Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 66 (2005) 991–999
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

A comparison of two strategies to modify the hydroxylation of condensed tannin polymers in *Lotus corniculatus* L.

Mark P. Robbins *, Adrian D. Bavage, Gordon Allison, Teri Davies, Barbara Hauck, Phillip Morris

Plant Cell Biology Team, Institute of Grassland and Environmental Research, Aberystwyth Research Centre, Plas Gogerddan, Aberystwyth, Ceredigion SY23 3EB, United Kingdom

Received 20 January 2005; received in revised form 16 March 2005
Available online 5 May 2005

Abstract

A full-length sense *Antirrhinum majus* dihydroflavonol reductase (*DFR*) sequence was introduced into birdsfoot trefoil (*Lotus corniculatus* L.) in experiments aimed at modifying condensed tannin content and polymer hydroxylation in a predictable manner. Analysis of transgenic plants indicated lines that showed enhanced tannin content in leaf and stem tissues. In contrast to previous data from root cultures, levels of propelargonidin units were not markedly elevated in lines with enhanced tannin content. RT-PCR analysis of four selected lines indicated a correlation between enhanced tannin content and expression of the introduced *DFR* transgene.

Using a contrasting approach we introduced a flavonoid 3′5′ hydroxylase (*F*3′5′*H*) sequence derived from *Eustoma grandiflorum* into *Lotus* root cultures. Expression of the transgene was associated with increased levels of condensed tannins and in this case there was also no alteration in polymer hydroxylation. These results suggest that additional mechanisms may exist that control the hydroxylation state of condensed tannins in this model species.

Keywords: *Lotus corniculatus*; Leguminosae; Metabolic engineering; Condensed tannins; Proanthocyanidins

1. Introduction

The genetic control of the condensed tannin (CT) pathway in higher plants has been studied by a number of research groups in recent years, and has recently been reviewed (Dixon et al., 2005; Marles et al., 2003; Tanner, 2004). Significant progress has been made regarding the genetic analysis of this pathway in a range of species including barley and *Arabidopsis*. One of the interesting aspects of this area of metabolism is that early steps in this pathway are common both to anthocyanin and flavonoid biosynthesis.

Biosynthesis of CT polymers involves the conversion of dihydroflavonol intermediates [dihydrokaempferol (DHK), dihydroquercetin (DHQ) and dihydromyricetin (DHM)] which are converted into corresponding flavan 3,4-diols *syn*. 3,4-*cis* leucoanthocyanidins by the action of dihydroflavonol reductase (*DFR*). Hydroxylation on the B ring of these intermediates is controlled by the action of two closely related enzymes; flavonoid 3′ hydroxylase (*F*3′*H*) and flavonoid 3′5′ hydroxylase (*F*3′5′*H*) (Heller and Forkmann, 1988) and a representation of this metabolic grid is shown diagrammatically in Fig. 1.

The stereochemistry of initiating units within polymers is defined by two independent metabolic pathways. (+)-Catechin and related subunits are derived from the activity of leucoanthocyanidin reductase (Tanner et al., 2003) while (−)-epicatechins are biosynthesised by the

* Corresponding author. Tel.: +44 1970 823113; fax: +44 1970 823242.
E-mail address: mark.robbins@bbsrc.ac.uk (M.P. Robbins).

0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.03.017

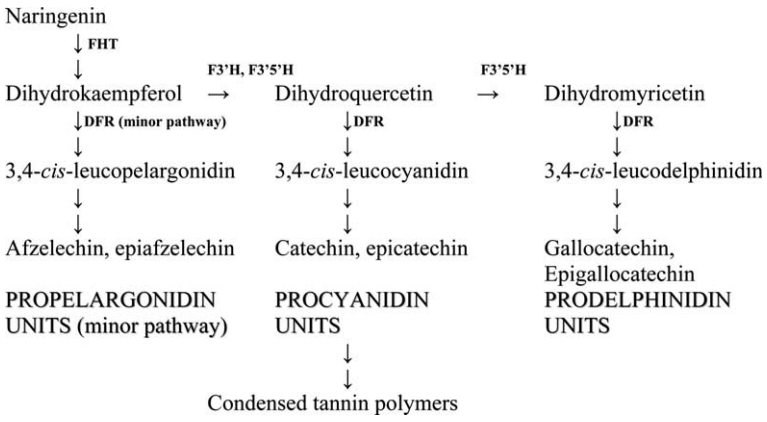

Fig. 1. A simplified version of the metabolic grid leading to the biosynthesis of CT polymers in *Lotus corniculatus*. Letters beside arrows indicate enzymes that convert pathway intermediates. **FHT**, flavanone 3-hydroxylase; **F3′H**, flavonoid 3′ hydroxylase; **F3′5′H**, flavonoid 3′5′ hydroxylase; **DFR**, dihydroflavonol 4-reductase. It should be noted that hydroxylation reactions may also occur at the flavanone level. Kaempferol and quercetin, the other major flavonoid end products in Lotus, are biosynthesised from dihydrokaempferol and dihydroquercetin, respectively, through the action of flavonol synthase.

concerted action of leucoanthocyanidin dioxygenase (Abrahams et al., 2003) and anthocyanidin reductase (Xie et al., 2003). Recent work on mutant analysis in *Arabidopsis* has elucidated aspects of CT polymerisation and has implicated a MATE transporter (Debeaujon et al., 2001) and a glutathione S-transferase (Kitamura et al., 2004) in the import of monomers prior to polymerisation in the vacuole.

One characteristic feature of CTs is that these molecules exist as mixed polymers made up of units with different levels of hydroxylation on the B-ring. Little is known about the genetic control of CT hydroxylation and the present study aims to address this question using transgenic technologies. Propelargonidin (PP), procyanidin (PC) and prodelphinidin (PD) units are a series with increasing levels of hydroxylation on the B-ring and this is of particular interest in forage crops where patterns of polymer hydroxylation have been reported to have significant effects upon ruminant nutrition (Min et al., 2003; Barahona et al., 2003).

Lotus corniculatus biosynthesises CT polymers with PC and PD units in a range of vegetative tissues including roots, stem, leaf and flower while PP units are only minor constituents in conventional germplasm. In view of the significant biomass that can be harvested from an individual transgenic plant, *L. corniculatus* has proved to be a popular model organism for the genetic engineering of the condensed tannin pathway in higher plants (e.g., Paolocci et al., 1999). There is considerable interest in increasing the condensed tannin content of *Lotus* and other forage legumes with a view to improving levels of protein protection in protein-rich, low-tannin crops fed to ruminant livestock.

Previous studies in this laboratory aimed at engineering CT structure, involved the ectopic expression of a dihydroflavonol reductase (*DFR*) sequence from *Antirrhinum majus* (Bavage et al., 1997). This transgene encodes a characterised enzyme activity utilising dihydrokaempferol (DHK) and dihydroquercetin (DHQ) substrates for the biosynthesis of propelargonidin and procyanidin subunits. High level expression of the *A. majus DFR* sequence resulted in enhanced levels of CT polymers in 'hairy root' cultures of *L. corniculatus* and in the most extreme example propelargonidin levels of up to 14% were noted.

In this current investigation, we have analysed a subset of transgenic lines expressing the *A. majus DFR* transgene with the aim of analysing phenotypes in regenerated *Lotus* plants. Additionally, employing an opposing strategy, we have introduced and expressed a *F3′5′H* cDNA sequence from *Eustoma grandiflorum*, which is predicted to increase polymer hydroxylation (Tanaka et al., 1998), and we report results derived from *Lotus* root cultures expressing the *E. grandiflorum F3′5′H* transgene.

2. Results

2.1. Molecular analysis of selected lines expressing *A. majus DFR* transgene

Northern analysis was insufficiently sensitive to detect transgene or endogenous gene expression in RNA extracted from *Lotus* leaves. However, reverse transcriptase (RT)-PCR did permit the analysis of transcripts in selected transgenic plants and this data is displayed in Fig. 2. When *DFR* primers specific for the *A. majus* transgene were used, no signal was noted in control lines but amplification products were noted in all four selected ADFR lines. Strong signals were noted in cDNA derived from leaves of ADFR02 and ADFR08. Ethidium bromide staining of RNA confirmed integrity of source RNA and also quantification of RNA prior to reverse

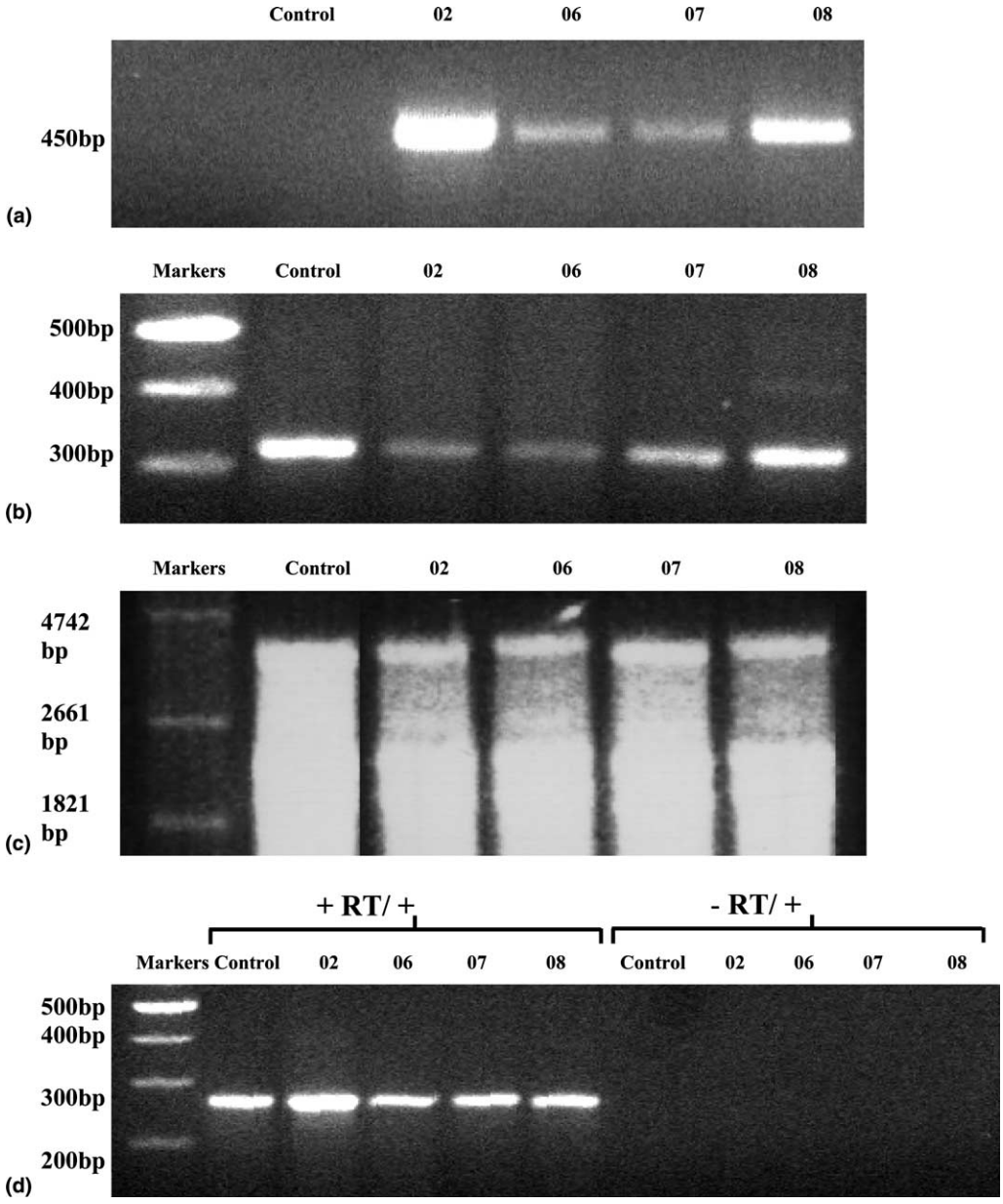

Fig. 2. Reverse transcriptase (RT)-PCR analysis of control and sense dihydroflavonol reductase lines. (a) RT-PCR of cDNA from shoot tips of a control line and lines ADFR02, 06, 07 and 08. Amplification for expression of *A. majus* DFR transgene; (b) as A but using primers specific for an endogenous Lotus DFR gene; (c) ethidium bromide staining of 10 μg of source RNA preparations; (d) RT-PCR of cDNA from leaves of control and sense dihydroflavonol reductase plants using actin primers [+RT, −RT; ± reverse transcriptase, respectively].

transcription. Actin amplifications were employed as an additional control. In actin amplifications, no signal was noted in the absence of reverse transcriptase which confirmed that amplification products derived from cDNA and not from contamination with genomic DNA.

In order to quantify the expression of endogenous *Lotus DFR* genes amplifications were carried using primers that amplify *DFRA* (Bavage et al., 1997). These primers are predicted to amplify the four additional members of the *DFR* gene family, *DFR1-4*, which have been cloned from the leaves of this *L. corniculatus* recipient genotype (Paolocci et al., 1999). Amplifications with these *Lotus DFR* primers showed expression of endogenous *DFR* genes in both control and sense *DFR* lines. Competitive PCR was then employed in order to make an estimate of the relative expression of *Lotus DFR* genes in control and sense *DFR* lines. In this analysis levels of endogenous *DFR* transcript were lower in all ADFR lines when compared with controls (data not shown). When combined with the RT-PCR for transgene expression, this data indicated that enhanced tannin levels in transgenic lines would derive from transgene expression rather than from an increase in endogenous *DFR* transcript.

2.2. Chemical analysis of lines expressing *A. majus* transgene

Chemical analysis was performed on the four selected lines described previously (ADFR02, ADFR06, ADFR07 and ADFR08) together with three control lines. The level of condensed tannins in leaves of control lines was 0.09% dry weight (Fig. 3). ADFR02 showed a clear enhancement of condensed tannin in leaf tissues with a value of 2.7% dry weight while ADFR06 and ADFR08 had values in excess of those noted in control leaves; 1.3% and 1.5% dry weight, respectively. Repeated analysis of the condensed tannin content of this plant material gave quantitatively consistent results. Levels of condensed tannins were also enhanced in stem tissues of ADFR02 (data not shown). Staining for cells containing condensed tannins using dimethylamino-cinnamaldehyde showed an increase in numbers of detectable CT-containing cells in the leaf blades of lines ADFR02 and ADFR08 (Fig. 4) and histochemical staining for GUS activity (Fig. 4(a)) confirmed that the CaMV promoter used to drive transgene expression in these experiments was active throughout the leaf in transgenic plants.

Flavonol levels were also determined in the leaves of selected lines (Fig. 5). Enzymatic hydrolysis confirmed that leaf flavonols were mainly kaempferol glycosides (ca. 98%) with the remainder being quercetin glycosides. Control levels were 6.7 ± 0.3% dry weight indicating that these end products are a significant product pool in *Lotus* leaves. Data suggested an enhancement of total flavonol levels in lines ADFR02 and ADFR08 which contained 7.9 ± 0.2% dry weight and 7.6 ± 0.35 dry weight, respectively. When leaf condensed tannin levels were plotted against total flavonol levels and subjected to regression analysis; an R^2 value of 0.70 was obtained which is indicative of a positive relationship between these two chemical parameters.

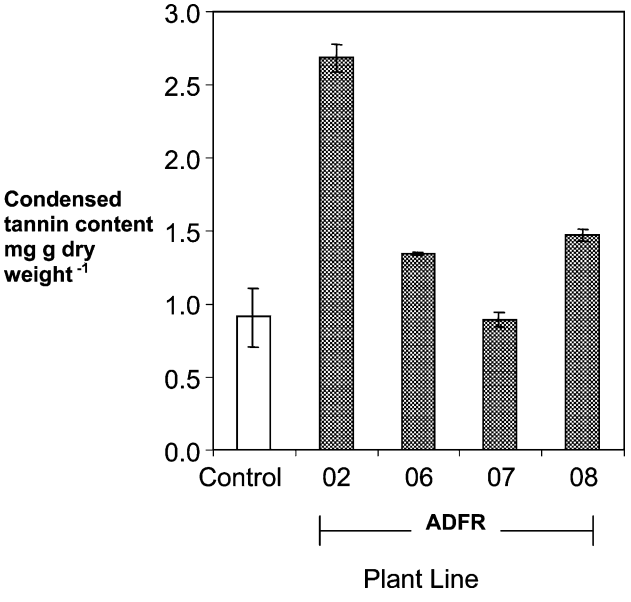

Fig. 3. Levels of condensed tannin in leaves of control and sense dihydroflavonol reductase lines. Values are means from duplicate determinations ± sem; control values are means from triplicate control lines ± sem.

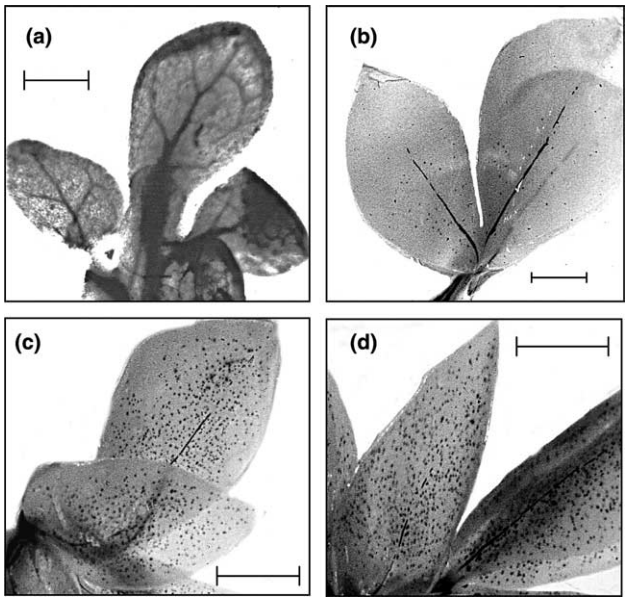

Fig. 4. Analysis of CaMV-sense dihydroflavonol reductase phenotypes in the leaves of transgenic Lotus lines. (a) histochemical staining for GUS enzyme activity in S50 genotype harbouring a CaMV-GUS construct; (b) DMACA staining for tannin containing cells in a control transformant; (c) DMACA staining of ADFR08; (d) DMACA staining of ADFR02. Size bar = 0.2 cm.

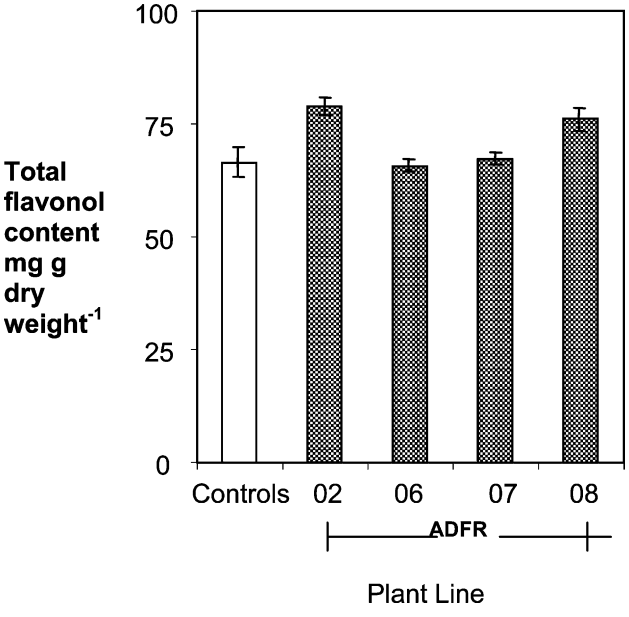

Fig. 5. Levels of flavonols (expressed as kaempferol-3-*O*-glucoside equivalents) in the leaves of control and sense dihydroflavonol reductase lines. Values are means from duplicate determinations ± sd; control values are means from triplicate control lines ± sem.

2.3. Condensed tannin hydroxylation in plants expressing *A. majus* transgene

In order to analyse hydroxylation levels of condensed tannins in control and transgenic lines methods based upon polymer hydrolysis were employed. The results of this polymer hydroxylation analysis are shown in Table 1. PC: PD: PP ratios for meaned control plants were 45:53:2. When ADFR lines were analysed and subjected to paired contingency χ^2 analysis only one sense *DFR* line had a significant difference in PC:PD:PP ratios when compared with meaned controls. This line, ADFR08, had a high PC:PD ratio that was significant at the 5% level.

2.4. Molecular analysis of selected lines expressing *E. grandiflorum F3′5′H* transgene

For studies relating to the over expression of a flavonoid 3′5′hydroxylase sequence, an authentic full-length sequence from *E. grandiflorum* was introduced and expressed in *L. corniculatus* genotype S50 using well established methodologies (Bavage et al., 1997).

We report here the analysis of four lines, one being a control line and three co-transformed lines LIS32, LIS38 and LIS39 with differing levels of expression of the introduced *F3′5′H* transgene (Fig. 6(a)). All lines were analysed as root cultures and two of the lines, LIS38 and39, showed enhanced levels of condensed tannins relative to control root cultures. In these experiments enhanced levels of condensed tannin occurred in the two lines with the highest steady state transcript levels of the introduced transgene, while LIS32 had CT levels lower than those found in controls.

In view of the increases in CT levels in two of these lines we hypothesised that endogenous *Lotus* genes in this metabolic sequence might also be up-regulated in these transgenic root cultures. In the absence of a cloned *F3′5′H* sequence we used competitive reverse transcriptase PCR to determine steady state transcript levels of adjacent genes in this metabolic pathway, i.e., flavanone 3-hydroxylase (*FHT*), flavonoid 3′hydroxylase (*F3′H*) and dihydroflavonol reductase (*DFR*). This data is presented in Fig. 6 and no obvious correlation was noted between transgene expression, CT levels and the measured levels of *FHT*, *F3′H* and *DFR* expression in these lines.

Table 1
Hydroxylation analysis of condensed tannins in leaves of selected *Lotus* lines harbouring *A. majus DFR* transgene

Line	Procyanidin (%)	Prodelphinidin (%)	Propelargonidin (%)	*P*
Controls	44.9 ± 4.9	53.5 ± 5.3	1.7 ± 0.6	–
ADFR02	56.4 ± 0.8	42.0 ± 1.1	1.6 ± 0.3	–
ADFR06	54.9 ± 0.9	44.1 ± 0.9	1.0 ± 0.4	–
ADFR07	47.9 ± 0.5	51.2 ± 0.5	0.9 ± 0.0	–
ADFR08	57.8 ± 0.1	40.8 ± 0.2	1.4 ± 0.2	*

Values are means of percentage determinations ± sem from duplicate analyses. Paired contingency χ^2 testing was performed against mean ratios derived from three independent control lines. –, not significantly different.

* $P < 0.05$.

2.5. Condensed tannin hydroxylation in lines expressing *E. grandiflorum* transgene

As discussed previously the predicted phenotype of *Lotus* root cultures over expressing a *E. grandiflorum F3′5′H* sequence should be an increase in CT polymer hydroxylation. These analyses were performed by direct hydrolysis of root tissues and subsequent quantification of anthocyanins. Control values for PC:PD:PP ratios were 81:14:5 which are typical for *Lotus* root cultures (Bavage et al., 1997) and correspond to lower levels of polymer hydroxylation than those in *Lotus* leaves (Table 1, this study; Foo et al., 1996).

In lines LIS38 and LIN39, enhanced CT levels were not accompanied by significant increases in polymer hydroxylation (Table 2). However, LIS 32 a co-transformed line with lower CT levels than controls and unusual growth kinetics had PC:PD:PP ratios that were significantly different from those found in controls.

3. Discussion

We can draw some general conclusions from the experiments outlined in this study. With regard to ADFR plants, expression of the *A. majus DFR* transgene resulted in an enhancement of CT content. Endogenous levels of *DFR* transcript were not elevated in any of the lines studied which implies that elevation of CT levels was likely to result directly from the expression of the transgene.

No obvious change in polymer hydroxylation was detectable in these lines. The most obvious explanation is that competing F3′H and F3′5′H enzymatic activities counteract any routing towards PP and PC pathways in these leaf tissues, which in any case, biosynthesise CTs with relatively high levels of polymer hydroxylation. Alternatively, the *A. majus DFR* gene might not have a significant dihydrokaempferol reductase activity although data from the *eosinea* mutant (Geissmann et al., 1954) and other transgenic and inhibitor experiments (Schwinn, unpublished) imply that dihydrokaempferol is a suitable substrate for the enzyme encoded by this transgene. Another possibility is that in *Lotus* leaves, which biosynthesise high levels of kaempferol, high activities of flavonol synthase may compete with the introduced dihydrokaempferol reductase activity for dihydrokaempferol intermediates. Also,

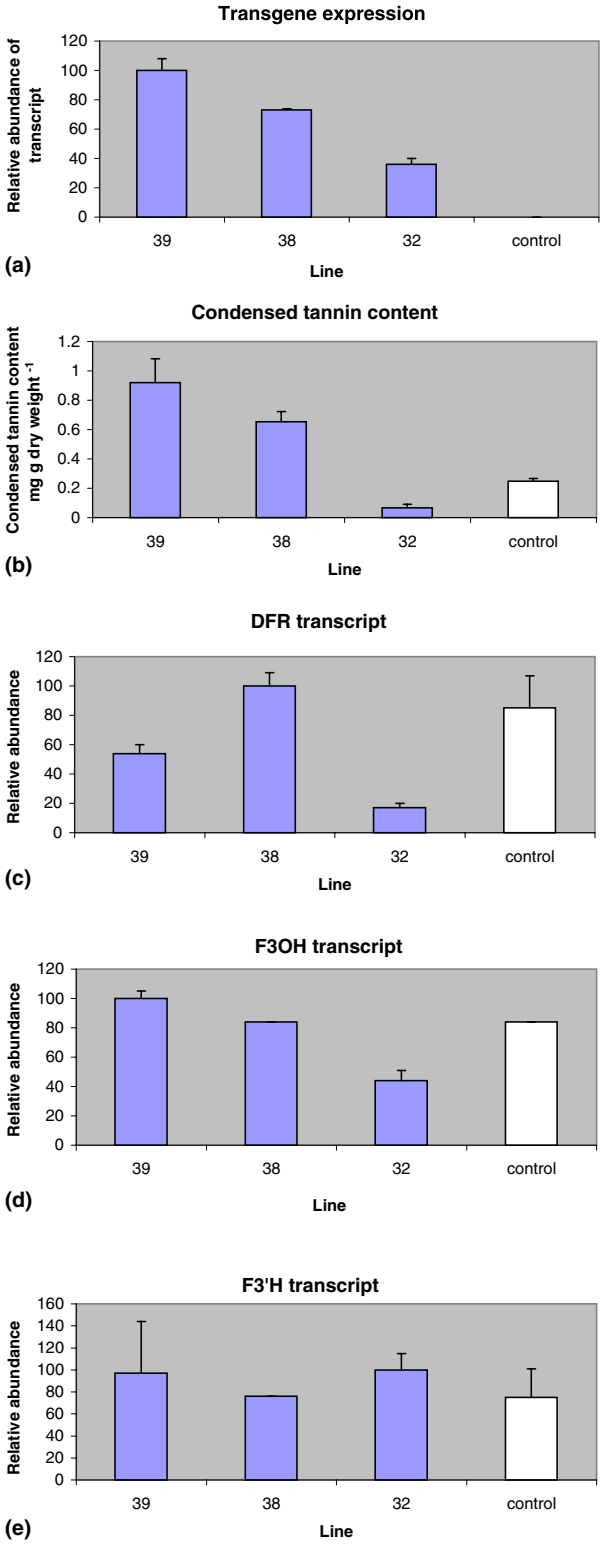

Fig. 6. Analysis of condensed tannin and transcript abundance in LIS3′5′H transgenic and control *L. corniculatus* root cultures: (a) relative abundance of transgene transcript; (b) abundance of CT. C-E, Relative abundance of transcripts from; (c) dihydroflavonol 4-reductase; (d) flavanone 3-hydroxylase; (e) flavonoid 3′-hydroxylase. Data in (a) and (c)–(e) normalised to levels of endogenous β-actin cDNA and expressed relative to highest transcript level (100%) in a given data set.

it is possible that dihydrokaempferol is not a metabolic intermediate in leaf cells that biosynthesise condensed tannins owing to carbon being routed through chalcone synthase using caffeoyl–CoA precursors rather than the conventional coumaroyl–CoA substrate (Heller and Forkmann, 1988). This hypothesis could be tested by

Table 2
Hydroxylation analysis of condensed tannins in leaves of selected *Lotus* lines harbouring *E. grandiflorum F3′5′H* transgene

Line	Procyanidin (%)	Prodelphinidin (%)	Propelargonidin (%)	*P*
Controls	80.7±	13.8±	5.5±	–
LIS32	68.0±5.6	28.3±5.9	3.7±0.3	***
LIS38	81.1±1.4	12.5±1.9	6.4±0.5	–
LIS39	78.2±0.6	16.1±0.8	5.7±0.5	–

Values are means of percentage determinations ± sem from duplicate analyses. Paired contingency χ^2 testing was performed against mean ratios derived from three independent control lines. –, not significantly different.
*** $P < 0.001$.

attempting to measure the levels of dihydrokaempferol intermediates in *Lotus* leaf tissues and comparing with levels in *Lotus* root cultures where the *A. majus DFR* transgene does appear to re-route carbon into the PP pathway. Finally, one cannot exclude the possibility that the metabolic channelling of intermediates involved in the synthesis of condensed tannins may make the predictive engineering of this pathway far from straightforward.

As regards the introduction and expression of an *E. grandiflorum F3′5′H* transgene in *Lotus* root cultures, this intervention also caused an increase in CT content as a result of transgene expression. Whilst one of the lines under study (LIS32) showed significant alterations in patterns of CT hydroxylation, we found no evidence correlating expression of the *F3′5′H* transgene with altered PC: PD: PP ratios in these experiments. In order to test for genetic compensation, RT-PCR technology was employed to monitor the transcript levels of genes that precede *F3′5′H* in this metabolic sequence (*FHT* and *F3′H*) and a gene that lies beyond this enzymatic step in CT biosynthesis (*DFR*). No obvious compensatory changes were noted at the transcript level, which might suggest that competing enzyme activities may be nullifying the effect of the predicted F3′5′H activity in these lines. We also note that a cytochrome *b5* activity has been reported as being necessary for the full activity of F3′5′H in experiments aimed at the transgenic modification of flower colour (De Vetten et al., 1999) and this activity may be present at low levels in *Lotus* root cultures.

In conclusion, in *L. corniculatus* we have not been able to produce wide-ranging changes in polymer htdroxylation by the expression of two well-characterised plant genes involved in low and high hydroxylation pathways, respectively. This is in contrast to experiments with lignin biosynthesis where transgenic interventions frequently result in changes in lignin structure but more infrequently cause changes in lignin content (Anterola and Lewis, 2002). Questions still remain about mechanisms that control of CT hydroxylation in leguminous species. There may be additional, currently uncharacterised genes, which control flux through this metabolic grid. An additional factor that impacts upon these experiments is the observation that predicting phenotypes in a metabolic grid formed by F3′H, F3′5H, FHT and DFR may be rendered difficult if there are tissue-specific isoenzymic forms of the above enzymes, some of which may be present exclusively in specific cells which accumulate condensed tannins. Future work may focus upon the phenotypic screening of mutant populations of *Lotus japonicus* with options for Targeting Induced Local Lesions IN Genomes (TILLING) and the cloning of candidate genes (Perry et al., 2003; Webb et al., 2004).

4. Experimental

4.1. Plant material and growth conditions

Lotus lines harbouring the *A. majus* dihydroflavonol reductase gene have been previously described in Bavage et al. (1997). For this study a subset of these lines, prescreened for CT phenotypes were propagated by vegetative cuttings and triplicate plants were established in John Innes No1/Levingtons 50:50 medium. Plants were glasshouse grown at IGER and CSIRO Canberra and analyses were performed on 20 cm regrowth after initial pruning.

Lotus root cultures harbouring a *F3′5′H* gene from *E. grandiflorum* were produced by introducing pLN60 into *Lotus* recipient genotype S50 using standard methodologies (Bavage et al., 1997). Southern analysis confirmed co-transformation and *Xba*1 digests indicated copy numbers of one or two for each transgenic line (data not shown). Sterile root cultures were cultured as Bavage et al. (1997) and root material was analysed after five subcultures.

4.2. Transcript analysis

Northern analysis was insufficiently sensitive to detect the expression of transgene or flavonoid pathway genes in these experiments. Therefore, all transcript estimations were performed using RT-PCR. Total RNA was isolated from *Lotus* leaves and root cultures using the method of Chang et al. (1993). cDNA synthesis was performed according to protocols outlined by GIBCO BRL after pre-treatment of RNA preparations with DNAase1 and reverse transcription was quality assessed by amplification using primers to an endogenous beta actin gene. Primer sequences for the amplification of *DFR* and *F3′5′H* transgenes and also *Lotus* beta actin, *F3H*, *F3′H* and *DFR* genes can be supplied on request to the authors.

For the data in Fig. 6, transcript measurements were estimated using semi-quantitative RT-PCR (Allison and

Shirazi-Beechey, 1997). Actin competimers were engineered by amplification of the cloned *L. corniculatus* beta actin cDNA (AF308733, AF308734) with a primer pair specifically designed to introduce a deletion in the amplified product. After band purification and quantification this competimer was used for quantification of beta actin in cDNA preparations. PCR reactions were set up containing 5 μl portions of cDNA together with actin primers and known quantities of competimer. The abundance of the amplimer bands was determined by digital analysis (Phoretix 1D) and used to calculate the corresponding abundance of beta actin template in cDNA preparations. Template abundance could be consistently estimated over 30–40 PCR cycles (data not shown). A normalisation factor was calculated for each cDNA preparation in this study to correct for differences in beta actin template.

Transcript levels for the *E. grandiflorum F3′5′H* and *L. corniculatus F3H*, *F3′H* and *DFR* were determined in a similar manner using competimers designed from the *F3′5′H* transgene and endogenous *DFR* (AF307300), *F3H* (AF308855) and *F3′H* (AF430127) sequences cloned in this laboratory.

4.3. Chemical analyses

Levels of condensed tannins were determined based upon their conversion to anthocyanidins on acid hydrolysis. Determinations on root cultures used the method of Colliver et al. (1997) while values were determined in freeze-dried leaf (and stem) samples using a modification of the method of Terrill et al. (1992) as described by Carter et al. (1999).

For the analysis of CT hydroxylation, constituent proanthocyanidins units were identified and quantified by HPLC analysis of butanol–HCl hydrolysates. Anthocyanidins were analysed following the removal of butanol under vacuum at 40 °C using the method of Bavage et al. (1997) except that HPLC was carried out with linear methanol:acetic acid (5%) gradient from 30–70% methanol in 20 min at a flow rate of 2 ml min^{-2}.

Methanol soluble flavonoid glycosides and aglycones were analysed by HPLC with photo-diode array detection essentially as described by Robbins et al. (1998). Enzymatic hydrolysis confirmed that the flavonols in *Lotus* leaves were mainly kaempferol glycosides (ca. 98%). Seven major glycosides were noted in unhydrolysed leaf extracts and relative proportions were similar in control and ADFR lines. Flavonol levels were determined by summing the quantities of flavonol glycosides, assuming identical extinction coefficients and expressing results as kaempferol-3-*O*-glucoside equivalents.

Histochemical localisation of condensed tannin containing cells was performed by staining fresh tissues with 4-dimethylamino-cinnamaldehyde–HCl (DMACA) as described by Li et al. (1996), except that chlorophyll was removed from foliar tissues by overnight treatment in methanol prior to histochemical staining.

Acknowledgements

IGER is funded by competitive strategic grant from BBSRC. ADB acknowledges funding from the BBSRC-PMB2 initiative and GA was funded by a European Union Grant FAIR CT 98-4068 (Compounds And Genes for Enhanced protein assimilation and Digestibility of forage legumes). Thanks are also extended to past and present members of the laboratory and also Dr. R. Evans. Some of the growth of plant material in this study was performed at CSIRO, Canberra and we would particularly like to thank Dr. G. Tanner and colleagues for assistance with these studies. The plant transformation vector pLN60 was donated by Dr. K. Davies of the Lewin Research Centre, NZ and this sequence has been used for research in compliance with the terms and conditions of limited licence agreement LL9607 existing between Florigene Ltd. (Victoria, Australia) and IGER.

References

Abrahams, S., Lee, E., Walker, A.R., Tanner, G.J., Larkin, P.J., Ashton, A.R., 2003. The *Arabidopsis TDS4* gene encodes leucoanthocyanidin dioxygenase (LDOX) and is essential for proanthocyanidin synthesis and vacuole development. The Plant Journal 35, 624–636.

Allison, G.G., Shirazi-Beechey, S., 1997. Measurement of ovine SGLT1 mRNA levels by competitive RT-PCR. Biochemical Society Transactions 25, 481S.

Anterola, A.M., Lewis, N.G., 2002. Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. Phytochemistry 61, 221–294.

Barahona, R., Lascano, C.E., Narvaez, N., Owen, E., Morris, P., Theodorou, M.K., 2003. In vitro degradability of tropical legumes. Journal of the Science of Food and Agriculture 83, 1256–1266.

Bavage, A.D., Davies, I.G., Robbins, M.P., Morris, P., 1997. Expression of an *Antirrhinum* dihydroflavonol reductase gene results in changes in condensed tannin structure and accumulation in root cultures of *Lotus corniculatus* (birds foot trefoil). Plant Molecular Biology 35, 443–458.

Carter, E.B., Theodorou, M.K., Morris, P., 1999. Responses of *Lotus corniculatus* to environmental change. 2. Effect of elevated CO2, temperature and drought on tissue digestion in relation to condensed tannin and carbohydrate accumulation. Journal of the Science of Food and Agriculture 79, 1431–1440.

Chang, S., Puryear, J., Cairney, J., 1993. A simple and efficient method for isolating RNA from pine needles. Plant Molecular Biology Reporter 11, 113–116.

Colliver, S.P., Morris, P., Robbins, M.P., 1997. Differential modification of flavonoid and isoflavonoid biosynthesis with an antisense chalcone synthase construct in transgenic *Lotus corniculatus*. Plant Molecular Biology 35, 509–522.

Debeaujon, I., Peeters, A.J.M., Léon-Kloosterziel, K.M., Koornneef, M., 2001. The *TRANSPARENT TESTA12* gene of *Arabidopsis* encodes a multidrug secondary transporter-like protein required

for flavonoid sequestration in vacuoles of the seed coat endothelium. Plant Cell 13, 853–871.

De Vetten, N., Horst, J.T., Van Schaik, H.P., Boer, A.D., Mol, J., Koes, R., 1999. A cytochrome *b5* is required for full activity of flavonoid 3′5′-hydroxylase, a cytochrome *p*450 involved in the formation of blue flower colours. Proceedings of the National Academy of Sciences 96, 778–783.

Dixon, R.A., Xie, D.-Y., Sharma, S.B., 2005. Proanthocyanidins – a final frontier in flavonoid research? New Phytologist 165, 9–28.

Foo, L.Y., Newman, R., Waghorn, G., McNabb, W.C., Ulyatt, M.J., 1996. Proanthocyanidins from *Lotus corniculatus*. Phytochemistry 41, 617–624.

Geissmann, T.A., Jorgensen, E.C., Johnson, B.L., 1954. The chemistry of flower pigmentation in *Antirrhinum majus*. Color genotypes. 1. The flavonoid components of the homozygous P, M, Y color types. Archives of Biochemistry and Biophysics 49, 368–388.

Heller, W., Forkmann, G., 1988. Biosynthesis of flavonoids. In: Harborne, J.B. (Ed.), The Flavonoids: Advances in Research Since 1980. Chapman and Hall, London, pp. 399–425.

Kitamura, S., Shikazono, N., Tanaka, A., 2004. *TRANSPARENT TESTA 19* is involved in the accumulation of both anthocyanins and proanthocyanidins in *Arabidopsis*. The Plant Journal 37, 104–114.

Li, Y.G., Tanner, G., Larkin, P., 1996. The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. Journal of the Science of Food and Agriculture 70, 89–101.

Marles, M.A., Ray, H., Gruber, M.Y., 2003. New perspectives on proanthocyanidin biochemistry and molecular regulation. Phytochemistry 64, 367–383.

Min, B.R., Barry, T.N., Attwood, G.T., McNabb, W.C., 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Animal Feed Science and Technology 106, 3–19.

Paolocci, F., Capucci, R., Arcioni, S., Damiani, F., 1999. Birdsfoot trefoil: a model system for studying the synthesis of condensed tannins. In: Gross, G.G., Hemingway, R.W., Yoshida, T. (Eds.), Plant Polyphenols 2. Kluwer Academic/Plenum Publishers, New York, pp. 343–356.

Perry, J.A., Wang, T.L., Welham, T.J., Gardner, S., Pike, J.M., Yoshida, S., Parniske, M., 2003. A TILLING reverse genetics tool and a web-accessible collection of mutants of the legume *Lotus japonicus*. Plant Physiology 131, 866–871.

Robbins, M.P., Bavage, A.D., Strudwicke, C., Morris, P., 1998. Genetic manipulation of condensed tannins in higher plants. 2. Analysis of birdsfoot trefoil plants harboring antisense dihydroflavonol reductase constructs. Plant Physiology 116, 1133–1144.

Tanaka, Y., Tsuda, S., Kasumi, T., 1998. Metabolic engineering to modify flower colour. Plant Cell Physiology 39, 1199–2126.

Tanner, G.J., 2004. Condensed tannins. In: Davies, K. (Ed.), Plant Pigments and Their Manipulation. Blackwell, Oxford, pp. 150–184.

Tanner, G.J., Francki, K.T., Abrahams, S., Watson, J.W., Larkin, P.J., Ashton, A.R., 2003. Proanthocyanidin Biosynthesis in Plants. Purification of legume leucoanthocyanidin reductase and molecular cloning of its cDNA. Biological Chemistry 278, 31647–31656.

Terrill, T.H., Douglas, G.B., Foote, A.G., Purchas, R.W., Wilson, G.F., Barry, T.N., 1992. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. Journal of the Science of Food and Agriculture 58, 321–329.

Webb, K.J., Robbins, M.P., Wang, T.L., Parniske, M., Meignez, A., 2004. EMS mutagenesis in *Lotus japonicus*. Lotus Handbook. Kluwer Academic Publishers.

Xie, D.-Y., Sharma, S.B., Paiva, N.L., Ferreira, D., Dixon, R.A., 2003. Role of Anthocyanidin Reductase, Encoded by BANYULS in Plant Flavonoid Biosynthesis Science 299, 396–399.