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Sn transgenic plants of *Lotus corniculatus* are utilised for isolating genes involved in the biosynthetic pathway of condensed tannins

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SUMMARY – *L. corniculatus* plants derived from transformation with the maize gene *Sn* of only one genotype resulted highly polymorphic for condensed tannins (CT) levels. Stable CT enhanced and suppressed plants have been screened and further analysed at molecular level for transgene insertion and for the expression of some genes of the biosynthetic pathway of phenylpropanoids. These selected individuals are utilised for isolating the genes responsible of the metabolic steps leading from flavonol to CTs.

Key words: Plant transformation, gene cloning, cDNA-subtraction.

RESUME – *"Des plantes de lotier corniculé transformées par insertion du gène* Sn *sont utilisées pour isoler les gènes de la voie de synthèse des tannins condensés". Des plantes de lotier dérivées par transformation d'un seul génotype avec le gène de maïs* Sn *résultent très polymorphiques pour le niveau de tanins condensés (TC). Les plantes stables pour la teneur en TC, ont été sélectionnées et évaluées es au niveau moléculaire. Ces plantes très polymorphiques pour la teneur en TC ont été utilisées pour identifier avec des analyses soustractives les gènes pour la synthèse de TC.*

Mots-clés : *Transformation, clonage, cDNA subtraction.*

Introduction

Condensed tannins (CT) are plant secondary metabolites with a significant effect on animal digestive processes. Their importance for nutritional purposes is well recognised since a CT-rich diet may have profitable and unprofitable drawbacks. CT, in fact, inhibit enzymatic activity and slow down digestive processes due to their ability to bind proteins and protect them from rapid degradation by bacterial flora. This is a common phenomenon occurring in ruminants, referred to as bloating. Bloating reduces the protein intake by animals and also promotes side effects such as water and air pollution due to ammonium excretion.

CT are compounds derived from the reduction of flavonols but information on which, and how many more genes are involved from this step till the formation of CT are still unavailable. This information is of paramount importance to be able to manipulate this pathway in order to modulate the level of CT in the forage legumes. In some of these, such as lucerne and clovers, CT are completely absent from edible tissues and, in some others, an excess of CT in leaves prevents the free intake of animals because of the bitter taste and the inhibitory effect on salivary enzymes that CT confer to these forages.

The aim of the work was to isolate the genes of the pathway utilising transgenic *Lotus* plants which resulted highly polymorphic for CT levels after transformation with a myc-gene (*Sn*) that in maize transactivates the anthocyanin pathway (Tonelli *et al*., 1991).

Materials and methods

Several transgenic individuals have been produced from *Sn* hairy-root transformation of a genotype of *Lotus corniculatus* cv. Leo (S50) characterised by low levels of CT in leaves. The transgenic plants plus two controls, the untransformed S50 and the S50 genotype transformed with the plasmid pBI121.1 (Jefferson *et al*., 1987) were evaluated for CT presence. For this purpose leaves of each plant were stained with DMACA as described by Li *et al*. (1996). Four cuttings for each plant were visually scored

for the intensity of CT staining and the assay was repeated monthly from March to June. The most contrasting plants were further evaluated for gene copy number, and for gene expression. Southern blotting and RT-PCR were performed as described by Damiani *et al*. (1999).

In order to isolate both regulatory and structural *L. corniculatus* genes involved in the pathway, degenerate primers were designed for DFR and bHLH genes based on sequence information of the orthologous genes cloned in other species. These primers were utilised for amplifying cDNA of *Lotus* from leaf tissues. Full-length cDNA both for DFR and an endogenous myc-gene were then obtained by 5' and 3' RACE (Paolocci, unpublished results).

Subtractive methods

Contrasting selected genotypes were utilised for cDNA subtractive experiments. Two methodologies were adopted. PCR-Select and cDNA-AFLP.

The first was based on a Clontech Kit and was performed as described in the "PCR-Select cDNA subtraction Kit User manual" utilising cDNA from the two pairs of contrasting plants in both directions of subtraction (i.e. CT+ minus CT- plants and *vice versa* plants, as control). Alternatively to this method cDNA-AFLP was performed. The protocol of Bachem *et al*. (1996) was applied with minor modifications.

All the bands isolated with both methods were cloned and sequenced through semi-automatic sequencer ABI-Prism 310 following manufacturer instructions using the "Big Dye terminator kit" (Applied Biosystems). Sequence similarities were searched using BLAST, and primers designed on the most interesting sequences were used for RT-PCR analyses to confirm their differential expression.

Results and discussion

Characterisation of transgenic plants *Sn*

Transgenic plants and the two controls were assayed for CT presence with a qualitative test based on DMACA staining which produces dark blue spot on CT-containing cell. *Sn* transformation greatly affected CT levels, some plants resulted highly enhanced for CT and hereafter will be labelled (E) some other resulted suppressed for CT synthesis (named S), see Fig. 1. The histogram of the CT score attributed to each plant is reported in Fig. 1A. A large variability was reported for CT levels within plants derived from the same genotype but no significant differences occurred between the untransformed control and the GUS-transformed plants. It indicates that the alterations of the CT phenotype are determined by the presence of *Sn* gene and are not related to transformation methodology, a further validation of this came from the observation that CT polymorphic transgenic plants do not show a consistent differential pattern of expression for *rol* genes; these genes are known to be transferred into the host genome following hairy root transformation and exhibit a hormonal activity.

A seasonal variability was observed for CT levels and some genotypes resulted unstable for CT levels showing a suppressed phenotype in the early analyses but displaying no differences with respect to the control on late assays. Nevertheless some genotypes resulted highly stable for CT enhancement (no. 10 and no. 11) and some highly stable for CT suppression (no. 6 and no. 9). Moreover, while some genotypes dramatically changed the level of CT in assays performed in successive years (plant 20 for example, Paolocci *et al*., 2000) the previously mentioned ones resulted stable over the seasons and over the year. *In vitro* cuttings produced from these plants maintained the original phenotype, therefore all the molecular analyses were concentrated on plants 6, 9, 10 and 11.

Transgene copy number and expression

In order to assess copy number and the presence of direct and inverted repeats plant DNA was restricted both with HindIII and EcoRV and probed with *Sn* and *NptII* fragments (for a detailed description of the method see Damiani *et al*., 1999). The number and the length of the hybridising bands permits the number and the type of insertions of T-DNA to be established. E plants resulted single copy transformant, S plant 6 had three copies and plant 9 six T-DNA insertions, two of them with truncated *Sn*.

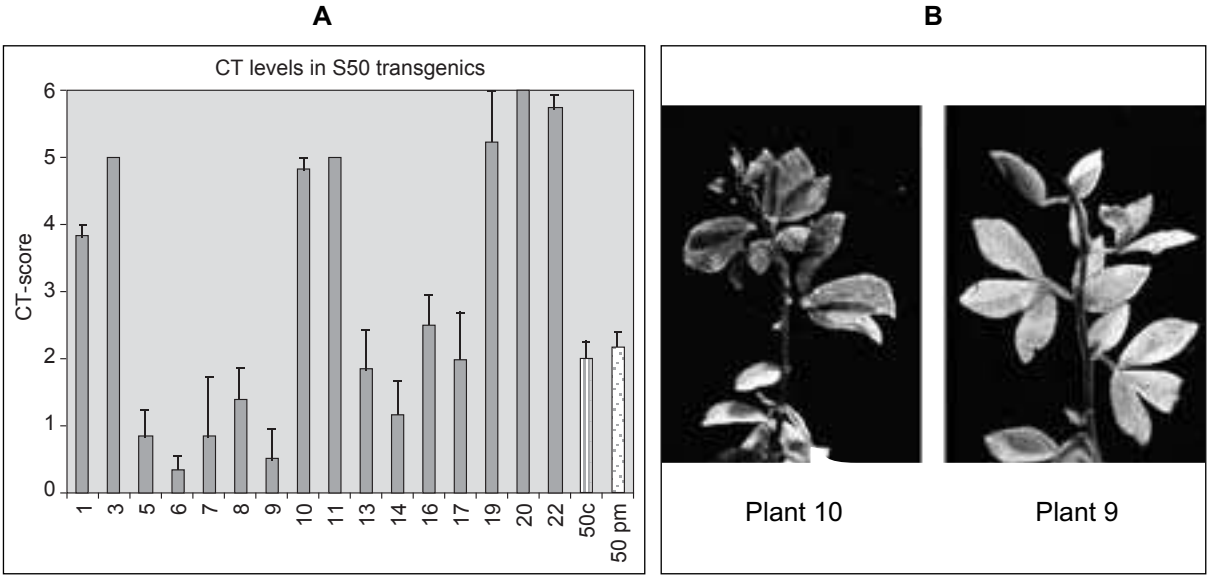

Fig. 1. Levels of condensed tannins in transgenic plants. (A) score 1-7 for controls and transgenics, (B) transactivated (plant 10) and suppressed (plant 9) transgenics.

The fact that enhanced plants contain only one copy of the transgene seems of importance for its expression, as matter of fact multicopy *Sn* transgenics resulted silenced. Expression of other genes of the pathway were assayed and DFR came out to be strongly expressed in E plants, weakly in controls and even less in S plants. It indicates that *Sn* when transcribed is able to transactivate DFR expression also in heterologous environments while *Sn* silencing should imply a negative effect on the endogenous counterpart that is deputed to transactivate DFR and probably other genes of the CT biosynthetic pathway. Interestingly, an endogenous bHLH gene was significantly down regulated in *Sn* silenced plants. On the basis of this observation it was hypothesised that as DFR, other genes of the pathway should have been alternatively transactivated or suppressed in E and S plants, respectively. This assumption was confirmed by the fact that the activity of LAR, the 1st enzymatic activity downstream DFR in the phenylpropanoid pathway, resulted suppressed in S plants produced with a previous experiment (Damiani *et al*., 1999).

Identification of differentially expressed genes

Two methodologies were applied, both requiring an amplification through PCR step, the utilisation of an amplification step allows a limited amount of RNA to be worked with, and, since the collection of large amounts of homogeneous tissue is quite difficult, it helps to overcome one of the main bottleneck steps of this subtractive strategy. Nevertheless PCR can add a new order of problems such as the non homogeneous amplification of cDNA pools and as a consequence the production of no real differentially expressed sequences. With the subtractive methods proposed by Clontech we made subtraction between plants 10 or 11 (E) and one S plant (6 or 9, alternatively). Utilising the suggested restriction enzyme (RsaI, a four cutter) the average size of the sequences resulted quite small (around 250 bp), in order to increase fragment size we utilised a different combination of restriction enzymes (PvuII and EcoRV, in the same restriction mix). This combination of six cutter enzymes is in fact expected to increase the size of fragments to 2048 bp respect to the 256 average sizes of RsaI fragments. This modification resulted successful and in fact cDNA fragments resulted significantly longer even though not as much as theoretically expected on the basis of the stochastic model. This is likely to be due to the amplification step that favours shorter sequences.

In conclusion 69 putative differentially expressed sequences were identified, sequenced and blasted. Twenty-eight fragments were further tested through RT-PCR analysis on S and E plants with sequence specific primers and only 3 sequences were confirmed to be differentially expressed. This result suggests that either mistakes occurred or that the methodology is not strong enough for removing the common sequences between the two cDNA pools.

The cDNA-AFLP strategy was thus performed. All the selected plants along with the control-untransformed one were used in the same experiment. In this way it is likely to get rid of sequences that resulted polymorphic also within the same class of individuals reducing the number of processed fragments. However, this method is slower than the previous one. In fact with PCR-Select, theoretically, one experiment is enough to recover all the differentially expressed genes containing the utilised restriction enzymes, while using cDNA-AFLP, independent experiments necessitate for each primer pair for a given couple of restriction enzymes. That is with our primers (each having two protruding nucleotides), 256 different amplification each involving at least 5 plants are necessary in order to cover all the possible fragments obtained with a pair of restriction enzymes.

At the moment we explored 96 primers combination of the couple of restriction enzyme EcoRi-MseI and we produced 55 polymorphic fragments. When checked for confirmation only 2 out of 11 tested resulted differentially expressed. These confirmed fragments result very promising and work is in progress to test their real involvement with CT synthesis.

Conclusions

The work reaffirms the ability of transactivator genes to affect heterologous systems both in terms of species and in terms of pathway. The usefulness of isogenic plants polymorphic for the CT pathway is evident, however the methods to exploit these plants for isolating genes are not totally reliable, this causes a delay in reaching the proposed goals and indicates the necessity of a significant improvement of subtractive methodologies.

Acknowledgements

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