High Polymorphism for Forage Production of *Lotus corniculatus Sn* Transformants

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HIGH POLYMORPHISM FOR FORAGE PRODUCTION OF LOTUS CORNICULATUS SN TRANSFORMANTS

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Abstract

Transgenic plants obtained from transformation of one individual of *Lotus corniculatus* with the maize gene *Sn*, a transactivator of anthocyanin pathway, were analysed for the expression of the transgene and for the accumulation of condensed tannins. A great variability was observed for these two traits. Some extreme individuals were clonally propagated and analysed for rooting ability and plant growth. Unexpectedly, a strong correlation between condensed tannin level, rooting ability and plant growth was observed. These results are discussed in order to explore new strategies to increase plant productivity in forage legumes.

Keywords: *Agrobacterium*, myc-like genes, rooting ability, plant growth, cosuppression, transactivation, *Lotus corniculatus*

Introduction

Sn is a maize gene of the myc family. It is a strong activator of the anthocyanin pathway and shows large homology at the level of the encoded protein with all genes of the same family (Quattrocchio 1994). This gene family has been demonstrated to affect other

pathways than anthocyanin biosynthesis, such as trichome development in transgenic *Arabidopsis* (Larkin et al., 1994).

Sn transgenic of *Lotus corniculatus* altered the pathway of condensed tannins (CT), compounds strictly related with anthocyanins (Damiani et al., 1999), and, interestingly, it was observed that within the same transgenic population, consisting of *Sn* independent transformations of the same genotype, a large variability was observed for the accumulation of CT (Paolocci et al. 2000). The multiplication of these plants and their growth in contrasting environments, performed with the purpose of isolating the CT genes, showed a large variability among these isogenic plants. The present work has the objective of analysing the direct and indirect effect of the transgene on plant growth.

Material and Methods

Agrobacterium rhizogenes binary vector was utilised for inserting the myc-like gene *Sn*, isolated in maize by Tonelli and co-workers (1991), into a well characterised genotype of *Lotus corniculatus*. The vector and the procedures for plant transformation and regeneration were described by Damiani et al. (1998, 1993). The genotype S50 of the cultivar Leo (Carron et al., 1994) was transformed. About 20 independent transgenic plants were produced. They were analysed for the presence and the expression of the transgene through Southern and Northern blotting**.** Protocols for DNA and RNA extraction, restriction electrophoresis and hybridisation were performed as reported by Damiani et al. (1999).

The transgenic plants were also analysed for the levels of condensed tannins in different tissues utilising the DMACA staining protocol described by Li et al. (1996).

Plant multiplication was performed in vitro, by culturing stem segments in Magenta vessel containing 50 ml MS medium ½strength. For this purpose, 5 Magenta vessels were prepared for each independent transformant, for the non-transformed mother plant and for the

plant transformed with the *uidA* gene (Jefferson et al., 1987). Each vessel contained an average of 10 cuts. The experiment was repeated three times.

The number of rooted cuts was assessed. Plants were then transferred to soil in the greenhouse. After plants were well established they were moved to a growth chamber, at 10°C with daylight cycle of 12 hours, light intensity 1500 μE $m^{-2}s^{-1}$. After 6 weeks, temperature was increased to 23°C. Plant growth was assessed.

Results and Discussion

A large variability for CT level in leaves was observed within transgenics. Some plants (labelled E, enhanced) showed an increased level of CT ($S50_{10}$, $S50_{11}$), some others (S, suppressed) a reduced level of leaf CT ($S50_6$, $S50_9$, $S50_{20}$). The molecular analyses of these plants showed that E plants had only one copy of the transgene while the S (suppressed) plants had a number of copies ranging from 3 to 5 (Table 1). The northern analysis of these plants indicated that E (enhanced) plants expressed the transgene and had the expression of a myc endogenous gene unaffected. S plants, on the contrary, showed the lack of expression of both genes (not shown).

Co-suppression events (Metzlaf et al., 1997) may offer an explanation for what was observed. These plants were particularly useful for isolating the gene committed to CT synthesis, through cDNA comparisons of E and S plants. Therefore to increase the amount of tissue available they were clonally propagated.

The method of propagation which allows for fast multiplication of *L.corniculatus* plants (Pupilli et al., 1991) was utilized. However a great variability for *in vitro* rooting was observed: $S50_{10}$, $S50_{11}$ derived cuttings easily rooted and, after 2 weeks from transplantation, all explants rooted. In contrast, only few S plants derived cuttings were rooted after 2 weeks of culture.

This was particularly evident for cuttings from plant $S50_9$ (see Table 1). By supplementing the medium with 3mg/l of Indoleacetic Acid (IAA), the differences in rooting ability were overcome.

Plant growth was evaluated after 4 weeks at 23°C, a typical temperature in the growing season of *L.corniculatus*. The results for 5 plants per type and three replication are reported in Table 2.

Data clearly indicate that S plants had a reduced growth potential and this is particularly true for plant $S50_9$. The hypothesis to explain these observations are related to the effect of the transgenes on plant morphology. Since the transformation has been carried out with the wild *A.rhizogenes* strain 1855, *rol* genes, the genes harboured in the *A.rhizogenes* plasmid and committed with hairy root formation, are integrated into the plant genome and since these genes exert an hormonal activity they can interfere with the plant growth (Schmülling et al., 1988). It is unequivocal that the supplementation of IAA in the rooting medium allowed all cuts to root; however the effect of the *myc* endogenous gene must not be ignored. Many experiments of genetic transformation through hairy root have been performed and although the *rol* genes affected significantly plant morphology such reduced growth ability was never reported. On the contrary, since the endogenous myc-like gene has been proved to be suppressed we can infer that this gene may affect the expression of genes in some way related to plant growth ability. The E plants featured by high growing ability and high levels of CT do not increase the myc endogenous activity but the transgene *Sn* is highly expressed and it could support the activity of the endogenous counter part.

No definite conclusions can be drawn yet, thus further study of the effect of such class of transactivator genes on plant growth is still needed.

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Table 1 - In vitro rooting of transformed plants classified as S (CT suppressed) and E (CT enhanced). Number of copies of Sn transgene and number of rooting cuts over total cuts plated (average of 15 magenta plates) are reported. The level of probability of $\chi 2$ calculated for the homogeneity for rooting ability respect to the control plants is reported on the last coloumn (P).

PLANT ID	N.Sn copies	N.cuts	N.rooted cuts	P*
S50 (C)**	-	10	8.2	
$S50_{121.1}$ (C)	-	11	7.5	n.s.
$S50_{6}$ (S)	3	10	4.3	0.05
$S50_{9}$ (S)	5	14	2.1	0.01
$S50_{10}$ (E)	1	10	9.1	n.s.
$S50_{11}$ (E)	1	11	8.7	n.s.
$S50_{20}$ (S)	4	13	5.9	0.05

*P= level of probability for the $\chi 2$ with one degree of freedom
** (C)= control, (S)=suppressed, (E)=enhanced

Table 2 - Average fresh weight (g/plant) of control (C) and transformed S50 plants, CT suppressed (S) and CT enhanced (E), evaluated in 5 plants each and three replicates.

PLANT ID	S50 (C)	S50-121 (C)	$S50_{-6}$ (S)	$S50_{-9}$(S)	$S50_{-10}$(E)	$S50_{-11}$(E)	$S50_{-20}$(S)
average f.w. (g/plant)	7.9 b*	7.4 b	7.2 b	2.1 c	12.1 a	12.7 a	3.5 c

* means followed by the same letter do not differ at $P \leq 0.05$