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KTRDC–developed genetic promoters for use in plant genetic engineering

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Introduction

There is continuing, and expanding, worldwide interest in developing improved crop plants exhibiting a wide variety of important traits, e.g. resistance to viruses, fungi, bacteria and insects, tolerance to herbicides for more efficient weed-control, manifestation of drought- or cold-tolerance, increased nutritional value, etc. These endeavors in agricultural biotechnology, together with the underlying research, create an ongoing need for materials and technologies for introduction and expression of transgenes in plants. Of particular interest is the desire for alternative gene-expression 'tools' that obviate technical problems resulting from multiple uses of the same genetic elements in the same plant, and that minimize risks of patent infringement. In recognition of this demand KTRDC research under the direction of Dr. Indu Maiti has designed and developed a range of novel, proprietary genetic promoters and plant genetic engineering tools for expression of single and multiple genes in plants. These include genetic promoters isolated from six different members of the plant-virus genus *Caulimovirus*, belonging to the *Caulimoviridae* family, a group of plant double-stranded DNA viruses. This Bulletin presents a summary and comparative analysis of these genetic promoters, together with a listing of relevant technical literature and information concerning access and licensing.

Why alternative promoters from *Caulimoviruses*?

Two major transcriptional promoters are present in the *Caulimovirus* genome. One transcribes the whole genome of the virus (a full-length transcript functionally equivalent to the familiar CaMV 35S transcript). The other transcribes only the open reading frame (ORF) VI (a subgenomic transcript equivalent to the CaMV 19S transcript). For plant genetic engineering the CaMV 35S promoter, a strong near-constitutive promoter, is widely used. This is a full-length transcript (FLt) promoter from *cauliflower mosaic virus*, a type member of the genus *Caulimovirus*, a subgroup of the *Caulimoviridae* family.

In plant metabolic engineering a number of traits may be required to be expressed in a cell simultaneously to achieve the desired phenotype. While two genes can be expressed under a single promoter using specific mechanisms (reviewed in: Hunt and Maiti, 2001), the level of expression of each gene of the pair may not be equal or adequate compared to that from a monocistronic construct under control of a strong promoter like CaMV 35S. A strong constitutive promoter can be used to achieve optimal expression of more than one gene by successive transformations. However, a risk of multiple transformations by a single promoter is that it might lead to silencing of both genes in successive generations through homologous recombination. (In nature, this may be one of the reasons why every gene in an organism is expressed from an individual promoter that has a different DNA sequence.) Thus the use of several strong promoters from *different* sources is highly advisable. For this reason, alternative FLt promoters from other member of the *Caulimoviruses* such as *figwort mosaic virus*, (FMV, Maiti et al 1997); *peanut chlorotic streak virus* (PCISV, Maiti and Shepherd, 1998); *mirabilis mosaic virus*, (MMV, Dey and Maiti, 1999a); *strawberry vein banding virus* (SVBV, Pattanaik et al., 2004); cassava vein mosaic virus (CAMV, Verdauger et al., 1996) have been isolated and characterized.

In this Bulletin we summarize a comparison of different *Caulimovirus* genetic promoters, several of which have been isolated at KTRDC, in terms of expression of the glucuronidase (GUS) gene as a 'reporter'. The results presented here were previously published in the scientific literature. Model dicotyledonous and monocotyledonous plant systems are employed, in combination with both transient and stable transformation methods.

Comparative expression analysis of *Caulimovirus* promoters in tobacco and corn (maize) protoplasts

Methods

Isolation of protoplasts from the tobacco (Xanthi 'Brad') and maize (BMS) cell suspension cultures, and electroporation of protoplasts with super-coiled DNA containing the promoter fragment fused with the GUS gene were essentially as described in the literature (e.g. Maiti et al., 1997; Dey and Maiti 1999a,b; Pattanaik et al., 2004). Caulimovirus promoter fragments were generated by PCR amplification as described previously (Dey and Maiti, 1999a) using appropriately designed primers. Constructs were introduced into *Agrobacterium tumefaciens* strain C58C1:pGV3850 by triparental mating. Tobacco plants (*Nicotiana tabacum* cv Samsun NN) were transformed with the engineered *Agrobacterium* as described earlier (Maiti et al., 1993). About 10 independent plant lines were generated for each construct. Regenerated kanamycin-resistant plants were grown under greenhouse conditions.

Total cellular RNA from electroporated protoplasts, or transgenic tobacco seedlings expressing the constructs, was isolated using the RNeasy plant mini kit (Qiagen, Chatsworth, USA). RNA dot-blot and Northern hybridization analysis were performed as described previously (Bhattacharyya et al., 2002; Dey and Maiti 1999a). The total RNA (2 µg) was treated with RNase-free DNase (Sigma, USA) per the manufacturer's instructions. The expression level of GUS mRNA in protoplasts electroporated with plasmids and plants developed for expression constructs was evaluated by real-time quantitative PCR (qRT-PCR) as described previously (Bhattacharyya et al., 2003; Pattanaik et al., 2004). Fluorometric GUS assays to measure GUS enzyme activity in plant tissue or protoplasts extracts, and chloramphenicol aminotransferase (CAT) activity were conducted according to the published methods (Maiti et al., 1997).

FLt promoters compared in transient expression

We have analyzed genetic promoters for the full-length transcript (FLt) from six different members of the family *Caulimoviridae*. These are *cauliflower mosaic virus* (CaMV), *figwort mosaic virus* (FMV), *peanut chlorotic streak virus* (PCISV), *mirabilis mosaic virus* (MMV), *cassava vein mosaic virus* (CVMV) and *strawberry vein banding virus* (SVBV). Chimeric constructs of these promoters with the GUS reporter gene were electroporated into tobacco (Xanthi) and corn (BMS) protoplasts to compare their strength (Figure 1).

In the tobacco protoplast transient assay (Figure 1A), GUS expression levels among PCISV, FMV and CVMV FLt promoters were almost same, but all showed higher GUS activity than the CaMV 35S promoter. The MMV FLt promoter was the strongest and showed approximately three-fold higher activity than the CaMV 35S promoter (Figure 1A, compare lanes 2 and 6). The SVBV FLt promoter was the weakest among all the promoters tested (Figure 1A, lane 3). Transcript levels for the reporter gene were also evaluated by dot-blot hybridization analysis of total RNA isolated from protoplasts, 10 h after electroporation. The differences in transcript levels were in good agreement with those in GUS activity (Figure 1B), suggesting transcription-level differences in promoter efficacy.

The GUS expression level in transient maize protoplast assays is shown in Figure 1C. A significant variation among promoter strengths was also noted, but the ranking was different from that observed with tobacco protoplasts (Figure 1A). The CaMV 35S promoter gave the highest expression in maize protoplasts (Figure 1C, lane 5). There was little difference in GUS activity among FLt promoters derived from the FMV, CVMV and PCISV genomes. Again, GUS activities ranked similarly to the respective transcript level as shown by RNA dot-blot analysis (Figure 1D). In the maize protoplast assay the MMV FLt promoter activity was about 30% compared to the CaMV35S promoter (Figure 1C, compare lanes 1 and 5). This is in contrast to the tobacco protoplast assay where the MMV FLt promoter showed about 3-fold higher activity than the CaMV 35S promoter (Fig 1A, compare lanes 2 and 6).

FLt promoters compared in stable expression

The relative expression activities of these promoters was also determined in stably transformed transgenic tobacco plants (*Nicotiana tabacum* cv Samsun NN, Figure 2). Twelve individual transgenic plant lines were developed with each of the six promoter-GUS constructs employing the FLt promoters derived from SVBV, FMV, CVMV, MMV, CaMV and PCISV respectively. Integrity of the GUS gene with the promoter was confirmed by PCR analysis of genomic DNA. R1 progeny seedlings giving a KanR:KanS = 3:1 segregation ratio were selected on the assumption that only a single locus had been integrated in transformation. Ten such selected R1 progeny plants were used for fluorometric analysis of GUS, with the results shown in Figure 2A.

In the transgenic plant assay, FLt promoters from SVBV, PCISV and FMV showed levels of expression that were favorably competitive with that from CaMV 35S (Figure 2A, lanes 2, 3 and 4, respectively). The MMV FLt promoter, as in the tobacco protoplast assay, was also showing about four times higher expression level compared with CaMV 35S (Figure 2A, compare lanes 5 and 6). Interestingly, the average GUS activity from the SVBV FLt promoter in transgenic plants was higher than from CaMV 35S (Figure 2A, compare lanes 2 and 5), although the SVBV FLt promoter showed 2-3 times lower GUS activity in protoplasts (Figure 1A compare lane 3 and 6). This difference in expression level may be due to a lack in protoplasts of the transcription factors needed for full promoter activity (Pattanaik et al. 2004). Northern analysis showed an expected c. 2kb size of GUS transcript from all promoters, and that the higher level of GUS activity was due to a higher abundance of GUS transcript (Figure 2B).

Overall, the MMV FLt promoter was the most active in tobacco, whereas in corn the CaMV 35S promoter showed the greatest activity. Space limitations here preclude discussion of plant-tissue specificity, but detailed information is available in the listed publications and by contacting us at KTRDC. For all these promoters GUS activity was typically highest in roots and lower in the aerial parts of the plant.

All six promoters have little sequence homology except for some small regulatory motifs. They can therefore be used for expression of multiple genes in transgenic plants. The *as-1* cis-motif (TGACG) of the CaMV 35S promoter has been studied in detail - mutation or deletion of this motif reduced promoter activity by 50% (Benfey and Chua, 1990). However, the 3-4-fold higher activities of the MMV FLt promoter relative to the CaMV 35S promoter suggest that other cis-elements are responsible for enhanced gene expression beside *as-1*, as both promoters have the *as-1* element located approximately at the same position upstream of the TATA box.

For examples of these FLt promoters 'driving' the expression of 'foreign' transgenes in plants with resulting dramatic phenotypes see Maiti et al. (2003) for PCISV and Hou et al. (2007) for MMV.

Subgenomic-transcript promoters

The subgenomic-transcript (Sgt) promoters from several *Caulimoviruses* were also analyzed. The MMV Sgt promoter fragment is a strong constitutive promoter, with strength comparable to that of the MMV FLt promoter (Dey and Maiti, 2002). The MMV Sgt promoter (Dey and Maiti, 2002) and FMV Sgt promoter (Bhattacharya et al., 2002) also demonstrated much greater activity compared to the CaMV 19S and 35S promoters, both in tobacco protoplasts and in transgenic tobacco plants. These Sgt promoters are therefore also useful for expressing foreign genes in transgenic plants. Again the presence of very little sequence homology between the Sgt promoters, in spite of the functional similarity, renders them very useful for multiple-transgene applications.

Enhancement of promoter activity

A genetic promoter has enhancer domains, i.e. cis-sequences that bind with the cognate nuclear proteins called transacting factors to form a functional complex. Duplication of the enhancer domain can result in higher levels of gene expression. We have developed enhancer-doubled versions of the FLt promoters PCISV, FMV and MMV, and obtained typically 3 to 4-fold greater expression of genes compared to the activities of the corresponding single-enhancer promoters (Maiti and Shepherd, 1998; Maiti et al., 1997; Dey and Maiti, 1999b).

The ability of natural introns to enhance gene expression has been well documented in various organisms, including plants (Callis et al. 1987; Luehrsen and Walbot 1991; Rose and Last 1997). We demonstrated that the antisense orientation of the PCISV ORF VII (p7R) in conjunction with the PCISV leader acts as an intron when incorporated into the transcription unit of chimeric gene constructs, and enhances the expression of reporter genes as much as 2-fold compared to the strong constitutive PCISV FLt promoter, both in protoplast transient expression experiments and in stably transformed transgenic plants (Bhattacharyya et al., 2003). We showed that the enhanced expression is due to higher accumulation of mature mRNA.

Access for research and commercial applications

The gene-expression applications of the FLt promoters from MMV, FMV, and PCISV, and the Sgt promoter from MMV are the subject-matter of patents issued to the University of Kentucky Research Foundation. The relevant patents are cited in the publications section below. For access to this intellectual property for research purposes, or to evaluate the promoters in a particular application prior to considering their commercial use, please contact Dr. Indu Maiti or Dr. H. Maelor Davies (Director, KTRDC) and request a Materials Transfer Agreement. To request a license to use the promoter technologies in commercial applications please contact the Intellectual Property Office at the University of Kentucky. The relevant contact information is:

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Publications describing the KTRDC-developed promoters

Bhattacharyya S, Dey N and Maiti IB (2002). Analysis of *cis*-sequence of subgenomic transcript promoter from the figwort mosaic virus and comparison of promoter activity with the cauliflower mosaic virus promoters in monocot. and dicot. cells. *Virus Res* 90: 47-62.

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Pattanaik S, Dey N, Bhattacharyya S and Maiti IB (2004). Isolation of full-length transcript promoter from the strawberry vein banding virus (SVBV) and expression analysis by protoplast transient assays and transgenic plants. *Plant Science* 167: 427-438.

Issued patents describing the KTRDC-developed promoters

Promoter (FLt) for the full-length transcript of peanut chlorotic streak caulimovirus (PCLSV) and expression of chimeric genes in plants.

Maiti et al. U.S. patent 5850019

Full length transcript (FLt) promoter from figwort mosaic caulimovirus (FMV) and use to express chimeric genes in plant cells.

Maiti & Shepherd. U.S. patent 5994521

Use of the full length transcript (FLt) from mirabilis mosaic caulimovirus to express chimeric genes in plants.

Maiti et al. U.S. patent 6420547

Composition and methods of using the Mirabilis mosaic caulimovirus sub-genomic transcript (Sgt) promoter for plant genetic engineering.

Maiti et al. U.S. patent 6930182

A related technology for expressing multiple genes in transgenic plants:

Methods and composition for expressing multiple genes in plants by alternate splicing of a polycistronic message.

Maiti et al. U.S. patent 7052905

Other cited literature

Benfey PN, and Chua NH (1990). The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. *Science* 250: 959-966.

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Contributors

The development and characterization of these gene expression technologies was conducted over several years with significant contributions from the following postdoctoral scholars who visited KTRDC and who are now at the following locations:

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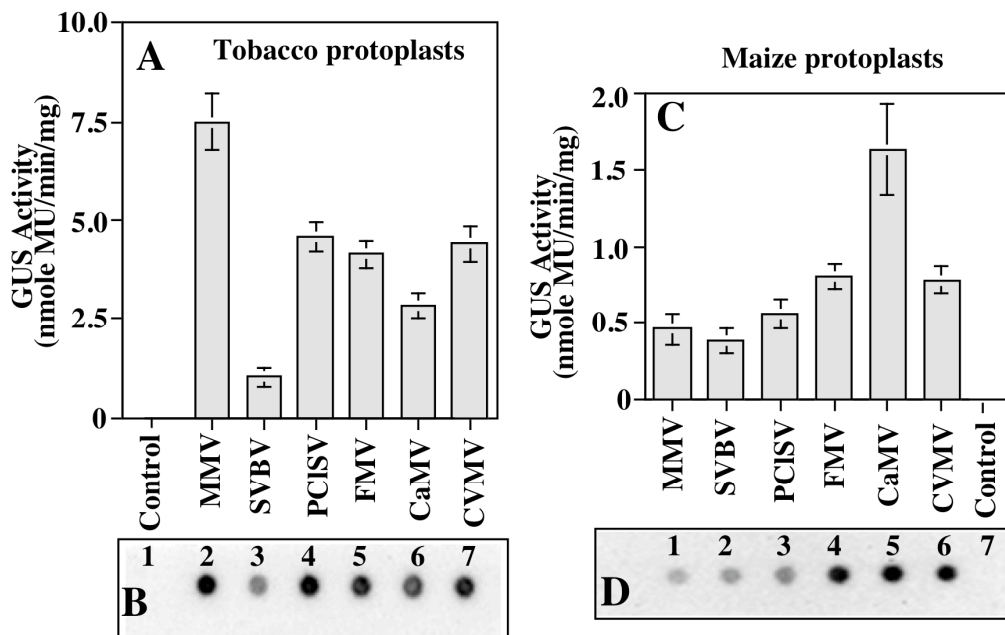


Figure 1. Comparative assessment of the full-length transcript (FLt) promoters in the protoplast transient-expression assay, using GUS as the 'reporter' gene. Results obtained with promoters isolated from the *Caulimoviruses*: MMV, SVBV, PCISV, FMV, CaMV and CVMV are shown, together with the equivalent result on untransformed control protoplasts. Average GUS activity with standard deviations is presented in the histogram for (A) tobacco and (C) maize protoplasts electroporated with the respective promoter constructs. The abundance of GUS transcript is shown by RNA dot-blot analysis for (B) tobacco and (D) maize protoplasts (lane numbers referred to in text). Each promoter construct was evaluated at least three times in three independent experiments. Error bars show the 95% confidence intervals on the means.

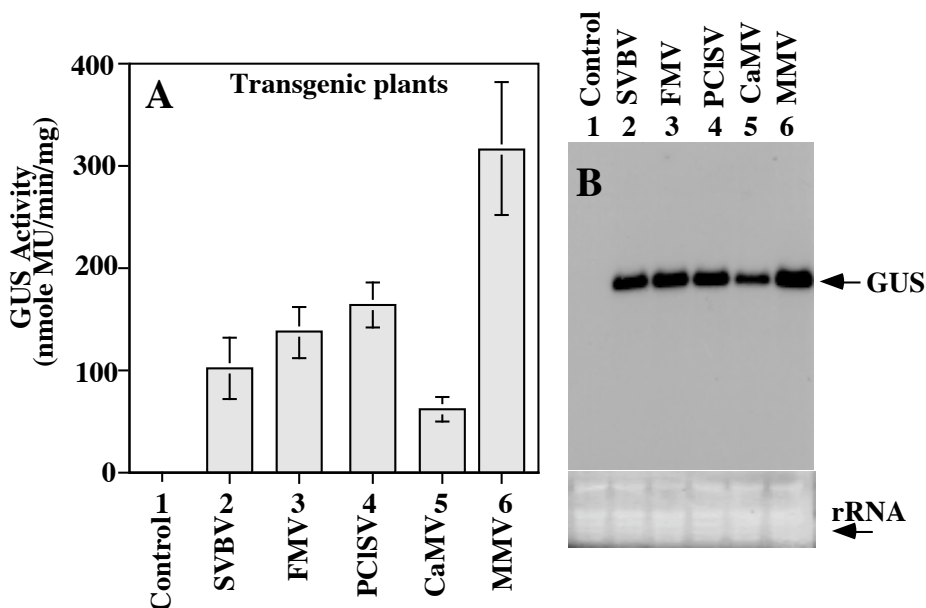


Figure 2. Comparative expression analysis of Caulimovirus full-length transcript promoters in transgenic tobacco plants. (A) Average GUS activity in the 4-week-old transgenic tobacco seedlings (R1 progeny) generated for each FLt promoter construct (lanes 2-6) and untransformed control Samsun NN (lane 1). (B) Relative abundance of GUS transcripts in transgenic tobacco seedlings determined by Northern-blot analysis (lanes 2-6, and untransformed control Samsun NN, lane 1). Total RNA was hybridized with ³²P-labeled GUS coding sequence.

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