LcTT2 Fw (5'-GACGTITGGAAGCCGTITGG-3') and LcTT2 Rev (5'-TGTATATATTTGCCCTTTTGATACCTGG-3') were designed with the help of the OligoExpress Software (Applera Biosystems) to produce a 64 bp long amplicon on LcTT2, an orthologue of the Arabidopsis TT2 MYB gene, cloned from L. corniculatus S41 genotype (F. Paolocci et al., unpublished results).

For each plant sample, two independent RNA isolations were performed and for each RNA sample two reverse transcription steps were performed and then pooled. An aliquot of 4 µl of 1:10 diluted pools of cDNA was used in the PCR that was made up using the Power SYBR Green PCR core mix (Applera Biosystems) according to the supplier’s instructions in a 25 µl final volume in the presence of 2.5 pmol of each primer. Cycling parameters were: two initial steps of 50 °C for 2 min and 95 °C for 10 min; a two-step cycle of 95 °C for 15 s and 60 °C for 1 min repeated 50 times, and a final step of 10 min at 60 °C. Afterwards, the dissociation protocol was performed. Amplifications were performed on an ABI PRISM 7300 SDS apparatus (Applied Biosystems). Four replicates for each RNA sample were amplified and two independent RT-PCR experiments were carried out for each RNA sample. For each transcript the average Ct (threshold cycle) was measured for each transcript the average Ct (threshold cycle) was calculated from the six dilution points, each one replicated four times to calculate the amplification efficiency of each primer pair. The gene quantification method based on the relative expression of the target gene versus the reference gene 1xEF was adopted as previously reported (Paolocci et al., 2007). Final figures from this analysis were then treated as quantitative traits and were statistically analysed. Analysis of variance (ANOVA) and Duncan tests for mean comparison were performed using the GLM Procedure of the SAS program (SAS Institute).

Quantification of transgene copy number was performed by RT-PCR analysis using the ABI 7300 apparatus and 7300 software according to the supplier’s instruction. 1xEF- and FaMYB1-specific primer pairs reported above were used to amplify genomic DNA from progeny of FaMYB1 and S41 control plants as well as the binary vector containing the FaMYB1 cDNA and the plasmid containing the 1xEF genomic fragment, used as standards. To this end, the genomic fragment resulting from the amplification of untransformed S41 plants with 1xEF-specific primers were cloned into pGEM-T Easy vector (Promega). Standard curves for target genes and the housekeeping gene (1xEF) were obtained by the amplification of serially diluted plasmids. The copy numbers of FaMYB1 in the DNA samples were determined from the corresponding standard curve, using the Ct values as reported in Lee et al. (2008).

**Analysis of flavonoid end-products**

Histological staining for the localization of PA-containing cells was performed using DMACA according to the protocol of Li et al. (1996). Levels of proanthocyanidins were measured in plant tissues by butanol-HCl hydrolysis using the method of Terrill et al. (1992).

Flavonoids and related phenolic end-products were analysed by reverse-phase HPLC with online photodiode array and electro-spray ionization-tandem mass spectrometry (HPLC-DAD-ESI/MS²). Typically 50 mg fresh weight (FW) of leaf tissue from representative control and transgenic lines were extracted four times in 5 ml of 70% methanol. The solvent was removed on a rotary evaporator, and the samples were partially purified using Waters Sep-Pak (500 mg) C18 reverse phase cartridges, dried down at 50 °C under air, and redissolved in 400 µl of 70% methanol.

HPLC/MS² analysis was performed on a Thermo Finnigan LC-MS system (Thermo Electron Corporation, USA) as described by Parveen et al. (2008) comprising a Finnigan Surveyor PDA Plus detector, a Finnigan LTQ linear ion trap with ESI source, and a Waters C18 reversed-phase Nova-Pak column (4 µm, 3.9 mm x 100 mm). The auto-sampler tray temperature was kept at 5 °C and the column temperature was maintained at 30 °C. Sample injection volume was 5 µl, the detection wavelength was set at 240–400 nm, and the flow rate was 1 ml min⁻¹, with 100 µl min⁻¹ going to the mass spectrometer. The mobile phase consisted of purified water–formic acid (A; 100:1, v/v) and HPLC grade methanol (B). The initial condition was A:B (10:90, v/v), and the percentage of B increased linearly to 60% over 65 min.

Mass spectra were acquired in negative ionization mode. Ionization parameters were optimized by infusion of chlorogenic acid standard at a constant rate into the LC flow. Interface and MSD parameters were as follows: sheath gas 30 arbitrary units, auxiliary gas 15 units, spray voltage 4 kV, capillary temperature 320 °C, capillary voltage −1 V, and tube lens offset −68 V. Initial MS/MS fragmentation was carried out at normalized collision energy 75% and isolation width 2.0 (m/z). Commercial standards for phenolic acids and flavonol aglycones were obtained from Sigma-Aldrich, UK.

**Results**

**FaMYB1 reduces leaf PA accumulation in primary L. corniculatus transgenic lines**

Eight transgenic lines harbouring the FaMYB1 transgene were obtained after regeneration of hairy roots resulting from independent transformation events of S41 stem explants. When amplified with a primer pair spanning the 35S promoter and the coding sequence of FaMYB1, all these T0 lines produced the expected amplicon. Then, these lines were propagated through cuttings and grown in the glasshouse under outdoor conditions along with cuttings of the S41 untransformed genotype and pBIN121.1- (CaMV-GUS) transformed lines. When stained with DMACA at monthly intervals to estimate PAs qualitatively in leaves, a subtle and consistent reduction in the number of PA-containing cells was observed only in FaMYB1 plant lines (data not shown). Three FaMYB1 transgenic plants named 3N, 4N, and 6N were then selected as representative of the different phenotypic classes resulting from FaMYB1 transformation, with 3N showing the most extreme PA reduction, 4N an intermediate phenotype, and plant 6N with a PA level similar to the control, untransformed plants.
Southern analysis of genomic DNA showed that plant 3N harboured a single insertion of the transgene, plant 4N three insertions, and plant 6N two (Supplementary Fig. S1A available at JXB online).

Notably, within individual cuttings and stems of line 3N, trifoliate leaves were observed with a number of PA-containing cells similar to the wild type along with trifoliate leaves where PA-containing cells were only distributed in the main leaf veins (Fig. 2A, B). Examples were also noted which showed asymmetric distribution of PA-positive cells with respect to the main (central) vein of the central leaflet (Fig. 2C). However, such a PA distribution pattern was not always observed; for example, in some leaves the half PA-positive lamina of the central leaf was not always contiguous with the half of the lateral leaf that showed PA cells. So, in summary for line 3N, trifoliate leaves with both symmetric and asymmetric patterns of PA depletion could be noted along a single stem.

RT-PCR analysis was carried out to detect the expression levels of \textit{FaMYB1} as well as those of two key flavonoid genes, \textit{DFR} and \textit{ANS}, and PA-specific genes, \textit{ANR} and \textit{LAR1}, in young leaves from the three selected transgenic lines and S41 control lines. Line 3N had \textit{FaMYB1} mRNA steady-state levels that were 15 times higher than 4N, while the transgene expression was not detectable in 6N and, as expected, in leaves of control untransformed or \textit{GUS}-transformed plants (Supplementary Fig. S1B at JXB online). Consistent with the \textit{FaMYB1} expression data, leaves from 3N plants showed the most severe down-regulation of \textit{DFR} and \textit{ANS} levels (90% and 95% reduction with respect to the control plants, respectively) whereas 4N showed a less marked reduction (75% and 90%, respectively) (Supplementary Fig. S2A). A slight but not significant increase in the mRNA steady-state levels for both \textit{DFR} and \textit{ANS} mRNAs was revealed in plant 6N. Likewise, the expression levels of PA-specific genes \textit{ANR} and \textit{LAR1} were down-regulated in both 3N and 4N plants, with a more marked reduction in the former, whereas their levels were similar to the control in the 6N line (Supplementary Fig. S2B). Thus, the severity of PA depletion correlated with expression of the transgene. In contrast to leaves, no modifications of PA distribution in flowers were noted between wild-type and transgenic lines as per DMACA staining (data not shown).

\textbf{Depletion of PA in leaves co-segregates with the \textit{FaMYB1} transgene}

Seeds from free pollination under isolation were recovered from S41 mother plants and from \textit{FaMYB1} transgenic plants and plated in an antibiotic-free Murashige and Skoog medium with the aim of producing two different segregating populations and studying the variation of PA levels in non-transgenic progeny of S41 (hereinafter referred to as \textit{S}$_1$ plants) and the variation in PA levels in correlation with the presence and expression of the transgene in the progeny of \textit{FaMYB1} plants (hereinafter referred to as \textit{T}$_1$ plants). Being a clonal genotype of an outcrossing species, segregation for the levels of PAs in leaves is an expected result for progeny from S41 plants, regardless of the pollen source, due to the recombination of endogenous genes regulating PA biosynthesis.

Progeny from \textit{FaMYB1} (\textit{T}$_1$) and S41 control (\textit{S}$_1$) plants were grown side by side in the glasshouse along with untransformed S41 plants and were periodically screened for leaf PAs with DMACA. Within the progeny from

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Trifoliate leaf adaxial surface stained for PA-containing cells with DMACA: (A) recipient genotype S41; (B and C) \textit{T}$_0$ 3N genotype; (D, E, F) \textit{T}$_1$(+) genotypes 16, 17, and 21, respectively; (G, H, I) leaf section of the same \textit{T}$_1$(+) genotypes.}
\end{figure}
transgenic plants, the severity of PA depletion ranged from plants with only a moderate decrease of leaf PA-containing cells to plants where no PA-containing cells were observed in the lamina (Fig. 2D–F). In contrast, the reduction of leaf PA was less pronounced in the progeny from S41. For a deeper insight into the genetic factors underlying the variation in the levels of leaf PAs, PA levels were determined spectrophotometrically and these values were calculated in mg g\(^{-1}\) dry weight (DW).

T\(_1\) progeny 1, 13, 15, 16, 17, 20, and 21, and the S\(_1\) progeny 2, 7, 8, 9, 14, and 19 were selected as being representative of the different phenotypes recovered from FaMYB1 and S41 plants, respectively. Overall, the average levels of PAs noted in the FaMYB1 progeny were 3.74±0.03 mg g\(^{-1}\) DW compared with 8.28±0.07 mg g\(^{-1}\) DW for the progeny of S41, while the original S41 genotype had a value of 11.1±0.02 mg g\(^{-1}\) DW (Fig. 3). T\(_1\) line 16 showed the most dramatic reduction of PA in leaves (1.2±0.07 mg g\(^{-1}\) DW) with a decrease of ~90% of total leaf PAs, and in this line PA-containing cells were confined to the basal part of the main veins of each trifoliate leaf and around the main vein in the central leaf (Fig. 2D). Four additional T\(_1\) lines, namely 13, 17, 20, and 21, showed a pronounced quantitative reduction of PAs in leaves relative to the control, with average levels ranging from 2.13 mg g\(^{-1}\) DW to 3.94 mg g\(^{-1}\) DW (line 20). Photographs of typical phenotypes are shown in Fig. 2D and E. Interestingly, line 21 had leaves that phenotypically resembled those of the primary transformant 3N line, with suppression of PA cells noted in sectors of trifoliate leaves and sometimes within a single part of the compound leaf. As far as concerns S\(_1\) plants, progeny 19 displayed the most severe leaf PA reduction (4.69 mg g\(^{-1}\) DW).

Quantitative RT-PCR was then performed to estimate the number of copies of the transgene among the selected FaMYB1 progeny. T\(_1\) lines 13, 16, 17, 20, and 21 were determined to have one, three, four, two, and two FaMYB1 copies, respectively, whereas lines 1 and 15 contained no transgene copies, which was consistent with the absence of a severe PA reduction phenotype (data not shown). As a further analysis, genomic DNA from lines 1, 15, 16, 17, and 21 was restricted with \(XbaI\) and hybridized against the FaMYB1 probe. As expected, no signals were detected in lines 1 and 15 (data not shown), whereas lines 16 and 21 showed a single insertion and line 17 three insertions. As regards the T\(_1\) lines 16, 17, and 21, all lines shared a common band probably derived from a common parental line, namely line 3N, whereas the other two FaMYB1 copies present in line 17 were probably contributed by the parental 6N (Supplementary Fig. S1 at JXB online). Overall, T\(_1\) plants where the transgene has segregated out (i.e. lines 1 and 15) have been labelled as T\(_1(\sim)\) plants, whereas those retaining the transgene (i.e. plants 16, 17, 20, and 21) were labelled as T\(_1(\sim+\)).

FaMYB1 down-regulates both catechin and epicatechin biosynthetic routes to PAs

Since histochemical and molecular analyses showed that the presence of one or more copies of the FaMYB1 transgene correlated with the reduction of PAs in leaves of T\(_1\) lines; the expression of the transgene was evaluated by RT-PCR analysis in leaves from lines 1, 15, 16, 17, 20, and 21. As a control, the untransformed S41 line was also analysed. FaMYB1 expression was not detected in control plants or in lines where the transgene had segregated out [T\(_1(\sim)\) lines 1 and 15]. The highest steady-state levels of transgene mRNA were noted in RNA derived from T\(_1(\sim+)\) line 16, the line that consistently exhibited the greatest reduction in leaf PAs (Supplementary Fig. S3 at JXB online).

To gain insight into the biosynthetic step(s) affected by FaMYB1 expression, the steady-state levels of key structural genes of this metabolic pathway, namely CHS, DFR, ANS, ANR, LAR1, and LAR2, were evaluated by RT-PCR analysis on the same lines, using primers previously designed to amplify all the different members cloned thus far for each of these gene families in L. corniculatus (Paolocci et al., 2005, 2007). The steady-state levels of CHS mRNA were significantly increased in lines 16 and 17 relative to line S41. Conversely, a significant reduction of the CHS mRNA level was shown by lines 15 and 20 (Fig. 4A). DFR and ANS steady-state levels were significantly reduced in lines 16, 17, 20, and 21 relative to the control line (Fig. 4A), these being transgene-positive lines with the most extreme reductions in leaf PAs. All the T\(_1(\sim+)\) progeny showed a significant reduction in steady-state levels for ANR and LARI (Fig. 4B). As regards the expression of LAR2, whose contribution to the biosynthesis of PAs is still under debate (Paolocci et al., 2007), only line 16 showed a significant down-regulation relative to the control (Fig. 4B).

The relationships between FaMYB1, LcTT2, the orthologue of AtTT2 in L. corniculatus, and PA accumulation were investigated to test whether the expression of the transgene affected the expression of the endogenous MYB gene. In L. japonicus, TT2 is arranged as a small multigene family made up of three gene members (Yoshida et al., 2008, 2010). In L. corniculatus a TT2 gene (LcTT2) whose expression accounts for most of the leaf PA variability.

![Fig. 3](https://academic.oup.com/jxb/article-abstract/62/3/1189/479876/5384249) A bar graph of leaf PA content in populations of control and transgenic plants. S41, host genotype used in this study; S1, progeny of S41 recipient line; T1, progeny of T0 plants.
were observed between groups of T1 plants that do or do not express \( LcTT2 \) in leaves. No significant differences in the steady-state levels of this MYB activator gene.

**Analysis of flavonoid and phenolic end-products in \( FaMYB1 \) transgenics**

The profiles of methanol-soluble flavonoids were determined from leaves of S41 (parental genotype), \( T1(–)1 \) (null for the \( FaMYB1 \) transgene), \( T1(+)17 \) (extreme PA suppression phenotype), and \( T1(+)20 \) (moderate phenotype). Figure 5 shows UV chromatograms of methanol extracts denoting the peaks which were tentatively identified by their UV and MS spectra (Table 1) and by comparison with aglycone standards and the literature. The compounds fall into two categories, with UV spectra consistent with either hydroxycinnamic acids (Bengoechea *et al.*, 1995) in the case of peaks 2, 3, and 4, or flavonols (Mabry *et al.*, 1970) for peaks 1, 5, 6, 7, 8, and 9.

Only subtle differences in flavonoids and hydroxycinnamic acids were noted between extracts from each of the lines. Even in line \( T1(+)17 \), changes in PA levels were not accompanied by corresponding changes in specific flavonoid and phenolic acid end-products.

Compound 4 had a UV spectrum similar to that of ferulic acid. Negative ESI-MS\(^2\) analysis revealed fragment ions at m/z 367 ([M-H-CO\(_2\)]\(^{-}\)) and 325 ([M-H-86]\(^{-}\)), indicating loss of malonate (Kachlicki *et al.*, 2008). Another abundant fragment ion at m/z 193 corresponded to ferulic acid as shown by MS\(^3\) analysis (data not shown). MS\(^3\) of the m/z 325 ion also yielded ferulate ([M-H-malonyl-132]\(^{-}\)), with a mass loss of 132 characteristic of pentose sugars, as reviewed recently by Vukics and Guttman (2010). This suggests that compound 4 consists of ferulic acid linked to a malonated pentose sugar. The UV spectrum of compound 2 was similar to that of \( p \)-coumaric acid. The MS\(^2\) fragmentation pattern of the pseudomolecular ion m/z 381 was identical to that of compound 4, with \( p \)-coumarate (m/z 163) as core. It was therefore concluded that this compound is \( p \)-coumaric acid linked to a malonated pentose sugar.

Compound 3 was very similar to compound 2 with respect to both UV and MS spectra. MS\(^2\) of m/z 381 yielded an additional ion at m/z 191 ([M-H-CO\(_2\)-146]\(^{-}\)) which fragmented further to m/z 131 ([M-H-malonyl-coumaroyl]\(^{-}\)). These data suggest that compounds 2 and 3 are isomers.

The UV spectra of flavonols are characterized by two major absorption peaks (Mabry *et al.*, 1970). Compounds 1, 6, 8, and 9 had a band II absorption maximum at \( \sim 265 \) nm, indicative of kaempferol derivatives, whereas compounds 5 and 7 had a band II absorption maximum typical of quercetin derivatives (255 nm). Furthermore, all flavonol genes in these progeny lines. The positive trend between \( LcTT2 \) expression and PA accumulation in \( FaMYB1 \)-negative plants was confirmed by the positive correlation between the expression of \( TT2 \) and the expression levels of \( DFR, ANS, ANR, \) and \( LAR1 \), this last in particular being highly significant (\( r=0.97 \)). These results not only confirmed the role of \( LcTT2 \) in controlling PA accumulation in \( L. corniculatus \) leaves (F. Paolocci *et al.*, unpublished results), but also showed that \( FaMYB1 \) does not interfere with the steady-state levels of this MYB activator gene.

![Graph](https://example.com/graph.png)  
**Fig. 4.** Data plotted from quantitative RT-PCR analysis of selected \( T1 \) progeny. (A) Early pathway genes; \( CHS \) (chalcone synthase), \( DFR \) (dihydroflavonol 4-reductase), \( ANS \) (anthocyanidin synthase). (B) Late genes in the PA pathway; \( ANR \) (anthocyanin reductase), \( LAR1, LAR2 \) (leucoanthocyanidin reductases 1, 2). The significance (\( P \leq 0.05 \)) of steady-state transcript levels is indicated by lower case on the figure.

exhibited by different \( L. corniculatus \) genotypes has been recently cloned (F. Paolocci *et al.*, unpublished results). Thus, the steady-state level of \( TT2 \) in \( L. corniculatus \) was evaluated here using primers designed based on \( LcTT2 \) and on the three \( TT2 \) members present in \( L. japonicus \), a species phylogenetically close to \( L. corniculatus \), to have representative amplification of all possible \( TT2 \) genes co-expressed in leaves. No significant differences in \( TT2 \) expression were observed between groups of \( T1 \) plants that do or do not express \( FaMYB1 \) (0.051 versus 0.027) and no significant correlation between \( LcTT2 \) and \( FaMYB1 \) expression was observed (\( r=0.1 \)) (Supplementary Fig. S3 at JXB online).

The mRNA steady-state levels of \( LcTT2 \) in plants \( T1(–)15 \), selected as the plant that exhibited the extreme PA depletion within \( T1 \) plants where the transgene segregated out, and \( S19 \), as the transgene-negative line with the lowest PA levels, were markedly lower than in \( S41 \), the original clonal genotype (Supplementary Fig. S4 at JXB online). The reduction of \( TT2 \) expression and, probably, of other endogenous regulators, due to gene recombination, can explain the decrease in the mRNA levels of some structural
conjugates described here displayed a considerable hypsochromic shift in their band I absorption maximum, suggesting substitution of the 3-hydroxyl group (Mabry et al., 1970; Santos-Buelga et al., 2003). Negative ESI-MS analysis of these compounds essentially showed fragmentation patterns typical of O-glycosides (Vukics and Guttman, 2010). The pseudomolecular ions m/z 609 and 593 (compounds 5 and 6) showed mass losses typical of one hexose ([M-H-162]−) and one deoxyhexose unit ([M-H-146]−), with quercetin (m/z 301) and kaempferol (m/z 285) aglycones, respectively. The ratio of the fragments shows that these compounds are di-O-glycosides with the sugar units attached at two different positions rather than O-diglycosides (Ferreres et al., 2004). As indicated by the UV spectra, one of the sugar units is linked to the 3-OH group, a common glycosylation site in flavonols (Vukics and Guttman, 2010). However, in the absence of a range of glycosylated flavonol standards, more detailed conclusions about the distribution of the sugar units cannot be drawn. Similarly, compounds 7 (m/z 593) and 8 (m/z 577) lost one or two deoxyhexose units ([M-H-146]− and [M-H-292]−) and had quercetin and kaempferol as aglycones.

The UV spectrum of compound 9 was identical to that of compound 8. MS² analysis of the pseudomolecular ion at

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**Fig. 5.** Diode array HPLC profiles of extracts from *Lotus* leaves. The lines selected for analysis were S41 (untransformed clonal genotype), T₁(−)₁ (transgene negative), T₁(+)₁₇ (an example of the extreme phenotype), and T₁(+)₂₀ (an example of moderate PA suppression). Major peaks were further subjected to mass spectral analysis.

**Table 1.** UV and negative ESI-MSⁿ characteristics of phenolic compounds extracted from *Lotus corniculatus*

<table>
<thead>
<tr>
<th>Peak</th>
<th>Approximate retention time (min)</th>
<th>ƛ_max (nm)</th>
<th>[M-H]⁻ (m/z)</th>
<th>Main MS² fragment ions in negative mode (base peak in bold)</th>
<th>Main MS³ fragment ions of MS² base peak in negative mode (base peak in bold)</th>
<th>Tentative structure assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.9</td>
<td>265, 347</td>
<td>739</td>
<td>593 264/285, 429, 255, 447, 327</td>
<td>Kaempferol-O-deoxyhexose-O-deoxyhexose-hexoside</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>21.3</td>
<td>300 (sh), 313, 381</td>
<td>381</td>
<td>337, 295, 163, 277</td>
<td>Coumaroyl-O-pentoside malonate</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>22.0</td>
<td>310 381</td>
<td>337</td>
<td>295, 163, 277, 191</td>
<td>Coumaroyl-O-pentoside malonate</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>23.4</td>
<td>298 (sh), 326, 411</td>
<td>411</td>
<td>367, 325, 193, 307</td>
<td>Feruloyl-O-pentoside malonate</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>24.1</td>
<td>256, 353</td>
<td>609</td>
<td>447/446, 463, 301</td>
<td>Quercetin-O-deoxyhexose-O-hexoside</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>28.2</td>
<td>265, 346</td>
<td>593</td>
<td>447, 431/430, 285</td>
<td>Kaempferol-O-deoxyhexose-O-hexoside</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>28.4</td>
<td>256, 349</td>
<td>593</td>
<td>447/446, 301</td>
<td>Quercetin-O-deoxyhexose-O-deoxyhexoside</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>33.0</td>
<td>264, 342</td>
<td>577</td>
<td>431/430, 285</td>
<td>Kaempferol-O-deoxyhexose-O-deoxyhexose malonate</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>38.6</td>
<td>264, 342</td>
<td>663</td>
<td>619 431/430, 285, 473</td>
<td>Kaempferol-O-deoxyhexose-O-deoxyhexose malonate</td>
<td></td>
</tr>
</tbody>
</table>
m/z 663 yielded m/z 619 ([M-H-CO₂]–) which was fragmented further to m/z 431 ([M-H-malonyl-deoxyhexosyl]–), the aglycone kaempferol ([M-H-malonyl-deoxyhexosyl-deoxyhexosyl]–), and m/z 473 ([M-H-CO₂-deoxyhexosyl]–). Thus, this compound is assumed to be a malonated derivative of compound 8.

Initial fragmentation of compound 1 (m/z 739) resulted in a product ion m/z 593 ([M-H-deoxyhexosyl]–). Negative ESI-MS² analysis of this ion resulted in mass losses not only of 146 and 308 (deoxyhexose and deoxyhexose plus hexose) but also of 164, indicating a diglycoside with deoxyhexose as the terminal sugar unit (Vukics and Guttman, 2010). Similar compounds to the ones reported here were found by Suzuki et al. (2008) investigating the metabolic profile of L. japonicus extracts, including several malonated flavonol glycosides.

**Discussion**

The engineering of PA biosynthesis in the leaves of forage legumes requires a profound understanding of the complex network of regulatory and structural genes involved. It was previously shown that both the epicatechin and catechin branches of PAs are co-ordinately regulated by the same bHLH transcription factor in leaves of L. corniculatus (Paolocci et al., 2007). Here it is shown that FaMYB1, an anthocyanin repressor gene from strawberry, specifically down-regulates the PAs in the leaves of L. corniculatus. These observations have at least two major implications. First, they show that FaMYB1 can act as a dominant repressor of PA biosynthesis in vegetative tissues, suggesting the use of FaMYB1 as a candidate gene to control not only flavonol and anthocyanin biosynthesis but also PAs in crop species. Additionally, the data presented here lend further support to the notion that the catechin and epicatechin PA pathways in the leaves of forage legumes are strictly co-regulated.

The expression of FaMYB1 causes a consistent down-regulation of leaf PAs in both primary transgenic lines and their progeny

FaMYB1 was originally described as a negative regulator of anthocyanin and flavonol biosynthesis in Fragaria and tobacco (Aharoni et al., 2001). The present study shows that FaMYB1 can cause a dramatic and specific down-regulation of another class of flavonoids, PAs. The ectopic expression of this gene in L. corniculatus in fact triggered a dramatic and specific down-regulation of the levels of PAs in leaves of both primary transgenic lines and their progeny. Spectrophotometric, HPLC, and HPLC/MS analyses indicated that neither leaf flavonols nor other end-products of the flavonoid pathway were affected by the transgene. Quantitative analysis of T₁ progeny harbouring the transgene showed a PA reduction ranging from 65% to 90% with respect to the recipient genetic background that naturally committed high levels (>10 mg g⁻¹ DW) of these metabolites. This dramatic reduction was not noted in T₁ lines where the transgene had segregated away [T₁(-)] or among S₁ lines derived from S41 control plants. Therefore, the expression of FaMYB1 clearly correlated with the net reduction of leaf PAs. However, according to PA-specific staining, the expression of FaMYB1 in Lotus does not appear to interfere with floral PAs, which suggests either that a different PA-specific transcriptional network operates between leaves and flowers in legumes or that the level of ectopic expression of FaMYB1 does not reach the threshold level to compete effectively with endogenous activators that should be highly expressed in floral tissues.

The magnitude of the leaf PA depletion is indeed related to the level of expression of the transgene. In turn, quantitative RT-PCR results showed that the expression of FaMYB1 was paralleled by a reduction of the steady-state levels of mRNAs relative to the key genes of the PA pathway, namely DFR, ANR, and LAR₁, in addition to ANS, as previously reported (Aharoni et al., 2001). Interestingly, these results provide further evidence for the hypothesis that in Lotus spp. there is differential regulation between early (i.e. CHS) and late genes of the flavonoid/proanthocyanidin pathway (Robbins et al., 2003; Paolocci et al., 2005). The changes in CHS mRNA level are in fact independent of the presence and expression of the transgene, whereas its marked increase in T₁(+) plants 16 and 17 could be interpreted as a result of either gene recombination or re-routing of the phenylpropanoid pathway in lines where PAs are highly reduced, or both. Nevertheless, the quantitative RT-PCR data suggest that the ANS, ANR pathway to proanthocyanidins appears to be co-regulated with the LAR pathway. This finding runs contrary to the thesis that the ANR and LAR pathways in Lotus are regulated by different transcriptional mechanisms (Yoshida et al., 2008).

**Possible PA suppression mechanisms mediated by FaMYB1**

The present data show that the ectopic expression of a flavonoid repressor is sufficient to nearly silence the PA pathway in leaves of a PA-rich L. corniculatus genotype without altering either other flavonoid end-products in leaves or PA in flowers. An increasing body of evidence shows the presence of flavonoid MYB repressors operating within the MBW complex in Arabidopsis (Dubos et al., 2008; Matsui et al., 2008). The ternary complex controlling anthocyanin and PA biosynthesis in Arabidopsis is disrupted by AtMYBL2, a R3MYB gene that negatively regulates the accumulation of these secondary metabolites in seedlings and seed coat, respectively. Strikingly, MYBL2 interacts with the bHLH proteins TT8, GL3, and EGL3, and appears to participate in a feedback loop to controlling both seed coat development and the anthocyanin biosynthetic pathway, where it controls TT8 transcription (Dubos et al., 2008; Matsui et al., 2008). The presence of a putative EAR repressor motif and a second repressor element in AtMYBL2 suggests that this protein acts as a dominant
transcriptional repressor. Notably, a chimeric PAP1 (Production of Anthocyanin Pigments 1) repressor, in which the EAR motif repression domain from SUPERMAN was fused to PAP1, the gene encoding one of the master regulator of anthocyanins in *Arabidopsis*, suppressed in *Arabidopsis* transgenic lines the expression of four flavonoid biosynthetic genes, namely CHS, DFR, LDOX, and ANR, to a significant extent, and, although to a lesser extent, the expression of the endogenous regulators TT2, PAPI, and PAP2 (Production of Anthocyanin Pigments 2) (Matsui et al., 2004). As a result, these chimeric genes depressed the accumulation of anthocyanin in seedlings and PAs in developing siliques (Hiratsu et al., 2003; Matsui et al., 2004). Even more recently, it has been shown by Velten and colleagues (2010) that a single spontaneous mutation within the coding region of the *Arabidopsis* 35S::AtMYB90 (PAP1) transgene is sufficient to convert the R2R3MYB activator of plant-wide anthocyanin production to a dominant-negative allele that inhibits normal pigment production within tobacco petals. The data produced by these authors support a model in which the mutant myb transgene product acts as a competitive inhibitor of a native R2R3MYB activator.

It was proposed that the R2R3FaMYB1 protein may act as a transcriptional repressor (Aharoni et al., 2001). Specifically, this protein presents at the C-terminus the short ‘C2’ (LNL[D/E] L-[G/S]) motif, which contains the core sequence (LXXLX) present in the EAR repressor motif. The ‘C2’ motif is also present in the repressor AtMYB4 and other members of the MYB protein subgroup 4 (Kranz et al., 1998). In this scenario, among the possible FaMYB1 target genes there would be endogenous MYB activators. To test this hypothesis, quantitative RT-PCR analyses using RNAs from plants that express different levels of FaMYB1 and from plants where the transgene segregated away were performed to assay LcTT2 mRNA levels. These analyses did not show any correlation between FaMYB1 and LcTT2 expression. LcTT2 is an endogenous gene highly expressed in S41 leaf cells, but much less in progeny plants that displayed low leaf PA levels even if they do not harbour FaMYB1. In these plants, down-regulation of LcTT2 is accompanied by a marked down-regulation of the key structural genes DFR, ANS, ANR, and, significantly, LAR1. Altogether, while confirming the involvement of LcTT2 in PA synthesis in *L. corniculatus*, these results do not directly support any interaction between FaMYB1 and LcTT2 genes at the level of mRNA regulation. Thus, it can be concluded that FaMYB1 does not act as a transcriptional repressor of the endogenous PA activator *MYB* gene. Conversely, the present data led to the modelling of FaMYB1 and LcTT2 proteins competing for a common site within the PA-associated MBW transcription complex. In keeping with this model, it has been shown that FaMYB1 can specifically bind to a number of bHLH partners in *vitro* (Aharoni et al., 2001). Thus, the present results suggest that FaMYB1 by competing with MYB activator(s) for binding to the bHLH component(s) of the ternary protein complex moves this complex away from the promoters of the late biosynthetic genes of the flavonoid pathways, thereby dampening the biosynthesis of these pigments.

It is however conceivable that FaMYB1 mediates PA inhibition in leaves of *L. corniculatus* not only because it competes with endogenous MYB activator(s) for binding to bHLH partners, but also because it represses the transcription of endogenous bHLHs through its EAR motif. The cloning of orthologue(s) of *Arabidopsis thaliana* bHLH genes such as AtTT8 and GL3 in *L. corniculatus* is under the way to test this hypothesis.

**Engineering PAs into foliar tissues of tannin-free forage legumes: models and perspectives**

Ectopic expression of specific transcription factors has been a problematical strategy for engineering foliar PAs in clover and *Medicago* spp. (Xie et al., 2006; Peel et al., 2009). As such, a single report claimed the successful accumulation of a modest amount of leaf PA in transgenic alfalfa over-expressing the maize LcMYC/bHLH transcription factor and only after prolonged plant exposure to abiotic stress (Ray et al., 2003). Indeed, in maize, tissue-specific pigmentation is caused in many cases by the tissue-specific expression of alleles of the bHLH genes (Ludwig and Wessler, 1990). However, the ectopic expression of Sn, a positive regulator of anthocyanin in maize that codes for a bHLH protein that can interact with MYBs of the anthocyanin and PA pathways without the partnership of the WD40 component (Baudry et al., 2004), was found to be insufficient to induce de novo PA synthesis in alfalfa leaves (F. Damiani et al., unpublished results). These data suggest that neither bHLH nor WD40 are the limiting components of the PA transcriptional machinery in this plant species. On the other hand, Gruber et al. (1998) reported on the attainment of transposon-tagged *L. japonicus* lines exhibiting tannin and LAR activity in their leaves and envisaged the presence of a repressor gene responsible for a blockage in flavonoid biosynthesis in legumes such as *L. japonicus*, alfalfa, and white clover which do not express leaf tannins. In turn, Yoshida et al. (2008, 2010) have more recently observed that the master regulator of PA biosynthesis in *L. japonicus* (LjTT2a) is only slightly expressed in leaves and stems in this species. It is therefore conceivable that at least in this model legume the control of PA biosynthesis could be mainly exerted at the transcriptional level, although the hypothesis that endogenous repressors also act at the post-transcriptional level cannot be ruled out. Besides the R3 and R2R3MYB repressors of the flavonoid pathway such as AtMYBL2 and FaMYB1, members of the LBD (lateral organ boundary domain) gene family negatively affect, at least in *Arabidopsis*, PA and anthocyanin accumulation by repressing the transcription of the MYB genes *PAPI* and *PAP2* (Rubin et al., 2009).

On these grounds, it is speculated that the synthesis of leaf PAs in forage legumes depends upon the balance between transcriptional activators and repressors operating within the MBW complex, with the activator MYB
component acting as the major PA-limiting factor in leaves of Medicago, clover, and many Lotus spp.

It should therefore be practically relevant to design bio-
safe forage legumes based on the expression of activators to
compete effectively with endogenous repressors. Strikingly,
the ectopic expression of TT2 from Arabidopsis has very
recently proven to activate genes implicated in the bio-
synthesis, modification, and transport of epicatechins in
transgenic M. truncatula hairy roots (Peel et al., 2009; Zhao
and Dixon, 2009).

Supplementary data

Supplementary data are available at JXB online.

Figure S1. (A) Southern blot of three primary trans-
formed lines (T0) and selected transgene-positive examples
(16, 17, and 21) from the T1 generation. (B) Quantitative
RT-PCR analysis of the FaMYB1 gene in primary trans-
formed lines. Data are expressed as means (±SEM). The
expression level of FaMYB1 in the T04N line, arbitrarily set
to 1, was used as the calibrator.

Figure S2. Quantitative RT-PCR analysis relative to DFR
and ANS (A) and ANR and LAR1 (B) genes in control and
primary transformed lines (T0). Data are expressed as means (±SEM). The expression level of each gene in the
S41 line, arbitrarily set to 1, was used as the calibrator.

Figure S3. Quantitative RT-PCR analysis relative to
dominant TT2 and FaMYB1 genes in transgene-positive
[T1+] and transgene-negative [T1−] plants. Data are expressed as means (±SEM). Gene expression is reported
as the value calculated in arbitrary units obtained from the
formula: x = 2−(ΔΔCT), ΔΔCT being the difference between
the target gene and the reference gene (1zEF) threshold cycles.

Figure S4. PA levels and expression of the endogenous
TT2 in the S41 recipient genotype and in two examples of
transgene-negative plants from S1 and T1 populations. Data
are expressed as means (±SEM). The expression level of
TT2 in the S41 line, arbitrarily set to 7, was used as the calibrator.

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2013. Asse II ‘Occupabilita’ Obiettivo specifico ‘e’ – Asse IV
‘Capitale Umano ‘Obiettivo specifico ‘f’.

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