INDUCTION OF MUTANTS WITH ECTOPIC EXPRESSION OF CONDENSED TANNINS

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ABSTRACT

Leaves of 47,000 *Lotus japonicus* plants were screened using a butanol:HCl histochemical test to select "gain of function" mutants. These plants were progeny from *L. japonicus* lines which were transformed with *T-DNA* constructs containing either the maize *Ds* or *Ac* transposon (Thykjaer *et al*., 1995). Among 21 putative leaf tannin mutants, five (*tan*1-5) were characterized for synthesis of condensed tannins, leucocyanidin reductase activity and the presence of *Ac* and the selectable marker gene, *npt*II . A range of leaf tannin content among other *Lotus* species was also characterized.

KEYWORDS

condensed tannins, *Lotus japonicus*, mutants, transgenic plants, *T-DNA* and Tn tagging

INTRODUCTION

Condensed tannins are important plant secondary metabolites with foam reducing, dietary protein protecting, insect resistant and antioxidant properties. As such, their synthesis in relevant plant organs is of particular interest for the industrial and agricultural use of legume crop plants. Particularly, it is an important breeding aim to express the condensed tannin pathway ectopically in leaves of the world's major forage legumes, alfalfa and white clover, as a means of eliminating pasture bloat and improving rumen protein-bypass and insect resistance. In an exploratory investigation, we have developed a system to obtain flower and leaf tannin mutants using *Lotus japonicus* transformed with maize transposable elements and a histochemical test for condensed tannins. The characterization of several mutants with leaf tannins is presented.

MATERIALS AND METHODS

Screening for leaf tannin mutants: Leaves of 32 species of *Lotus* were assayed for condensed tannin content using a modified butanol:HCl:PVPP assay (Skadhauge *et al*., 1997) to determine candidate species for mutant development. Floral, leaf, stem and root tissues of the selected species, *L. japonicus*, were also tested for tannin content. *L. japonicus* lines were transformed with *Agrobacterium tumefaciens* binary vectors and selected as *T-DNA* lines (containing a disabled maize transposable element) or *Ac* excision lines (containing an active maize *Ac* transposable element) (Thykjaer *et al*., 1995). Approximately 47,000 self pollinated progeny seeds from 1230 independent transformed lines were sown in flats and grown in greenhouses. A subset of seedlings was cultivated under field conditions as outlined below. Seed grown for mutant selection from *T-DNA* insertion lines originated from the 2nd generation of self-pollinated transgenic plants (T2). Seed from Ac lines arose from T3 and T4 transgenic plants selected for transposon excision (Thykjaer *et al*., 1995). Two leaves per progeny from 7-10 week old plants and two flag petals per progeny from 3.5 month old plants were tested histochemically for variation in tannin content (Skadhauge *et al*., 1997). Lines were also assessed for variation in morphology. It was most efficient to test leaves for tannins in the greenhouse prior to transplanting, but f lowers were easily collected from field plots. Variants which gave similar results repeatedly were designated mutants and cultivated in the greenhouse. Subsequently, several tannin mutants were tested genetically and biochemically as follows. Monomeric and dimeric flavonoids were isolated from mutants as an ethyl acetate fraction according to Koupai-Abyazani *et al.* (1992), separated by HPLC on a *u*Bondapak phenyl column (30 cm x 3.9 cm, Waters Assoc.) (2 to 10% v/v linear acetic acid gradient, 60 min, 2 ml.min^{-1} flow rate) and detected by UV absorption. Condensed tannins were purified from the remaining aqueous fraction by elution from a Sephadex LH20 column (15 cm x 4 cm, Pharmacia) using 75% acetone (Koupai-Abyazani *et al*., 1992), and their molecular weights determined according to Koupai-Abyazani *et al*. (1993). Southern blot hybridization with *Ac* and *npt*II genes was performed according to standard molecular biology protocols (Sambrook *et al*., 1989). Condensed tannin content was determined for self pollinated S1 and S2 generations of selected mutants by the butanol:HCL:PVPP method above. A transgenic field trial (#94-ACS1-LOJ01-SK01-01) was undertaken at the AAFC Saskatoon Research Centre farm. Approximately 6,500 seedlings from 76 independently transformed lines were manually transplanted to the field, two months after germination. The row/plot design was equally useful for manual harvesting of seed from individual plants, manual harvesting of bulked seed from each line or mechanical harvesting of bulked seed. A misting system was devised to reduce excessive seed shattering under dry prairie conditions. A black polyethylene woven ground cloth protected the plot from weed growth and aided in the harvest of valuable seed from any shattered pods. Each transgenic line was covered by a translucent row crop cover cloth to protect against insect and wind damage and to prevent cross pollination among the 76 transgenic lines and with other *Lotus* species planted on the farm. Plant lines were monitored for insect damage and growth.

Since *L. japonicus* flowers indeterminantly, ripe (brown) pods were manually harvested in several rounds from 4-5 month old field plants, then stored in paper bags for up to 5 months at temperatures ranging from 10-20^{o} C until seed was cleaned. At the end of the season (September 30, 1994), the forage from each plant was harvested manually and placed into paper bags. Plot condition, the number of nonflowering plants and top growth, (forage length and weight of 15 plants) were recorded for each line. All remaining green pods were collected manually from the cut forage, stored in a cool forage shed in paper bags and dried at 50^{o} C in a forced air forage dryer. Seeds were removed manually from dry pods, weighed, and then packaged as green or ripe seeds (from brown pods) into paper envelopes, and stored at 4^{o} C. Seed weight and germination rate were compared among 10 lines. Seed was germinated by treatment for two minutes in conc. H_2SO_4 to promote imbibition, followed by several washes in sterile water placement on damp filter paper for 5 days.

RESULTS AND DISCUSSION

A range of leaf tannin concentrations were present among the 32 *Lotus* species tested (Table 1). Diploid *L. japonicus* was identified as a good candidate for development of mutant legume populations with altered tannin content. Flowers of this species contained large amounts of tannin; levels in leaves were undetectable (Table 2). Subsequently, progeny lines of *L. japonicus* transformed with either an active *Ac* transposon or a disabled transposon (Thykjaer *et al*., 1995) were screened for variation in tannin content and growth traits. The design and nature of the field trial enabled us to recover and sort all seed from ripe, unripe and shattered pods with reduced risk of plant loss through early freezing damage, an important consideration when undertaking a field increase of such

valuable germplasm in Canada. Although seed was bulked from each line, the design could be used easily to collect seed from individual plants. Germination rates of seed from fully ripe pods were consistently >80% (measured on 10 lines). When hard seeds which did not imbibe were subsequently scarified with a scalpel and incubated for a further 5 days, germination rates rose to >95% (measured on 3 lines). Seedlings grown from the ripened field seed were very vigorous. Seed from green pods germinated inconsistently at a frequency ranging from 3-30 % (measured on 8 lines). Seed stocks are maintained at the University of Aarhus. Several plants in one line had a dried brown curled leaf at node 4. Three lines had dwarf plants. In total, 23 plants had detectable levels of leaf tannin and were designated *tan* mutants. One line had a plant with a pleiotrophic mutation leading to very small flowers, hairy leaves and leaf tannins (*tan*-1). With the exception of *tan*- 1, all *tan* mutants resembled untransformed *L. japonicus* morphology. Flower variants without tannins were not observed under either greenhouse or field screening conditions. Condensed tannin levels in field-grown flowers were generally much higher than in greenhouse grown, but the levels were more variable. Conditions across the field plot varied and were less easy to control compared with greenhouse growth. Hence, selection of variants with subtle genetic differences in tannin expression was impossible in the field trial. Five mutants (*tan*1-5) were chosen for further analysis (Table 2). These mutants all have the requisite *T- DNA*/ transposon insertion elements. *Tan*-1 was the only one selected after *Ac* excision selection, i.e. from the T_4 generation; the rest were selected from lines with disabled transposons from the T_2 generation (Table 1). Leucocyanidin reductase (LCR), the first enzyme in the flavonoid pathway uniquely committed to condensed tannin biosynthesis, was easily detected in the leaves of the five mutant genotypes. LCR activity correlates well with the level of leaf tannin. The leaf tannin content of *tan*1 was uaually stable in plants of two subsequent generations (S_1 and S_2) after self pollination, although the tannin phenotype had reverted in 7% of the S_1 plants (Table 1). Phenotype reversion occurs on average in 10-15% of progeny in *L. japonicus Ac* excision lines (Thykaer *et al*., 1995). In contrast, leaf tannins could only be detected in 4-8% of the S_1 progeny of *tan*-2 to *tan*-5. This low heritability improved slightly in the S_2 generation, such that 7-11% had detectable leaf tannins. The masking of the mutant phenotype in *tan*2-5 was not due to transposition of the stable *Ds* element through the activity of a native *L. japonicus* transposase (data not shown). These plants are now being backcrossed to untransformed *L. japonicus* to try and improve *tan*2-5 expression and to stabilize the mutations. The flavonoid content and morphology of *tan*-1 was compared with *L. angistissimus*, a species with morphology unlike *L. japonicus*. *Tan*-1 and *L. angustissimus* look very similar with small hairy leaves, short internodes and small flowers. Untransformed *L. japonicus* has large flowers, large smooth leaves and longer internodes. The leaf flavonoid profile of *tan*-1 and its S_2 progeny appeared more like *L. angustissimus* than like untransformed *L. japonicus*. Preliminary molecular weight determinations on purified condensed tannin polymer fractions from *tan*-1 and *L. angustissimus* were also similar. We consider *tan*-1 to be a pleitrophic mutant, since both morphology and tannin content were altered. At this point, it is not clear whether the *tan*1-5 phenotypes arose directly from transgene insertion events or, alternatively, from some other mutation event. For example, recent observations of the generation and disappearance of the *tan*-1 phenotype in untransformed *L. japonicus* and untransformed *L. angistissimus* (Gruber, unpublished) lend support to a mechanism involving a natural transposon system in *Lotus* species. However, the suppression of the *tan2-5* phenotype, followed by improvement of expression during subsequent generations, parallels what has been observed with transgenes in *Arabidopsis thaliani* (Mittelsten Scheid *et al*., 1991; Dehio and Schell, 1994).

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Table 1
Leaf Tannin Variation Among *Lotus* Species

Lotus Species Name	%FW Tannin (+/- SD)
L. purshianus (Benth.) Clem.& Clem.	4.1 (0.3)
L. palestinus (Boiss.) Bornum	0.5 (0.3)
L. palustris Willd	3.4 (0.7)
L. arabicus L.	0.4 (0.3)
L. angustissimus	3.2 (0.7)
L. peregrinus L.	0.4 (0.2)
L. corniculatus var. Hirsutus	2.5 (0.3)
L. decumbens	0.4 (0.2)
L. parviflorus Desf.	2.4 (0.7)
L. campylocladus Webb & Berth	0.3 (0.1)
L. hispidus	2.3 (0.5)
L. jacobeus L.	0.3 (0.2)
L. creticus L.	1.9 (0.5)
L. hirsutus	0.2 (0.2)
L. scoparius (Nutt.) Ottley	1.5 (0.7)
L. cytisoides	0.2 (0.2)
L. caucasicus Kupr.	1.2 (0.8)
L. krylovii Schischk and Serg.	0.1 (0.2)
L. discolor	1.0 (0.6)
L. schoelleri Schweinf.	0.0 (0.0)
L. macroccanus Ball	1.0 (0.9)
L. borbasii Ujhelyi	0.0 (0.0)
L. conjugatus L.	0.9 (0.3)
L. burttii Sz. Borsos	0.0 (0.0)
L. ornithododiodes L.	0.8 (0.3)
L. cruentus Court.	0.0 (0.0)
L. sulphareus	0.7 (0.4)
L. edulis L.	0.0 (0.0)
L. siliquosis L.	0.6 (0.5)
L. filicaulis Dur.	0.0 (0.0)
L. carmeli Boiss.	0.6 (0.02)
L. japonicus (Regel) Larsen	0.0 (0.0)

Table 2
Leaf Tannin Mutant Biochemistry and Genetics (*tan*1-5)

S_0 Genotype (screening generation)	Tannin Content in S_0 Generation ($ug \cdot mg^{-1}FW$)			LCR Activity (leaves) ($DPM \cdot 10^{-1} \cdot mg^{-1}FW \cdot h^{-1}$)	Segregating Plants with Leaf Tannin (%)	
	Flowers	Leaves	Stems		S_1	S_2
Untransformed	188	N.D.	6	N.D.	-	-
***tan*-1 (T_4)**	137	118	5	16.8	93.2	100.0
***tan*-2 (T_2)**	222	59	9	9.3	8.5	11.3
***tan*-3 (T_2)**	172	37	7	5.1	6.8	9.8
***tan*-4 (T_2)**	183	25	5	1.6	3.7	7.3
***tan*-5 (T_2)**	196	12	8	2.1	3.9	6.9