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Composition and Methods of Using the *Mirabilis Mosaic* Caulimovirus Sub-Genomic Transcript (SGT) Promoter for Plant Genetic Engineering

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(54) **COMPOSITION AND METHODS OF USING THE *MIRABILIS MOSAIC* CAULIMOVIRUS SUB-GENOMIC TRANSCRIPT (SGT) PROMOTER FOR PLANT GENETIC ENGINEERING**

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(52) **U.S. Cl.** **536/24.1**; 435/320.1; 435/419; 536/23.1

(58) **Field of Search** 536/24.1, 23.1; 435/320.1, 419

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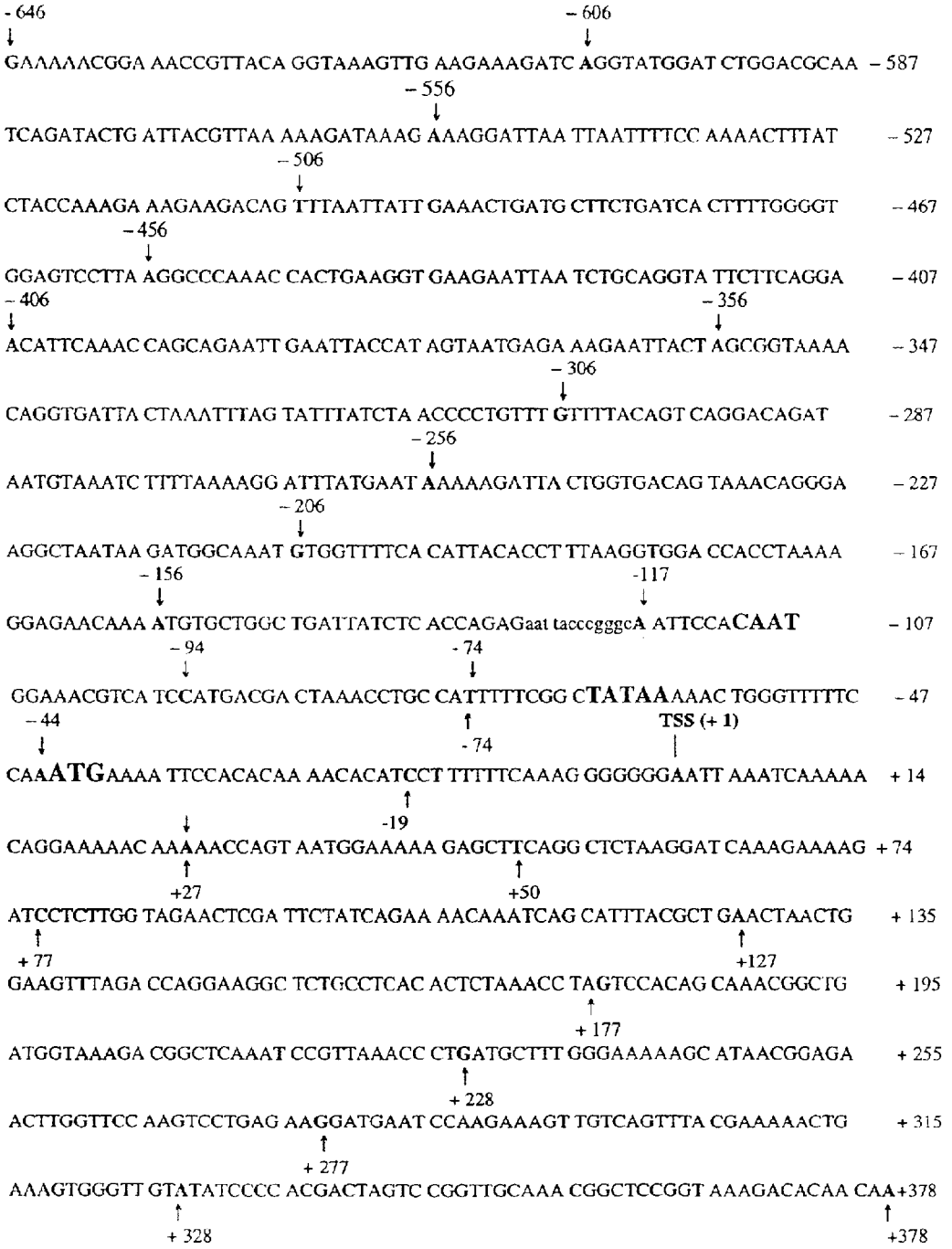
(57) **ABSTRACT**

The isolation of and methods of using a sub-genomic transcript (Sgt) promoter from *Mirabilis mosaic* virus (MMV) are described. A 333 bp MMV Sgt promoter fragment (sequence −306 to +27 from the transcription start site, TSS) was found to be sufficient for strongest promoter activity. This MMV Sgt promoter fragment shows comparable promoter activity to the MMV FLt promoter both in transgenic plants and in protoplasts. The MMV Sgt promoter also demonstrates much greater activity compared to *Cauliflower mosaic* virus (CaMV) 19S promoter and 35S promoter. The MMV Sgt promoter fragment and any chimeric gene to which it may be linked are usefull for plant geneic engineering to obtain transgenic plants, plant cells and seeds.

32 Claims, 8 Drawing Sheets

Figure 1


- 646
- 606
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- 556
TCAGATACTG ATTACGTTAA AAAGATAAAG AAAGGATTAA TTAATTTTCC AAAACTTTAT - 527
- 506
CTACCAAAGA AAGAAGACAG TTTAATTATT GAAACTGATG CTTCTGATCA CTTTTGGGGT - 467
- 456
GGAGTCCTTA AGGCCCAAAC CACTGAAGGT GAAGAATTAA TCTGCAGGTA TTCTTCAGGA - 407
- 406
- 356
ACATTCAAAC CAGCAGAATT GAATTACCAT AGTAATGAGA AAGAATTACT AGCGGTAAAA - 347
- 306
CAGGTGATTA CTAAATTTAG TATTTATCTA ACCCCTGTTT GTTTTACAGT CAGGACAGAT - 287
- 256
AATGTAAATC TTTTAAAAGG ATTTATGAAT AAAAAGATTA CTGGTGACAG TAAACAGGGA - 227
- 206
AGGCTAATAA GATGGCAAAT GTGGTTTTCA CATTACACCT TTAAGGTGGA CCACCTAAAA - 167
- 156
-117
GGAGAACAAA ATGTGCTGGC TGATTATCTC ACCAGAGaat tacccgggcA ATTCCACAAT - 107
- 94
- 74
GGAAACGTCA TCCATGACGA CTAAACCTGC CATTTTTCGG CTATAAAAAC TGGGTTTTTC - 47
- 44
TSS (+ 1)
- 74
CAAATGAAAA TTCCACACAA AACACATCCT TTTTTCAAAG GGGGGGAATT AAATCAAAAA + 14
-19
CAGGAAAAAC AAAAACCAGT AATGGAAAAA GAGCTTCAGG CTCTAAGGAT CAAAGAAAAG + 74
+27
+50
ATCCTCTTGG TAGAACTCGA TTCTATCAGAA AACAAATCAG CATTTACGCT GAACTAACTG + 135
+ 77
+127
GAAGTTTAGA CCAGGAAGGC TCTGCCTCAC ACTCTAAACC TAGTCCACAG CAAACGGCTG + 195
+ 177
ATGGTAAAGA CGGCTCAAAT CCGTTAAACC CTGATGCTTT GGGAAAAAGC ATAACGGAGA + 255
+ 228
ACTTGGTTCC AAGTCCTGAG AAGGATGAAT CCAAGAAAGT TGTCAGTTTA CGAAAAACTG + 315
+ 277
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+ 328
+378

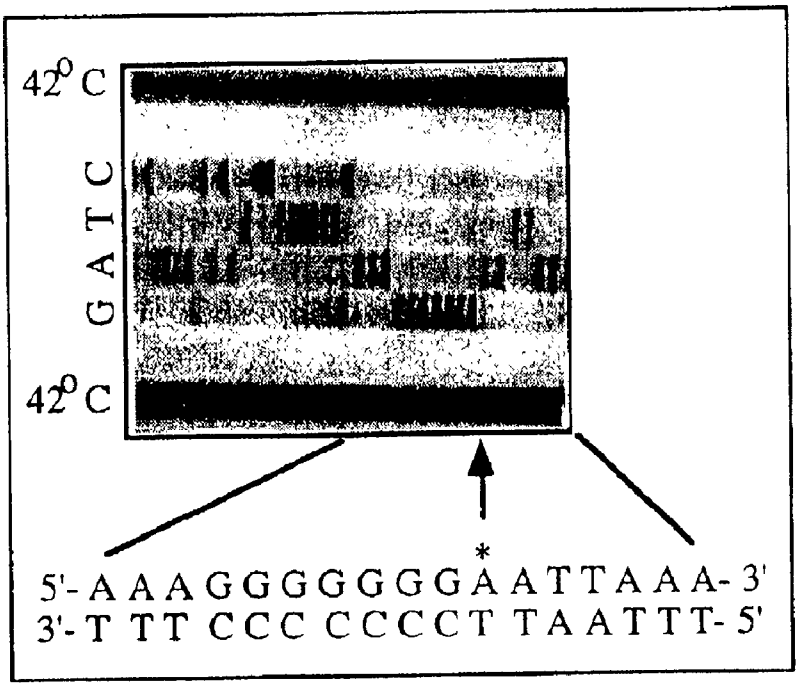


Figure 2

Figure 3, A and B

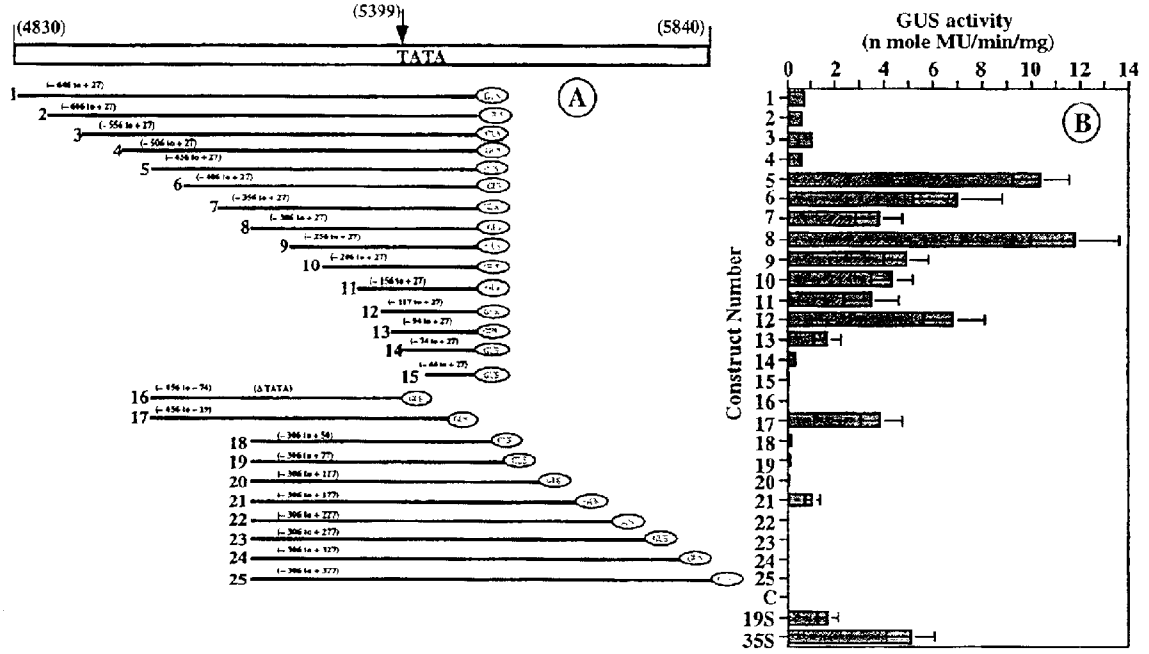


Figure 4A and 4B

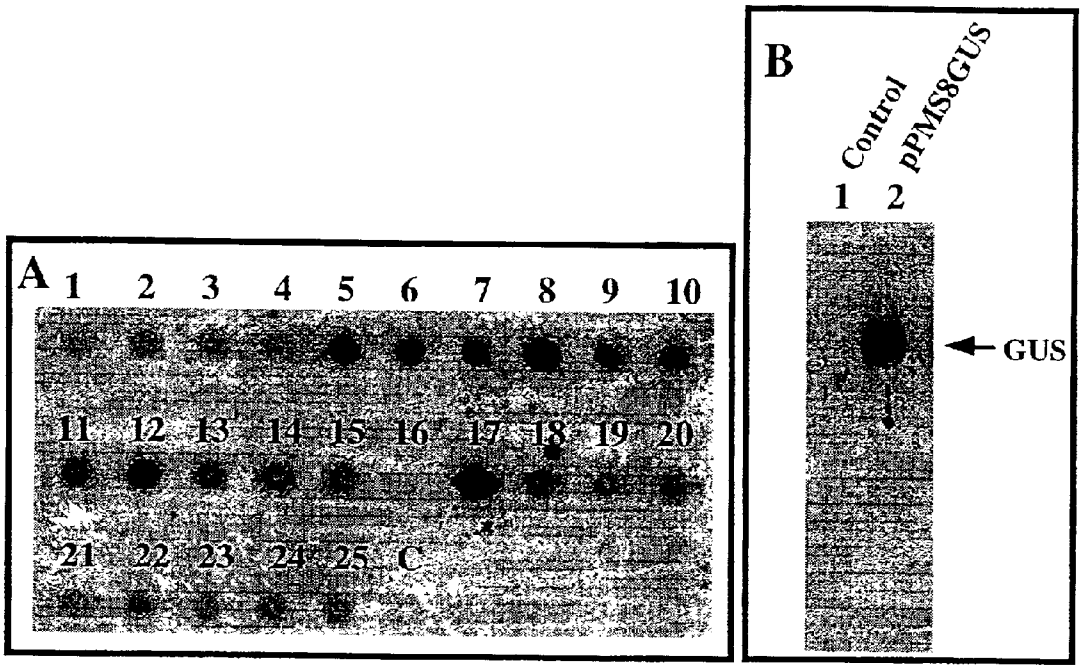


Figure 5 A and B

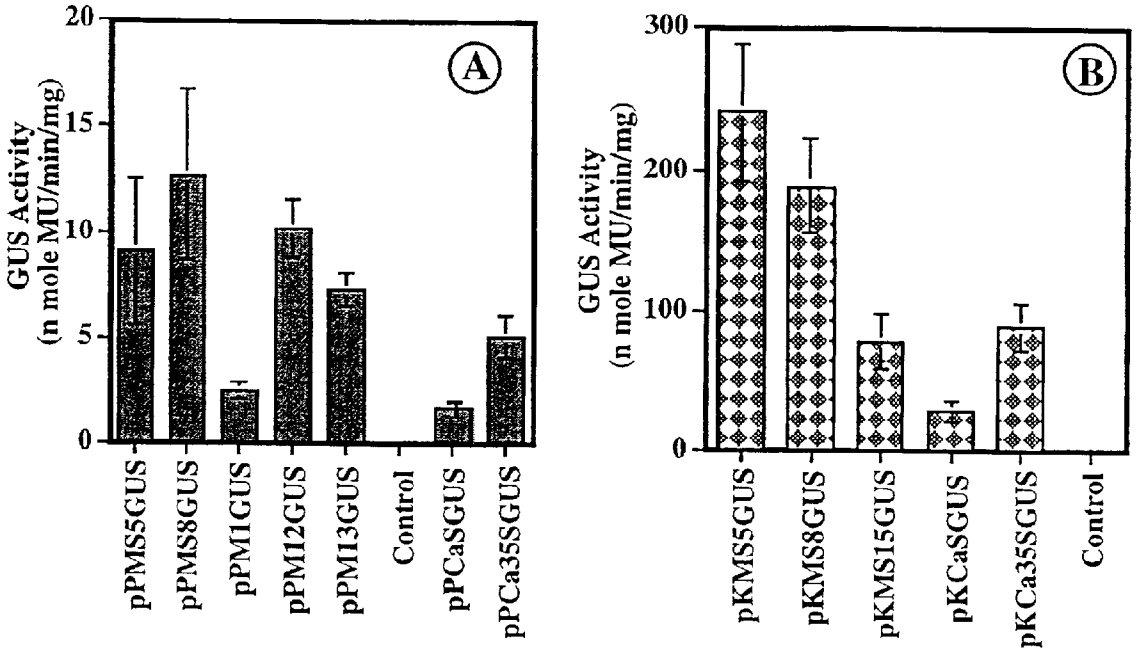
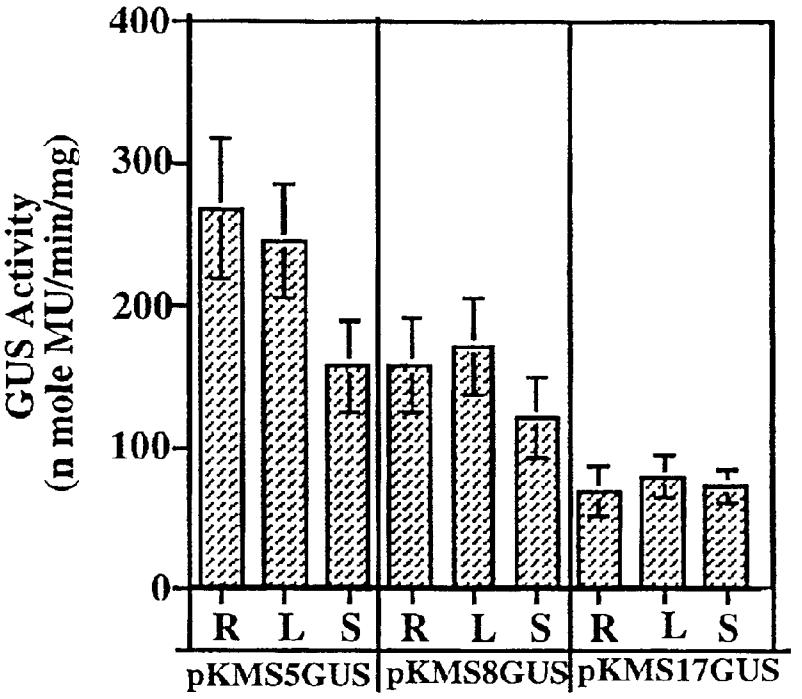


Figure 6


400
300
200
100
0
GUS Activity
(n mole MU/min/mg)
R L S R L S R L S
pKMS5GUS
pKMS8GUS
pKMS17GUS

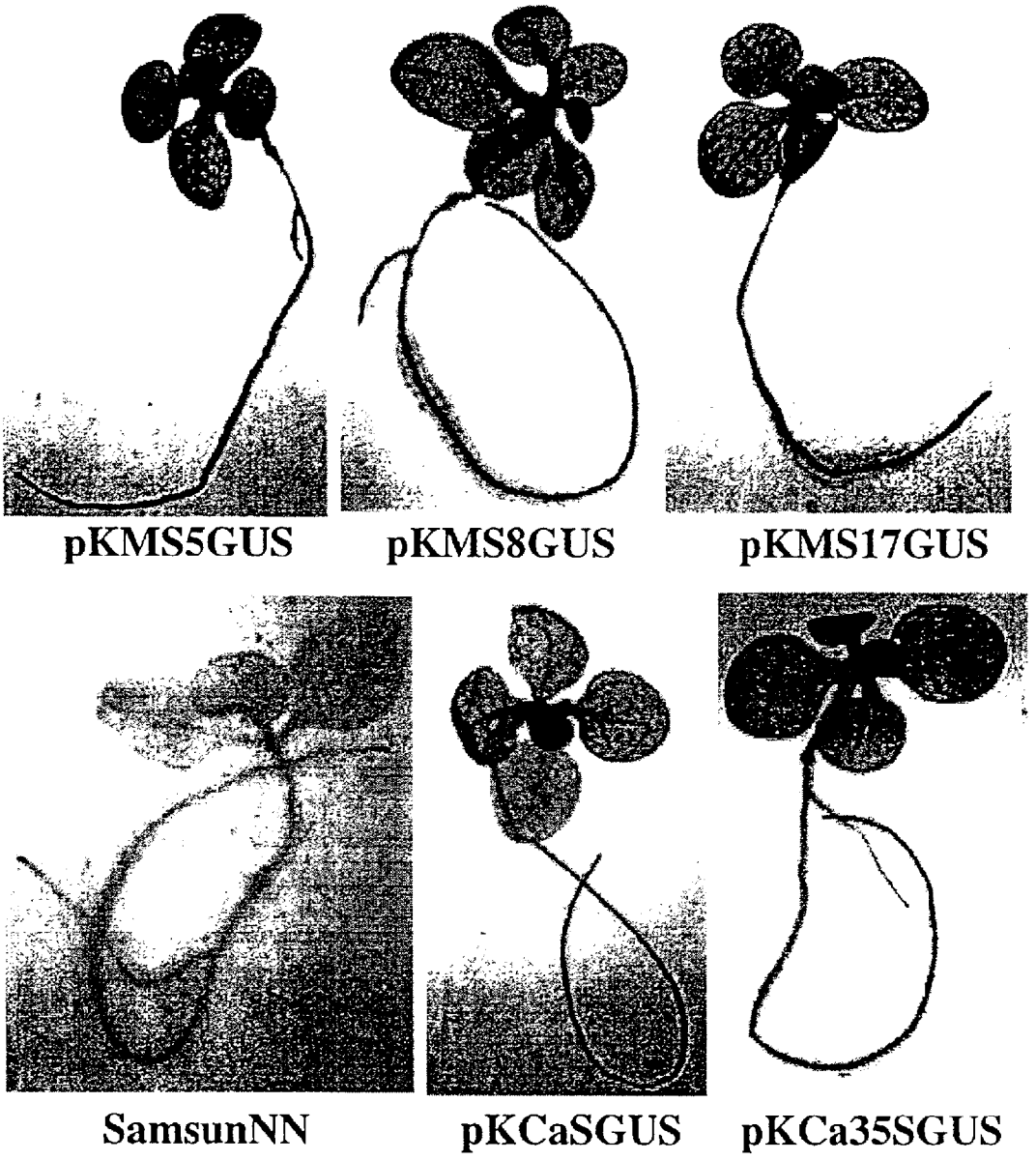

pKMS5GUS
pKMS8GUS
pKMS17GUS
SamsunNN
pKCaSGUS
pKCa35SGUS

Figure 7

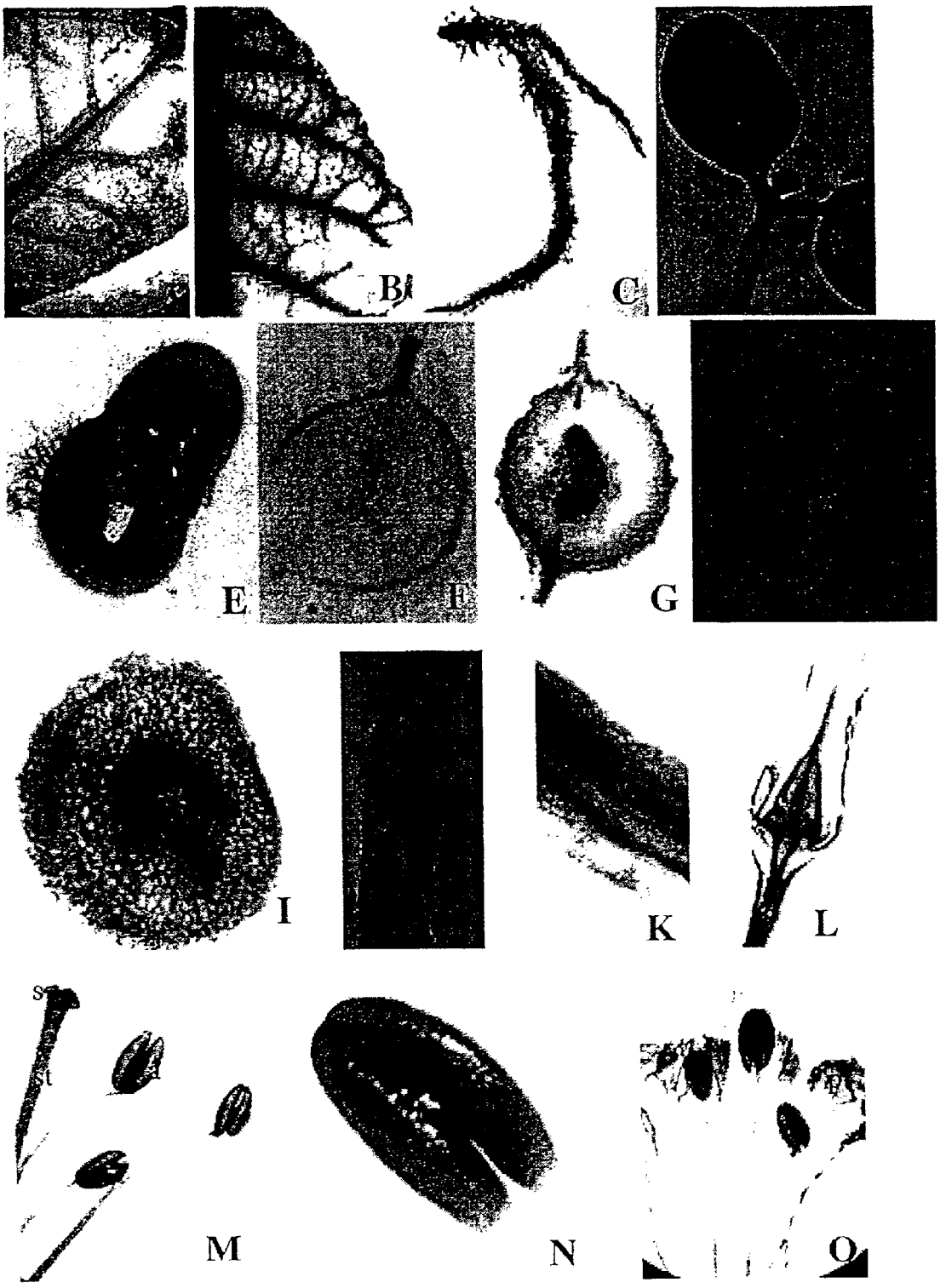


Figure 8

COMPOSITION AND METHODS OF USING THE *MIRABILIS MOSAIC* CAULIMOVIRUS SUB-GENOMIC TRANSCRIPT (SGT) PROMOTER FOR PLANT GENETIC ENGINEERING

This application claims priority to U.S. application Ser. No. 60/295,566, filed Jun. 5, 2001.

FIELD OF THE INVENTION

This invention relates generally to plant genetic engineering and plant molecular biology. More particularly, the invention relates to the isolation and use of a new plant promoter for driving the expression of plant genes.

BACKGROUND OF THE INVENTION

The *Mirabilis mosaic* virus (MMV) infects *Mirabilis* plant species (family *Nyctaginaceae*), a member of the *Caulimoviridae* family. The virus has a circular double-stranded DNA genome of about 8 Kb with four single-stranded discontinuities in the DNA, one in the alpha strand and three in the complementary strand [1]. The restriction map of the MMV genome is quite different from that of the other members of the genus *Caulimovirus* [1]. The MMV virus was characterized as a member of the genus *Caulimovirus* based upon the morphology of its virions and inclusion bodies [2].

Recently, MMV has been fully sequenced, and homology analysis of its genomic DNA has shown that it is a definitive member of the genus *Caulimovirus* [Maiti, unpublished]. However, MMV is serologically distinct from the *Cauliflower mosaic* virus (CaMV), the type species of this genus [2].

Several *Caulimoviridae* genomes have been fully sequenced and characterized. These include *Cauliflower mosaic* virus (CaMV) [3], *Carnation etched ring* virus (CERY) [4], *Figwort mosaic* virus (FMV) [5], *Soybean chlorotic mottle* virus (SoCMV) [6], *Peanut chlorotic* streak virus (PCISV) [7], *Casava vein mosaic* virus (CVMV) [8], *Strawberry vein* banding virus (SVBV) [9], *Petunia* vein clearing virus PVCV) [10], and *Mirabilis mosaic* virus (MMV) [Maiti, unpublished].

The *Caulimovirus* genome generally contains two transcriptional promoters, one for the full-length transcript and the other for the subgenomic transcript. These transcripts are equivalent to the CaMV 35S and 19S transcript respectively [6, 11, 12]. A number of strong constitutive promoters have been derived from viruses of the *Caulimoviridae* family, particularly from the *Cauliflower mosaic* virus (CaMV): CaMV35S and 19S promoter[13, 14]. Genetic promoters have also been isolated from other members of this family, namely *Rice tungro* bacilliform virus RTBV) [15], *Commelina yellow* mottle virus (CYMV) [16], *Soybean chlorotic* mottle virus (SoCMV) [6], *Figwort mosaic* virus (FMV, strain DxS) [17, 18]), FMV strain M3 [19], *Cassava vein* mosaic virus (CVMV) [20], *Peanut cholotic* streak virus (PClSV) [21] and *Mirabilis mosaic* virus (MMV) [22, 23] and used for the construction of plant transformation vectors. Transcript promoters from *Caulimoviruses,* such as CaMV, FMV, PCISV, MMV and FMV are active in all plant organs [13, 18, 21–23], whereas, transcript promoters from *Badnaviruses,* such as CYMV and RTBV are phloem-specific [15, 16] in expressing genes in transgenic plants.

The CaMV 35S promoter has been well characterized [13, 24–30] and widely used in chimeric gene constructs for heterologous gene expression in transgenic plants [31–33].

The CaMV 35S promoter is also active in bacteria [34], yeast [35], Hela cells [36] and *Xenopus oocytes* [37].

The expression of useful foreign traits in plants is a major focus in plant biotechnology. There is a need for a variety of different (e.g., constitutive, tissue specific and/or inducible) promoters that meet the different potential applications in this field of plant genetic engineering. Introduction of heterologous genes of interest into plant cells generates the desired qualities in the plants of choice (Maiti and Hunt, 1992; Wagner, 1992). Plant biotechnology is leading a rapid progress in production of economically valuable germplasm with improved characteristics or traits such as insect resistance, virus resistance, fungal resistance, herbicide resistance, bacterial or nematode pathogen resistance, cold or drought tolerance, improved nutritional value, seed oil modification, delayed ripening of fruits, and male sterility, to name a few. These germplasms provide an enhanced development in breeding programs for crop improvement as well as a better understanding of gene regulation and organization in transgenic plants.

Plant metabolic engineering is the application of genetic engineering methods to modify the nature of chemical metabolites in plants. For metabolic engineering where multiple genes need to be inserted into a single cell, the use of different strong constitutive promoters is desirable in order to avoid genetic instability caused by recombination between identical or closely related promoter sequences, for example, those taken from plants themselves. Through use of these promoter sequences the introduced genes can be transcribed to messenger RNA and then translated to resultant proteins to exhibit new traits or characters.

Besides developing useful traits in crops, plant molecular engineering will lead to further understanding of molecular pathways involved in disease development and secondary metabolism in plants. Moreover, by engineering plants with specific foreign genes, the responses of plants to abiotic and biotic stress and stress related metabolism can be analyzed.

Thus, there is a need in the art for plant promoters that can be used to drive the expression of genetically engineered genes in plants.

SUMMARY OF THE INVENTION

In one aspect of the invention there is provided an isolated DNA molecule comprising a *mirabilis mosaic* virus (MMV) subgenomic transcript (Sgt) promoter comprising the nucloetide sequence of SEQ ID NO:1 or a fragment thereof having at least about 30% MMV Sgt promoter activity. In a preferred embodiment of this aspect of the invention the Sgt promoter is a fragment comprising the nucleotide sequence of −306 to +27 of SEQ ID NO:1. In another preferred embodiment, the MMV Sgt promoter is a fragment comprising the nucloetide sequence of −456 to +27 of SEQ ID NO:1. In a most preferred embodiment, the MMV Sgt promoter of the invention is operably linked to a nucleotide sequence which encodes a polypeptide. In yet another preferred embodiment, the MMv Sgt promoter has the nucleotide sequence of SEQ ID NO:2.

In another aspect of the invention, there is provided an intermediate plant transformation plasmid comprising a region of homology to an *Agrobacterium tumefaciens* gene vector, an *Agrobacterium tumefaciens* T-DNA border region and a chimeric gene located between the T-DNA border and the region of homology, said chimeric gene comprising a MMV Sgt promoter comprising MMV Sgt promoter comprising the nucloetide sequence of SEQ ID NO:1 or a fragment thereof having at least about 30% MMV Sgt

promoter activity, wherein the MMV Sgt promoter or fragment thereof is operably linked to a nucleotide sequence encoding a polypeptide. In a preferred embodiment of this aspect of the invention, the chimeric gene further comprises a MMV leader sequence operably linked to said nucleotide sequence. In another preferred embodiment of this aspect of the invention the MMV Sgt promoter comprises at least one enhancer domain.

In yet another aspect of the invention, there is provided a plant transformation vector comprising a disarmed *Agrobacterium tumefaciens* plant tumor-inducing plasmid and a chimeric gene, said chimeric gene comprising a MMV Sgt promoter comprising the nucleotide sequence of SEQ ID NO:1 or a fragment thereof having at least about 30% MMV Sgt promoter activity, wherein the MMv Sgt Promoter or fragment thereof is operably linked to a nucleotide sequence encoding a polypeptide. In a preferred embodiment of this aspect of the invention, the MMV Promoter comprises at least one enhancer domain.

In another aspect of the invention, there is provided an intermediate plant transformation plasmid comprising a region of homology to an *Agrobacterium tumefaciens* gene vector, an *Agrobacterium tumefaciens* T-DNA border region and a chimeric gene located between the T-DNA border and the region of homology, said chimeric gene comprising a MMV Sgt promoter comprising the nucleotide sequence of SEQ ID NO:1 or a fragment thereof having at least about 30% MMV Sgt promoter activity, wherein the MMV Sgt promoter or fragment thereof is operably linked to a nucleotide sequence encoding a polypeptide.

In a further aspect of the invention, there is provided a transgenic plant or transgenic plant part comprising a plant transformation vector comprising a disarmed *Agrobacterium tumefaciens* plant tumor-inducing plasmid and a chimeric gene, said chimeric gene comprising a MMV Sgt promoter comprising the nucleotide sequence of SEQ ID NO:1 or a fragment thereof having at least about 30% MMV Sgt promoter activity, wherein said MMV Sgt promoter or fragment thereof is operably linked to a nucleotide sequence which encodes a polypeptide; and wherein said chimeric gene is expressed in the plant. In a preferred embodiment of this aspect of the invention, the chimeric gene encodes a plant metabolic polypeptide or a polypeptide that confers pathogen resistance to the transgenic plant. In another preferred embodiment, the plant part is selected from the group consisting of a root, leaf, stem, flower petal, pollen, callus and cell.

In another aspect of the invention, there is a transformed plant protoplast comprising a plant transformation vector comprising a disarmed *Agrobacterium tumefaciens* plant tumor-inducing plasmid and a chimeric gene, said chimeric gene comprising a MMV Sgt promoter comprising the nucleotide sequence of SEQ ID NO:1 or a fragment thereof having at least about 30% MMV Sgt promoter activity, wherein said MMV Sgt promoter or fragment thereof is operably linked to a nucleotide sequence which encodes a polypeptide.

In yet another aspect of the invention, there is provided a method of making a transgenic plant comprising (1) transforming a plant cell with a chimeric gene comprising a MMV Sgt promoter comprising the nucleotide sequence of SEQ ID NO:1 or a fragment thereof having at least about 30% MMV Sgt promoter activity, wherein said MMV Sgt promoter or fragment thereof is operably linked to a nucleotide sequence which encodes a polypeptide; (2) culturing the plant cell under growing conditions to produce a regenerated plant; and (3) and expressing the chimeric gene in the regenerated plant.

There is also provided a promoter enhancer element having the sequence set forth in SEQ ID NO: 10.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the DNA sequence of the subgenomic transcript promoter from *mirabilis mosaic* virus (MMV) (SEQ ID NO:1). The nucleotide sequence of the MMV Sgt promoter (coordinates −646 to +377 in respect of transcription start site; corresponding coordinates in MMV genome 4829 to 5840), and a 1023 bp fragment including the 3' end of gene V, followed by the small intergenic region and 5' portion of gene VI presented from left to right in the 5' to 3' direction of the transcript. Modification of the promoter sequence resulted from insertion of an 'EcoRI to SmaI' adapter is shown in lower case. The end points for the 5' or the 3' deletion constructs are also indicated above or below the sequence, respectively. The TATA-box, (TATAA), CAT box (CAAT) and initiator ATG codon for gene VI are shown in bold. The transcriptional start site (TSS) is indicated as +1.

FIG. **2** is an autoradiograph showing the location of the transcriptional start site (TSS) of the MMV Sgt promoter by primer extension analysis. Primer extension was carried out as described in Example 4. Primer extension reaction products were subjected to electrophoresis on denaturing polyacrylamide gel containing urea, alongside with the sequence reaction of GUS gene construct (lane G, A, T and C). The process was performed with the same labeled primer. The minus strand DNA sequence read on the gel is shown, and the transcriptional start site (A*) in the corresponding plus strand is indicated by an arrow.

FIGS. **3**A and **3**B: FIG. **3**A is a schematic map of the GUS constructs (number 1 to 25) developed for deletion analysis of the MMV Sgt promoter. The coordinates of the relative deletion fragments are given in parenthesis. The end points for the 5' or the 3' deletion constructs are also indicated above or below the sequence, respectively in FIG. **1**. At the top, the relative position of the TATA box and the MMV genomic coordinates are shown. FIG. **3**B is a bar graph showing MMV Sgt promoter expression analysis in a protoplast transient expression assay using a GUS reporter gene. Soluble protein extracts (5 μg) from transformed protoplasts were used for the GUS assay. Each construct was assayed at least three times in three independent experiments. The average GUS activity is presented in the histogram. Error bars show a 95% confidence interval of the means. The statistical (one-way analysis of variance, ANOVA) analysis showed an extremely significant P value of <0.001. C., untransformed control, extract from untransformed protoplast; 19S, 35S, GUS gene is directed by the CaMV 19S and 35S promoter, respectively.

FIGS. **4**A and B: FIG. **4**A is an autoradiograph of RNA dot-blot analysis of total RNA (10 μg) obtained from transformed protoplasts with construct No 1 to 25 as indicated in FIG. **3**A. FIG. **4**B is a Northern blot analysis of total RNA (10 μg) obtained from pPMS8GUS with 32P labeled GUS as probe (lane 2) and RNA obtained from untransformed Samsun NN plant (lane 1).

FIGS. **5**A and B: FIGS. **5**A and **5**B are a bar graphs showing comparative expression analyses of the MMV Sgt promoter with the MMV FLt promoters and CaMV promoters (35S and 19S) in (A) transient expression in protoplasts and (B) stable expression in transgenic plants.

(5A): GUS constructs: pPMS5GUS, pPMS8GUS with the MMV Sgt promoter; pPM1GUS, pPM12GUS and

pPM13GUS with MMV FLt promoter, as described earlier [22]; and pPCaSGUS and pPCa35SGUS with CaMV 19S and 35S promoters, respectively, were assayed in protoplast transient expression experiments. Each construct was assayed at least three times in three independent experiments. The average GUS activity is presented in the histogram. Error bars show a 95% confidence interval on the means. The statistical ANOVA analysis showed a P value <0.001; this is considered to be extremely significant.

(5B): The MMV Sgt promoter (GUS-constructs pKMS5GUS, pKM8GUS and pKMS17GUS) and CaMV 19 S and 35S promoter (GUS constructs pKCaSGUS and pKCa35SGUS respectively) were compared. The promoter activity was measured in four-week-old seedlings (R1 progeny) grown aseptically on an MS-agar medium in the presence of kanamycin (200 mg/ liter) and 3% sucrose. Soluble protein extract from the whole seedlings were used for the GUS assay. The data are means of five independent experiments for each construct; eight to ten independent transgenic lines developed for each construct were assayed. The average GUS activity is presented for each construct in the histogram, with standard deviation from the mean indicated by an error bar. Error bars show a 95% confidence interval on the means. The statistical ANOVA analysis indicated that the P value <0.001 means extremely significant. Untransformed control (Control), tissue extract from wild-type *N. tabacum* cv. SamsunNN.

FIG. **6** is a histogram showing expression of MMV Sgt promoters in various parts (roots, R; leaves, L; stems, S) of four-week-old seedlings developed for pKMS5GUS, pKMS8GUS and pKMS17GUS. GUS activity was measured fluorometrically using soluble protein extract (5 μg) from roots, stems and leaves of seedlings. The presented value in the histogram, with standard deviation indicated by an error bar, is the average of six samplings from each of the eight independent lines developed for each construct. Error bar shows a 95% confidence interval on the means. The statistical ANOVA analysis showed that a P value <0.001 means extremely significant.

FIG. **7** provides photographs of six transgenic plants of the invention. Shown are histochemical assays of GUS expression in transgenic tobacco (*N. tabacum* cv. Samsun NN) seedlings (R1 progeny, 24-day old) developed for the following constructs: pKMS5GUS, pKMS8GUS, pKMS17GUS, pKCaSGUS (19S-GUS) and pKCa35SGUS (35S-GUS). These data were derived from pools of transformed lines with best expressing independent lines shown representing each construct. Untransformed control is shown wild type *N. tabacum* Samsun NN.

FIG. **8** provides photographs of parts of transgenic plants of the invention. Histochemical localization of GUS activity in developing transgenic tobacco plants expressing the GUS reporter gene directed by MMV Sgt promoter is shown. All sections are at 15× magnification. A. Samsun NN tobacco plant (non-transformed) as control; note no GUS staining. B. Matured leaf section from thirteen-weeks-old plants (R1 progeny) developed for the construct pKMS5GUS; note more GUS staining in vascular tissues (v), midrib and veins. C. Root from four-week-old seedlings (pKMS5GUS, R1 progeny) showing intense staining at the tip and in vascular (v) tissue. D. Top portion of ten-day old seedling (pKMS5GUS, R1 progeny); most GUS activity localized in leaves and apical meristematic (m) region. E. Transgenic tobacco seedling (pKMS5GUS, R1 progeny) at day 7 after imbibition, grown axenically on agar plate; GUS activity is localized primarily in root tips, root hairs and in the lower hypocotyls. F and G. Transverse cross section of petiole from control non-transformed Samsun NN (F), no GUS staining; and from four week-old seedlings (pKMS5GUS, R1 progeny), GUS staining is most intense in the vascular (v) cells (G). H, I, J and K. Transverse cross section (H and I) and longitudinal cross section (J and K) of stem from four week old control seedlings nontransformed Samsun NN (H and J respectively), note no GUS activity; and from four week old transformed seedlings (pKMD5GUS, R1 progeny); GUS activity localized mostly in vascular (v) tissues (I and K). L. Transverse section of tobacco flower pedicel and ovary; M. Stigma (s) and style (St); N. Anther (a); and O. The petal (p) and another (a) in flower tissues display GUS staining.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to (i) the isolation and characterization of the promoter for the subgenomic transcript (Sgt) from a genornic clone of *mirabilis mosaic* virus (Richins and Shepherd, 1983) as described below in Example 1, (ii) use of the MMV Sgt promoter to transform plant cells, plant expression vectors including a MMV Sgt promoter, chimeric genes including a MMV Sgt promoter sequence, and transgenic plants, plant cells, and plant parts, including seeds, which contain the MMV Sgt promoter in a chimeric gene.

Disclosed herein is the identification and characterization of a subgenomic transcript (Sgt) promoter from the *Mirabilis mosaic* virus (MMV), newly described species of the genus *Caulimovirus*. The optimal boundries required for maximal promoter activity and boundries that provided sub-maximal promoter activity have been defined by 5'-and 3'-end delation analysis of the promoter/leader region, both in transgenic plants and in protoplast transient expression experiments. The nucleotide sequence of the MMV Sgt promoter, containing a 12 nucleotide insert between nt −130 and −117 is shown in FIG. **1** (SEQ ID NO:11). It is understood that the MMV Sgt promoter described herein does not contain the 12 nt insert in nature and that the isolated Sgt promoter and promoter fragments described herein do not require the 12 nt insert for function. The insert was added for ease of manipulation of the isolated promoter and fragments. It is also understood, that all references to the nucleotide sequence of the isolated promoter and fragments thereof include the 12 nt insert as shown in FIG. **1**, unless otherwise indicated. However, the invention also encompasses the MMV Sgt promoter and fragments thereof that lack the 12 nt insert and whose sequence numbering is adjusted accordingly, i.e., nt−130 is linked to nt−117. For example, SEQ ID NO:12 is the sequence of the MMV Sgt promoter from nt−646 to +377, but lacking the 12 nt insert.

A 333 nt MMV Sgt promoter fragment (sequence −306 to +27 from transcription start site (SEQ ID NO:2); 321 nt without the 12 nt insert shown in FIG. **1**) (SEQ ID NO:13) was found to be sufficient for maximal promoter activity in a protoplast transient expression system. (FIG. **3**). A larger promoter fragment containing nucleotides −456 to +27 (SEQ ID NO:9) showed even higher activity in stably transformed transgenic plants. (FIG. **5**). Various other Sgt promoter fragments that retain promoter activity, albeit at a lower level than the full length or 333 bp fragment, have also been identified. (FIG. **3**). Fragment 1 (−646 to +27) is SEQ ID NO:14. Fragment 2 (−606 to +27) is SEQ ID NO:15. Fragment 3 (−556 to +27) is SEQ ID NO:16. Fragment 4 (−506 to +27) is SEQ ID NO:17. Fragment 5 (−456 to +27) is SEQ ID NO:9. Fragment 6 (−406 to +27) is SEQ ID

NO:18. Fragment 7 (−356 to +27) is SEQ ID NO:19. Fragment 8 (−306 to +27) is SEQ ID NO:2. Fragment 9 (−256 to +27) is SEQ ID NO:20. Fragment 10 (−206 to +27) is SEQ ID NO:21. Fragment 11 (−156 to +27) is SEQ ID NO:22. Fragment 12 (−117 to +27) is SEQ ID NO:23. Fragment 13 (−94 to +27) is SEQ ID NO:24. Fragment 14 (−74 to +27) is SEQ ID NO:25. Fragment 15 (−44 to +27) is SEQ ID NO:26. Fragment 16 (−456 to −74) is SEQ ID NO:27. Fragment 17 (−456 to −19) is SEQ ID NO:28. Fragment 18 (−306 to +50) is SEQ ID NO:29. Fragment 19 (−306 to +77) is SEQ ID NO:30. Fragment 20 (−306 to +127) is SEQ ID NO:31. Fragment 21 (−306 to +177) is SEQ ID NO:32. Fragment 22 (−306 to +227) is SEQ ID NO:33. Fragment 23 (−306 to +277) is SEQ ID NO:34. Fragment 24 (−306 to +327) is SEQ ID NO:35. Fragment 25 (−306 to +377) is SEQ ID NO:36.

The strength of the MMV Sgt promoter is compatible with the MMV FLt promoter and greater than that of the CaMV 19S and CaMV 35S promoters. This is the first report, to our knowledge, documenting the strong and constitutive expression characteristic of the MMV Sgt promoter.

The MMV Sgt promoter sequence contains several regulatory domains found in other caulimovirus promoters: the TATAA sequence (coordinates −65 to −61 from the Transcription Start Site (TSS) in FIG. **1**) and the CAAT sequence (coordinates −110 to −70 from TSS in FIG. **1**) located 41 bp upstream of the TATA box. In the MMV Sgt promoter sequence, an 'as-1'-like enhancer element (TGACG; coordinates −90 to −88 from TSS in FIG. **1**) and an 'as-2'-like motif (GATT; coordinates −145 to −142 from TSS in FIG. **1**) are located at the 22 bp and 76 bp upstream of the TATA box, respectively. The MMV Sgt promoter has only one copy of an 'as-1'-like or 'as-2'-like motif; whereas, duplicated copies are present in full-length transcript promoters of CaMV, FMV and MMV [13, 18, 22]. In addition, several direct repetitive sequences are present in the MMV Sgt promoter. These are: TCAGGA (−412 to −407 and −297 to −292), GAATTAC (−386 to −380 and −364 to −358), GGTGA (−244 to −240 and −344 to −340), CC(A/T) TTTTTC (−77 to −69 and −19 to −11) and AAACA (−28 to −24, +12 to +16, and +21 to +25) (FIG. **1**). These repetitive sequences may have some regulatory function.

An EcoRI site located at 48 bp upstream of the TATA sequence was modified to a SmaI site using a SmaI adaptor. This change inserted 12 additional nucleotides (5'-AATTACCCCGGGC-3') (SEQ ID NO:11), into the MMV Sgt promoter sequence as shown in lowercase (FIG. **1**),—but did not affect promoter activity.

A comparison of the activity of a promoter fragment containing nucleotides −406 to +27 (SEQ ID NO:18) to that of a fragment containing nucleotides −356 to +27 (SEQ ID NO:19) shows that the inclusion of the nucleotide sequence from −406 to −356 enhances promoter activity. (FIG. **3**A and B). Thus, it appears that the sequence −406 to −357 (SEQ ID NO:10) contains a promoter enhancer. This enhancer sequence can be used with other plant promoters to enhance promoter activity. For example, the enhancer sequence can be operably linked to a plant promoter either alone, or in combination with other promoter enhancer elements. Several enhancer elements may be operably linked, preferably in tandem, or may be spaced apart from one another, depending on the particular level of activity desired. The enhancer element may also be used with fragments of the present promoter to increase activity thereof.

In *Caulimovirus,* both subgenomic and full-length transcript promoters share the same 3'-ends by using the same poly (A) signal.

The transcriptional start site (TSS) of the MMV Sgt promoter was determined by primer extension analysis using total RNA isolated from transgenic plants developed with the construct pKMS5GUS (FIG. **2**). The major extension product was detected and mapped to an adenine residue located 63 nucleotide downstream of the TATA box in the MMV Sgt sequence and, most likely, it represents the 5'-end of the MMV Sgt transcript (FIG. **2**). The location of the TSS reported for other caulimoviruses: CaMV 35S [46], FMV34S [47], FMV FLt [18], PCISV FLt [7] and MMV FLt [22] is at the 32, 37, 45, 29 and 24 nucleotides downstream of respective TATA boxes. The transcription start site of the MMV Sgt promoter shows no sequence homology with that of other caulimovirus promoters.

A deletion analysis scheme of the MMV Sgt promoter is shown in FIG. **3**A. A series of 5'-and 3'-end -deleted promoter fragments (total of 25 fragments) were included to map the optimal boundries required for maximal expression from the promoter/leader region and also to analyze the influence of the upstream and downstream cis-sequences with respect to the TATA box. The designed deletion promoter fragments 1 to 25 (FIG. **3**A) were amplified by PCR and cloned into the expressing sites of vector pUCPMAGUS, as described in the Examples. Results of the expression analysis of the MMV Sgt promoter are shown in FIG. **3**B. In a transient expression assay, construct 8 (pPMS8GUS), which contains the promoter fragment (coordinate−306 to +27 from TSS (SEQ ID NO:2)) gave maximum activity in protoplasts. The expression level of 5' deletion constructs 1, 2, 3 and 4 was 6%, 5%, 9% and 5%, respectively relative to construct 8. This suggest that the upstream sequence region (coordinates −646 to −455 from TSS in FIG. **1**) may contain repressor elements. However, in this context, to obtain maximal promoter activity this region (coordinates −646 to −455 from TSS in FIG. **1**) is not essential. The 5' deletion construct 5, 6 and 7 showed 88%, 60% and 32% of maximal activity, respectively. In construct 7, 5' deletion of 50 bp of sequence (−406 to −356 from TSS in FIG. **1**) reduced promoter activity by 46% relative to construct 6 (compared construct 6 and 7) and by 63% relative to construct 5 (compare construct 5 and 7). These deletion results clearly show the importance of this region (−406 to −357 from TSS; SEQ ID NO:10) in overall promoter activity. There are also two direct repeat sequences GAATTC (coordinates −386 to −380, and −364 to −358 from TSS) in this region. Although, constructs 8 with promoter sequences (−306 to +27 from TSS (SEQ ID NO:2)) showed maximum activity in protoplast transient expression experiments, construct 5 with promoter coordinates (−456 to +27 from TSS (SEQ ID NO:9)) exhibited more activity in stable expression assay in transgenic plants. Constructs 9, 10 and 11 gave 42%, 37% and 29%, respectively, of maximal activity compared to construct 8, demonstrating the importance of cis sequences between −306 to −255 from TSS, as deletion of this stretch reduced maximal promoter activity by 58% (compare construct 9 with construct 8). Construct 12, containing a CAAT sequence (coordinates −110 to −107 from TSS in FIG. **1**), showed 58% of construct 8 activity. However, further deletion of 22 bp (coordinates −117 to −93 from TSS in FIG. **1**) in construct 13 (which contains an 'as-1'-motif) reduced promoter activity to 14% of that of construct 8, and to 24% of that of construct 12, suggesting the importance of the CAAT box in this region for promoter function. The 5' deletion construct 13, which contains the 'as-1'-motif, construct 14, which contains the TATA like element, and construct 15, which lacks a TATA region, showed 14%, 3% and

1%, respectively, of maximal activity (compare with construct 8). This demonstrates the requirement of further additional TATA upstream sequences for full promoter activity. The 3' deletion-construct 16 (promoter coordinates −456 to −74 from TSS in FIG. **1** (SEQ ID NO:27), which is devoid of a TATA box showed no appreciable promoter activity, suggesting the importance of the TATAA sequence in the MMV Sgt promoter function. Although, the MMV Sgt promoter does not contain a eukaryotic consensus regulatory sequence, TATATAA, this result indicates that the TATAAA sequence in MMV Sgt promoter functions as a TATA box. The 3' deletion construct 17 (−456 to −19 in respect to TSS in FIG. **1** (SEQ ID NO:28)), showed about 33% of maximal promoter activity. In this context, construct 17 may produce transcripts with different TSS. Construct 18, 19, 20, 21, 22, 23, 24 and 25, with successively extended 3' leader sequence, gave significantly less activity (2%, 1%, 0.7%, 9%, 0.25%, 0.3%, 0.4%, and 0.3%, respectively) of full promoter activity. These results suggest that, in this context, the longer leader sequence +50 to +378 has a significant inhibitory effect on promoter function.

A 333 bp MMV Sgt promoter/leader fragment, sequence −306 to +27 from TSS (See FIG. **1**)(SEQ ID NO:2), was found to be sufficient for maximal GUS expression. In contrast, in the FLt promoter from FMV and MMV, an extended leader sequence is required for maximum promoter activity [18,22].

The relative strength of various MMV Sgt promoter fragments that were operably linked to the GUS reporter gene was evaluated by hybridization analysis of total RNA. Total RNA extracted from the transformed protoplasts with each of the constructs (No. 1 to 25, as described in FIG. **3**A) was used for RNA dot-blot analysis (FIG. **4**A). The P32-labeled GUS coding sequence was used as a probe. Construct 8 gave the strongest signal, and was followed by construct 5, 6, 7, 9, 10 and 17. A minimum signal was obtained from construct 16, which is devoid of a TATA-box (FIG. **4**A). The relative transcript level obtained with these constructs, in general, is in good agreement with the observed GUS activity. The level of transcript in constructs 18 to 25 containing longer leader sequence (+50 to +378) was relatively less, and promoter function was reduced, probably through the effect of longer untranslated leader sequences on transcription and subsequent translation. Northern analysis of total RNA isolated from tobacco protoplasts transformed with construct 8 showed a single discrete band corresponding to GUS transcripts of the expected size (2100 nt) (FIG. **4**B).

The present invention provides plant expression vectors and intermediate transforming vectors containing the subgenomic transcript (Sgt) promoter from MMV. The MMV Sgt promoter sequence is useful for directing and expressing foreign genes of interest, e.g., pathogen resistance genes, genes encoding metabolic proteins, gene encoding stress resistance factors, etc., in plants to confer useful properties to those transgenic plants, such as pathogen or stress resistance, for example. For example, the MMV Sgt promoter sequence may be operably linked to an insect resistance gene, such as the Bt toxin gene of *Bacillus thuringiensis,* a gene encoding phenol oxidase, a proteinase inhibitor, an alpha-amylase inhibitor, a chitinase, lectin, tobacco peroxidase, VIP1 or VIP2 of *Bacillus cereus*, tryptophan decarboxylase, cholesterol oxidase, or the wasp teratocyte secretory protein (TSP 14); an herbicide resistance gene, such as Mutated Acetolatate Syntase (ALS) from tobacco, bar gene (phosphinothricin acetyl tansferase from *Streptomyces hygroscopius,* 5-enolpyruvylshikimate-3 phosphate synthase, nitrilase gene (bxn) from *K. ozaenae,* or 2,4-dichlorophenoxyacetate monooxygenase from the soil bacterium *Alcaligenes eutrophus* (JMP 134); a fungal resistance gene, such as ribosome inhibiting proteins (RIP), ricin-A chain, wheat tritin, KP4 gene, or chitinase, or combinations thereof. In addition, there may be a transcriptional termination signal downstream of the coding sequence.

Where inhibition of gene expression is desired in a plant, the MMV Sgt promoter or fragments thereof can be operably linked to a polynucleotide coding sequence in antisense orientation so that the transcribed RNA is complementary in sequence to MRNA of the gene whose expression is to be inhibited. In addition, there may be a transcription termination sequence downstream of the polynucleotides directing synthesis of the antisense RNA.

These vectors are useful for both direct DNA uptake by isolated protoplasts and Ti plasmid-mediated gene transfer. Thus, the invention is applicable to plant genetic engineering in general.

For example, the MMV Sgt promoter constructs of the invention may comprise an intermediate plant transformation plasmid which has a region of homology to an *Agrobacterium tumefaciens* gene vector, an *Agrobacterium tumefaciens* T-DNA border region and a chimeric gene located between the T-DNA border and the region of homology. The chimeric gene prferably contains a MMV Sgt promoter of SEQ ID NO:1 or SEQ ID NO:12, or a fragment of SEQ ID NO:1 or SEQ ID NO:12 operably linked to a nucleotide sequence encoding a polypeptide in sense or antisense orientation. Fragments of the MMV Sgt promoter used in these constructs preferably have at least about 30% promoter activity (relative to the full length promoter), more preferably about 32% promoter activity, and most preferably, from about 60% to 100% promoter activity. The nucleotide sequence that is linked to the MMV Sgt promoter preferably encodes a heterologoud gene (heterologous relative to the promoter). Preferably, the construct does not include any sequence encoding MMV polypeptide.

The present invention also provides plant transformation vectors, comprising a disarmed *Agrobacterium tumefaciens* plant tumor-inducing plasmid and a chimeric gene. The chimeric gene contains a MMV Sgt promoter of SEQ ID NO:1 or a fragment thereof having at least about 30% promoter activity relative to a full length promoter, preferably about 32% activity, and most preferably, about 60% to about 100% promoter activity. The promoter is operably linked to a nucleotide sequence which encodes a polypeptide, preferably a heterologous peptide or polypeptides (relative to the MMV promoter). In another preferred embodiment, the chimeric gene contains a MMV Sgt promoter of SEQ ID NO:12, or a fragment thereof operably linked to a nucleotide sequence encoding a polypeptide, wherein the fragment has at least about 30% promoter activity relative to the full length promoter of SEQ ID NO:12).

The MMV Sgt promoter-containing DNA molecules, plasmids and vectors of the invention can be used to generate stable transgenic plants or transform plant protoplasts. Methods for transforming plant protoplasts and generating transgenic plants are well known in the art of plant molecular engineering. For generating transgenic plants or plant protoplasts, the MMV Sgt promoter of the invention, which has a nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:12, or a fragment of SEQ ID NO:1 or SEQ ID NO:12 that has at least about 30% promoter activity, preferably

about 32% promoter activity, more preferably about 60% promoter activity, and most preferably up to 100% promoter activity, is operably linked to an open reading frame encoding a polypeptide of interest. In a preferred embodiment, the MMV Sgt promoter-open reading frame construct also includes a 5'-leader sequence and/or a non-translated polyadenylation site operably linked thereto. Preferably, promoter-containing constructs of the invention include at least one enhancer domain in the promoter region, and most preferably, the promoter-containing constructs of the invention include two or more enhancer domains.

The MMV Sgt constructs of the invention can be used to generate transgenic plants in any type of plant, i.e., monocots or dicots. Preferably, transgenic plants of the invention are crop plants, such as tobacco, hemp, or food crops, such as tomato, corn, soy, wheat, rice, etc. Transgenic plants of the invention may also be flowering plants, such as carnations, roses, and the like. The skilled practitioner, using methods known in the art, can readily transform any plant type using the isolated DNA molecules and vectors of the invention. For example, a transgenic plant of the invention can be produced using *Agrobacterium tumefaciens* mediated DNA transfer, preferably with a disarmed T-DNA vector, electroporation, direct DNA transfer, and particle bombardment. Techniques are well known in the art for introducing nucleic acids into monocots as well as dicots, as are the techniques for culturing such plants and plant tissues and regenerating them.

The MMV Sgt promoter constructs of the present invention can be used to generate transgenic plants, seeds or protoplasts, and may be used to drive expression of a gene or genes of interest in any plant tissue, e.g., roots, stems, leaves, flowers, stems, pollen, or seeds.

The studies presented herein demonstrate that the MMV Sgt promoter is a strong constitutive promoter capable of directing foreign gene expression in heterologous systems, including transgenic plants, at a greater level than that of both CaMV35S and CAMV 19S promoters. There is very limited sequence homology between the MMV Sgt promoter with other caulimovirus promoters, although they are functionally analogous.

The present MMV Sgt promoter and fragments of the promoter are useful for generating transgenic plants and, for example, studying plant metabolism. For metabolic engineering, expression of multiple genes in a single cell may be necessary to gain an understanding of plant metabolic pathways. The use of different promoters having non-homologous sequences may be useful in order to avoid genetic instability due to recombination between identical promoter sequences. Thus, the present MMV Sgt promoter constructs will lend themselves handily to such studies, when used to drive the expression of a plant metabolic gene or genes in conjunction with other chimeric constructs driven by other, nonhomologous promoters.

The present invention is illustrated by the following examples, but is not intended to be limited thereby.

EXAMPLE 1

Protoplasts, Plants and Enzymes

Isolation of protoplasts from tobacco cell suspension cultures (Xanthi 'Brad') and electroporation of protoplasts with supercoiled DNA containing a promoter fragment fused with the GUS gene were done essentially as described by [38]. Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were used for plant transformations. Restriction enzymes, antibiotics, components of plant tissue culture medium, and RNA isolation kits were purchased from commercial sources. Nitrocellulose and Nytran membranes were obtained from Schleicher & Schuell, USA.

Isolation of a MMV Sgt Promoter and Modification of its DNA Sequence

A full-length genomic clone of *mirabilis mosaic* virus (MMV) in plasmid pMMV-B10 [1] has been fully sequenced (Maiti, unpublished results), and was used as template for PCR reactions. A 1012 bp segment (coordinates 4829 to 5840 of the MMV genome) was selected for promoter deletion analysis. The fragment was isolated as a BamHI-HindIII fragment by PCR using the following primers: (i) Forward primer: 5'-GCG GGC GGA TCC GAA AAA CGG AAA CCG TTA-3' (SEQ ID NO:3) and (ii) Reverse primer: 5'-ATG CAG AAG CTT TTG TTG TGT CTT TAC CGG-3' (SEQ ID NO:4). Promoter sequences in the primers are shown in bold and restriction enzymes sites are underlined. The PCR-fragment was gel-purified using QIAGEN procedure (Valencia, Calif. 91355, USA). After digestion with BamHI and HindIII, the PCR fragment was cloned into the corresponding sites of pBS(KS+) (Strategen, La Jolla, Calif. 92037, USA). The plasmid was designated as pBSMS1011.

Modification of EcoRl site to a SmaI site

The pBSMS1011 has an EcoRI site at the MMV genome coordinate 5345. This EcoRI site located 52 bp upstream of a TATA sequence was modified with a SmaI site using two primers having the following sequences: Forward primer has sequence 5'-AATTACCCGGGC-3' (SEQ ID NO:5) and reverse primer has sequence 5'-AATTGCCCGGGT-3' (SEQ ID NO:6). These two primers were mixed in a 1:1 molar ratio and allowed to anneal by slow cooling (2° C./min) from 94° to 24° under a programmed PCR cycle. The plasmid pBSMS1011 was digested with EcoRI and ligated with the 'EcoRI to SmaI' adapter, followed by transformation. The resulting plasmid was designated as pBSMS1011E. This change inserted 12 additional nucleotides (5'-AATTACCCGGGC-3') (SEQ ID NO:5) into the promoter sequence (FIG. **1**).

DNA Sequencing

Automated DNA sequencing was done with an Applied Biosystem ABI Prism 310 Genetic Analyzed (Perkin Elmer) using ABI Prism Dye terminator cycle sequencing ready reaction kit containing Ampli Taq DNA polymerase. Primer extension was carried out by using PCR (denaturation 96° C. for 30 seconds; annealing 50° C. for 30 seconds and extension at 60° C. for four minutes; 27 cycles five nmole template DNA and 50 pmole specific primer in 20 μl of reaction mix. After PCR excess terminators were removed by 95% ethanol wash. The pellet containing the purified extension products was finally suspended in 25 μl of template suspension reagent (P/N401674; Perkin Elmer), vortexed, and heat-denatured at 95° C. for two minutes before loading into a capillary tube.

Glucuronidase (GUS) Assay

Fluorometric GUS assays to measure GUS activity in plant tissue or protoplasts extracts and histochemical GUS staining to localize the distribution of GUS activity in plants were performed according to Jefferson et al., [42], as described earlier [18]. Protein in plant extract was determined according to the method of Bradford [43], using BSA as a standard.

RNA Extraction, RNA Dot Blot and Northern Blot Analysis

Total RNA was prepared from transformed protoplasts containing individual MMV Sgt promoter constructs by extracting with guanidine thiocyanate [44] solution using an Ambion RNA extraction kit (RNAqueous), as described earlier [22]. The RNA dot blot and Northern blot analysis

were performed using a 32P-labelcd GUS-probe essentially, as described previously [22].

Determination of Transcriptional Start Site (TSS) of MMV Sgt Promoter

The transcriptional start site was determined by primer extension analysis. The extension product was separated on a 7.5% polyacrylamide gel containing 7M-urea [45]. Sequencing reactions were carried out according to Sanger et al. [39], using Sequenase Version 2.0, USB, as described earlier [22].

EXAMPLE 2

Construction of Vectors for Transient Expression Experiment in Protoplasts

A series of promoter fragments used in construction of the plant transformation vector with the subgenomic promoter of MMV were designed to study the influence of upstream and downstream sequences with respect to the TATA box on promoter activity. The defined MMV Sgt promoter sequence, of length as indicated (in FIG. **1**) was amplified by PCR using pBSMS1011E as template and appropriately designed primers to tailor an EcoRI site at the 5'-end and HindIII site at the 3'-end of the amplified products. PCR amplification was carried out for 31 cycles under the following standard conditions: denaturation (92° C. for 1 minute), annealing (55° C. for 1 minute), extension (72° C. for 2 minutes) using ELONGASE enzyme mix (recombinant high fidelity TaqDNA polymerase mix with proof reading 3'–5' exonuclease activity from Gibco-BRL, Maryland, USA. Each of PCR amplified fragments 1 to 25 was restricted with EcoRI and HindIII; the restricted fragments were gel-purified and cloned into the corresponding sites of pUC119 vector and sequenced by dideoxy chain terminator method [39] using synthetic primers.

Subcloning of the MMVSgt Promoter Fragments from pUC119 into pUCPMAGUS Vector

The sequence of each of the MMV Sgt promoter fragments cloned in pUC119 was verified before subcloning to pUCPMAGUS, a protoplast expression vector (Dey and Maiti, 1999). MMV Sgt promoter fragments were individually gel purified from the corresponding pUC119 clone after restriction digestion with EcoRI and HindIII and subcloned into the corresponding sites of pUCPMAGUS (Dey and Maiti, 1999). The following deletion plasmids were developed (see FIG. **2**). The 5' and 3' coordinates of the promoter fragment with respect to TSS are given in parenthesis: pPMS1GUS (−646 to +27 (SEQ ID NO:14)), pPMS2GUS (−606 to +27(SEQ ID NO:15)), pPMS3GUS (−556 to +27 (SEQ ID NO:16)), pPMS4GUS (−506 to +27(SEQ ID NO:17)), pPMS5GUS (−456 to −27(SEQ ID NO:9)), pPMS6GUS (−406 to +27(SEQ IN NO:18)), pPMS7GUS (−356 to +27(SEQ ID NO:19)), pPMS8GUS (−306 to +27 (SEQ ID NO:2)), pPMS9GUS (−256 to +27(SEQ ID NO:20)), pPMS10GUS (−206 to +27(SEQ ID NO:21)), pPMS11GUS (−156 to +27(SEQ ID NO:22)), pPMS12GUS (−117 to +27(SEQ ID NO:23)), pPMS13GUS (−94 to +27 (SEQ ID NO:24)), pPMS14GUS (−74 to +27(SEQ ID NO:25)), pPMS15GUS (−44 to +27(SEQ ID NO:26)), pPMS16GUS (−456 to −74(SEQ ID NO:27)), pPMS17GUS (−456 to −19(SEQ ID NO28)), pPMS18GUS (−306 to +50(SEQ ID NO:29)), pPMS19GUS (−306 to +77(SEQ ID NO:30)), pPMS20GUS (−306 to +127(SEQ ID NO:31)), pPMS21GUS (−306 to +177(SEQ ID NO:32)), pPMS22GUS (−306 to 227(SEQ ID NO:33)), pPMS23GUS (−306 to 227(SEQ ID NO:34)), pPMS24GUS (−306 to +327(SEQ ID NO:35)), pPMS25GUS (−306 to +377(SEQ ID NO:36)),

Construction of Vectors with MMV Sgt Promoter Fragments for Expression of Genes in Plants

The MMV Sgt promoter fragments from constructs pPMS5GUS (−456 to +27), pPMS8GUS (−306 to +27) and pPMS17GUS (−456 to −19) were isolated by EcoRI and HindIII digestion followed by gel purification and cloned into the plant expression vector pKYLX71 [40] individually at its unique restriction EcoRI and HindlII sites that flank the promoter. The following plant gene expression vectors were developed: pKMS5, pKMS8 and pKMS17. These plant gene expression vectors have multiple cloning sites (MCS): 5'-HindIII-BamHI-Xhol-Sstl-XbaI-3') with the following unique sites: HindlII, XhoI, SstI and Xbal. The reporter GUS gene from pBSGUS was inserted as an XhoI-SstI fragment into these pKYLX-based expression vectors separately, and the resulting plasmids were designated as pKMSSGUS, pKMS8GUS and pKMS17GUS.

Isolation of CaMV 19S Promoter, and Construction of Vectors with the CaMV 19S and 35S Promoters for Transient and Stable Expression of Genes in Plant Cells

A 990 bp SalI to SstI fragment containing the CaMV 19S promoter was isolated from pCaMV10, a full length genomic clone of the CaMV strain CM1841 [3]. This SalI to SstI restricted fragment, corresponding to the CaMV genomic coordinates 4833 to 5822, was gel-purified and cloned into the corresponding sites of pBS(KS+). The resulting plasmid was designated as pBSCaMV (4833–5822). An internal EcoRI site in the pBSCaMV (4833–5822) corresponding to the CaMV genomic coordinate 5646 was modified to SmaI site using the synthetic adaptor, and the modified plasmid was designed pBSCaMV (4833–5822)E. Using this clone as a template, a 412 bp CaMV 19S promoter fragment (CaMV coordinates 5380 to 5773) was isolated by PCR with the designed primers. The forward primer, 5'CAAGAATTCGTTAAC AAGCTGCAGAAAGGAATTACC-3' (SEQ ID NO:7), contains EcoRI and HpaI sites (underlined) and CaMV sequence (shown in bold). The reverse primer 5'-CTTAAGCTTGCTTGGAGGTCTGATTTT-3' (SEQ ID NO:8), has a HindIII site (underlined) and CaMV sequence (indicated in bold). The PCR-amplified promoter fragment has EcoRI and HpaI sites at the 5'-end and a HindIII site at the 3'-end to facilitate cloning. The fragment has the general structure 5'-EcoRI-HpaI-promoter sequence SmaI-TATA-promoter sequence-HindIII-3'.

The PCR amplified CaMV 19S promoter fragment (412 bp) was cloned into the corresponding EcoRI-HindlII sites of the vector pUCPMAGUS [22] for the transient expression in protoplasts, and also into the corresponding sites of the vector pKYLXGUS for stable transgene expression. The resulting expression vectors were named pPCaSGUS and pKCaSGUS, respectively.

Similarly, the CaMV 35S promoter (−940 to +27 from TSS; corresponding to the CaMV genomic coordinates 6493 to 7459), was cloned as an EcoRI-HindIII fragment into the corresponding sites of the transient expression vector (pUCPMAGUS) for the expression of GUS gene in protoplasts. The resulting plasmid was named pPCa35S-GUS. The GUS reporter gene was inserted as an XhoI-SstI fragment into the corresponding sites of PKYLX71 [40]. In the resulting plant expression vector pKCa35S-GUS, the GUS reporter gene is directed by the CaMV 35S promoter (coordinates −940 to +27 from TSS).

EXAMPLE 3

Transient Expression Analysis of MMV Sgt Promoter Deletion Constructs in a Protoplast System

Isolation of protoplasts from the tobacco cell suspension cultures (Xanthi 'Brad') and electroporation of protoplasts

with supercoiled DNA containing the MMV Sgt promoter fragment and GUS gene were done essentially as described earlier (Maiti et al., 1998). Electroporation was carried out by using the GenePulser II Apparatus (BioRad) with the Capacitance Extender II (Model 165-2107). An aliquot of 800 1 containing 2X10 ^{6} protoplasts in an electroporation cuvette (0.4 cm electrode gap) was electroporated (200V used for charging 960 F capacitance for 40 milliseconds) with 5 g of supercoiled plasmid DNA containing GUS reporter gene. After 20 hours, two billion electroporated protoplasts were individually harvested for GUS assay. A deletion analysis scheme of MMV Sgt promoter is shown in FIG. **3**A. A series of 5'-and 3'-end deleted promoter fragments (total 25 fragments) were included in order to map the optimum boundaries required for maximal expression from promoter/leader region and also to analyze the influence of the upstream and downstream cis-sequence with respect to the TATA box. The designed deletion promoter fragments 1 to 25 (as shown in FIG. **3**A) were amplified by PCR and cloned into protoplasts expressing vector pUCPMAGUS as in Example 2. Results of the expression analysis of the MMV Sgt promoter are shown in FIG. **3**B. In a transient expression assay, the construct pPMS8GUS, which contains the promoter fragment (coordinates −306 to +27 from TSS) gives maximum activity. The construct pPMS5GUS, pPMS7GUS, pPMS10GUS and pPMS17GUS showed 76%, 70%, 65% and 45% of GUS activity compared to the constructs pPMS8GUS (with highest GUS activity). Construct pPMS16GUS, which is devoid of the TATA box, showed very little of GUS activity indicating the importance of TATA element in MMV Sgt promoter activity. A 320 bp MMV Sgt promoter/leader fragment, sequence −306 to +27 from TSS, was found to be sufficient for maximal GUS expression. Constructs pPMS5GUS, pPMS8GUS and pPMS17GUS were selected for their stable expression analysis in transgenic tobacco plants (see Example 4).

EXAMPLE 4

Stable Expression Analysis of MMV Sgt Promoter in Transgenic Plants

The MMv Sgt promoter fragments from constructs pPMS5GUS (−456 to +27), pPMS8GUS (−306 to +27) and pPMS17GUS (−456 to −19) were isolated by EcoRI and HindIII digestion followed by gel purification and cloned into the plant expression vector pKYLX71 (Schardl et al., 1987) separately at its unique EcoRI and HindIII restriction sites that flank the promoter. The following plant expression vectors were developed: pKMS5, pKMS8 and pKMSl7. These plant expression vectors have multiple cloning sites (MCS): 5'-HindIII-BamHI-XhoI-SstI-XbaI-3') with the following unique sites: HindIII, XhoI, SstI and XbaI. The reporter GUS gene from pBSGUS as XhoI-SstI fragment was inserted into these pKYLX-based plant expression vectors separately and the resulting plasmids were designated as pKMS5GUS, pKMS8GUS and pKMS17GUS.

On average, twelve to fourteen independent primary transgenic tobacco (*Nicotiana tabacum* cv. Samsun NN) lines (R_o progeny) were developed for each of these constructs and grown under greenhouse condition. Leaf extract from these R_O plants was used for fluorometric GUS assays. Analysis of these lines from R_O progeny showed that the GUS expression level in transgenic plant lines obtained from pKMSSGUS construct is maximum followed by plant lines obtained from construct pKMS8GUS (82% of pKMS5GUS activity) and pKMS17GUS (48% of pKMS5GUS activity), (data not presented for Ro plants)). Seeds were collected from self-fertilized independent Ro lines. Segregation analysis for the marker gene (Kan^R) were performed. About 8 to 9 individual R_1 transgenic lines showing expected segregation ratio (Kan^R:Kan^S=3:1) for the marker Kan^R for each construct were further analyzed. Whole seedling extract was used for fluorometric GUS assays. The GUS activity in R_1 transgenic plants (FIG. **3**B). is however 5 to 8 times higher than the GUS activity obtained in R_O plants. Transgenic plants (R1 progeny) developed for construct pKMS5GUS showed highest activity followed by pKMS8GUS (−306 to +27, 58% of pKMS5GUS) and pKMS17GUS (−456 to −19, 32% of pKMS5GUS). Histocherical GUS staining was carried out with whole seedlings separately from these three constructs showed comparable intensity of GUS activity (FIG. **4**).

EXAMPLE 5

Comparative Expression Analysis of MMV Sgt Promoter with MMV FLt, CaMV 35S and CaMV19S Promoters.

In pPCaSGUS or pKCaSGUS, the GUS reporter gene is directed by the CaMV sub-genornic transcript promoter sequence (corresponding to CaMV genomic coordinates 5380 to 5773). The MMV Sgt promoter constructs pKMS5GUS, pKMS8GUS and pKMS17GUS were compared with the CaMV promoters (19S and 35S) and three MMV FLt promoter constructs pKM1GUS, pKM12GUS and pKM13GUS (Dey and Maiti 1999a) both in protoplasts assay (FIG. **5**A), and transgenic plant expression analysis (FIG. **5**B). In protoplast assays, the MMV Sgt promoter fragments in constructs, pPMS5GUS and pPMS8GUS, showed more activity (5 and 7 fold, respectively) as compared to pPCaSGUS, and about two fold greater activity than CaMV 35S promoter (FIG. **5**A). MMV Sgt promoter in pPMS5GUS and pPMS8GUS showed comparable activity with MMV FLt promoter in pPM12GUS and pPM13GUS (FIG. **5**A), suggesting that the strength of the MMV Sgt promoter is comparable to or greater that of the MMV FLt promoter. The CaMV 19S is a weakpromoter, as compared to the 35S promoter [14]. This suggests that the MMV Sgt promoter may have a different functional mechanism, as compared to the CaMV 19S promoter.

For stable transformation assays, a number of independent transgenic tobacco (*Nicotiana tabacum* cv. Samsun NN) lines were generated. Flurometric GUS assays were carried out with whole seedling (R1 progeny) extracts. The results of transgenic plant analysis shown in FIG. **5**B. In protoplast assays the GUS expression with construct pPMS8GUS was highest. Three MMV Sgt promoter fragments analyzed in transgenic plants showed strong GUS expression compared to both CaMV 19S and 35S promoter. The level of expression of GUS reporter gene in pKMS5GUS (highest expressing construct) is about 8 fold more than CaMV 19S promoter and 2 fold stronger than CaMV 35S promoter (FIG. **5**B).

The relative intensity of histochemical GUS staining of the young seedlings developed for the constructs, pKMS5GUS and pKMS8GUS, pKMS17GUS showed strong promoter activity compared to pKCaSGUS (with CaMV35S promoter) and pKCa35SGUS (with CaMV 35S promoter) (FIG. **4**).

EXAMPLE 6

Constitutive Expression of MMVSgt Promoter in Different Parts of Transgenic Seedlings

The MMV Sgt promoter activity was measured in various tissues during seedling (R1 progeny, second generation) development. Transgenic seedlings were aseptically grown on MS-agar medium in presence of kanamycin (240μg/ml) supplemented with 3% sucrose. Seedlings from the independent lines showing segregation ratio (Kan^s:Kan^r=1:3) for

the Kan^r gene were selected for further analysis. Eight independent lines for each construct were examined. The relative expression of GUS reporter gene in 28 day (4 weeks) old seedlings (R_1 progeny) transformed with pKMS5GUS, pKMS8GUS and pKMS 17GUS were monitored by fluorometric GUS assay and by histochemical staining. A relative level of GUS activity in roots, leaves and stems is shown in FIG. **5**. On average GUS activity was maximum in roots followed by in leaves and stems in seedling developed for pKMS5GUS. Seedlings developed with pKM8GUS showed more activity in leaves followed by in roots and stems. Seedlings developed for construct pKMS15GUS showed more uniform GUS activity in different parts of seedlings although slightly more activity in leaves followed by stems and roots (FIG. **5**).

EXAMPLE 7

Histochemical Localization of GUS Activity in Transgenic Plants

MMV Sgt promoter activity was measured in various tissues during seedling (R1 progeny, second generation) development. The level of intensity of GUS activity was measured by histochemical staining of hand-cut fresh tissue sections of various organs of transgenic plants developed for the construct pKMS5GUS shown in FIG. **8**. Strong GUS activity was detected in vascular tissues in midrib and lateral secondary veins of matured leaves (FIG. **8**B), in young leaves and in the apical meristem region of young seedlings (FIG. **8**D). Cross section of stems (FIG. **8**I) and petioles (FIG. **8**G) showed intense staining of the phloem cells. Strong GUS accumulation was detected in vascular tissues of roots and root tips (FIG. **8**C). The non-transformed tobacco showed no GUS staining in mature leaves (FIG. **8**A), in root tissues (data not shown) or in cross sections of stems FIG. **8**H), and petioles (FIG. **8**F). Histochemical GUS staining of different floral tissues was performed. The petal (corolla) portion of the flower showed light GUS staining (FIG. **8**O). Another, plant section containing pollen grains exhibited intense GUS activity (FIG. **8**N). The stigma and style portion of the flower showed much less GUS staining (FIG. **8**M). The longitudinal cross-section of the flower pedicel and ovary (6 days after opening of the flower) showed intense staining of the pedicel and the basal vascular part of the ovary (FIG. **8**L). Differential GUS staining in various floral organs may be due to tissue specific expression of MMV Sgt promoter. A similar tissue specific expression pattern was documented for the FLt promoter from CaMV [30], FMV [18], PCISV [21] and MMV [22,23].

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gttttacagt caggacagat aatgtaaatc ttttaaaagg atttatgaat aaaaagatta 360

ctggtgacag taaacaggga aggctaataa gatggcaaat gtggttttca cattacacct 420

ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc tgattatctc accagagaat 480

tacccgggca attccacaat ggaaacgtca tccatgacga ctaaacctgc cattttcgg 540

ctataaaaac tgggtttttc caaatgaaaa ttccacacaa aacacatcct tttttcaaag 600

gggggaatt aaatcaaaaa caggaaaaac aaa 633

<210> SEQ ID NO 16
<211> LENGTH: 583
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 16

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gaaactgatg cttctgatca cttttggggt ggagtcctta aggcccaaac cactgaaggt 120

gaagaattaa tctgcaggta ttcttcagga acattcaaac cagcagaatt gaattaccat 180

agtaatgaga aagaattact agcggtaaaa caggtgatta ctaaatttag tatttatcta 240

acccctgttt gttttacagt caggacagat aatgtaaatc ttttaaaagg atttatgaat 300

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aaaaagatta ctggtgacag taaacaggga aggctaataa gatggcaaat gtggttttca 360

cattacacct ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc tgattatctc 420

accagagaat tacccgggca attccacaat ggaaacgtca tccatgacga ctaaacctgc 480

cattttcgg ctataaaaac tgggtttttc caaatgaaaa ttccacacaa aacacatcct 540

tttttcaaag ggggggaatt aaatcaaaaa caggaaaaac aaa 583

<210> SEQ ID NO 17
<211> LENGTH: 533
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 17

tttaattatt gaaactgatg cttctgatca cttttggggt ggagtcctta aggcccaaac 60

cactgaaggt gaagaattaa tctgcaggta ttcttcagga acattcaaac cagcagaatt 120

gaattaccat agtaatgaga aagaattact agcggtaaaa caggtgatta ctaaatttag 180

tatttatcta acccctgttt gttttacagt caggacagat aatgtaaatc ttttaaaagg 240

atttatgaat aaaaagatta ctggtgacag taaacaggga aggctaataa gatggcaaat 300

gtggttttca cattacacct ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc 360

tgattatctc accagagaat tacccgggca attccacaat ggaaacgtca tccatgacga 420

ctaaacctgc cattttcgg ctataaaaac tgggtttttc caaatgaaaa ttccacacaa 480

aacacatcct tttttcaaag ggggggaatt aaatcaaaaa caggaaaaac aaa 533

<210> SEQ ID NO 18
<211> LENGTH: 433
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 18

acattcaaac cagcagaatt gaattaccat agtaatgaga aagaattact agcggtaaaa 60

caggtgatta ctaaatttag tatttatcta acccctgttt gttttacagt caggacagat 120

aatgtaaatc ttttaaaagg atttatgaat aaaaagatta ctggtgacag taaacaggga 180

aggctaataa gatggcaaat gtggttttca cattacacct ttaaggtgga ccacctaaaa 240

ggagaacaaa atgtgctggc tgattatctc accagagaat tacccgggca attccacaat 300

ggaaacgtca tccatgacga ctaaacctgc cattttcgg ctataaaaac tgggtttttc 360

caaatgaaaa ttccacacaa aacacatcct tttttcaaag ggggggaatt aaatcaaaaa 420

caggaaaaac aaa 433

<210> SEQ ID NO 19
<211> LENGTH: 383
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 19

agcggtaaaa caggtgatta ctaaatttag tatttatcta acccctgttt gttttacagt 60

caggacagat aatgtaaatc ttttaaaagg atttatgaat aaaaagatta ctggtgacag 120

taaacaggga aggctaataa gatggcaaat gtggttttca cattacacct ttaaggtgga 180

ccacctaaaa ggagaacaaa atgtgctggc tgattatctc accagagaat tacccgggca 240

attccacaat ggaaacgtca tccatgacga ctaaacctgc cattttcgg ctataaaaac 300

tgggtttttc caaatgaaaa ttccacacaa aacacatcct tttttcaaag ggggggaatt 360

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aaatcaaaaa caggaaaaac aaa 383

<210> SEQ ID NO 20
<211> LENGTH: 283
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 20

aaaaagatta ctggtgacag taaacaggga aggctaataa gatggcaaat gtggttttca 60

cattacacct ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc tgattatctc 120

accagagaat tacccgggca attccacaat ggaaacgtca tccatgacga ctaaacctgc 180

cattttcgg ctataaaaac tgggtttttc caaatgaaaa ttccacacaa aacacatcct 240

tttttcaaag ggggggaatt aaatcaaaaa caggaaaaac aaa 283

<210> SEQ ID NO 21
<211> LENGTH: 233
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 21

gtggttttca cattacacct ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc 60

tgattatctc accagagaat tacccgggca attccacaat ggaaacgtca tccatgacga 120

ctaaacctgc catttttcgg ctataaaaac tgggtttttc caaatgaaaa ttccacacaa 180

aacacatcct tttttcaaag ggggggaatt aaatcaaaaa caggaaaaac aaa 233

<210> SEQ ID NO 22
<211> LENGTH: 183
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 22

atgtgctggc tgattatctc accagagaat tacccgggca attccacaat ggaaacgtca 60

tccatgacga ctaaacctgc catttttcgg ctataaaaac tgggtttttc caaatgaaaa 120

ttccacacaa aacacatcct tttttcaaag ggggggaatt aaatcaaaaa caggaaaaac 180

aaa 183

<210> SEQ ID NO 23
<211> LENGTH: 144
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 23

aattccacaa tggaaacgtc atccatgacg actaaacctg ccatttttcg gctataaaaa 60

ctgggttttt ccaaatgaaa attccacaca aaacacatcc ttttttcaaa ggggggggaat 120

taaatcaaaa acaggaaaaa caaa 144

<210> SEQ ID NO 24
<211> LENGTH: 121
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 24

catgacgact aaacctgcca tttttcggct ataaaaactg ggtttttcca aatgaaaatt 60

ccacacaaaa cacatccttt tttcaaaggg ggggaattaa atcaaaaaca ggaaaaacaa 120

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a 121

<210> SEQ ID NO 25
<211> LENGTH: 101
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 25

tttttcggct ataaaaactg ggtttttcca aatgaaaatt ccacacaaaa cacatccttt 60

tttcaaaggg ggggaattaa atcaaaaaca ggaaaaacaa a 101

<210> SEQ ID NO 26
<211> LENGTH: 71
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 26

aatgaaaatt ccacacaaaa cacatccttt tttcaaaggg ggggaattaa atcaaaaaca 60

ggaaaaacaa a 71

<210> SEQ ID NO 27
<211> LENGTH: 383
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 27

aggcccaaac cactgaaggt gaagaattaa tctgcaggta ttcttcagga acattcaaac 60

cagcagaatt gaattaccat agtaatgaga aagaattact agcggtaaaa caggtgatta 120

ctaaatttag tatttatcta acccctgttt gttttacagt caggacagat aatgtaaatc 180

ttttaaaagg atttatgaat aaaaagatta ctggtgacag taaacaggga aggctaataa 240

gatggcaaat gtggttttca cattacacct ttaaggtgga ccacctaaaa ggagaacaaa 300

atgtgctggc tgattatctc accagagaat tacccgggca attccacaat ggaaacgtca 360

tccatgacga ctaaacctgc cat 383

<210> SEQ ID NO 28
<211> LENGTH: 438
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 28

aggcccaaac cactgaaggt gaagaattaa tctgcaggta ttcttcagga acattcaaac 60

cagcagaatt gaattaccat agtaatgaga aagaattact agcggtaaaa caggtgatta 120

ctaaatttag tatttatcta acccctgttt gttttacagt caggacagat aatgtaaatc 180

ttttaaaagg atttatgaat aaaaagatta ctggtgacag taaacaggga aggctaataa 240

gatggcaaat gtggttttca cattacacct ttaaggtgga ccacctaaaa ggagaacaaa 300

atgtgctggc tgattatctc accagagaat tacccgggca attccacaat ggaaacgtca 360

tccatgacga ctaaacctgc catttttcgg ctataaaaac tgggtttttc caaatgaaaa 420

ttccacacaa aacacatc 438

<210> SEQ ID NO 29
<211> LENGTH: 356
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 29

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gttttacagt caggacagat aatgtaaatc ttttaaaagg atttatgaat aaaaagatta 60

ctggtgacag taaacaggga aggctaataa gatggcaaat gtggttttca cattacacct 120

ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc tgattatctc accagagaat 180

tacccgggca attccacaat ggaaacgtca tccatgacga ctaaacctgc catttttcgg 240

ctataaaaac tgggtttttc caaatgaaaa ttccacacaa aacacatcct tttttcaaag 300

gggggaatt aaatcaaaaa caggaaaaac aaaaaccagt aatggaaaaa gagctt 356

<210> SEQ ID NO 30
<211> LENGTH: 383
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 30

gttttacagt caggacagat aatgtaaatc ttttaaaagg atttatgaat aaaaagatta 60

ctggtgacag taaacaggga aggctaataa gatggcaaat gtggttttca cattacacct 120

ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc tgattatctc accagagaat 180

tacccgggca attccacaat ggaaacgtca tccatgacga ctaaacctgc catttttcgg 240

ctataaaaac tgggtttttc caaatgaaaa ttccacacaa aacacatcct tttttcaaag 300

gggggaatt aaatcaaaaa caggaaaaac aaaaaccagt aatggaaaaa gagcttcagg 360

ctctaaggat caaagaaaag atc 383

<210> SEQ ID NO 31
<211> LENGTH: 433
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 31

gttttacagt caggacagat aatgtaaatc ttttaaaagg atttatgaat aaaaagatta 60

ctggtgacag taaacaggga aggctaataa gatggcaaat gtggttttca cattacacct 120

ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc tgattatctc accagagaat 180

tacccgggca attccacaat ggaaacgtca tccatgacga ctaaacctgc catttttcgg 240

ctataaaaac tgggtttttc caaatgaaaa ttccacacaa aacacatcct tttttcaaag 300

gggggaatt aaatcaaaaa caggaaaaac aaaaaccagt aatggaaaaa gagcttcagg 360

ctctaaggat caaagaaaag atcctcttgg tagaactcga ttctatcaga aaacaaatca 420

gcatttacgc tga 433

<210> SEQ ID NO 32
<211> LENGTH: 483
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 32

gttttacagt caggacagat aatgtaaatc ttttaaaagg atttatgaat aaaaagatta 60

ctggtgacag taaacaggga aggctaataa gatggcaaat gtggttttca cattacacct 120

ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc tgattatctc accagagaat 180

tacccgggca attccacaat ggaaacgtca tccatgacga ctaaacctgc catttttcgg 240

ctataaaaac tgggtttttc caaatgaaaa ttccacacaa aacacatcct tttttcaaag 300

gggggaatt aaatcaaaaa caggaaaaac aaaaaccagt aatggaaaaa gagcttcagg 360

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ctctaaggat caaagaaaag atcctcttgg tagaactcga ttctatcaga aaacaaatca 420

gcatttacgc tgaactaact ggaagtttag accaggaagg ctctgcctca cactctaaac 480

cta 483

<210> SEQ ID NO 33
<211> LENGTH: 533
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 33

gttttacagt caggacagat aatgtaaatc ttttaaaagg atttatgaat aaaaagatta 60

ctggtgacag taaacaggga aggctaataa gatggcaaat gtggttttca cattacacct 120

ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc tgattatctc accagagaat 180

tacccgggca attccacaat ggaaacgtca tccatgacga ctaaacctgc catttttcgg 240

ctataaaaac tgggtttttc caaatgaaaa ttccacacaa aacacatcct tttttcaaag 300

gggggggaatt aaatcaaaaa caggaaaaac aaaaaccagt aatggaaaaa gagcttcagg 360

ctctaaggat caaagaaaag atcctcttgg tagaactcga ttctatcaga aaacaaatca 420

gcatttacgc tgaactaact ggaagtttag accaggaagg ctctgcctca cactctaaac 480

ctagtccaca gcaaacggct gatggtaaag acggctcaaa tccgttaaac cct 533

<210> SEQ ID NO 34
<211> LENGTH: 583
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 34

gttttacagt caggacagat aatgtaaatc ttttaaaagg atttatgaat aaaaagatta 60

ctggtgacag taaacaggga aggctaataa gatggcaaat gtggttttca cattacacct 120

ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc tgattatctc accagagaat 180

tacccgggca attccacaat ggaaacgtca tccatgacga ctaaacctgc catttttcgg 240

ctataaaaac tgggtttttc caaatgaaaa ttccacacaa aacacatcct tttttcaaag 300

gggggggaatt aaatcaaaaa caggaaaaac aaaaaccagt aatggaaaaa gagcttcagg 360

ctctaaggat caaagaaaag atcctcttgg tagaactcga ttctatcaga aaacaaatca 420

gcatttacgc tgaactaact ggaagtttag accaggaagg ctctgcctca cactctaaac 480

ctagtccaca gcaaacggct gatggtaaag acggctcaaa tccgttaaac cctgatgctt 540

tgggaaaaag cataacggag aacttggttc caagtcctga gaa 583

<210> SEQ ID NO 35
<211> LENGTH: 633
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 35

gttttacagt caggacagat aatgtaaatc ttttaaaagg atttatgaat aaaaagatta 60

ctggtgacag taaacaggga aggctaataa gatggcaaat gtggttttca cattacacct 120

ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc tgattatctc accagagaat 180

tacccgggca attccacaat ggaaacgtca tccatgacga ctaaacctgc catttttcgg 240

ctataaaaac tgggtttttc caaatgaaaa ttccacacaa aacacatcct tttttcaaag 300

gggggggaatt aaatcaaaaa caggaaaaac aaaaaccagt aatggaaaaa gagcttcagg 360

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ctctaaggat caaagaaaag atcctcttgg tagaactcga ttctatcaga aaacaaatca    420

gcatttacgc tgaactaact ggaagtttag accaggaagg ctctgcctca cactctaaac    480

ctagtccaca gcaaacggct gatggtaaag acggctcaaa tccgttaaac cctgatgctt    540

tgggaaaaag cataacggag aacttggttc caagtcctga gaaggatgaa tccaagaaag    600

ttgtcagttt acgaaaaact gaaagtgggt tgt                                633


<210> SEQ ID NO 36
<211> LENGTH: 683
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 36

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ctggtgacag taaacaggga aggctaataa gatggcaaat gtggttttca cattacacct    120

ttaaggtgga ccacctaaaa ggagaacaaa atgtgctggc tgattatctc accagagaat    180

tacccgggca attccacaat ggaaacgtca tccatgacga ctaaacctgc catttttcgg    240

ctataaaaac tgggtttttc caaatgaaaa ttccacacaa aacacatcct tttttcaaag    300

ggggggaatt aaatcaaaaa caggaaaaac aaaaaccagt aatggaaaaa gagcttcagg    360

ctctaaggat caaagaaaag atcctcttgg tagaactcga ttctatcaga aaacaaatca    420

gcatttacgc tgaactaact ggaagtttag accaggaagg ctctgcctca cactctaaac    480

ctagtccaca gcaaacggct gatggtaaag acggctcaaa tccgttaaac cctgatgctt    540

tgggaaaaag cataacggag aacttggttc caagtcctga gaaggatgaa tccaagaaag    600

ttgtcagttt acgaaaaact gaaagtgggt tgtatatccc cacgactagt ccggttgcaa    660

acggctccgg taaagacaca aca                                           683


<210> SEQ ID NO 37
<211> LENGTH: 471
<212> TYPE: DNA
<213> ORGANISM: Mirabilis mosaic virus (MMV)

<400> SEQUENCE: 37

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cagcagaatt gaattaccat agtaatgaga aagaattact agcggtaaaa caggtgatta    120

ctaaatttag tatttatcta acccctgttt gttttacagt caggacagat aatgtaaatc    180

ttttaaaagg atttatgaat aaaaagatta ctggtgacag taaacaggga aggctaataa    240

gatggcaaat gtggttttca cattacacct ttaaggtgga ccacctaaaa ggagaacaaa    300

atgtgctggc tgattatctc accagagaat tccacaatgg aaacgtcatc catgacgact    360

aaacctgcca tttttcggct ataaaaactg ggtttttcca aatgaaaatt ccacacaaaa    420

cacatccttt tttcaaaggg ggggaattaa atcaaaaaca ggaaaaacaa a             471
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We claim:

1. An isolated DNA molecule comprising a *mirabilis mosaic* virus (MMV) subgenomic transcript (Sgt) promoter comprising the nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 12 or a fragment of SEQ ID NO: 1 or SEQ ID NO:12 having at least 32% MMV Sgt promoter activity.

2. An isolated DNA molecule comprising a fragment of the MMV Sgt promoter, said fragment comprising SEQ ID NO:2.

3. An isolated DNA molecule comprising a fragment of the MMV Sgt promoter, said fragment comprising SEQ ID NO:9.

4. An isolated DNA molecule comprising a fragment of the MMV Sgt promoter, said fragment comprising SEQ ID NO:13.

5. An isolated DNA molecule comprising a fragment of the MMV Sgt promoter, said fragment comprising SEQ ID NO:37.

6. An isolated DNA molecule of claim **1**, **2**, **3**, **4** or **5** further comprising a nucleotide sequence encoding a polypeptide operably linked thereto.

7. An isolated DNA molecule of claim **6** further comprising a 3' non-translated polyadenylation signal sequence operably linked to said nucleotide sequence.

8. An isolated DNA molecule of claim **6** wherein the nucleotide sequence encoding a polypeptide is in antisense orientation relative to the promoter or promoter fragment.

9. An isolated DNA molecule of claim **1** comprising a MMV 5' non-translated leader sequence operably linked thereto.

10. An isolated DNA molecule of claim **6**, wherein said DNA molecule is free of nucleotide sequences encoding MMV polypeptide.

11. An isolated DNA molecule of claim **6** wherein said nucleotide sequence encodes a plant protein.

12. An isolated DNA molecule of claim **6** wherein said nucleotide sequence encodes a heterologous protein relative to the MMV Sgt promoter.

13. An intermediate plant transformation plasmid comprising a region of homology to an *Agrobacterium tumefaciens* vector capable of transferring a gene into a cell, an *Agrobacterium tumefaciens* T-DNA border region and a chimeric gene located between the T-DNA border and the region of homology, said chimeric gene comprising a MMV Sgt promoter of claim **1** operably linked to a nucleotide sequence encoding a polypeptide.

14. An intermediate plant transformation plasmid of claim **13** wherein said polypeptide is heterologous with respect to the MMV Sgt promoter.

15. An intermediate plant transformation plasmid of claim **13**, further comprising a MMV leader sequence operably linked to said nucleotide sequence.

16. An intermediate plant transformation plasmid of **13**, wherein said MMV Sgt promoter comprises at least one enhancer domain.

17. A plant transformation vector comprising a disarmed *Agrobacterium tumefaciens* plant tumor-inducing plasmid and a chimeric gene, said chimeric gene comprising a MMV Sgt promoter of claim **1** operably linked to a nucleotide sequence which encodes a polypeptide.

18. A plant transformation vector of claim **17** wherein said MMV Sgt promoter comprises at least one enhancer domain.

19. A plant transformation vector of claim **17** wherein said nucleotide sequence encodes a polypeptide that is heterologous relative to the MMV Sgt promoter.

20. A plant transformation vector of claim **18** wherein the MMV Sgt promoter comprises a single enhancer domain.

21. A plant transformation vector of claim **18** wherein the MMV Sgt promoter comprises two enhancer domains.

22. A transgenic plant or transgenic plant part comprising a plant transformation vector comprising a disarmed *Agrobacterium turmefaciens* plant tumor-inducing plasmid and a chimeric gene, said chimeric gene comprising a MMV Sgt promoter comprising the nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 12 or a fragment of SEQ ID NO: 1 or SEQ ID NO: 12 having at least 32% MMV Sgt promoter activity, wherein said MMV Sgt promoter or fragment thereof is operably linked to a nucleotide sequence which encodes a polypeptide; and wherein said chimeric gene is expressed in the plant.

23. A transgenic plant or transgenic plant part of claim **22** wherein said plant transformation vector further comprises a 3' non-translated polyadenylation signal sequence operably linked to said nucleotide sequence.

24. A transgenic plant or transgenic plant part of claim **22** wherein said nucleotide sequence encodes a plant metabolic protein or a polypeptide that confers pathogen resistance to said transgenic plant.

25. A transgenic plant or transgenic plant part of claim **22** wherein said MMV Sgt promoter comprises at least one enhancer domain.

26. A transgenic plant or transgenic plant part of claim **22** wherein said plant is a tobacco plant.

27. A transgenic plant or transgenic plant part of claim **22** wherein said polypeptide confers pathogen resistance to the transgenic plant.

28. A transgenic plant part of claim **22**, wherein said plant part is selected from the group consisting of a root, leaf, flower, stem, seed, petal, pollen, callus and cell.

29. A transformed plant protoplast comprising a plant transformation vector comprising a disarmed *Agrobacterium tumefaciens* plant tumor-inducing plasmid and a chimeric gene, said chimeric gene comprising a MMV Sgt promoter comprising the nucleotide sequence of SEQ ID NO: 1 or a fragment thereof having at least 32% MMV Sgt promoter activity, wherein said MMV Sgt promoter or fragment thereof is operably linked to a nucleotide sequence which encodes a polypeptide.

30. A method of making a transgenic plant comprising (1) transforming a plant cell with a chimeric gene comprising a MMV Sgt promoter comprising the nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 12 or a fragment of SEQ ID NO: 1 or SEQ ID NO: 12 having at least 32% MMV Sgt promoter activity, wherein said MMV Sgt promoter or fragment thereof is operably linked to a nucleotide sequence which encodes a polypeptide; (2) culturing the plant cell under growing conditions to produce a regenerated plant; and (3) and expressing the chimeric gene in the regenerated plant.

31. A method of making a transgenic plant of claim **30** wherein said chimeric gene encodes a polypeptide conferring pathogen resistance to the transgenic plant.

32. A plant promoter enhancer element comprising the nucleotide sequence of SEQ ID NO: 10.

* * * * *