ALTERATION OF CONDENSED TANNIN SYNTHESIS IN TRANSGENIC FORAGE LEGUMES

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ABSTRACT

The transformation of *Lotus corniculatus* plants with the maize gene Sn, reorganizes the tissue specificity of condensed tannins accumulation. In particular the transformed plants show an increase of tannin content in roots and a decrease in leaves. Molecular and enzymatic analyses suggest that the transgene can functionally substitute an endogenous unknown gene not expressed in roots and induces its silencing when it is expressed. These findings could have applications for reducing tannin content in unpalatable plants and for cloning genes involved in tannin synthesis.

KEYWORDS

Forage legumes, bloating, condensed tannins, transformation, regulatory genes, cosuppression

INTRODUCTION

The concentration of condensed tannins in forage legumes is a critical point for their quality; as a matter of fact a high amount of tannins reduces the voluntary intake of a forage (Butler, 1979) but the presence of a moderate amount of tannins in legumes improves the assimilation of proteins and prevents bloating (Tanner et al., 1995). The strategy for bettering forage quality of legumes is to improve the aminoacidic composition of proteins and to induce the synthesis of moderate amount of condensed tannins in leaves. The most important forage legumes, lucerne and clovers, do not contain tannins and therefore works are in progress in order to clone the genes responsible for their synthesis and to transfer such genes into the target species.

METHODS

Plants of *Lotus corniculatus* cv. Leo have been transformed with the coding sequence of a maize gene which transactivates the anthocyanin pathway (Sn) under the constitutive promoter CaMV35S and with the reporter gene NptII, conferring kanamycin resistance (Damiani et al., 1996).

Several plants have been regenerated and analysed for the presence of condensed tannins (CT) in various tissues (leaves, veins, stems, root) as described by Li et al. (1996).

Three key enzymes (Chalcone synthase, CHS; Dihydroflavanol reductase, DFR; Leucoanthocyanidin reductase, LAR) of CT pathway have been analysed in the most contrasting tissues (leaves and roots). CHS has been quantified by dot-blot immunological analysis as described by Jahnen and Halhbrock (1988), while DFR and LAR by their enzymatic activities through radio-HPLC.

Expression of the transgene has been detected through RT-PCR and Northern Blot.

Kanamycin resistance has been assayed culturing explants of transformants (Damiani et al., 1993) at different doses of the antibiotic. Regenerants have been tested again for leaf tannin, analysed for gene expression through RT-PCR and for promoter methylation by the amplification with primers complementary to flanking sequences of the CaMV35S promoter of DNA samples cleaved with methylation sensitive restriction enzymes.

RESULTS AND DISCUSSIONS

Seven plants have been regenerated and tested for tannin concentration in leaves and roots and compared with a control plant transformed with the plasmid pBI121.1 (Table 1). Five plants showed a significant reduction in leaf CT, six plants showed an increase in root CT. Enzymatic analysis showed a strict parallelism between DFR (limited to leaves) and LAR activity and CT content, conversely no clear relations among CHS content and CT concentration have been observed (Table 2).

Analysis of Sn expression, through RT PCR, indicates that in CT negative leaves no mature Sn-mRNA is detectable but run-on analysis showed that the transcription of the transgene has been started also in Sn-mRNA negative plants (Table 1). This observation resembles the described mechanism of homologous gene silencing (Flavell, 1994) and induces to account for the strong decrease of leaf CT to the insurgence of a cosuppression mechanism between an unknown *Lotus* gene and Sn. This hypothesis is confirmed by the ability of Sn to increase CT in roots indicating its efficiency in a heterologous system. Other supports to the cosuppression hypothesis are added by the behaviour of somaclones, in fact the regenerants from leaf CT negative plants although still resistant to kanamycin show a large variability for CT content and most of the somaclones revert to the original phenotype; this is a typical feature of cosuppressed plants. Preliminary analysis seems to indicate that the methylation of Sn promoter is responsible for the reversion to the wild phenotypes.

These results offer tools for improving forage quality: in fact with this strategy or perhaps using some other regulatory genes of the tannin pathway it should be possible to reduce tannin content when necessary. The most intriguing result is however the availability of CT negative mutants to be utilized for cloning, through subtractive strategies, those genes (regulatory and structural), still unknown, involved in the CT synthesis and then to isolate the respective promoters. Having those genes and promoters available, a strategy of genetic transformation to induce the CT synthesis in bloating species, as the most cultivated forage legumes, becomes conceivable.

REFERENCES

Butler, L.G. (1989) Effects of condensed tannin on animal nutrition *In Chemistry and Significance of Condensed* Tannins, Hemingway R.W. and Karchesy J.B eds (New York: Plenum Press) pp. 391-402.

Damiani, F., Nenz, E , Paolocci, F., and Arcioni, S. (1993) Introduction of hygromycin resistance in *Lotus* spp. through *Agrobacterium rhizogenes* transformation. *Transgenic Research* **2**, 330-335

Damiani, F., Paolocci, F., Consonni, G., Crea, F., Tonelli, C., and Arcioni, S. A maize anthocyanin transactivator induces pigmentation in several transgenic dicotyledonous species. Submitted to *Plant Cell Report*

Flavell, R.B. (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. *Proc. Natl. Acad. Sci. USA* **91**, 3490-3496.

Jahnen, W. and Hahlbrock, K. (1988) Differential regulation and tissue-specific distribution of phenylpropanoid pathways in developing parsley seedlings. Planta **173**, 453-458.

Li, Y.G., Tanner, G.J., and Larkin, P.J. (1996) The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *J. Sci. Food Agric* **70**, 89-101

Table 1
Leaf and root CT content and Sn expression of control and Sn transgenic plants of *L. corniculatus* (-= not detected; +=present; nd= not determined)

Plant	CT (mg Leaf	/g DM Root Root	RT PCR	Run-on
Control	20	23	-	-
LSN32	0.5	55.1	-	ND
LSN39	0.2	76.4	-	ND
LSN50	0.3	72.6	-	+++
LSN60	16	71.3	+++	+++
LSN63	0.8	21.3	-	ND
LSN64	20	80.4	+++	ND
LSN65	6.2	76.9	++	ND

Table 2
CHS protein amount (% of control) and DFR and LAR activity measured om ;eaves pf Sn trasfpr,ed amd cpmtrp; [;amts pf *L. corniculatus.* LAR[1] has been measured in roots.

Plant	CHS (prot %)	DFR (pmol	LAR /min/	LAR[1] mg)
Control	100	31±1	10±1	27±3
LSN32	173	0±0	0±0	100±2
LSN39	88	0±0	0±0	96±3
LSN50	98	4±1	0±0	45±8
LSN60	82	38±4	4±1	153±6
LSN63	56	4±2	1±1	82±21
LSN64	75	38±6	14±1	26±4
LSN65	77	153±3	6±1	64±13