

## Isolation and Expression Analysis of Peanut Chlorotic Streak Caulimovirus (PCISV) Full-Length Transcript (FLt) Promoter in Transgenic Plants

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**A promoter fragment from peanut chlorotic streak caulimovirus (PCISV) full-length transcript (FLt) was identified and later modified to have duplicated enhancer domain. The FLt promoter with its single or double enhancer domains, fused with the GUS reporter gene to form chimeric gene constructs, showed a high level of expression of these genes in cells and transgenic plants. The FLt promoter with its double enhancer domain gives an average threefold greater expression of genes compared to the FLt promoter with its single enhancer domain in transgenic plants. In young seedlings the expression was in the order root > leaf > stem. The histochemical GUS assay in young seedlings showed more activity in root tips and leaf midribs, veins, and other vascular tissues. The expression from the PCISV FLt promoter was compared with that from the figwort mosaic virus promoter in transgenic plants. These constitutive promoters were comparable in respect to GUS expression level.** © 1998

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Peanut chlorotic streak caulimovirus (PCISV), a newly described member of caulimovirus group, (plant pararetrovirus) is unique, with a broad host-range compared to other caulimoviruses. The host range for PCISV includes peanut (*Arachis hypogaea*), beans (*Phaseolus vulgaris*), cowpeas (*Vigna unguiculata*), soybean (*Glycin max*), *Datura stramonium*, and several *Nicotiana* species (1). It is an economically important virus in tropical countries. In tobacco and *Datura innoxia*, PCISV is more productive at relatively higher temperature (30-37°C), a temperature at which other caulimoviruses fail to infect their host (2, 3).

The caulimoviruses are a group of small DNA viruses with an unusual strategy for expression of their 6 - 8 genes. Their members, as exemplified by cauliflower mosaic virus (CaMV) and figwort mosaic viruses (FMV), have small circular DNA genomes that repli-

cate in the cytoplasm *via* reverse transcription (4, 5) but transcription occurs in the nucleus, where up to 1000 copies of the viral DNA may accumulate as mini chromosomes (6).

The genomes of CaMV (7) and six other members of this group, namely carnation etched ring virus (CERV), (8), figwort mosaic virus (FMV), (9) soybean chlorotic mottle virus (SoCMV), (10), peanut chlorotic streak virus (PCISV), (11), cassava vein mosaic virus (CVMV), (12), and strawberry vein banding virus (SVBV), (13), have been fully sequenced.

The genomes of caulimoviruses generally harbor at least two active transcriptional promoters. FMV and PCISV have been shown to have transcript similar to the 19S and 35S RNAs found in CaMV infected plant cells (2, 14-16) Two major RNA species result from transcription by host RNA polymerase II. One transcript, which serves as a replicative intermediate (pre-genomic RNA), spans the entire circular genome whereas a subgenomic species spans just the gene VI region. The exact mechanism of translation of the genes of the full-length transcript is still obscure but evidence has been accumulating which suggests that the major transcript functions as a polycistronic messenger RNA (14, 17, 18).

Several transcriptional promoters have been isolated from the genomes of two pararetrovirus subgroups namely caulimovirus (19-22) and badnavirus (23, 24). The CaMV 35S promoter has been well characterized (25-31) and it has been extensively used in chimeric gene constructs in a wide variety of plants (19, 32-34).

Single or multiple copies of enhancer sequences from the CaMV 35S promoter can increase homo- and heterologous promoter activity in an orientation-independent manner (25, 35, 36). Similar observation was made when single or multiple copies of the enhancer sequence was inserted upstream of the TATA element of the CaMV 19S promoter (25, 37), rbcS-3A promoter (26), the nos promoter (19). The duplication of enhancer

sequence of FMV FLt promoter also increase the promoter activity (22).

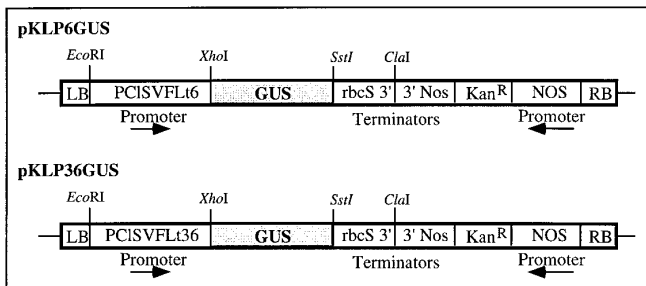
In this paper, we report on the isolation and characterization of the strong constitutive full-length transcript (FLt) promoter from the peanut chlorotic streak caulimovirus (PCISV). The PCISV FLt promoter with its single or double enhancer domains when linked to heterologous sequences to form chimeric genes construct showed high levels of expression of these genes in cells and transgenic plants.

## MATERIALS AND METHODS

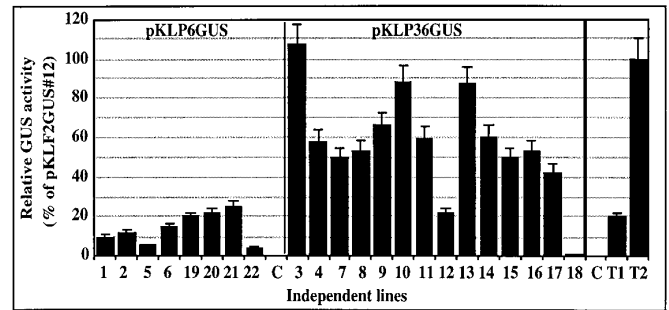
**Plant and enzymes.** Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were used for transformation. Restriction enzymes and DNA modifying enzymes were from commercial sources.

**Construction of PCISV FLt promoter with its single and double enhancer elements.** The basic FLt promoter of PCISV, 250 bp (position 5852 to 6101 of the PCISV sequence) was isolated after amplification by PCR using oligonucleotides containing the appropriate sites to generate an *EcoRI-HincII* sites at the 5' end and a *HindIII* site at the 3' end of the fragment. The promoter sequence was inserted as an *EcoRI-HindIII* fragment into the corresponding sites of the plant expression vector pKYLX71 (38) and the plasmid pUC119. The resulting plasmids were designated pKLP6 and pUCPFLt6, respectively. The upstream sequence containing enhancer elements, a 178 bp (position 5852 to 6029) of the PCISV FLt promoter was amplified by PCR with oligonucleotides engineered for the *EcoRI-HincII* sites at the 5' end and the *SmaI-HindIII* sites at the 3' end of the fragment. The enhancer element fragment was cloned into *EcoRI* and *HindIII* sites of PUC119 and the plasmid designated as pUCP-enhancer. The PCISV FLt basic promoter fragment as a *HincII-HindIII* fragment (isolated from pUCPFLt6) was inserted into the pUCP-enhancer plasmid after digestion with *SmaI* and *HindIII*. The resulting plasmid designated as pUCPFLt36 contains two copies of the enhancer elements. The PCISVFLt promoter with its double enhancer domain was inserted into the plant expression vector pKYLX71 at its unique *EcoRI* and *HindIII* sites that flank the promoter. The resulting plasmid was designated as pKLP36. The PCISV basic FLt promoter and enhancer elements was amplified from a full length clone of PCISV (2).

**Stable transformation and analysis of transgenic plants.** The reporter gene GUS was tailored by PCR to include just the coding



**FIG. 1.** Schematic representation of chimeric GUS constructs used for assaying PCISVFLt promoter expression activity in transgenic plants. The identity of the respective promoter is shown for each plasmid. GUS represents the gene for  $\beta$ -glucuronidase of *E. coli*. The position of restriction sites *XhoI*, *SacI*, *EcoRI*, *HindIII*, *ClaI* used to assemble these plasmids are shown. The position of the left and right T-DNA borders (LB and RB respectively) the *rbcS* polyadenylation signal (Terminators) and the *Kan<sup>R</sup>* gene are illustrated.



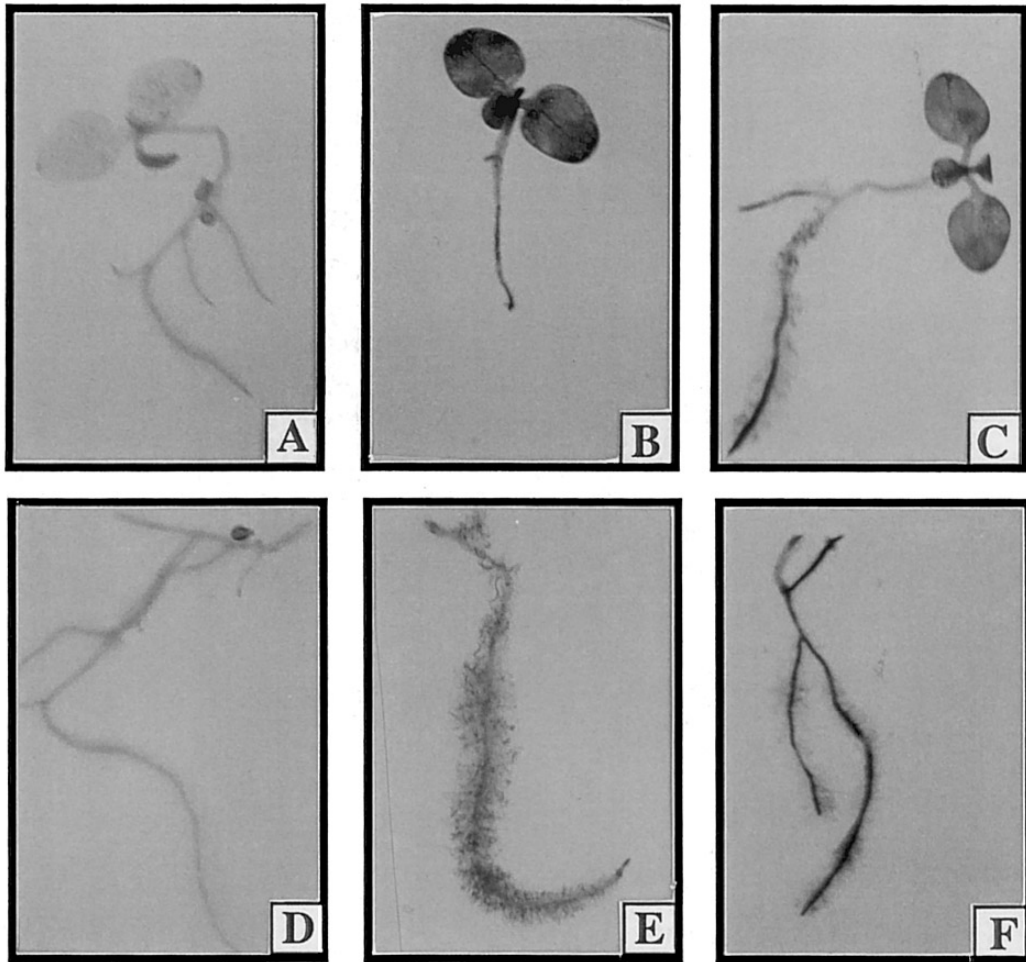
**FIG. 2.** PCISV FLt promoter activity in transgenic plants expressing GUS reporter gene. Comparative analysis of the PCISV FLt promoter activity in independent transgenic plants *Nicotiana tabacum* cv Samsun NN (2 week old seedlings, R1 progeny/ second generation) expressing a GUS reporter gene. Independent transgenic lines were developed with PCISV FLt promoter in construct pKLP6GUS containing a single enhancer domain, and in construct pKLP36GUS containing a double enhancer domains. Seeds obtained from transgenic plants were germinated in presence of kanamycin (200  $\mu$ g/ml). GUS activity was determined in tissue extracts from whole seedlings. Independent plants were developed for constructs pKLP6GUS line # 1, 2, 5, 6, 19, 20, 21, and 22; and for construct pKLP36 line # 3, 4, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 and 18. (C) Negative control, tissue extract from Samsun NN (wt). Positive control, one of the best expressing lines either from (T1) pKLF GUS line #11 with FMV FLt promoter with single enhancer domain or (T2) pKLF2GUS line #12 with double enhancer domain compared with PCISV FLt promoter. GUS activity are presented as % activity of the best expressing pKLF2GUS#12 line with FMV FLt double enhancer domain. The presented data is the mean of three samples from at least two independent experiments, variation was within 10% of the presented value.

sequence with the initiation and termination codons, flanked by a *Xho I* site at the 5' end and a *Sst I* site at the 3' end. The PCR isolated fragment for the reporter gene (GUS) was digested with *Xho I* and *Sst I*, gel purified and cloned into the corresponding sites of the plant expression vectors pKLP6 and pKLP36 and the resulting constructs pKLP6GUS and pKLP36GUS (Fig. 1) were introduced into *Agrobacterium tumefaciens* strain C58C1:pGV3850 by triparental mating. Tobacco (cv. Samsun NN) was transformed with the engineered *Agrobacterium* as described earlier (39).

**$\beta$ -Glucuronidase (GUS) assay and histochemical GUS staining.** Fluorometric GUS assays to measure GUS activity of plant tissue extracts and histochemical GUS assays to determine the distribution of GUS activity in plants, embryos and seedlings, were performed according to published procedures (40) as described earlier (22). Protein in plant extracts was estimated (41) using BSA as a standard.

## RESULTS AND DISCUSSION

**Testing the Expression Vectors with a GUS reporter gene.** To examine the integration of genes in transgenic plants, genomic DNA was isolated following the procedure (42) for PCR analysis. The integration of reporter GUS gene in the genome of transgenic plants (R0 and R1 progeny) was detected by PCR amplification using appropriately designed oligonucleotides specific for the GUS gene sequence. Specificity of each PCR product was tested by Southern hybridization with a GUS probe (data not shown).



**FIG. 3.** Histochemical localization of GUS activity in developing transgenic tobacco. (A) Transgenic tobacco seedlings (X4), (pKLP36CAT, R1 progeny) with CAT gene, no GUS activity was detected. (B) Seedling (X4), (pKLP6 GUS #21, R1 progeny) 14 DAI; GUS activity was localized in root and leaves. (C) Seedling (X4), (pKLP36 GUS #3, R1 progeny) 14 DAI; GUS activity was localized in roots, roots hairs and leaves. (D) Roots (3X) from six week old plants pKLP36CAT, R1 progeny, no GUS activity was detected. (E) Roots (3X) from six week old plants pKLP6GUS #21, R1 progeny, GUS activity was detected in roots. (F) Roots (X3) from six week old plants pKLP36GUS #3, R1 progeny, GUS activity was detected in roots.

*Comparative functional analysis of FMV and PCISV Flt promoters in transgenic plants.* The constructs shown in Fig. 1 were transformed into tobacco plants via the *Agrobacterium* co-cultivation method as described earlier (39). Transformations were done using *Nicotiana tabacum* cv Samsun NN. Primary transformants of tobacco were selected for resistance to kanamycin (300 mg/ml) and these were grown to maturity in the greenhouse. At least 8-10 independent lines were generated for each construct tested. The expression levels of the GUS reporter gene in independent transformants developed for pKLP6GUS, pKLP36GUS are shown in Fig. 2. The expression from the PCISV Flt promoter was compared with that from the previously studied FMV promoter (22). Individual plant lines generated from independent calli expressing the same gene showed variable GUS activity. Similar patterns of

plant-to-plant variations in gene expression have been reported with many other plant promoters as pointed out earlier (22). Most of the plant lines developed with pKLP36GUS showed more activity than any of the plants transformed with pKLP6GUS (Fig. 2). On average, about 3 fold higher activity was exhibited by plants transformed with pKLP36GUS, which has a duplicated enhancer domain as compared to plants transformed with pKLP6GUS which has a single enhancer domain. Hence, the PCISV Flt promoter with a duplicated enhancer domain is more active than the Flt promoter with a single enhancer domain. Similar effects have also been reported with enhancer sequences from the CaMV 35S promoter (25, 26, 35,36, 37) and also from the FMV Flt promoter (22).

In earlier studies (22) we compared the relative strengths of the CaMV 35S and the FMV Flt promot-

ers. We tested different vector constructs with the GUS gene in transient expression experiments in protoplasts of *Nicotiana edwardsonii*. In present study we compare the expression of the FMV FLt promoter with the PCISV FLt promoter in transgenic plants. These constitutive promoters developed from PCISV and FMV FLt promoter were comparable in respect to expression of reporter genes in transgenic plants.

*Expression levels in seedlings (R1 progeny) and young tobacco plants.* To examine the promoter activity in various tissues during seedling development, the expression of the GUS reporter gene in seedlings (R1 progeny) transformed with pKLP6GUS, or pKLP36GUS was examined by fluorometric assay of tissue extracts and by histochemical staining of sections of leaves, stems and roots. The PCISV promoter activity was monitored in 15 day old seedlings grown aseptically on an MS-agar medium in the presence of kanamycin (300  $\mu$ g/ml) and 3% sucrose. Several independent lines for each construct were studied. Comparison of activities of the FLt promoter indicated a gradient of expression in the following order; the highest level of activity was found in roots followed by leaves and stems (data not shown). The histochemical staining shown in Fig. 3, is representative of the staining patterns analyzed in plants expressing high levels of GUS activity. In seedlings and sections of young leaves stained for GUS, the intensity of staining was markedly greater in vascular tissues of young leaves, petioles, stems and roots. The intensity of GUS staining observed in vascular tissue was in the following order : roots > leaves > stems. The histochemical GUS assay in leaves showed more activity in midribs, veins and other vascular tissue, and in trichomes, than in leaf mesophyll and palisade cells (data not shown). No GUS activity was detected in transgenic plants containing the construct pKLP36CAT with CAT gene (Fig. 2, Fig 3 A). Unlike caulimovirus promoters which are active in all plant organs (19, 22), the promoters from badnavirus like *Commelina* yellow mottle virus (CoYMV) and rice tungro bacilli form virus (RTBV) are phloem-specific in expressing genes in transgenic plants (23, 24).

We have isolated a promoter from the plant viral genome of newly characterized peanut chlorotic streak caulimovirus (PCISV). Our results indicate that it is a strong constitutive promoter. The comparative expression analysis from the PCISV FLt promoter with that from figwort mosaic viruses (FMV) promoter in transgenic plants indicated that these constitutive promoters were comparable in respect of GUS expression level and showed similar pattern of expression in transgenic plants. Although functionally analogous, the nucleotide sequence of PCISV promoter has limited homology with other caulimovirus promoters.

We have adapted plant expression vectors with the PCISV FLt promoter with its single and duplicated enhancer domains. We are interested in developing plant

expression vectors with promoter from other caulimovirus. In plant metabolic engineering where multiple genes need to be expressed in a single cell, the use of different promoters with nonhomologous sequence will be useful in order to avoid genetic instability due to recombination between identical promoter sequences. Promoters from other caulimoviruses such as FMV, PCISV as well as CaMV 35S promoter will be very useful in plant genetic engineering application.

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