# GENETIC MANIPULATION OF SAINFOIN LEAF TANNINS

M.Y. Gruber, P. Auser, G. Lees, A.D. Muir and M. Wood
Agriculture & Agri-Food Canada, 107 Science Place, Saskatoon, SK, S7N 0X2, Canada.

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**ABSTRACT**

An isogenic population of sainfoin (*Onobrychis viciifolia* Scop.) plants was developed by two methods; regeneration after long term tissue culture and plant transformation with an antisense chalcone synthase gene. Tannin (proanthocyanidin) content could be dramatically increased in untransformed callus by long term culture, but was not significantly altered in regenerating plantlets. Some of the mature transgenic plants had dramatically reduced levels of leaf tannin. The antisense gene was eliminated from all transgenic plants later in development.

**KEYWORDS**

condensed tannins, proanthocyanidins, transformation, antisense chalcone synthase, tissue culture

## INTRODUCTION

In order to determine optimal concentrations of condensed tannin when breeding for forage quality (bloat resistance, rumen protein bypass and insect resistance), we elected to develop an isogenic population of sainfoin plants with varied concentrations of leaf tannin. Some natural variation exists among sainfoin populations, but they always contain large quantities of leaf tannin (Koupai-Abyazani *et al*., 1993). In this study, we explored the potential of using either long term callus culture or transformation with an antisense chalcone synthase (CHS) gene to develop tannin variability.

## MATERIALS AND METHODS

Sainfoin callus cultures were established and induced to regenerate from seedling or mature plant explants using existing protocols (Lees, 1988). Each callus was divided bimonthly over a period of 1.5 years. One callus half was transferred to fresh CIM (Table 1); the other callus and any regenerating plants were assayed for condensed tannins using a butanol:HCl histochemical test (Skadhauge *et al*., 1997). Conditions for regeneration were optimized by testing media outlined in Table 1. A transformation protocol for sainfoin was developed as follows. A binary vector was constructed to express the *Petunia hybrida* chalcone synthase (CHS-A) gene in antisense orientation as transcript fused to the detectable β-glucuronidase (GUS) gene (Fig. 1A). This vector was used to transform *Agrobacterium tumefaciens* MP90 and form strain B71. Asceptically grown four week old seedlings were cut into 2-3 mm explants and cultured for 3-4 days on callus inducing medium (CIM). Cultured explants were submerged in an *A. tumefaciens* B71 suspension for 15 min and cultured on CIM for 2 days, then transferred to shoot inducing medium (SIM). SIM contained a range of kanamycin concentrations (35-50 $mg.L^{-1}$) when optimizing selection of transformants. Regenerating kanamycin resistant shoots were removed from calli after 6-12 weeks, subcultured on fresh changes of SIM until they were 2-3 cm, and then transferred to root inducing medium (RIM). SIM and RIM contained 250-300 mg. $L^{-1}$ cefotaxime and either carbenicillin (250-300 $mg.L^{-1}$), cloxacillin (250-300 $mg.L^{-1}$) or Timentin™ (250 $mg.L^{-1}$) to inhibit *Agrobacterium* growth. When 2-4 cm roots had developed, plants were potted in a soiless mix and grown in growth cabinets. The presence of the petunia CHS-A transgene was detected specifically in some cases by hybridizing a $^{32}$P-labelled 1.35 kb DNA fragment containing the petunia gene (Fig. 1A) to 10 µg BamHI digested genomic DNA from kanamycin resistant sainfoin under stringent conditions (Ausubel *et al*., 1994). Alternatively, the polymerase chain reaction (PCR) was used to amplify a 380 kb genomic DNA fragment from kanamycin resistant sainfoin using primers highly specific for the 3' end of the petunia gene (Fig 1B). Sainfoin CHS genes were detected by hybridizing untransformed sainfoin genomic DNA to the petunia gene under less stringent conditions. β-glucuronidase (GUS) activity was assayed fluorimetrically (Jefferson, 1987). Protein was quantified for GUS specific activity determinations by the method of Bradford (1976). Condensed tannin was assayed histochemically as above in tissues of transgenic and untransformed somaclonal variant plants, as well as in 1-6 week untransformed seedlings. Tannin was also determined quantitatively on leaves from two and three year old transgenic plants using a modification of the histochemical assay (Skadhauge *et al.*, 1997). Plant leaves were harvested before flowering and ground to a fine powder in liquid $N_2$ for quantitative assays.

## RESULTS AND DISCUSSION

**Manipulation of tannin content by long term tissue culture:** Genotypically unique sainfoin callus cultures were established and a portion regenerated into plants. After 3-4 weeks on CIM, explants began to form small calli along the whole length of the tissue. The fastest and healthiest callus production occurred using explants from seedling tissues. After 2-8 weeks on SIM, 10-35% of the callused explants produced shoots. RIM with 2-iP stimulated rooting far more than media with BAP or without hormones, although induction of rooting was slow in all cases. Roots usually developed after 8 months. Initially, tannin formation was genotype dependent, i.e. 56 out of 100 calli each arising from a different seedling were alive after four months, but only one callus synthesized tannins. After 14 months in culture, 23 of the 56 calli produced tannins, but lengthy subculturing also reduced the ability of calli to survive and to regenerate into plants (35 calli were alive after 14 months). The amount of tannin did not appear to change significantly during callus subculturing, but long term tissue culture slowed the development of tannin in regenerating shoots compared with seedling shoots. However, the tannin content was already high by the time regenerating shoots were 4 cm, and shoots of two and three year old regenerated plants contained levels of tannin similar to those present in plants which had never been through a tissue culture process.

**Manipulation of tannin content using antisense CHS plant transformation:** Since tissue culture was unsuccessful as a protocol to form low tannin leaf variants, we turned to antisense technology. Antisense transformation is known to produce populations of plants with a range of product depression (Kooter and Mol, 1993) by expressing transgene RNA in opposite orientation to transcipts of "sense" genes. As a result, RNA duplex formation between an antisense CHS gene and native sainfoin CHS genes should prevent translation of CHS enzyme. Since antisense RNA is a "mobile effector", the strategy should enable detection of phenotypes with low and medium leaf tannin content in an outcross pollinating tetraploid species such as sainfoin directly after transformation, without the need for segregation analysis. Consequently, an *A. tumefaciens* transformation method was developed for sainfoin using a petunia antisense CHS-A binary vector (Fig. 1A). The optimum kanamycin concentration to select transformed sainfoin tissue was 50 $µg.ml^{-1}$ (Fig. 1B). *Agrobacterium* infected leaf blades and petioles were more responsive to callus and shoot formation on kanamycin containing media, compared with infected cotyledon and stem explants. Root tissue occasionally produced callus on kanamycin

media, but never shoots. All kanamycin resistant plantlets developed roots after 8-10 months in culture, and roots formed quite quickly after initiation. Media containing Timentin™ was most efficient at inhibiting *Agrobaterium* growth on the medium. In an initial experiment, Southern hybridization of the petunia CHS gene (Fig. 1A) to genomic DNA from uninoculated control plants under low stringency conditions resulted in the detection of multiple fragments. None of these were the same size as the 1.35 kb petunia CHS fragment. Southern hybridization to genomic DNA from several kanamycin resistant plantlets under high stringency conditions indicated a single 1.35 kb band was present (data not shown). These results indicate that CHS is multigenic in sainfoin and can be easily distinguished from the petunia CHS transgene.

In subsequent experiments, DNA from shoots from more than 100 different plantlets arising from independently inoculated explants was tested by PCR amplification using oligonucleotide primers specific for the petunia CHS (Fig. 1B). A total of 45 cloned plants, representing 27 independent insertions, were found to amplify the 380 bp petunia fragment. Several larger bands were also found for many of the transgenics, likely due to amplification from incompletely digested genomic DNA. No fragments were amplified from DNA of untransformed plants.

The sequence of a typical amplified fragment had 100% homology with the petunia gene. Transformants exhibited a wide range of foliar condensed tannins during shoot regeneration, unlike wildtype seedlings and somaclonal variants which routinely contained large amounts of tannin. For example, as late as 10 months after shoot establishment, 22 out the 45 kanamycin resistant plantlets which amplified the CHS transgene did not synthesize leaf or stem tannin, while 9 plantlets synthesized only enough to be barely detectable. These levels were maintained long after kanamycin selective pressure had been eliminated and after larger plants had been moved to pots in a growth cabinet. Still others had intermediate or wildtype levels of tannin while still growing in Magenta™ boxes, indicating that the culture conditions, per se, did not inhibit tannin expression. The varied phenotypes likely arose due to differences in the chromosomal insertion sites among the transformants, some of which could change the efficiency of transgene expression.

Our results indicate that antisense initially was an effective strategy to use when "down regulating" the tannin pathway in sainfoin. However, it was disappointing to observe later that the sainfoin low tannin transgenic phenotype was actually unstable. Specifically, over a 6 month period and before transfer to soil, a few initially tannin-free plantlets were found to accumulate tannin during random leaflet testing, while other plantlets remained completely tannin-free.

Tannin content was then tested more methodically in transgenic plants using leaflets at identical positions on five different leaves, including plants undergoing transfer to new culture media and plants adapted to growth in soil. On occasion during the more methodical testing, several shoots which appeared at the same stage of development on a plant were found to accumulate tannins, while other shoots had no tannin. In addition, large amounts of tannin were sometimes observed only in the large adaxial mesophyll cells of a leaf, with little in the abaxial network (Lees *et al*., 1993). Later, no differences were observed in flower colour and flower tannin content among transformed and untransformed plants. GUS activity which would indicate the production of antisense CHS could not be detected in two year old transgenic sainfoin plants. Tissue which was cultured from these latter plants had also reverted to kanamycin sensitivity. Finally, two year old transgenic plants were unable to amplify the 380 bp PCR fragment containing the antisense CHS gene and could not hybridize with the 1.35 kb CHS gene under stringent conditions. By three years, all plants regardless of their origin, produced high levels of leaf tannin. These findings support gene elimination as the major mechanism for reversion to the untransformed phenotype.

## REFERENCES


**Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl.** 1994. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York.

**Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248-254.

**Gruber, M.Y. and W.F. Crosby.** 1993. Vectors for plant transformation. Pages 89-119 *in* B.R. Glick and J.E.Thompson, J.E. eds. Methods in Plant Molecular Biology and Biotechnology. CRC Press, Boca Raton.

**Jefferson, J.** 1987. GUS gene fusion system. User's Manual. Clonetech Laboratories.

**Kooter, J.M. and J.N.M. Mol.** 1993. Trans-inactivation of gene expression in plants. Curr. Opin. Biotech. **4:** 166-171.

**Koupai-Abyazani, M.R., A.D. Muir, B.A. Bohm, G.H.N. Towers and M.Y. Gruber.** 1993a. The proanthocyanidin polymers in some species of *Onobrychis*. Phytochem. **34:** 113-117.

**Lees, G.L.** 1988. Influence of benzyladenine on condensed tannin formation and callus growth in cultures from sainfoin (*Onobrychis viciifolia* Scop.) cotyledons. Plant Cell Rep. **7:** 166-169.

**Lees, G.L., N.H. Suttill and M.Y. Gruber.** 1993. Condensed tannins in sainfoin. 1. A histological and cytological survey of plant tissues. Can. J. Bot. **71:** 1147-1152.

**Lindsey, K.** 1991. Plant tissue culture manual. Kluwer Acad. Publ., Boston.

**Singh, S., J. McCallum, M.Y. Gruber, G.H.N. Towers, A.D. Muir, B.A. Bohm, M.R. Koupai-Abazani and A.D.M. Glass.** 1997. Biosynthesis of flavan-3-ols by leaf extracts of *Onobrychis viciifolia*. Phytochemistry **44:** 425-432.

**Skadhauge, B., M.Y. Gruber, K.K. Thomsen and D. von Wettstein.** 1996. Leucocyanidin reductase activity and accumulation of proanthocyanidins in developing legume tissue. Am. J. Botany. **In Press.**

**van der Krol, A.R., P.E. Lenting, J. Veenstra, I.M. van der Meer, R.E. Koes, A.G.M. Gerats, J.N.M. Mol and A.R. Stuitje.** 1988. An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. Nature **333:** 866-869.

**Table 1**
Conditions for regeneration of sainfoin plants.

| CIM | SIM | RIM(1-3) | RIM(4) | RIM(5) |
|---|---|---|---|---|
| MS | MS | MS | SHE | Kao's |
| 2 mg.L$^{-1}$ 2,4-D | 0.1 mg.L$^{-1}$ NAA | | 1/2 strength | 1/2 strength |
| 0.4 mg.L$^{-1}$ BAP | 1 mg.L$^{-1}$ BAP | | | |
| sucrose (3%) | sucrose (3%) | sucrose (1.5%) | sucrose (1.5%) | sucrose (1.5%) |

All media contained 0.8% agar and was prepared as outlined in Lindsey, 1991.
**CIM**, callus inducing medium; **SIM**, shoot inducing medium; **RIM**, root inducting medium; **RIM(1)**, 0.5 mg.L$^{-1}$ BAP, 2 mg.L$^{-1}$ NAA; **RIM(2)**, 0.1 mg.L$^{-1}$ NAA; **RIM(3)**, 1 mg.L$^{-1}$ IBA; **RIM(4)**, 1 mg.L$^{-1}$ 2-iP

**Figure 1**
Plant binary vector used to transform sainfoin with petunia antisense CHS gene.

A) Binary vector insert. GUS, β-glucuronidase; 35S-35S AMV, enhanced constitutive plant promoter (Gruber and Crosby, 1993); antiCHS, antisense chalcone synthase (van der Krol *et al*., 1988); NOS-T, nopaline synthase 3' terminus. The *Hin*dIII-*Eco*RI fragment was inserted into *BIN*19 to develop the kanamycin resistant binary vector pB69 which was used to transform *A. tumefaciens* to strain B71. The *Bam*HI fragment was used as a CHS hybridization probe.

B) Oligonucleotide primers used to amplify a 380 bp DNA fragment by PCR specifically containing the 3' end of the petunia CHS gene.