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M. Bellucci · A. Alpini · F. Paolocci
F. Damiani · S. Arcioni

Transcription of a maize cDNA in *Lotus corniculatus* is regulated by T-DNA methylation and transgene copy number

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Abstract *Lotus corniculatus* plants transformed with a maize cDNA (G1L) encoding a sulphur-rich γ-zein were obtained by using two fusion genes: one with the CaMV 35S promoter, the other with the ribulose bisphosphate carboxylase small subunit (*rbcS*) promoter. The highest expression of G1L mRNA was found in plants transformed with G1L under the *rbcS* promoter. The steady state level of G1L mRNA in the leaves was generally directly correlated with the G1L copy number. However, due to a transcriptional block, no G1L mRNA was detected in some of the 35S-G1L multicopy transformants. Analyses with methylation-sensitive restriction enzymes revealed that the T-DNA of the silenced 35S-G1L transformants was methylated. T-DNA copy number, G1L silencing activity, and the state of methylation were strictly correlated in primary transformants. A cross between two 35S-G1L transformed plants, one (S) with the T-DNA methylated and the other (NS) without, showed that: (1) the methylated state of T-DNA passed through meiosis; and (2) when T-DNA copies from the two parents were combined in the progeny, the unmethylated T-DNA copies of parent NS became methylated at different levels and G1L mRNA production was dependent on the degree of methylation.

Key words CaMV 35S promoter · DNA methylation · Gene silencing · *Lotus corniculatus* · *rbcS* Promoter

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M. Bellucci · A. Alpini · F. Paolocci · F. Damiani
S. Arcioni (✉)
Istituto di Ricerche sul Miglioramento Genetico delle Piante Foraggere, CNR, via Madonna Alta, 130, 06128 Perugia, Italia
Fax: +39-075-5005228
E-Mail: s.arcioni@irmgpf.pg.cnr.it

Introduction

The improvement of the agronomic value of crops through genetic transformation with heterologous genes is a major focus of plant biotechnology. This usually requires abundant transgene expression, and the rate of transcription of the introduced gene contributes to the expression level. The transgene is generally driven by a strong promoter, for example the CaMV 35S promoter, even if a large mRNA pool does not guarantee a large pool of protein product. The number of transgene copies can also affect the steady state level of transgene RNA in the cell. The correlation between transgene copy number and transgene mRNA expression has been often reported to be negative (Jorgensen 1990; Mittelsten et al. 1991; Assaad et al. 1993; Hobbs et al. 1993; Meyer et al. 1993; Ingelbrecht et al. 1994; Matzke et al. 1994). Gene silencing in transgenic plants, referred to as homology dependent gene silencing, can be due to either decreased transcription of the affected sequences (Meyer et al. 1993) or to post-transcriptional mechanisms (Ingelbrecht et al. 1994; Pang et al. 1996), and may affect endogenous genes and/or transgenes. Silencing mechanisms, either transcriptional or post-transcriptional, are often associated with DNA methylation (Linn et al. 1990; Assaad et al. 1993; Hobbs et al. 1993; Meyer et al. 1993; Ingelbrecht et al. 1994; English et al. 1996), but de novo methylation does not appear to be restricted to the symmetrical sequences CG and CNG responsible for cytosine methylation in plants (Gruenbaum et al. 1981). Transposable elements, such as endogenous genes (Spena et al. 1983; Ronchi et al. 1995), are also frequently transcriptionally inactivated and methylated (Chandler and Walbot 1986; Schwarz and Dennis 1986). Several models have been postulated to explain homology based silencing, including ectopic pairing between homologous genes (Jorgensen 1990), antisense RNA (Grierson et al. 1991) and biochemical switch mechanisms (Hart et al. 1992). Recently, English

et al. (1996) proposed that transgenic virus resistance and post-transcriptional silencing of nonviral genes operate through similar mechanisms, and suggested that DNA-based transgene-methylation and RNA-based gene-silencing process are linked.

The present study was initiated with the objective of analyzing the RNA expression of a maize cDNA encoding a sulphur-rich γ-zein in leaves of transformed *Lotus corniculatus* plants to establish whether constitutive CaMV 35S or light-regulated *rbcS* was the better promoter. The long-term goal of this research is to increase the content of sulphur amino acids (SAAs) in the aerial part of forage legumes. *L. corniculatus* was chosen as a model legume because it is tractable to genetic transformation, tissue-culture, and the regeneration of transgenic plants (Damiani and Arcioni 1993), as demonstrated by the wide use of this species in other studies (Petit et al. 1987; Damiani et al. 1996; Bavage et al. 1997). Several 35S-G1L multi-copy plants with no detectable G1L mRNA were investigated to explore the reasons for this lack of expression. A functional relationship between transgene silencing and T-DNA methylation was noted in the silenced 35S-G1L multi-copy plants.

Materials and methods

Plasmid construction

The construction of the chimaeric G1L genes, under the control of the CaMV 35S and *rbcS* promoters, that form the plasmids p121.G1L and pROK.G1L have already been described (Bellucci et al. 1997). Plasmids p121.G1L and pROK.G1L were both transferred from *Escherichia coli* strain JM83 into *Agrobacterium rhizogenes* strain NCPPB 1855 by triparental mating using the conjugative strain RK 2013 of *E. coli* (Ditta et al. 1980).

Plant transformation

L. corniculatus cv Franco plants (seeds from our laboratory), were transformed (Damiani and Arcioni 1993) with *A. rhizogenes* harbouring either p121.G1L or pROK.G1L. Eleven transformants of the first, and nine of the second, were selected on kanamicin 50 mg/l and examined. Plantlets were grown in the greenhouse and maintained by cuttings.

DNA and RNA analysis

L. corniculatus genomic DNA was isolated from young leaves (Cluster 1996), digested (10 μg) using a 5-fold excess of the appropriate restriction enzyme and separated by electrophoresis in 1% agarose gels. When DNA methylation analyses were performed, an unmethylated plasmid (pSP72, Promega) was included as an internal digestion control in all DNA samples and its complete digestion was verified by Southern analysis. The DNA was transferred to Hybond-N^+ membranes (Amersham), according to the manufacturers' instructions. Two G1L probes were used; one a 318-bp *Pvu*II/*Nae*I fragment of the G1L coding region of plasmid pBSKS.G1L (Bellucci et al. 1997), the other the entire G1L cDNA (a *Hinc*II/*Sac*I fragment of the same plasmid). The *npt*II probe was from a *Bam*HI fragment of pCaMVNEO (Fromm 1986). Probes of the promoters 35S and *rbcS* were from a *Hin*dIII fragment of p121.G1L and a *Hin*dIII/*Xba*I fragment of pROK8 (a gift of Dr. T. A. Kavanagh, Trinity College, Dublin), respectively.

Total RNA was isolated from young leaves (Chandler and Walbot 1986). RNA was electrophoretically fractionated in 1.4% formaldehyde agarose gels and transferred to Hybond-N membranes (Amersham). The entire G1L cDNA was used as a probe. Hybridization was performed for both DNA and RNA filters, as indicated by the membrane supplier, with ^{32}P-labelled probes (ready-to-go kit, Pharmacia). Autoradiograms of Northern analyses were scanned and the intensity of the bands quantified by a suitable software (Phoretix 1D).

Isolation of nuclei and nuclear run-on assay

Isolation of nuclei and nuclear run-on assay were basically performed according to Van Blokland et al. (1994). Eight to ten grams of leaves were used to isolate nuclei. Labelled RNA was hybridized to Southern-blot membranes. Each membrane contained 1 μg of the PCR-amplified genes G1L, *npt*II, 18S rDNA (from *M. sativa*), and *rbcS* (from *L. corniculatus*).

Results

Integration of G1L into the genome and RNA expression

L. corniculatus plants transformed with a maize cDNA (G1L) were obtained using the *A. rhizogenes* system. G1L was under two different promoters, CaMV 35S and *rbcS* from tobacco, in p121.G1L and pROK.G1L respectively (Fig. 1). To determine the number of integrated transgenes, leaf DNA was digested with *Hin*dIII and hybridized with the G1L and *npt*II probes. p121.G1L contains two *Hin*dIII sites in its T-DNA region and pROK.G1L one, and all sites are located between the G1L and *npt*II genes. As both probes hybridize to one T-DNA end fragment from each single-copy insertion, the number of G1L and *npt*II copies can be established. The rbcS-G1L transformants had from one to three T-DNA copies, the 35S-G1L transformants from one to nine. Figure 2 shows Southern analyses of three plants carrying 1–4 copies of the two genes. When necessary, other restriction enzymes were used to investigate for the presence of contiguously duplicated T-DNA structures.

The presence of G1L mRNA was analysed by Northern blotting. Figure 3 A shows that 35S-G1L transformants carrying more than three G1L copies had less mRNA than transformants with one or three transgene copies. In contrast, there was a direct correlation between transgene copy number and the mRNA level in rbcS-G1L transformants, but no transformants with more than three copies were obtained (Fig. 3 B). Two different-sized transcripts were detected. All rbcS-G1L transformants produced a 1.18-kb transcript, as did maize endosperm, while 35S-G1L transformants

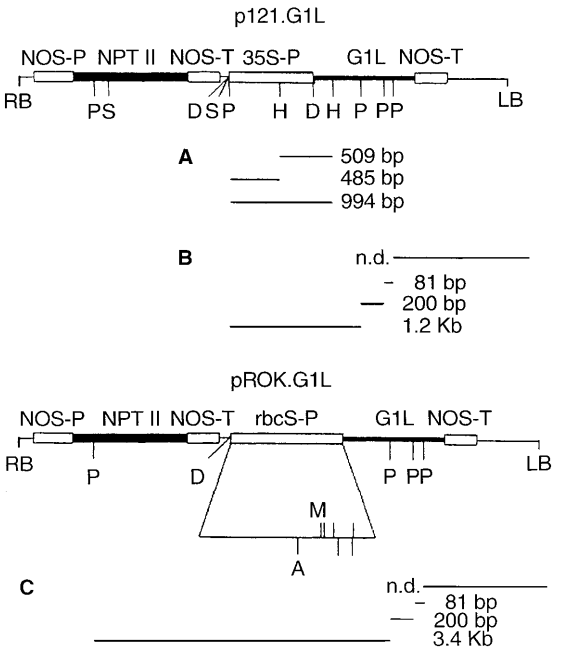

Fig. 1 T-DNA from plasmids p121.G1L and pROK.G1L. Structures of chimaeric genes and restriction sites. *A to C*, length of restriction fragments obtained by digestion with: *Hpa*II together with *Sph*I when probed with 35S, the 994-bp fragment indicates methylation of the *Hpa*II site in the promoter (*A*); *Pst*I when probed with the entire G1L (*B, C*). *LB* and *RB* left border and right border of T-DNA; *NOS-P* nopaline synthase promoter; *rbcS-P* rbcS promoter; *NOS-T* NOS terminator; *NPTII* neomycin phosphotransferase gene; *G1L* G1L cDNA; *P Pst*I; *S Sph*I; *H Hpa*II (only in the 35S-P and the first restriction site in G1L); *D Hin*dIII; *A Alu*I and *M Mnl*I (only the restriction sites in the rbcS-P); *n.d.* not determined

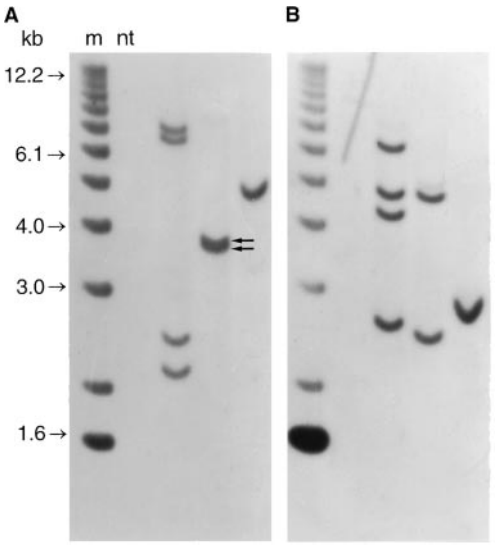

Fig. 2A, B Southern-blot analyses of *Hin*dIII-restricted DNA from leaves of transformants with four T-DNA copies (a 35S-G1L plant), two and one T-DNA copies (two rbcS-G1L plants). **A** DNA was probed with the 318-bp G1L fragment. The two *arrows* indicate two bands (a doublet) not clearly visible in the figure due to the low resolution of the picture. **B** The same filter probed with nptII. *m* a 1-kb molecular size marker (Gibco, BRL); *nt* not transformed

produced a 1.28-kb transcript. The G1L gene had two transcription-termination sites in both constructs: one from the maize cDNA, the other from the NOS terminator. The maize cDNA termination site located in the 3′UTR of G1L, which was apparently used when the gene was driven by the *rbcS* promoter, produced the short transcript. Figure 3 C shows a comparison between the G1L mRNA levels of transformants containing 35S-G1L and rbcS-G1L. Reproducible data indicated that, in presence of the same G1L copy number (three copies), mRNA expression was highest in transformants bearing the rbcS-G1L construct, up to 30% more than that of the 35S-G1L transformants.

To answer the question of whether silencing occurred transcriptionally or post-transcriptionally, run-ons were performed on four multi-copy 35S-G1L transformants, two of which produced G1L mRNA, while the other two did not. Figure 4 shows the results of two plants: the transcriptional activity of G1L and the *npt*II genes was suppressed in silenced plant S (Fig. 3 A, transformant with four copies), but not suppressed in nonsilenced plant NS (Fig. 3 A, transformant with three copies). The same results were obtained with the other two transformants examined (Fig. 3 A, transformants with one and eight copies).

Methylation analyses

Digestions with methylation-sensitive endonucleases were performed to establish whether methylated nucleotides were present on T-DNA. The *Pst*I enzyme was used to reveal methylation on the G1L coding sequence (Fig. 5); the absence of the expected length fragments (Figs. 1 B and C) denotes methylation of *Pst*I cleavage sites. The 81-bp fragment was never clearly visible on the figures. A 3.2-kb fragment appeared in plant S but it was almost imperceptible in plant NS (Fig. 5 A). As demonstrated by using *npt*II and G1L probes (data not shown), this fragment was located between the *Pst*I site in the *npt*II gene and the first *Pst*I site of the G1L coding region, which indicates methylation of the *Pst*I site 5′ of the 35S promoter. All the rbcS-G1L transformants were unmethylated (Fig. 5 B), while only the 35S-G1L transformants with more than three copies had methylated *Pst*I sites; but the degree of methylation was not the same. As can be seen in Fig. 5 A, the plant with eight T-DNAs was more heavily methylated than the plant with four T-DNAs. The hybridization of the *Pst*I-restricted DNA with the *npt*II probe indicated that the transformants with methylated *Pst*I sites on the G1L sequence were also methylated on the *npt*II sequence (data not shown).

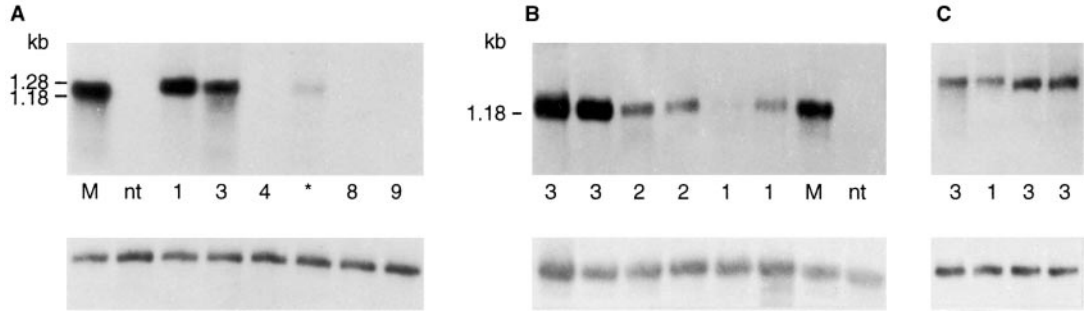

Fig. 3A–C Northern analyses on total RNA from leaves with a G1L probe (*upper portion*) and a 18S rDNA probe from alfalfa (*lower portion*). **A** 35S-G1L transformants. **B** rbcS-G1L transformants. In panels **A** and **B** each lane was loaded with 10 μg of RNA, except for lane *M*, where 0.2 μg of maize endosperm were loaded together with 10 μg of RNA from a non-transformed plant. **C** *First two lanes* total RNA from 35S-G1L transformants; *last two lanes* RNA from rbcS-G1L transformants (2.5 μg of RNA in all lanes). *M* maize endosperm; *nt* not transformed; * plant with an undetermined number of T-DNA copies, its *Hin*dIII-restricted DNA showed one normal-intensity band and five or six weak bands with the *npt*II probe, while with the G1L probe only the weak bands were visible. *Numbers under lanes* indicate the number of T-DNA copies in each transformant

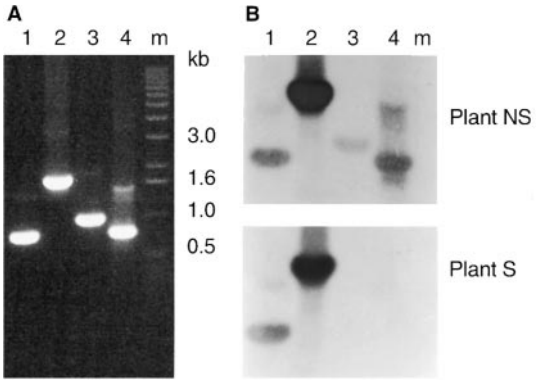

Fig. 4A, B Transcription run-on analysis. **A** Ethidium bromide-stained gel. The DNA samples loaded from *lane 1 to m* were, respectively: *rbcS*, 18S rDNA, *npt*II, G1L, and the 1-kb molecular size marker (Gibco, BRL). Two identical gels like this were blotted on membranes. **B** Nuclei isolated from the silenced plant S and the nonsilenced plant NS were used to synthesize labelled run-on transcripts. The two membranes in **A** were hybridized with the labelled RNA. The 1-kb marker in *lane m* was a negative control, because the 1.6-kb band and all bands less than 1 kb were *Hin*fI fragments of the vector pBR322. *Lanes 1 and 4* of plant NS, and *lane 1* of plant S, gave the expected signals, corresponding to the *rbcS* and G1L genes, and two weaker signals coming from two aspecific PCR-products

Methylation of the 35S promoter was investigated with *Hpa*II/*Sph*I double digestion (Fig. 6) and the two isoschizomers *Hpa*II and *Msp*I (data not shown). Since *Sph*I was not methylation-sensitive, the presence of two co-migrating bands of 485- and 509-bp denoted the absence of methylation of the *Hpa*II site in the 35S promoter; by contrast the presence of a 994-bp DNA fragment (Fig. 1 A) confirmed the methylation of this site. The frequent appearance of larger fragments in Fig. 6 indicated the degree of methylation on the *Hpa*II sites located inside the G1L coding region and regions further downstream (Fig. 1 shows only one of the *Hpa*II sites in the G1L sequence). As already seen for the G1L coding region in Fig. 5 A, the transformant with the highest number of T-DNAs was the more methylated. The results of the *Hpa*II/*Sph*I combination were confirmed by DNA restrictions with the two isoschizomers *Hpa*II and *Msp*I. When the 35S promoter was used as a probe, no differences in the restriction patterns of the two endonucleases were detected in any plant examined, which indicates that both cytosines in the recognition sequence were methylated. The *rbcS* promoter was not methylated, as revealed by separate DNA digestions performed with two different enzymes, *Alu*I and *Mnl*I. Due to the large number of restriction sites on the *rbcS* promoter (Fig. 1), the hybridization profiles were difficult to interpret and there was uncertainity as to which nucleotides were methylated and which were not. However, as all rbcS-G1L transformants and the pROK.G1L plasmid yielded the same hybridization pattern when probed with the *rbcS* promoter sequence, there was no methylation in the promoter region investigated (data not shown).

In summary, only 35S-G1L plants with more than three T-DNAs were methylated. Methylation involved the 35S promoter, as well as the G1L and *npt*II genes. Furthermore, these plants did not transcribe G1L mRNA and there was a direct correlation between the degree of methylation and the T-DNA copy number.

G1L silencing in the cross-progeny

A cross between two 35S-G1L transformed plants, one (S) with methylated T-DNA and the other (NS) without, produced a progeny of 15 individuals. Parent S had four independent T-DNA copies, while parent NS had three (Fig. 7). The T-DNA copies segregated in the progeny as independent insertions and the total number for each individual varied from two to five. The

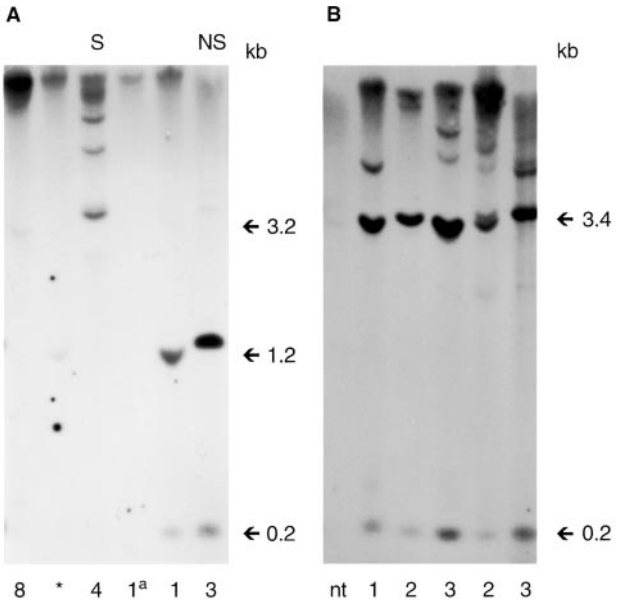

Fig. 5A, B Southern analyses of *Pst*I-restricted DNA probed with the entire G1L sequence. **A** 35S-G1L transformants. **B** rbcS-G1L transformants. *nt* not transformed; * this plant has an undetermined number of T-DNAs, 1^a = the T-DNA is truncated between the 35S and the G1L coding region, so this plant does not have the G1L gene; capital letters *S* and *NS* denote the same two plants of Fig. 4. *Numbers under lanes* indicate the number of T-DNA copies in each transformant

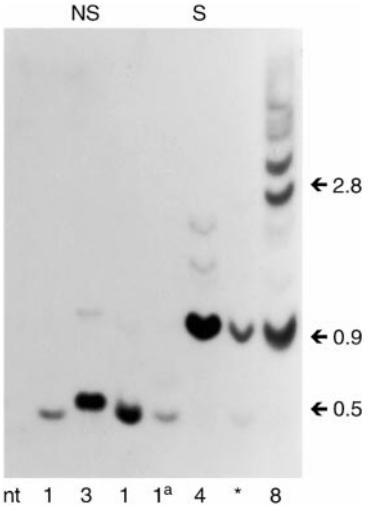

Fig. 6 Southern analysis of DNA from 35S-G1L transformants restricted with *Hpa*II/*Sph*I double digestion and probed with the 35S promoter sequence. The two fragments of 485 and 509 bp (Fig. 1 **A**) co-migrate in this figure in one band of 0.5-kb. Abbreviations as in Fig. 5

15 individuals were characterized for T-DNA methylation and G1L mRNA production, as already done for the primary transformants (Fig. 8). We expected G1L mRNA transcription in the individuals of the progeny

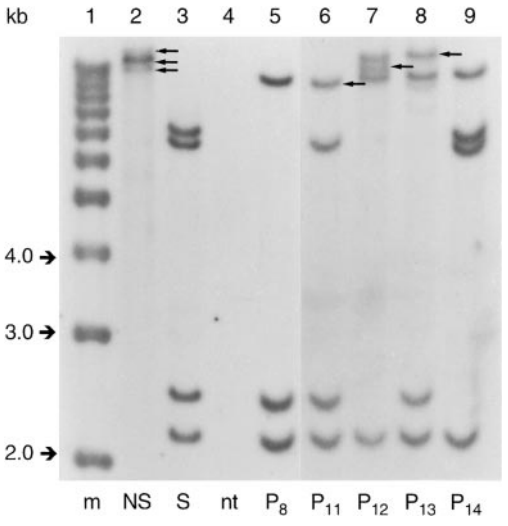

Fig. 7 Southern-blot analysis of *Hin*dIII-restricted DNA from cross-progeny individuals and parents. The DNA was probed with the 318-bp G1L fragment. *Lanes 2 and 3* show the female parent NS and the male parent S; *lanes 5 to 9* show 5 out of 15 progeny individuals. *m* a 1-kb molecular size marker (Gibco, BRL); *nt* not transformed; *capital letters* parents; *letter P plus number* cross-progeny individuals. The *arrows in lane 2* show the three bands of parent NS, while the *arrows in lane 6,7 and 8* show the position of each of these bands in the progeny

because all, except one (plant P10, Fig. 8), inherited unmethylated T-DNA copies from parent NS, and so was potentially transcribing. In contrast, only 1/3 of the progeny variably transcribed G1L mRNA. Moreover, the degree of *Pst*I methylation reflected the degree of G1L-silencing for each progeny plant (compare Fig. 8 A and B). Irrespective of the total number of G1L copies, the higher the intensity of the 0.2- and 1.2-kb *Pst*I fragments, the higher the G1L mRNA steady state level. The same correlation was seen with *Hpa*II/*Sph*I methylation analysis of the 35S promoter (data not shown). The steady state level of G1L mRNA in the progeny was intermediate with respect to the parents (Fig. 8 B). Similarly, the progeny individuals had intermediate levels of methylation with respect to the parents. A 3.2-kb band was clearly visible in some progeny plants. The parents displayed this *Pst*I-fragment during the first year of the study (Fig. 5 A), but the intensity of the signal in the figure was weak, particularly in parent NS. In the second year, when the DNA extraction and methylation analyses were repeated, the 3.2-kb band was absent in both parents (Fig. 8 A).

We hypothesized that, in the progeny, the unmethylated T-DNA copies from parent NS were methylated differently under the influence of the methylated T-DNA copies from parent S. This would explain why, for example, progeny plants P5 and P12 did not produce, or produced very little, G1L mRNA, even though they received two and three G1L copies from parent NS. The attempts to determine a different

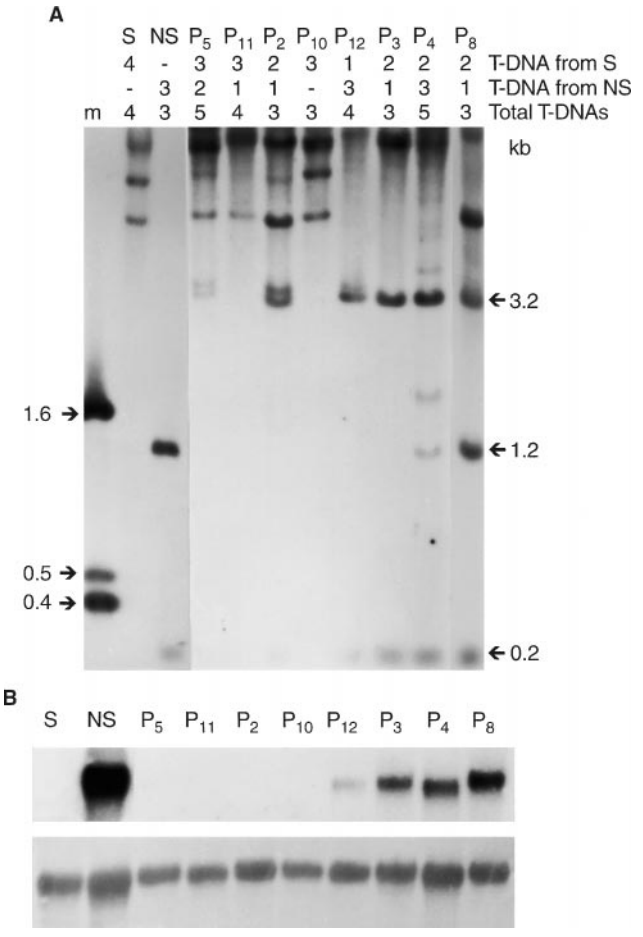

Fig. 8A, B G1L silencing in the cross-progeny. **A** Southern analysis of *Pst*I-restricted DNA probed with the entire G1L. **B** Northern analyses on total RNA with the G1L probe (*upper portion*) and a 18S rDNA probe from alfalfa (*lower portion*). Abbreviations as in Fig. 7

ability of the T-DNA loci of plant S in inducing de novo methylation (i.e. G1L silencing) on the T-DNAs of the other parent were unsuccessful; in fact we were not able to establish if there was a T-DNA locus that was a more-potent silencer with respect to the other three copies of plant S. The analysis of the progenies of the self-fertilized plant NS confirmed that, in the absence of methylated T-DNA copies from parent S, all the individuals should have transcribed G1L mRNA. In fact, seven out of seven transformants, with T-DNA copies from one to three, transcribed G1L mRNA (data not shown). Nevertheless, the degree of T-DNA methylation in two plants of the selfed progeny seemed to be slightly higher than in the parent but this did not compromise the G1L transcription.

Discussion

As plant transgene technology spreads, the control of transgene expression becomes even more crucial. Transgene inactivation involving the duplication of homologous sequences has occurred in many plants. Homology dependent gene silencing has received considerable attention not only because it inhibits transgene expression but, even more so, because transgene research has lead to the discovery of an endogenous multiple-sequence control mechanism, that may have a biological function in genome organization (Meyer and Saedler 1996; Matzke and Matzke 1997). We studied the silencing of the G1L gene, which codes for a maize γ-zein that is rich in sulphur amino acids, in some 35S-G1L multi-copy *L. corniculatus* transformants.

G1L mRNA expression was examined because the gene was under the control of two different promoters: CaMV 35S and *rbcS*. The highest level of G1L mRNA was detected in transformants bearing the gene driven by the *rbcS* promoter, as has been reported in alfalfa with *rbcS* promoters from tobacco and *A. thaliana* directing the expression of the SFA8 gene (Tabe et al. 1995). Two different-sized transcripts were produced, presumably due to preferential utilization of the two termination sites located in each chimaeric gene (Bellucci et al. 1997).

The silencing of G1L transcription and T-DNA methylation were strictly correlated in the 35S-G1L transformants. The addition of a methyl group to position 5 of the cytosine ring was analyzed in 5′-CG-3′ or 5′-CC-3′ di-nucleotides (*Hpa*II and *Msp*I enzymes used) in the 35S promoter sequence and in 5′-CNG-3′ sequences (*Pst*I enzyme) in G1L and *npt*II coding regions. The greater the number of 35S-G1L copies, the more extensive the T-DNA methylation (Figs. 5, 6 and unpublished data). Therefore, the T-DNA copy number, the silencing activity, and the state of methylation were closely associated. Matzke et al. (1994) reported similar results for three transgene loci that variably inactivated and methylated a partially homologous locus and demonstrated that the silencing loci comprised multiple methylated copies of the transgene construct, whereas there was a single unmethylated copy in the non-silencing locus. We show that the methylated T-DNA copies are able to silence other unlinked homologous transgenes following a cross between parent NS, which has high G1L mRNA levels, and parent S, which does not transcribe G1L mRNA. The silencing of the T-DNA copies inherited from parent NS, and their de novo methylation, indicates that the unlinked transgenes in the progeny interacted in trans. This interaction was probably unidirectional and changeable, which means that the methylation state and the inhibition of transcription were variably transmitted from parent S to parent NS transgenes. Moreover, the unmethylated parent used in the cross was self-pollinated and the transgenes in the self-progeny were unsilenced. Only two individuals showed a T-DNA methylation slightly higher, and a G1L mRNA steady state level slightly lower, than in the parent. The

achievement of the homozygous condition of the single T-DNA copy, that can sometimes reduce transgene expression (Neuhuber et al. 1994), would explain why these self-progeny individuals have less G1L mRNA with respect to the parent NS.

Post-transcriptional silencing of *npt*II transgenes in the progeny of tobacco transformant GVCHS(320)-1 has been associated with cytosine methylation (Van Houdt et al. 1997) and an inverse correlation between steady state *npt*II mRNA levels and the degree of *Bam*HI methylation was found. Although an inverse correlation between G1L mRNA steady state levels and the degree of *Pst*I and *Hpa*II/*Sph*I methylation was documented in our study, it was a case of transcriptional silencing. It is a generally accepted rule that homology dependent gene silencing occurs at the transcriptional level, if silencing primarly involves promoter homology, or post-transcriptionally, if silencing involves coding-region homology. It has been suggested that promoter homology dependent and post-transcriptional silencing are not mechanistically distinct and that both could be based on an RNA-DNA interaction (Park et al. 1996).

In the case of trans-inactivation, due to homologous transgene inserts and the fact that the entire T-DNA is usually hyper-methylated, it is difficult to establish which T-DNA region is responsible for the homology dependent silencing. For example, lack of expression of the maize A1-gene in transgenic petunia plants seems to be exclusively due to inactivation of transcription associated with hyper-methylation within the 35S promoter region (Linn et al. 1990). Analysis of de novo DNA methylation in epigenetic variants obtained from the transgenic petunia line R 101-17 has revealed that transgene-specific methylation is not limited to the 35S promoter, but also occurs within the A1 coding region and the *nos* promoter (Meyer et al. 1993; Meyer and Heidmann 1994). In our study, too, transgene methylation was detected within both the promoter (35S) and coding (G1L and *npt*II) regions. As run-on experiments showed, 35S-G1L plants with more than three copies did not transcribe either G1L or *npt*II mRNA, so that the silencing acted transcriptionally and probably primarily involved the promoter region. It remains to be seen why high-copy number 35S-G1L transformants were frequently obtained, while the rbcS-G1L transformants had no more than three T-DNAs.

A possible explanation for our transgene inactivation would be the DNA-DNA pairing model. It has been postulated that a DNA-DNA interaction can function in fungi and plants as a signal for de novo methylation (Goyon et al. 1994; Matzke and Matzke 1995). According to this model there would be a greater chance of a T-DNA copy in the 35S-G1L multi-copy primary transformants undergoing cis-inactivation and de novo methylation. The hyper-methylated T-DNA would then silence the un-methylated, homologous target sequences located at unlinked sites in the genome. This model would also function when, in a cross progeny, hyper-methylated and un-methylated transgenes copies were inserted together into the same genome.

Doubts about the above explanation (DNA-DNA pairing model) provided for G1L silencing were elicited by the *npt*II gene behaviour. The silencing of the *npt*II gene in the 35S-G1L plants was regulated in a time-dependent manner because the primary transformants were selected on kanamicin, whereas later run-on experiments showed the absence of *npt*II mRNA in silenced 35S-G1L multi-copy plants. In the same way, the state of T-DNA methylation was not constant in the plants S and NS, as demonstrated by the disappearance of the 3.2-kb fragment in the 2nd year of this study (see Results section and Figs. 5 and 8). Therefore, we can infer that, at least in the case of nptII gene, DNA methylation and the lack of transcript would be a consequence of a silencing mechanism that involves a RNA-directed methylation of T-DNA (Wassenegger et al. 1994), leading to the inhibition of transgene transcription. Recently, it was also reported that silencing of the *npt*II transgenes in tobacco transformants was reinforced by ageing (Van Houdt et al. 1997).

The 35S promoter and silencing effects seem to be frequently associated in a number of species. In our study, 35S-G1L transformants often gave rise to plants with multi-copy insertions and G1L was usually silenced in these transformants. In addition, when the RNA polymerase transcribed the mRNA by using the G1L gene under control of the 35S promoter, a strong terminator (e.g. NOS terminator) was required to stop the transcription. It would, therefore, seem that, when the aim is to accumulate high levels of heterologous proteins in *Lotus* species, the 35S promoter should be avoided.

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