

Poster

Functional analysis of the cassava vein mosaic virus promoter and its usage for cassava genetic engineering

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Cassava vein mosaic virus (CsVMV) is a plant pararetrovirus infecting cassava plants in Brazil. The promoter which directs the synthesis of the terminally redundant genome length viral ribonucleic acid (RNA), was isolated and used to express heterologous genes in transgenic plants. A deletion analysis of the upstream region of the promoter was carried out in order to study its functional structure. Effects of the deletions were examined in tobacco plants using the *uidA* reporter gene. Additional data were obtained by protoplast transfection experiments and *in vitro* deoxyribonucleic acid (DNA) binding assays. The constitutive pattern of the promoter expression is due to the promoter sequence of organo-specific *cis* elements. The specificity of the virus for cassava plants and the constitutive as well as the molecular function of its promoter, make it a good alternative to the 35S.

Keywords: Cassava mosaic vein virus; Genetic engineering; Promoters; Pararetrovirus; Brazil

Cassava vein mosaic virus (CsVMV) is a double-stranded deoxyribonucleic acid (DNA) virus that infects cassava plants in Brazil. Electron microscopy studies of infected plants showed isometric viral particles localized in all cell types and accumulated in cytoplasmic inclusion bodies (Kitajima and Costa, 1996). Based on these data, CsVMV was listed as a putative member of the *Caulimovirus* genus. Consensus sequences of pararetroviruses were found in the CsVMV genome suggesting that this virus has a replication mechanism similar to that of the badnaviruses and caulimoviruses. However, the genomic organization of the CsVMV exhibits specific features not found in other pararetroviruses (Calvert *et al.*, 1995; de Kochko *et al.*, 1997). The CsVMV genome is 8159 nucleotides long and is organized in 4 (maybe 5) open reading frames (ORF) (GenBank accession # U59751). Consequently, it was suggested that the CsVMV might be representative of a new genus of plant pararetroviruses.

Different transcriptional promoters used to express foreign genes in transgenic plants have been isolated from plant pararetrovirus genomes. The 35S promoter of the cauliflower mosaic virus (CaMV) directs a constitutive gene expression in both monocotyledonous and dicotyledonous transgenic plants (Odell *et al.*, 1985; Terada *et al.*, 1990; Yang and Christou, 1990). Similarly, the promoter 34S from the

figwort mosaic virus (FMV) is active in all tissues of transgenic plants and is of comparable strength to the 35S promoter (Sanger *et al.*, 1990). Promoters were also isolated from the rice tungro bacilliform virus (RTBV) and the commelina yellow mottle virus (ComYMV). These two promoters isolated from badnaviruses displayed a vascular specific gene expression pattern in transgenic plants (Medberry *et al.*, 1992; Yin and Beachy, 1995). As a pararetrovirus infecting cassava, the CsVMV was a strong candidate to isolate a new promoter to express genes in transgenic cassava plants.

Isolation and Expression in Transgenic Plants of the CsVMV Promoter

Sequence analysis of the CsVMV genome allowed the identification of a consensus TATA box similar to that present in plant pararetrovirus promoters. Specific primers were used to amplify, by polymerase chain reaction, a 511 nucleotides fragment containing the TATA motif. This fragment was fused to the coding sequence of the *uidA* reporter gene (coding for the β -glucuronidase, GUS; Jefferson *et al.*, 1987) and the resulting chimeric construct was used to study promoter expression. A primer extension experiment showed that this CsVMV



DNA fragment could initiate the transcription 35 nucleotides downstream of the putative TATA box. According to this transcription start site, the CsVMV promoter fragment extends from position -443 to +72. This fragment was able to cause a high level of gene expression in protoplasts isolated from tobacco or cassava cell suspension. These preliminary experiments showed the CsVMV promoter was of similar strength to the enhanced 35S promoter. Genetic transformation of cassava plants was not accomplished when this project was initiated. Consequently, the expression pattern of the CsVMV:*uidA* fusion gene was first analysed in transgenic tobacco and rice plants (Verdaguer *et al.*, 1996). The results suggested that the CsVMV promoter is more active in vascular elements, in cells containing chloroplasts, and in meristematic zones. The CsVMV promoter was active in all organs tested and in different cell types independently of the developmental stage. The expression pattern was similar in transgenic tobacco and rice plants, suggesting that promoter activity is not dependent on transcriptional factors specific to a plant species.

This study shows that the CsVMV promoter is a strong promoter capable of expressing genes in transgenic plants. Because of its constitutive properties, the CsVMV promoter can be used in plant biotechnology as an alternative to the widely used 35S CaMV promoter.

CsVMV Promoter Expression in Cassava Plants

Micro-bombardment experiments on cassava tissues using the CsVMV:*uidA* fusion gene have been carried out (Verdaguer *et al.*, 1996). These experiments provided the first evidence of promoter activity in cassava plants. Preliminary results of transgenic cassava plants carrying the CsVMV:*uidA* gene showed that the CsVMV promoter is very active in the vascular elements. The GUS staining was also detectable in leaf tissues and was somehow stronger in the younger leaves. Efficiency of the CsVMV promoter to express genes of impact in cassava plants was confirmed by transformation experiments using a plasmid containing the *nptII* gene that confers resistance to the aminoglycoside family of antibiotics. Transgenic cassava plants that carry a CsVMV:*nptII* fusion gene were regenerated after selection on medium containing paramomycin. Also, the CsVMV promoter was used to express the coat protein of the cassava common mosaic virus (CsCMV) in transgenic cassava. Western analysis performed on leaf extracts from regenerated plants showed a high level of accumulation of the CsCMV coat protein.

Promoter Elements and Deletion Constructs

A deletion analysis of the region upstream of the TATA box of the CsVMV promoter was carried out to identify important *cis*-regulatory elements. A better control of gene expression in transgenic plants could be achieved through a better understanding of the mechanisms of promoter regulation. Likewise, an enhanced version of the 35S CaMV promoter was constructed after the identification of its enhancer region (Kay *et al.*, 1987). It has also been shown (Benfey and Chua, 1990) that the 35S CaMV promoter pattern of expression is controlled by different modules which have different tissue-specific functions. Accordingly, it was possible to alter the promoter's profile of expression by specific deletions or duplication of tissue-specific elements. The CsVMV promoter has very little sequence homology with the 35S CaMV promoter and the 34S FMV promoter. This suggests the presence of different *cis*-elements and therefore the possibility of different regulation mechanisms. The deletion analysis of the CsVMV promoter was thus undertaken to address this question.

A set of deleted promoters were engineered and cloned upstream of the *uidA* gene. The promoter activity of the different deletions was monitored in transgenic tobacco plants using the expression of the reporter gene. Different staining patterns as well as significant and reproducible differences in the staining intensity were detected between promoter constructs. These differences indicated the effect of the deletions on promoter function. The results showed that the constitutive pattern of the CsVMV promoter in transgenic plants is due to the interaction between distinct specific domains. A domain that control promoter expression in the vascular elements was identified between the position -173 to -63. This domain, when associated with the TATA box region is sufficient to direct a high level of gene expression in vascular elements of transgenic tobacco. The region spanning from nucleotides -222 to 173 contains *cis*-elements that control promoter expression in green tissues and in root tips. In this region, the present results suggested that a sequence homologous to the activating sequence 1 (as1) previously identified in the 35S CaMV promoter (Lam *et al.*, 1989) is involved in the root tissue expression. Expression in mesophyll cells might be controlled by both the as1 element and a GATA motif. Also, it was shown that additional sequence elements located between positions -149 and -64 are required for promoter activation in green tissues. Since these latter elements cannot direct gene expression in mesophyll cells by themselves, it is probable that synergistic interactions are involved in the

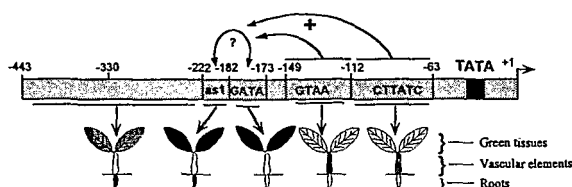


Figure 1 Schematic representation of the functional map of the CsVMV promoter. Effects of the different promoter regions on *uidA* gene expression in transgenic plant are represented. The darkened areas on the plant model represent a strong level expression, while grey areas indicate low level expression. Arrows at the top of the figure symbolize the synergistic interactions of the different *cis* elements defined. Motifs identified that might play an important role for CsVMV promoter regulation are also mentioned. Positions relative to the transcription start site are indicated

regulation of promoter activity in green tissues.

The expression pattern as well as the type of molecular organization suggested that the CsVMV promoter is related to caulimovirus promoters. However, specific features such as a *cis*-element not identified in the 35S CaMV promoter or in the 34S FMV promoters, might indicate different regulation mechanisms.

Different promoter constructs that exhibited distinct tissue-specific profiles of expression in transgenic tobacco plants have been obtained by the authors. A promoter construct with lower expression than the full length in leaf mesophyll cells was isolated. Since this construct is active in all cell types, this pattern of expression was designated as 'constitutive weak'. Vascular-specific promoters that displayed variable levels of expression were also developed. A promoter construct which was nearly inactive in the aerial tissues of transgenic plants displayed a strong GUS staining in root tips and vascular tissues of the roots. This result suggests that this construct is preferentially expressed in root tissues.

Acknowledgements

The authors are grateful to Dr R.J. Shepherd (University of Kentucky) for providing the CsVMV genomic clone. They wish to acknowledge C. Fux, S.L. Leitner, and L. Chen for great assistance in plant transformation and plant maintenance. They also thank Dr C. Fauquet and Pr R. Beachy co-directors of ILTAB where the work was done. This work

was supported by the Rockefeller Foundation and the French Institute for Scientific Research for Development in Cooperation (ORSTOM).

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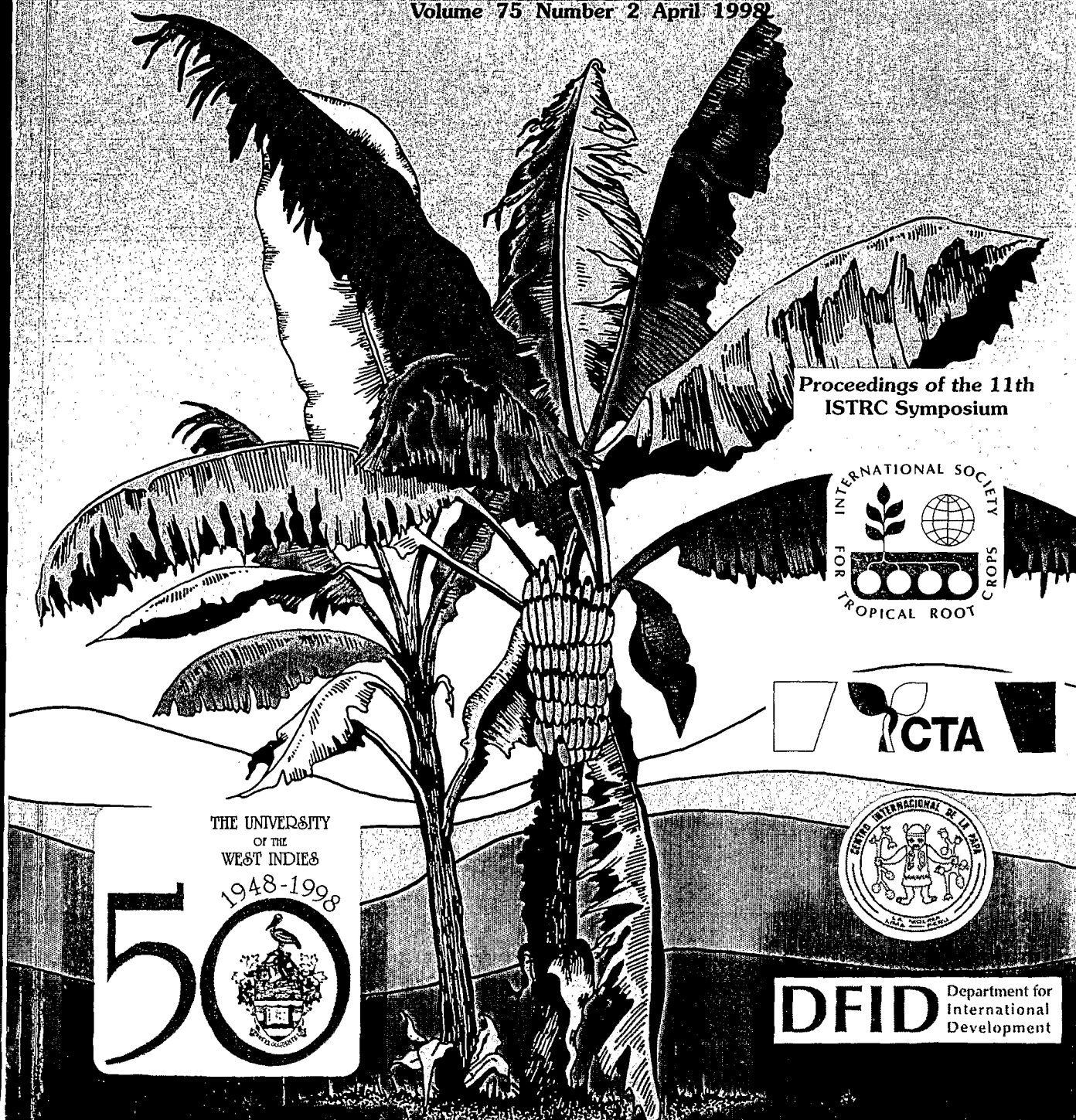
The Journal of the Faculty of Agriculture (Imperial College of Tropical Agriculture),
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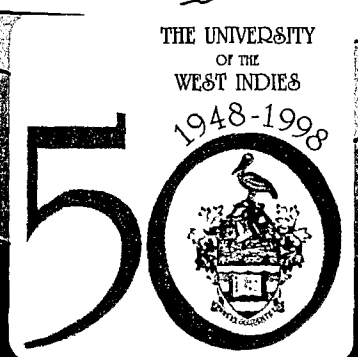
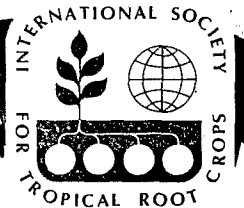
Volume 75 Number 1 January 1998

and

Volume 75 Number 2 April 1998



Proceedings of the 11th
ISTRC Symposium



TROPICAL AGRICULTURE

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Editorial Offices: Office of the Editor-in-Chief, Tropical Agriculture Journal, UWI, St. Augustine, Trinidad. Telephone (868) 645-3640; facsimile (868) 645-3640.

Tropical Agriculture is published by the School of Agriculture on behalf of The University of the West Indies Press.

General Managing and Publishing responsibilities: F.A. Gumbs

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Copyediting and typesetting: Office of the Journal of Tropical Agriculture, UWI, St. Augustine, Trinidad

Printers: Trinprint Ltd., Barataria, Trinidad