

Dihydroflavonol reductase a *Lotus corniculatus* L. tannin biosynthesis gene: Isolation of a partial gene clone by Polymerase Chain Reaction.

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One aim of this group is to manipulate tannin biosynthesis in forage legumes, by the use of molecular genetic techniques. A key step in the biosynthetic pathway culminating in the production of condensed tannin, is the reduction of dihydroquercetin and dihydromyricetin by dihydroflavanol-4-reductase (DFR).

Using *Agrobacterium rhizogenes* mediated transformation it has been possible to introduce antisense DFR-gene constructs into *L.corniculatus* (Carron, Robbins and Morris 1994). Whilst it has been possible to monitor the expression of the introduced heterologous antisense gene, analysis of the expression of the native gene has proved difficult.

We report the use of the polymerase chain reaction to amplify a fragment from genomic *L.corniculatus* DNA which corresponds to part of a native DFR gene.

Materials and methods

Genomic DNA from *L.corniculatus* lines S33 and S50 (Carron, Robbins and Morris 1994) was isolated as described by Robbins et al., 1991.

Degenerate primers for PCR were designed based on the known sequences of DFR protein from *Antirrhinum majus*, *Petunia hybrida* and *Gerbera hybrida*. The 5' primer was a modification of that used by Helariutta et al., (1993) and comprised: AGAATGAAGT(G/T/A)AT(A/C/T)AA(A/G)CC (Primer 1).

Two 3' Primers were employed whose sequences were:

GGGTCGAC(A/G)CA(G/A/T/C)A(A/G)(A/G)TC(A/G)TC(G/A/T/C)A(A/G)(A/G)TG or

GGGTCTACCAT(A/G)TC(C/T)TC(G/A/T/C)A(A/G)(G/A/T/C)GT(A/T)TA

(Primers 2 & 3). The latter being located nearest to the 3' end of the gene.

Conditions for PCR product generation were optimised using a *Petunia hybrida* DFR cDNA clone (Clone pTIP1 supplied by J.Kooter).

Each reaction contained in a volume of 50µl:

5µl 100mM Tris-HCl pH8.5

5µl 500mM KCl

5µl 1mg/ml Gelatin

2µl 50mM MgCl₂

1µl 10mM deoxynucleotides

0.5µl 15µM Primer 1

0.5µl 15µM Primer 2 or 3

0.2µl AmpliTaq DNA polymerase (1 unit)

10 μ l Target DNA: 10-50ng Plasmid DNA or
100-300ng *Lotus* genomic DNA.

Reactions were run in 0.5ml microcentrifuge tubes with the reaction mixture overlaid with 50 μ l liquid paraffin. DNA was always made up in sterile distilled water. Liquid transfers were carried out using Aerosol Resistant Tips. All manipulations were conducted in a clean class 2 flow cabinet to reduce the risk of contamination from outside sources.

The following cycling conditions were used on a Perkin-Elmer 480 thermal cycler:

Hot start denaturation of: 94 $^{\circ}$ C 3 minutes,
35 cycles of: 94 $^{\circ}$ C 30 seconds, 55 $^{\circ}$ C 1 minute, 72 $^{\circ}$ C 2 minutes.
Final extension of: 72 $^{\circ}$ C 10 minutes.

A 25 μ l aliquot was taken from each reaction and run on a 0.5% agarose gel and visualised with ethidium bromide. When a product was detected a 1 μ l aliquot of the reaction mixture was used as a target for a second round of amplification using both primer 1/2 and primer 1/3 combinations. After photography gels were blotted onto Hybond-N membrane (Amersham) and then probed with an *Antirrhinum majus* DFR cDNA clone (Clone pJAM212 Cathie Martin).

PCR products were cloned as follows:

A 25 μ l aliquot (100ng-1 μ g product) from the reaction mixture was precipitated by adding 0.1 volumes 3M sodium acetate pH 5.2, 2 volumes ethanol and incubating on ice for 1 hour. The DNA was precipitated by centrifugation 12000g for 15 min in a microfuge. The supernatant was removed and the pellet washed with 200 μ l 70% ethanol (pre-cooled -20 $^{\circ}$ C). After centrifugation for 5 minutes the pellet was air dried until all traces of ethanol had evaporated. The pellet was resuspended in 50 μ l 1x one-phor-all buffer plus (Pharmacia). The ends of the fragments were blunted by the addition of 10 μ l dNTP solution (2mM dATP, 2mM dCTP, 2mM dGTP, 2mM dTTP, Pharmacia) and 2 μ l (2 units) T4 DNA polymerase (Boehringer-Mannheim). The reagents were gently mixed and incubated at 12 $^{\circ}$ C for 30 minutes.

An aliquot of 100-500ng blunt ended PCR product was mixed with 200ng *Sma*I digested pUC18 and ethanol precipitated as described above. The pelleted DNA was resuspended in 7.0 μ l water, 1 μ l 10x T4 DNA ligase buffer and 2 μ l (2 units) T4 DNA ligase (Gibco-BRL) and incubated 12 $^{\circ}$ C overnight. Half of the ligation mixture was used to transform CaCl₂ competent *E.coli* strain DH5 α .

Results

Using primers 1 and 2 a fragment of approximately 750bp was amplified from both S33 and S50 genomic DNAs. With primers 1 and 3 a fragment of approximately 1.5kb was generated (Fig). Both of these fragments were around the predicted size for DFR gene products assuming that the introns in the *L. corniculatus* gene were similar to those of the published *Antirrhinum majus* and *Arabidopsis thaliana* sequences. When the large fragment from primers 1 and 3 was used as a

template for re-amplification with primers 1 and 2 a 750 bp fragment was produced. The 750bp fragment produced in both primary and re-amplification reactions cross-hybridised with the *A.majus* DFR probe (Fig).

The 750bp fragment from S50 was cloned into pUC18. Sequence analysis revealed homologies between 71.6% and 68.3% over a 110bp overlap with the *A. majus*, *Arabidopsis thaliana*, *Hordeum vulgare*, *Petunia hybrida*, *Vitis vinifera* and *Zea mays* DFR RNA sequences in the Geneml database.

Discussion

Amplifications using degenerate primers for DFR initially produced a series of fragments from *L.corniculatus* genomic DNA (data not shown). After optimisation of the reaction conditions a single product was obtained with each pair of primers. This product was isolated and shows homology to a DFR gene from *A.majus* both by cross hybridisation and sequence analysis. To the authors knowledge this is the first tannin biosynthesis gene fragment to be cloned from a Lotus species. The isolation of this fragment should enable the entire gene to be isolated more easily. The partial DFR gene clone will be useful in investigating the expression of the native gene in *L.corniculatus*, both in wild-type lines and transgenic lines harbouring heterologous DFR gene constructs.

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References

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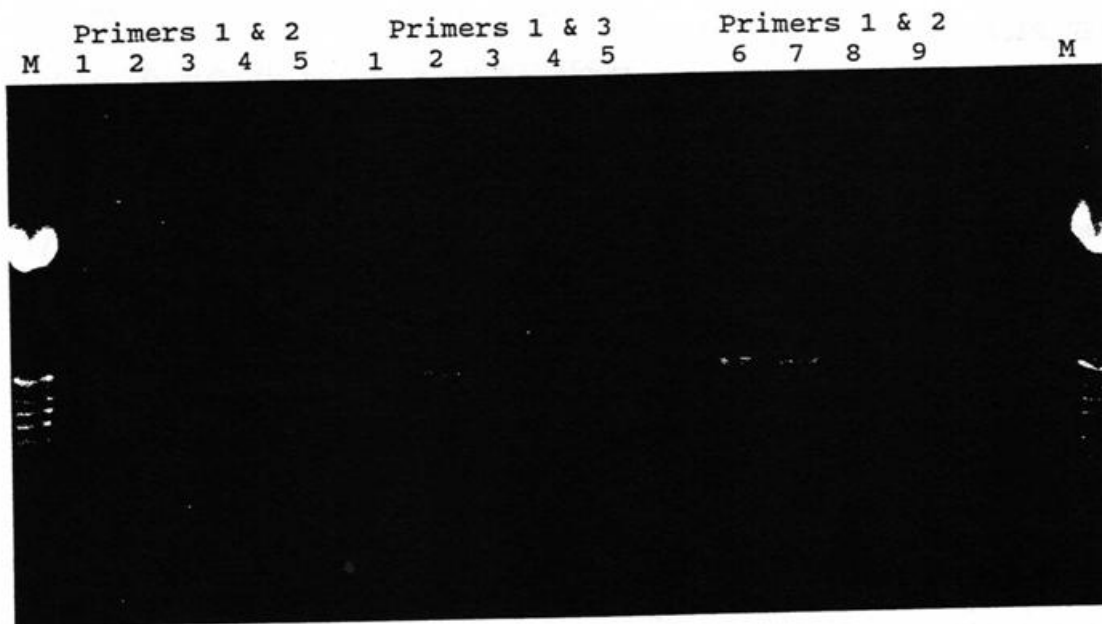
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Figure:

Analysis of PCR amplification products from *Lotus corniculatus* genomic DNA.

PCR products obtained from target DNAs:

1 No target DNA, 2 *P.hybrida* DFR cDNA clone, 3 *H. vulgare* DFR cDNA clone, 4 *L. corniculatus* line S33 genomic DNA, 5 *L. corniculatus* line S50 genomic DNA, 6 product from primers 1&2 x 4, 7 product from primers 1&2 x 5, 8 product from primers 1&3 x 4, 9 product from primers 1&3 x 5.
M 100bp size marker ladder.



Hybridisation of *Petunia hybrida* DFR cDNA clone to *L. corniculatus* PCR products.

