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Genetic modification of condensed tannin biosynthesis in *Lotus corniculatus*. 1. Heterologous antisense dihydroflavonol reductase down-regulates tannin accumulation in "hairy root" cultures

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Abstract. An antisense dihydroflavonol reductase (DFR) gene-construct made using the cDNA for DFR from *Antirrhinum majus* was introduced into the genome of a series of clonal genotypes of *Lotus corniculatus* via *Agrobacterium rhizogenes*. After initial screening, 17 antisense and 11 control transformation events were analysed and tannin levels found to be reduced in antisense root cultures. The effect of this antisense construct, (pMAJ2), which consisted of the 5′ half of the DFR cDNA sequence, was compared in three different recipient *Lotus* genotypes. This construct effectively down-regulated tannin biosynthesis in two of the recepient genotypes (s33 and s50); however, this construct was relatively ineffective in a third genotype (s41) which accumulated high levels of condensed tannins in derived transgenic root cultures. Four pMAJ2 antisense and three control lines derived from clonal genotypes s33 and s50 were selected and studied in greater detail. The antisense DFR construct was found to be integrated into the genome of the antisense "hairy root" cultures, and the antisense RNA was shown to be expressed. Tannin levels were much lower in antisense roots compared to the controls and this reduction in tannin levels was accompanied by a change in condensed tannin subunit composition.

Key words: *Lotus corniculatus* – Condensed tannins – Antisense RNA – *Agrobacterium rhizogenes*

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Introduction

Condensed tannins (proanthocyanidins) are polymeric phenylpropanoid compounds which accumulate in various tissues of many plant species. They are polymers of flavan-3,4-diols (catechin-4-ols), typically joined by 4 → 8 interflavan bonds, with flavan-3-ols (catechins) attached to the 4′ terminal end of the chains. Both the tannin monomers are C15 flavonoid based and are derived from naringenin chalcone via the phenylpropanoid pathway (Fig. 1). Flavan-3,4-diols are common intermediates in the biosynthesis of both condensed tannins and anthocyanins, and their biosynthesis is well documented (Doone et al. 1991; Tunen and Mol 1991). The enzymatic conversion of flavan-3,4-diols to flavon-3-ols was described by Stafford and Lester (1985). A number of models for the condensation of the tannin subunits have been proposed (Haslam 1989; Stafford 1989) but the exact nature of the condensation process has not been determined.

Tannins are of agronomic significance because many plant species that are used as animal feeds contain tannins in their vegetative tissue, and the ability of these tannins to bind dietary protein can have profound effects on animal nutrition. In ruminants, tannins have been shown to be effective anti-bloat agents (Reid et al. 1974). This property is thought to be due to reduced foaming of the rumen as a result of binding of the tannins to protein causing an effective decrease in rumen protein concentration. Tannins have also been shown to act as protein protectants, reducing bacterial deamination in the rumen (Reid et al. 1974). In non-ruminant animals, dietary tannin can lead to reduced nitrogen uptake and weight loss (Griffiths 1989). Condensed tannins have also been implicated in fungal pathogen resistance (Brownlee et al. 1992) and as insect

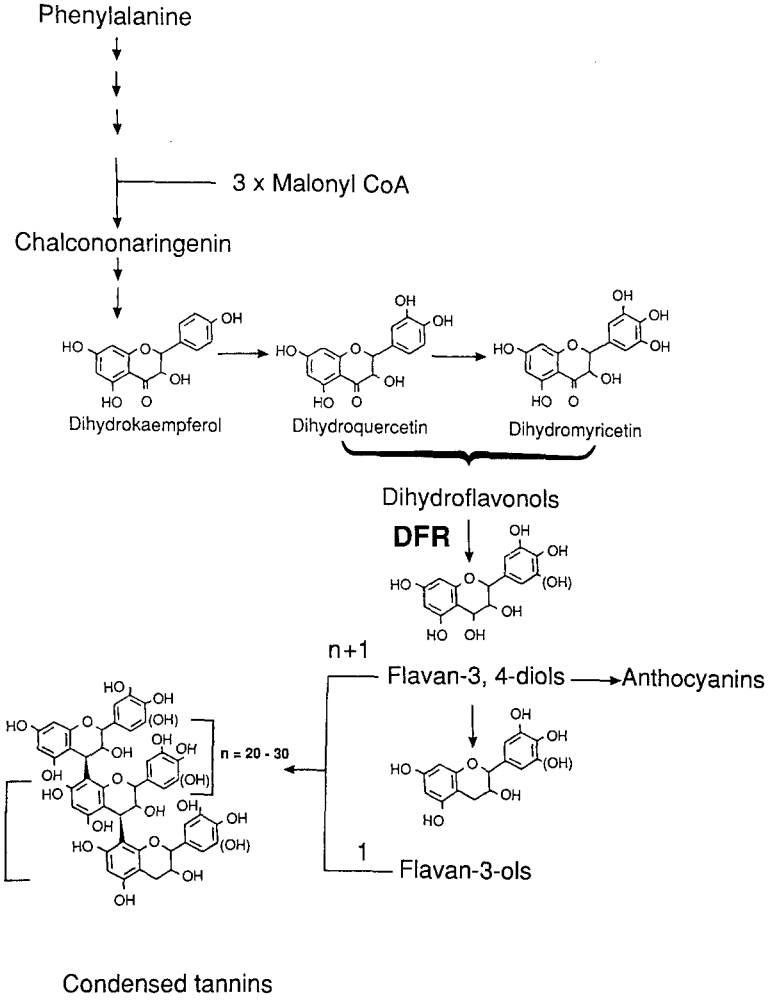

Fig. 1. Pathway for the biosynthesis of condensed tannins in *Lotus corniculatus*. The enzyme dihydroflavonol reductase (*DFR*) is marked

antifeedants (Scalbert and Haslam 1987). The major forage legumes of European and U.S. agriculture, *Trifolium repens* (white clover) and *Medicago sativa* (alfalfa), do not contain tannins in their vegetative tissue whereas some of the lesser grown forage legumes, *Lotus corniculatus* (birds foot trefoil) and *Onobrychis viciifolia* (sainfoin), do accumulate tannins in these tissues.

L. corniculatus has a number of features that make it suitable as a model organism in the molecular study of condensed tannin accumulation. It produces polymeric, mostly insoluble, tannins in its stems, roots, leaves, flowers and seeds (Morris and Robbins 1992), at levels as high as 10% dry weight. *L. corniculatus* is also very amenable to genetic transformation and tissue culture. *Agrobacterium rhizogenes* will readily infect wounded *L. corniculatus* tissue and produce "hairy roots". These roots can be cultured on solid or liquid growth media, and can be propagated with regular subculture for many years. The root cultures will spontaneously produce shoots when illuminated, and these can be propagated as shoot cultures (Morris and Robbins 1992), or regenerated into plants which are phenotypically similar to non-transformed plants (Webb et al. 1990).

A. rhizogenes-transformed root cultures of *L. corniculatus* have already proven to be useful tools in the study of condensed tannin biosynthesis. Morris and Robbins (1992) showed that root cultures produce tannins in a similar manner to non-transformed roots and that the tannins are accumulated in specific "tannin cells". Additionally, they have shown that tannin accumulation could be modified by certain plant growth regulators. *A. rhizogenes*-transformed *L. corniculatus* has also been used in studies of phenylpropanoid metabolism related to plant defence (Robbins et al. 1991) as well as nitrogen fixation (Petit et al. 1987) and nitrogen metabolism (Forde et al. 1989).

Antisense RNA, used as a method of blocking the transcription/translation pathway of a particular gene,

and so producing an organism with an absent or a much reduced gene product, has proved to be a useful tool in the genetic manipulation of metabolic pathways. The exact nature of the mechanism of this inhibition is a subject of debate (Grierson et al. 1991; Krol et al. 1991; Mol et al. 1991). In some systems it has been shown that the formation of sense: antisense RNA duplexes is involved (Melton 1985), though in other systems no such duplexes have been found. Antisense transcripts of as little as 41 bp have been shown to be effective (Cannon et al. 1990), and it has also been shown that an excess of antisense transcript is not necessary for the effective inhibition of gene expression (Krol et al. 1991). Antisense RNA has already proven to be a powerful tool in the manipulation of plant metabolism. Tomatoes with modified fruit ripening characteristics have been produced using this technique (Smith et al. 1988), and in two cases this has lead to the identification of function in genes whose function was previously unknown.

Phenylpropanoid metabolism has also been a target for manipulation using antisense RNA. *Petunia hybrida* plants with altered levels of anthocyanin in their flowers have been produced by introducing the gene for chalcone synthase into the plants in antisense orientation (Krol et al. 1988). The gene for chalcone isomerase has also been used in antisense experiments, but these did not produce plants with altered flavonoid levels (Tunen and Mol 1991). The gene for dihydroflavonol reductase (DFR) has not, to our knowledge, been used in antisense experiments, though it has been shown to co-suppress (Tunen and Mol 1991), a phenomenon thought to be due to antisense RNA expression (Grierson et al. 1991; Mol et al. 1991).

Heterologous antisense has also been shown to be effective in a number of systems. Krol et al. (1988) demonstrated that the antisense CHS A gene effectively down-regulated the expression of the CHS B gene in *P. hybrida*, despite having only 87% homology to it, whereas Visser et al. (1991) found that the *Zea mays* granule-bound starch synthase (GBSS) down-regulated potato GBSS activity, though not as effectively as an homologous GBSS.

In the present study we have brought together the techniques of *A. rhizogenes* transformation and antisense RNA technology to modify condensed tannin biosynthesis in *L. corniculatus* root cultures.

Materials and methods

Plasmid DNA manipulations

Restriction enzymes, bovine alkaline phosphatase, and T4 ligase were supplied by Boehringer Mannheim UK, and reactions performed using the manufacturer's recommended conditions. *E. coli* transformations were carried out using the $CaCl_2$ method described by Maniatis et al. (1982) and all plasmids were propagated and maintained in *E. coli* host JM83. Plasmid DNA was prepared using the alkaline-lysis method [Maniatis et al. (1982), for small scale; Lev (1987), for large scale].

Antisense gene cassette construction

Three different antisense constructs were produced by ligating fragments of the *Antirrhinum majus* cDNA for DFR, [pJAM 212 (Beld et al. 1989)], in the antisense orientation, between the CaMV35S promoter and the nos terminator of the binary vector pROK2 (Hemon et al. 1990). The first construct, pMAJ1, was produced by cloning the 330-bp *Bam*HI fragment from pJAM212 into the *Bam*HI site of pROK2 and selecting for the correct orientation. The second construct, pMAJ2, was produced by first cloning the 838-bp *Kpn*I/*Eco*RV fragment of pJAM212 into *Kpn*I/*Sma*I-cut pIC19H (Lawrence-Marsh et al. 1984) to give the plasmid pICMAJA, followed by the cloning of the *Kpn*I/*Bgl*II fragment of pICMAJA into the *Kpn*I, *Bam*HI sites of pROK2. The third construct, pMAJ3 was produced by first cloning the 771-bp *Eco*RV (complete digest)/*Eco*RI (partial digest) fragment into the *Eco*RI/*Sma*I sites of pIC19H to give the plasmid pICMAJB, and then cloning the *Bam*HI/*Bgl*II fragment of pICMAJB into the *Bam*HI site of pROK2 and selecting for the correct orientation. Hence, three constructs were produced spanning the first quarter, first half, and second half of the cDNA for DFR (Fig. 2). In this paper we present results relating to pMAJ2, a gene construct that includes the first half of the *A. majus* cDNA sequence.

Introduction of plasmids into A. rhizogenes

Binary vectors were introduced into *A. rhizogenes* strain LBA9402 by triparental mating using a modification of the method of Herrera-Estrella and Simpson (1988). One-hundred microliters of a culture of *A. rhizogenes* LBA9402 (grown for 2 days in YEB plus 50 µg/ml rifampicin at 28 °C with shaking),

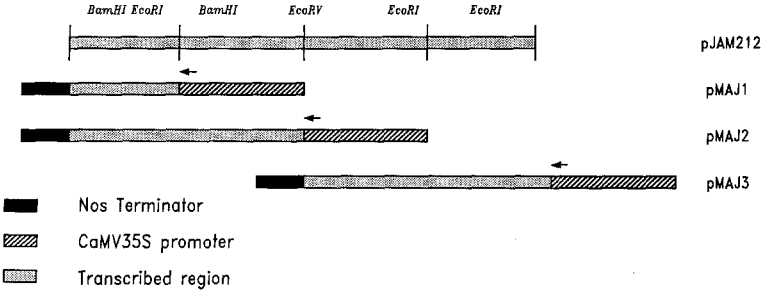

Fig. 2. Diagram representing the three DFR antisense gene constructs

100 µl of *E. coli* JM83 containing the binary vector (grown overnight in LB plus 50 µg/ml kanamycin at 37 °C with shaking), and 100 µl of *E. coli* containing the mobilisation plasmid pRK2013 (grown overnight in LB plus 50 µg/ml kanamycin at 37 °C) were combined in an Eppendorf tube, vortexed gently to mix, and pelleted in a microcentrifuge. The supernatant was discarded and the cells resuspended in 200 µl of 10 mM TRIS (pH7.2), 10 mM $MgSO_4$. Ten microliters of this was spotted onto YEB agar plates and grown overnight at 28 °C. The cells were then resuspended in 1 ml of λ buffer and 100 µl of this spread on a YEB agar plate containing 50 µg/ml rifampicin and 50 µg/ml kanamycin and grown for 2 days. The resulting bacterial growth was streaked to single colonies on YEB plates containing 50 µg/ml rifampicin and 50 µg/ml kanamycin.

Selection and micropropagation of plants for transformation with A. rhizogenes

To select a series of clonal genotypes that were sensitive to *A. rhizogenes*, *L. corniculatus* cv Leo seeds were surface sterilized as described by Webb et al. (1987) and germinated on 1.5% water agar (20 °C 125 µEm^{-2} sec^{-1} for 14 days). The shoots were excised and transferred to 30-ml universal tubes containing 10 ml of MS medium with 3% sucrose, without hormones, and grown as above. The cut surface of the residual hypocotyl was then immediately inoculated with *A. rhizogenes* LBA 9402. The infected hypocotyls were incubated in the light at 20 °C (under the above conditions), and "hairy root" production scored after 26 days. Eight of the genotypes producing roots were selected, and the uninfected cut shoots grown and propagated on MS medium, 3% sucrose, without hormones in universal tubes, under the temperature and light conditions as described above. Three of these selected clonal genotypes were used as recipients for antisense constructs in the experiments described in this paper.

Plant transformations and maintenance of root cultures

The stems of the micropropagated clonal genotypes were stripped of leaves and side branches and cut into pieces approximately 3 mm long with a scalpel that had been infected with *A. rhizogenes*. The stem pieces were then placed on sterile Whatman filter paper on the surface of a 60-mm Petri dish (Sterilin) containing 0.5 × Gamborgs B5 media (Flow Laboratories) 3% sucrose and 1% agar without hormones. These were incubated at 20 °C in the light (100 µE m^{-2} s^{-1}). After 7 days the stem pieces (some of which had begun to develop roots) were transferred to 60-mm Petri dishes containing solid 0.5 × B5 media with 500 µg/ml ampicillin. As "hairy roots" developed individual root tips were excised from the explant and maintained according to Webb et al. (1990), except that ampicillin was the only antibiotic used in the media unless bacterial growth persisted, in which case roots were grown on ampicillin/cefotaxime/carbenicillin [concentrations as in Webb et al. (1990)]. Roots were transferred into liquid culture (Gamborgs B5 media, 3% sucrose, no hormones) once free of bacterial contamination, and maintained as described by Morris and Robbins (1992).

Kanamycin selection

Individual 0.5-cm root tips from cultures grown on solid media were transferred to a compartment of a 5 × 5 repli dish (Sterilin) containing 1 ml of solidified B5 media. Four replicates of each culture were prepared. These were then incubated for 14 days at 25 °C in the dark. Two replicates growing at approximately equivalent rates were selected and the cube of agar containing the growing root was removed from the dish and placed on solid Gamborgs B5 media in a 60-mm Petri dish, one replicate of each on media containing 25 µg/ml kanamycin, and one on media without antibiotic. The cultures were grown at 25 °C in the dark and growth scored after 21 days.

DNA and RNA extraction and hybridisation

Genomic DNA was isolated according to the method of Dellaporta et al. (1983) with the following modifications; after the first isopropanol precipitation the pellet was resuspended in 10 × concentration TE buffer and extracted with phenol:chloroform:isoamyl alcohol (50:49:1), the DNA was then precipitated with isopropanol and the final pellet was resuspended in 1 × concentration TE buffer plus 50 µg/ml RNase A. The DNA concentration was determined fluorometrically (Cesarone et al.1979). RNA was extracted from root cultures by the method of Ougham and Davies (1990). Blotting of nucleic acids was performed using Zetaprobe (for Southern- and DNA slot-blots) (Biorad Ltd), or Hybond-N (for Northern blots) (Amersham International). Slot-blots were performed using a Biorad slot-blot apparatus and Southern-, Northern- and slot-blots carried out according to the relevant manufacturer's instructions. Hybridisations were performed using a Hybaid blot processing system, under conditions as described by Webb et al. (1990) (Southern- and slot-blots) or Bettany (1988) (Northern-blots). Double-stranded DNA probes were prepared using a random primed DNA labelling kit (Boehringer Mannheim UK), and single-stranded RNA probes using a SP6/T7 transcription kit (Boehringer Mannheim UK). Antisense T-DNA was detected using the insert from pJAM212 as a probe, T_L using the *Bam*HI fragment 8a (derived from pRi15834) as a probe, and T_R using the *Sma*I fragment 5 (derived from pRiHRI) as a probe. Antisense RNA was detected using labelled complementary RNA transcribed from pTC101 [the insert of pJAM212, cloned into the SP6/T7 transcription vector, pSPT18 (Boehringer Mannheim UK)].

Estimation of tannin content

Tannins were estimated based on their conversion to anthocyanidins upon acid hydrolysis. Between 100 mg and 400 mg fresh weight of root tissue was placed in a screw-capped 15 ml glass tube, and 2 ml of butanol:HCl (95:5) was added. The tubes were heated to 100 °C for 1 h and then allowed to cool. The spectrum of the hydrolysate was determined between 400 nm and 700 nm and the absorption at 550 nm measured. The absorption at 550 nm not due to anthocyanidins was estimated by interpolation of the underlying curve, not part of the 550 nm peak, and subtracted from the total absorption at 550 nm. The resulting value was used to calculate the tannin content of the sample using the $E^{1\%}_{550}$ of 150 determined by Stafford and Cheng (1980). This butanol:HCl hydrolysis method for the determination of condensed tannins gives an accurate and linear determination of total (i.e., soluble and insoluble) condensed tannins and has recently been validated in a range of forage species by Terrill et al. (1992). Estimation of percentage procyanidin (% PC) of condensed tannins was performed as described by Morris and Robbins (1992).

Results

Selection of sensitive seedlings and plant transformation

To produce a series of clonal genotypes sensitive to *A. rhizogenes* 50 seedlings were selected, 25 of which were

inoculated with *A. rhizogenes*, and 25 of which were untreated. Of the inoculated group 15 (60%) produced "hairy roots" after 26 days, while none of the untreated group produced "hairy roots". Of the 15 genotypes that produced "hairy roots", eight were micropropagated and used as recipients for the antisense constructs. These were designated s26, s28, s33, s35, s41, s46, s47, and s50; this paper discusses results using three of these genotypes, s33, s41 and s50.

Transformations were performed on these three genotypes, using *A. rhizogenes* LBA 9402 harbouring the antisense construct, pMAJ2. Control transformations were carried out using either wild-type LBA9402 or LBA9402 housing the binary plant transformation vector, pROK2. Initially 60 antisense transformants were tested for kanamycin sensitivity and 45 showed the ability to grow on media containing 25 mg/ml of kanamycin, indicating a co-transformation rate of 75%.

Analysis of condensed tannin levels in transgenic root cultures

Condensed tannin levels were determined in transgenic root cultures derived from three different recipient *Lotus* genotypes. Comparisons were made between independent control lines transformed with wild-type *A. rhizogenes* LBA9402 and kanamycin-resistant lines transformed with LBA9402 harbouring the antisense vector construct, pMAJ2. After initiation in liquid culture, control (C) and antisense (RFD) root cultures were subcultured at 14-day periods and harvested for analysis after the third subculture. Analysis of control transformed lines indicated that independent control lines derived from individual genotypes showed consistent patterns of tannin accumulation (Fig. 3). Three control lines derived from genotypes s33 had a mean tannin level of 0.39 ± 0.06 mg/g fresh weight (FW), three control lines derived from s50 accumulated 0.51 ± 0.15 mg/g FW of condensed tannin, while for five lines derived from s41 mean tannin levels were 3.29 ± 0.66 mg/g FW. Tannin levels of lines transformed with *A. rhizogenes* LBA9402 harbouring the unmodified plant transformation vector pROK2 were similar to values for wild-type LBA9402 (data not shown).

In subsequent analyses, tannin levels were analysed in kanamycin-resistant *Lotus* root cultures transformed with the antisense DFR construct, pMAJ2. In genotype s33 two antisense lines, RFD 7 and RFD4, had tannin levels statistically indistinguishable from control lines; however, all the remaining six antisense lines showed a marked reduction in tannin accumulation in comparison to controls (Fig. 3). The most extreme example of low tannin levels was line RFD38 which accumulated condensed tannin levels of 0.03 ± 0.03 mg/g FW in comparison to mean s33 control values of 0.39 mg/g FW. In genotype s50 a different distribution of tannin levels was found in individual antisense lines. One line, RFD 3, had significantly-higher levels of condensed tannins in root culture material than controls; two lines, RFD37 and RFD31, had similar levels to controls; while two further lines, RFD40 and RFD28, had significantly-lower levels

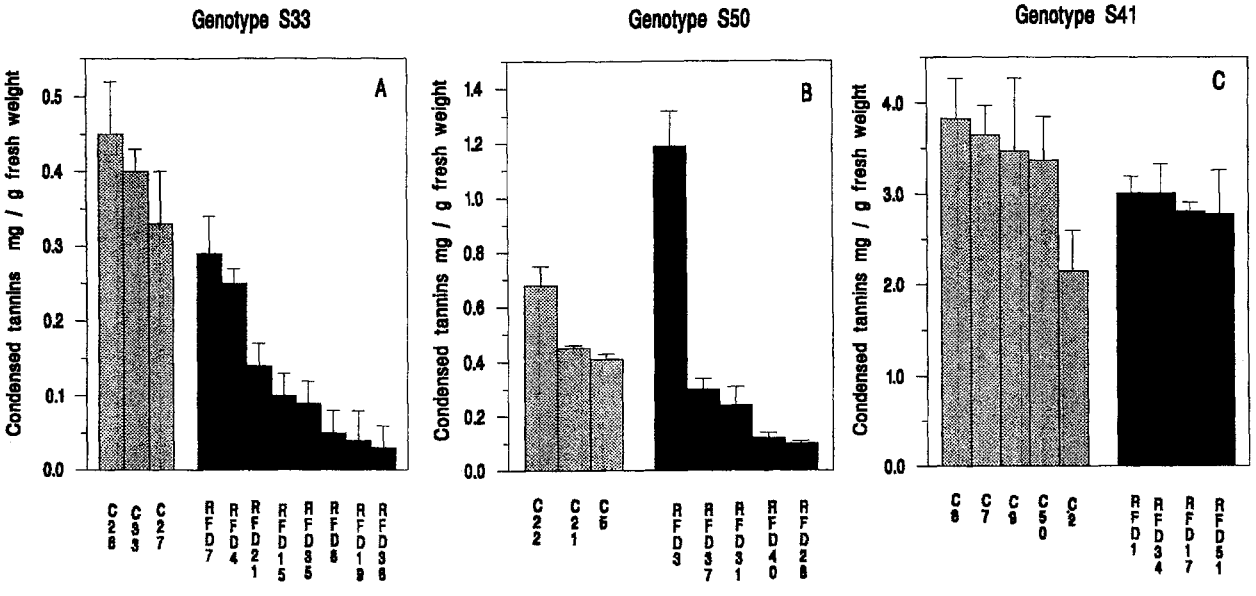

Fig. 3. Condensed tannin levels in control and antisense (pMAJ2) transformed root cultures of three different clonal genotypes of *L. corniculatus*. Tannin levels were determined from triplicate flasks harvested after the third liquid subculture

than s50 control lines. In genotype s41 antisense lines accumulated condensed tannins with similar levels to s41 control lines (Fig. 3).

In order to analyse whether the reductions in tannin levels noted in genotypes s33 and s50 resulted in alterations in tannin polymer structure, the degree of hydroxylation of tannin polymers was determined in tissue from control and antisense root cultures at the third subculture. The results of the PC:PD analysis are shown in Fig. 4. In genotype s33, control PC:PD values lay in the region of 70–80% procyanidin, while four antisense lines with reduced tannin levels had PC:PD values of 80–100%, with one line which yielded 100% procyanidin on hydrolysis. In s50, control lines had PC:PD ratios of 70–80%, while three low-tannin antisense lines had PC:PD values of greater than 80%. In s41, PC:PD values were indistiguishable between control and antisense lines and ranged between 50 and 80%.

Molecular analysis of control and antisense lines

In view of the condensed-tannin results for control and antisense lines in genotypes s33 and s50, detailed molecular analysis was carried out on three typical control lines and four selected antisense lines. The s33 lines selected for study were: C26, C27, C33, RFD8, RFD19, RFD21 and RFD38; and for s50 the selected lines were: C5, C21, C22, RFD28, RFD31, RFD37 and RFD40. Genomic DNA was extracted from each of these lines and analysed for the presence of three classes of T-DNA sequences (the binary antisense T-DNA and two T-DNAs from the *A. rhizogenes* Ri plasmid, T_L and T_R). DNA slot-blots were used to determine the presence or absence of three T-DNAs, and to estimate their copy number and the results are shown in Fig. 5. All of the control and antisense transformed lines were shown to contain the T_L T-DNA, indicating that all the lines were *A. rhizogenes*-transformed, and all but one (RFD28) of the antisense transformants, but none of the controls, were shown to contain the antisense T-DNA, indicating that these lines were co-transformed. Though no antisense DFR sequences were detected in the genome of RFD28, it was strongly kanamycin-resistant, indicating the transfer of some of the binary T-DNA, and so was treated as an antisense line. Four of the six controls, and six of the eight antisense lines were shown to contain the T_R T-DNA. A greater proportion of the s50 transformants than the s33 transformants contained the T_R T-DNA (Fig. 5e), and this may reflect a greater susceptibility to T_R transfer in s50 than in s33. A correlation, significant at the 5% level (coefficient 0.613), was found between the number of T_R copies transferred and the number of T_L copies transferred, though no significant correlation was found between either the number of T_R or T_L copies transferred and the number of antisense T-DNA copies transferred. The slot-blot was also hybridised to total *L. corniculatus* genomic DNA to ensure even-loading of the samples. No large differences between amounts of genomic DNA loaded for each sample were found (Fig. 5d). The presence and integration into the genome of the antisense T-DNA was further confirmed by Southern hybridisation (Fig. 6d).

The antisense lines were also analysed for expression of the antisense gene by Northern hybridisation. Single-stranded RNA probes specific to the antisense RNA were used to detect antisense RNA transcript in

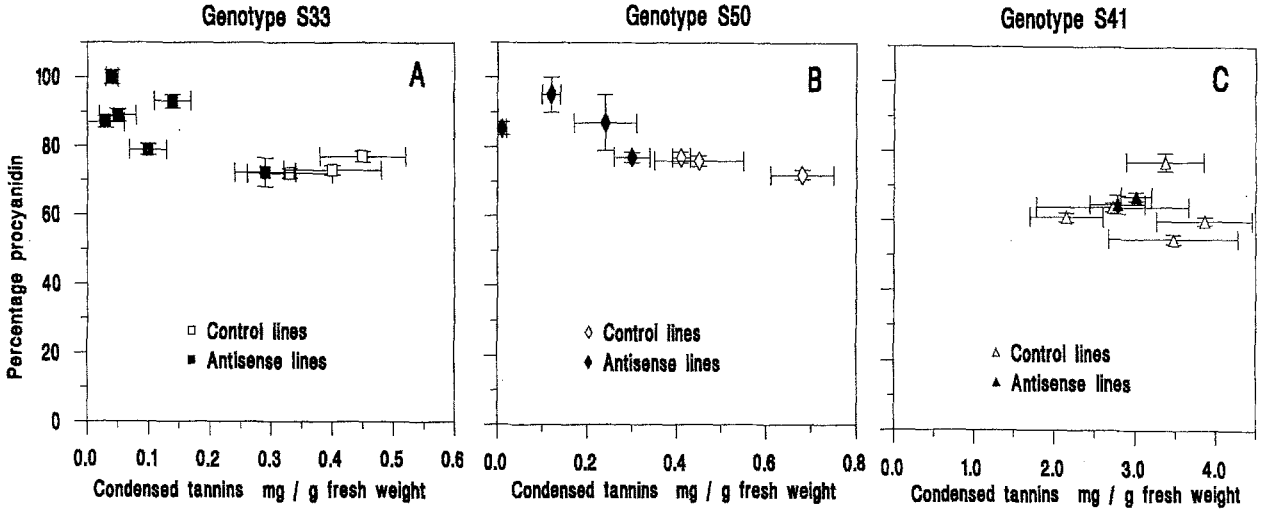

Fig. 4. Relationship between condensed tannin levels in root cultures of *L. corniculatus*, and the percent procyanidin content of the tannin, in antisense and control root cultures produced from three different clonal genotypes. Standard errors of the means (three determinations) are shown for both tannin level and percent procyanidin

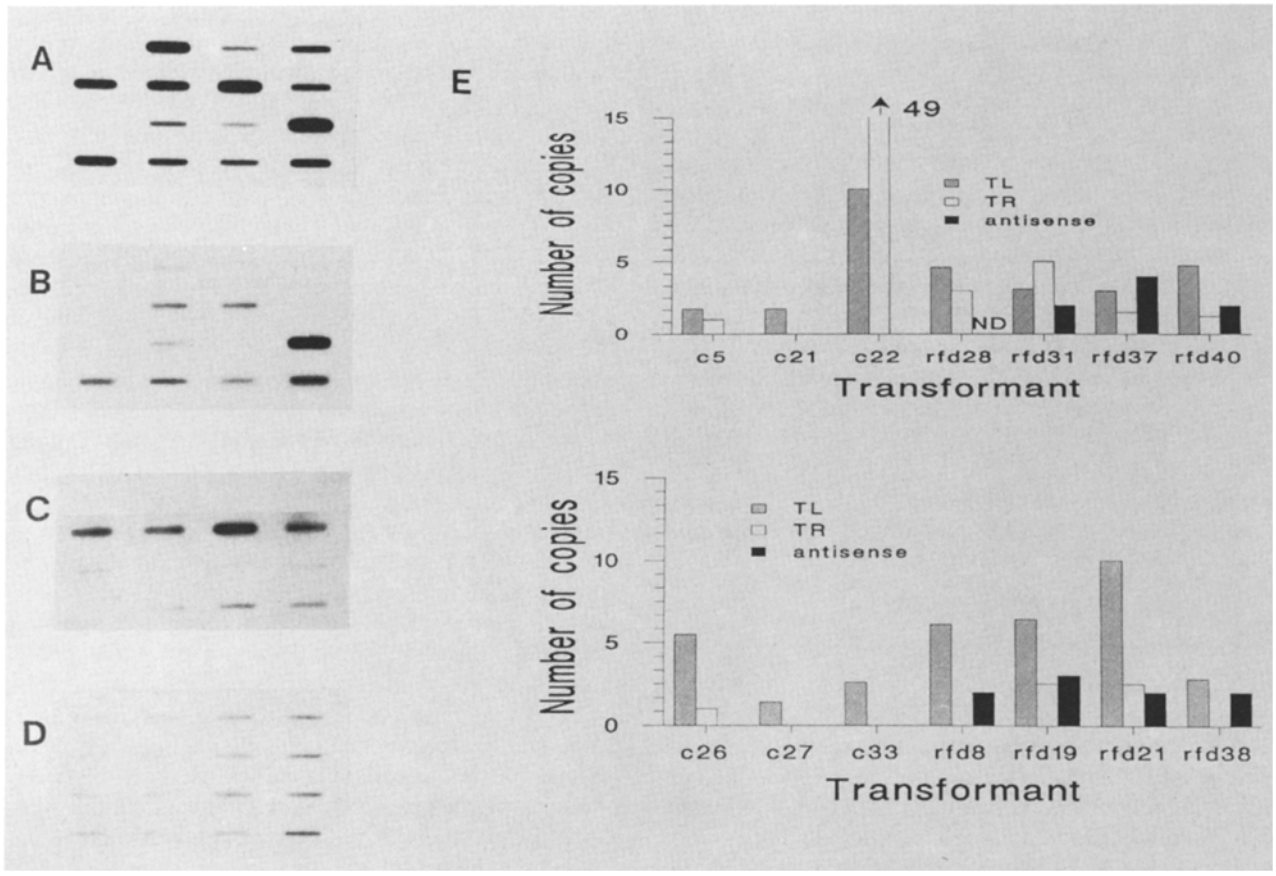

Fig. 5a–e. Analysis of the copy number for T_L, T_R, and the antisense T-DNA in selected antisense and control root cultures. **a–d** show a *L. corniculatus* genomic DNA slot-blot hybridised to probes for; T_L (**a**), T_R (**b**), the antisense T-DNA (**c**), and total *L. corniculatus* DNA (**d**). The slots are, from left to right, top row: s33 (non-transformed), c26, c27, c33; second row: RFD8, RFD19, RFD21, RFD38; third row: s50 (non-transformed), c5, c21, c22, bottom row: RFD28, RFD31, RFD37, RFD40. **e** is a bar chart showing the copy numbers of the three T-DNAs, as determined by comparison to copy number reconstructions. ND, not detected

total RNA isolated from the root cultures. Antisense transcript was found in six of the eight antisense transformants, but not in the control transformants (Fig. 6a–c).

Discussion

As an outbreeding crop, *L. corniculatus* shows much heterogeneity in phenotype, including its chemical composition (Roberts et al. 1989), and for this reason it was considered necessary to produce a series of clonal genotypes which were sensitive to *A. rhizogenes* infection and which gave good "hairy root" formation and regeneration. This allowed antisense transformants to be compared directly with control transformants of the same genotype. Subsequent data showed this to be an essential prerequisite for the detailed analysis of the experiment as tannin levels varied greatly between genotypes (Fig. 3).

The exact nature of the mechanism of antisense RNA suppression of gene expression is not fully understood (Grierson et al. 1991; Krol et al. 1991; Mol et al. 1991) and the features of the antisense transgenes that confer optimum suppression of gene expression are not well defined. Comparison of effects of different portions of transcripts used in antisense experiments give different results in different biological systems. Krol et al. (1990) demonstrated that the 3′ end of the CHS coding region was enough to confer antisense suppression, whereas the 5′ sequence alone did not. In contrast Melton (1985) showed that the 5′ sequence of frog oocyte globin was essential for antisense-mediated down-regulation of the protein. Short oligonucleotides have also proven to be effective in down-regulating gene expression in some systems. Cannon et al. (1990) showed effective inhibition of *gus* expression using a 41-bp antisense gene homologous to the 5′ end of the *gus* cDNA, whereas Smith et al. (1986) showed that

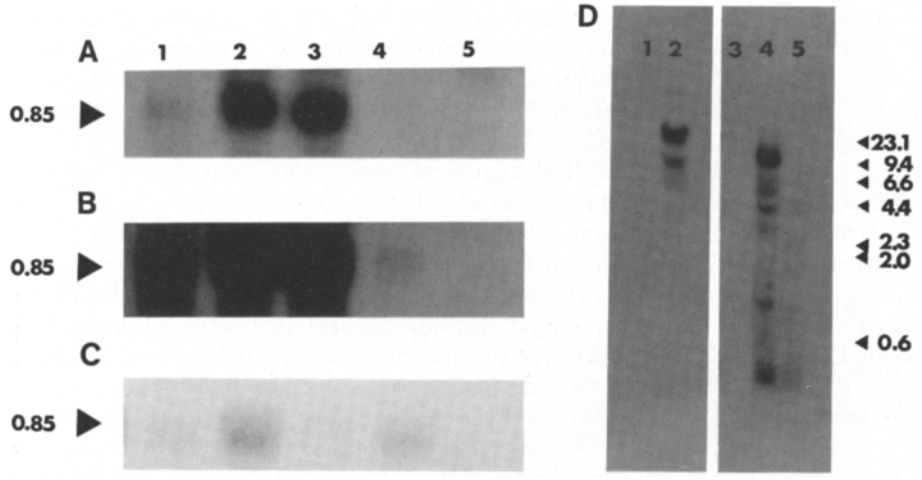

Fig. 6. Southern and Northern analysis of selected transformed root cultures. **a** shows a Northern-blot of RNA from, *lane 1*, RFD8; *2*, RFD19; *3*, RFD21; *4*, RFD38; *5*, c26, probed with an RNA probe specific for the antisense RNA, and exposed for 16 h. **b** is as **a** except that it is a 5-day exposure. **c** shows a Northern of RNA from root cultures of, *lane 1*, RFD28; *2*: RFD31; *3*, RFD37; *4*, RFD40; *5*, c5, probed with an RNA probe specific for the antisense RNA, and exposed for 16 hours. **d** is a Southern-blot of DNA from root cultures of lane *1*, c26; *2*, RFD19; *3*, c5; *4*, RFD37; *5*, RFD40, probed with the construct pMAJ2. Sizes of antisense transcript and DNA molecular weight markers are in kbp

antisense treatment directed to the intron-exon splice sites of herpes simplex I reduced virus production by 99% in mammalian cell culture.

These contrasting observations make it difficult to predict whether or not a given antisense construct will be effective. In these experiments we have used a construct consisting of the 5′ half of a dihydroflavonol reductase cDNA from *A. majus*. Two other constructs consisting of the first quarter and the second half of the *A. majus* DFR cDNA have also been produced and results comparing the effectiveness of these three different constructs will be published elsewhere. With regard to homologies required for effective antisense phenotypes there are a number of pieces of evidence that have suggested that the *A. majus* DFR gene would be a suitable heterologous sequence. Firstly, stringency washes have indicated an homology of approximately 80% between the *A. majus* cDNA sequence and sequences present in the genome of *L. corniculatus* (Robbins, unpublished data). In fact we have detected a small family of 2–4 genes in a range of *Lotus* genotypes. Other evidence supporting heterologous DFR experiments comes from the outstanding homologies noted between DFR gene sequences in different plant species. Sequence comparisons of the *A. majus* DFR cDNA with other cloned DFRs has shown significant homologies to cDNA sequences from a range of other species including *P. hybrida* (Beld et al. 1989), *Z. mays* (Schwartz-Sommer et al. 1988) and *Arabidopsis thaliana* (Shirley et al. 1992). Comparisons of these sequences indicate between-species homologies of up to 85%, with these homologies being most marked in the 5′ half of cDNA sequences.

Initial analysis of antisense and control root cultures showed that the presence of the antisense construct, pMAJ2, was correlated with reduced tannin levels strongly suggesting that the construct was effectively down-regulating tannin accumulation. Comparison of tannin levels between antisense and control transformants in genotypes s33 and s50 indicated a number of antisense transformants which exhibited reductions in relative tannin levels. In addition there were examples of transformants in which there was no detectable antisense phenotype, e.g., RFD7 and RFD4 in genotype s33 and RFD37 and RFD31 in genotype s50, and these may be regarded as ineffective antisense transformation events. In s41, there were no examples of antisense transformants with reduced tannin levels relative to the controls. This observation, that the pMAJ2 T-DNA has a more significant effect on tannin accumulation in low-tannin genotypes than in the high-tannin genotypes, is novel. To the authors' knowledge this is the first published report of differential effects of antisense inhibition of secondary metabolic pathways noted with genotypes of different biosynthetic capacities.

The analysis of antisense transformants of the two selected genotypes, s33 and s50, showed the binary DNA to be integrated into the genome of the antisense-transformed plants (Figs. 5c, 6d), that the antisense RNA was expressed (Fig. 6a–c), and that the presence of the antisense T-DNA was strongly associated with a reduction in tannin levels. Southern analysis of *Hin*dIII digests of genomic DNA from three of the antisense lines, (RFD19, RFD37, and RFD40), indicated the presence of a diagnostic 1.3-kbp fragment (Fig. 6d),

comprising the CaMV35S promotor and 530 bp of the *A. majus* DFR gene, demonstrating that the antisense construct was intact in these lines. This band was clearly visible in the DNA from RFD37, a line containing five antisense copies as determined by slot-blot analysis (Fig. 5), but less visible in the DNA from RFD19 and RFD40 (containing four and two antisense copies respectively). Other hybridising bands were found in all three antisense lines examined. These were of varying molecular weight, suggesting, that they were border fragments, comprising DNA from the pMAJ2 plasmid with *Lotus* genomic DNA surrounding the integration sites (Fig. 6d). The presence of these border fragments is strong evidence for the integration of the binary vector into the genome of *Lotus*. In one antisense line, RFD28, we were unable to detect any antisense sequence in genomic DNA using slot-blots (Fig. 5); however, this line has shown consistantly low tannin levels both in root cultures and in regenerated plants (unpublished data). We suspect that pMAJ2 may have undergone some sort of structural rearrangement and we are currently continuing with a genetic analysis of RFD28 in order to clarify the details of this transformation event.

The observation that antisense RNA is easily detectable in the transformed root cultures may seem to contradict the findings of other workers (Krol et al. 1988; Smith et al. 1988) that antisense RNA is not detectable in tissue where the sense message is also expressed. However, in this case the RNA was isolated from whole-root cultures, whereas there is strong evidence that tannin biosynthesis (and presumably DFR expression) occurs only in certain cell lineages, and only in a region of the root directly behind the meristem (Morris and Robbins 1992). Therefore, the RNA extracted from antisense transformants may represent the RNA from cells in which both the antisense and the sense gene are active (those cells actively synthesising the DFR protein), and cells in which only the antisense gene is active (those cells not actively synthesising the DFR protein). Observations from other systems (Krol et al. 1988; Smith et al. 1988) would suggest that antisense RNA would be easily detected in RNA extracted from cells in which the sense gene is inactive.

Despite the strong correlation between the presence of the antisense T-DNA and the reduction in tannin levels no correlations could be found between the degree of reduction in tannin levels and either the number of copies of the antisense T-DNA integrated into the genome or the steady-state levels of antisense RNA. This is in accordance with the findings of other workers who showed that an excess of antisense RNA is not necessary for the antisense phenotype (Krol et al. 1991). Also the presence and copy number of the *A. rhizogenes* T-DNAs did not appear to affect the tannin levels in the transformed root cultures. The correlation between the number of T_L copies and the number of T_R copies was significant, but not great, and may reflect differences in the level and duration of *A. rhizogenes* Ri plasmid vir activity during different transformation events.

The changes in tannin composition associated with the antisense-mediated reduction in tannin accumulation probably reflect alterations in the regulation of tannin biosynthesis. These alterations are not specific to cultures with genetically-modified tannin levels but also hold true for cultures that have naturally low, genotype-dependant, tannin levels. However, the use of some chemical inhibitors of tannin biosynthesis have also been shown to reduce tannin levels by similar amounts to the antisense constructs but do not cause any change in the percent of procyanidin (unpublished data). These observations may have implications for the mechanisms of developmental control of condensed tannin biosynthesis and these phenomena are currently under investigation.

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