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ORIGINAL ARTICLE

Efficient chimeric plant promoters derived from plant infecting viral promoter sequences

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Abstract In the present study, we developed a set of three chimeric/hybrid promoters namely FSgt-PFlt, PFlt-UAS-2X and MSgt-PFlt incorporating different important domains of *Figwort Mosaic Virus* sub-genomic transcript promoter (FSgt, −270 to −60), *Mirabilis Mosaic Virus* sub-genomic transcript promoter (MSgt, −306 to −125) and *Peanut Chlorotic Streak Caulimovirus* full-length transcript promoter (PFlt-, −353 to +24 and PFlt-UAS, −353 to −49). We demonstrated that these chimeric/hybrid promoters can drive the expression of reporter genes in different plant species including tobacco, *Arabidopsis*, petunia, tomato and spinach. FSgt-PFlt, PFlt-UAS-2X and MSgt-PFlt promoters showed 4.2, 1.5 and 1.2 times stronger GUS activities compared to the activity of the CaMV35S promoter, respectively, in tobacco protoplasts. Protoplast-derived recombinant promoter driven GFP showed enhanced accumulation compared to that obtained under the CaMV35S promoter. FSgt-PFlt, PFlt-UAS-2X and MSgt-PFlt promoters showed 3.0, 1.3 and 1.0 times stronger activities than the activity of the CaMV35S^2 (a modified version of the CaMV35S promoter with double enhancer domain) promoter, respectively, in tobacco (*Nicotiana tabacum*, var. Samsun NN). Alongside, we observed a fair correlation between recombinant promoter-driven GUS accumulation with the corresponding *uidA-mRNA* level in transgenic tobacco. Histochemical (X-gluc) staining of whole transgenic seedlings and fluorescence images of ImaGene Green™ treated floral parts expressing the *GUS* under the control of recombinant promoters also support above findings. Furthermore, we confirmed that these chimeric promoters are inducible in the presence of 150 μM salicylic acid (SA) and abscisic acid (ABA). Taken altogether, we propose that SA/ABA inducible chimeric/recombinant promoters could be used for strong expression of gene(s) of interest in crop plants.

Keywords Chimeric promoter · PClSV · MMV · FMV · GFP · GUS

Abbreviations

GUS	β-Glucuronidase
GFP	Green fluorescent protein
X-gluc	5-bromo-4-chloro-3-indolyl β-D-glucopyranosiduronic acid
CLSM	Confocal laser scanning microscope
Kan^r	Kanamycin^{resistant}
Kan^s	Kanamycin^{susceptible}
FMV	Figwort mosaic virus
MMV	Mirabilis mosaic virus
PClSV	Peanut chlorotic streak virus
SA	Salicylic acid
ABA	Abscisic acid
ROI	Region of interest

Introduction

In general, genome of plant infecting pararetroviruses particularly the *Caulimoviruses* (Viz. *Cauliflower Mosaic*

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Virus, Mirabilis Mosaic Virus, *Figwort Mosaic Virus, Peanut Chlorotic Streak Virus*) typically contains two transcriptional promoters, one for the full-length transcript (Flt-) and other for the sub-genomic transcript (Sgt-); these promoters are equivalent with 35S and 19S transcripts of *Cauliflower Mosaic Virus* (CaMV), respectively. Both 35S and 19S promoters comprise a distal (upstream activation sequence; UAS) and a TATA element containing proximal region (core promoter). Several small DNA regulatory sequence motifs like enhancers, silencers, insulators and *cis*-motifs (*cis*-elements) are distributed across both distal and proximal parts of the promoter. The combinatorial interactions between specific trans-protein factors with respective *cis*-elements in association with other transcriptional factors (TBPs; TATA box binding protein) usually determine the transcriptional destiny of transgene in plant cell. The cross-talks among different *cis*-elements distributed throughout the promoter-backbone and different nuclear factors also play major role in imparting the tissue specificity and strength of the eukaryotic promoter (Dvir et al. 2001; Hartwell et al. 2000; Potenza et al. 2004; Roeder 1996; Singh et al. 2002; Zawel and Reinberg 1995). Moreover, the finer structure of *Caulimovirus* promoter is modular; for example, the CaMV35S core promoter consists of domain A and domain B (Benfey et al. 1989; Fang et al. 1989; Odell et al. 1985; Ow et al. 1987). Domain A was further subdivided into two subdomains: domain A1 and the minimal promoter (mp). On the other hand, domain B was subdivided into five sub-domains: B1–B5 (Benfey et al. 1989). Functional characterization of both A and B domain of the CaMV35S promoter was carried out in detail earlier (Benfey et al. 1989; Bhullar et al. 2003, 2007). The modularity in structure of plant-promoter (*Caulimovirus* promoter) open up the opportunity to design unique recombinant transcriptional machinery (chimeric/hybrid promoter) with altered clustering of *cis* elements by moving/exchanging/swapping of specific segment of a particular promoter with corresponding counterpart from other homologous/non-homologous promoter. Recombinant promoter thus developed demonstrates characteristic properties combining the intrinsic properties of both parent promoters (Bhullar et al. 2003; Comai et al. 1990; de Boer et al. 1983; Kumar et al. 2012; Lee et al. 2007; Patro et al. 2012; Ranjan et al. 2011). In addition to above, by inserting point/frame-shift mutation employing site-directed mutagenesis, it is possible to manipulate the distribution of number/s and position/s of the key *cis*-elements present in the promoter sequence for developing *cis*-rearranged 'better' promoter with enhanced strength, stress-inducibility and tissue specificity (Ranjan et al. 2012; Rushton et al. 2002; Venter 2007). Furthermore, use of such novel *cis*-shuffled chimeric promoters in plant biotechnology is advantageous particularly in cases of "gene-pyramiding" based plant metabolic engineering and development of transgenic plant expressing particular trait of interest. Unfortunately, access to such efficient chimeric promoter/s with specific attributes is insufficient in plant molecular biology at this time.

With a prospect for designing and testing efficient chimeric plant promoters with enhanced activity and salient *cis*-distributions, in the present study, we developed above-mentioned three chimeric promoters. PFlt-UAS-2X developed by fusing the PFlt-UAS (−353 to −49) to the upstream of the TATA box containing PFlt promoter fragment (−353 to +24). Likewise, FSgt-PFlt and MSgt-PFlt promoters developed by coupling the UAS of the sub-genomic transcript promoter of the *Figwort Mosaic Virus* (FSgt-, −270 to −60) and *Mirabilis Mosaic Virus* (MSgt-, −356 to −125) upstream to the TATA containing core promoter domain of *Peanut Chlorotic Streak Virus* (PFlt, −353 to +24) individually. We carried out transient expression analysis of the intra- (PFlt-UAS-2X) and inter- (FSgt-PFlt and MSgt-PFlt) molecularly shuffled recombinant promoters coupled to the *GUS* reporter using tobacco and *Arabidopsis* protoplasts individually; also in whole plants like *Solanum lycopersicum, Petunia hybrida* and *Spinacia oleracea* by *Agrobacterium* infiltration assay (Imogen et al. 2006). We performed an in-depth comparative expression analysis of the activities of PFlt-UAS-2X, FSgt-PFlt, MSgt-PFlt, CaMV35S and CaMV35S^2 promoter, in transgenic tobacco plants (T_1 generation) and determined the relation between GUS accumulation with the corresponding *uidA-mRNA* levels in transgenic plants expressing the above promoters, individually. Furthermore, we recorded histochemical X-gluc-staining of whole transgenic seedlings (21 days old) and ImaGene Green™ staining of different floral parts/organs expressing the *GUS* under the control of above promoter constructs. Moreover, we examined the comparative performance of above promoter constructs under 150 μM exogenous SA and ABA treatment.

Chimeric/hybrid promoters (FSgt-PFlt, PFlt-UAS-2X and MSgt-PFlt) developed in this study could become potential tools for efficient expression of transgenes in a wide variety of plant cells for promoting agricultural biotechnology.

Materials and methods

Materials

General reagents including MS-salt, antibiotics, salicylic acid (SA), abscisic acid (ABA), 4-methylumbelliferyl-beta-D-glucuronide (MUG), 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc), 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) and diethyl pyrocarbonate (DEPC) were purchased from Sigma-Aldrich (St. Louis,

USA). Platinum high fidelity Taq DNA polymerase and ImaGene Green™ C_{12} FDGlcU GUS gene expression kit were purchased from Invitrogen (California, USA). Restriction and modifying enzymes were purchased from Promega (Madison, WI, USA), and were used according to the manufacturer's instructions.

Finer 5′, 3′ and 3′–5′ deletion analysis of 1,600 bp (−900 to +700) of PClSV-Flt promoter

Construction of 5′, 3′ and 3′–5′ deletion PClSV-Flt promoters fragments

A total number of twenty-seven (27) 5′, 3′ and 3′–5′ deletion PClSV-Flt promoter fragments Viz. P1 (−858 to +24), P2 (−758 to +24), P3 (−653 to +24), P4 (−608 to +24), P5 (−553 to +24), P6 (−498 to +24), P7 (−448 to +24), P8 (−401 to +24), P9 (−353 to +24), P10 (−303 to +24), P11 (−253 to +24), P12 (−226 to +24), P13 (−176 to +24), P14 (−128 to +24), P15 (−77 to +24), P16 (−37 to +24), P17 (−226 to −49), P18 (−226 to +53), P19 (−226 to +88), P20 (−226 to +113), P21 (−226 to +263), P22 (−226 to +323), P23 (−226 to +433), P24 (−226 to +433), P25 (−226 to +523), P26 (−226 to +570) and P27 (−226 to +623) were PCR amplified individually using promoter-specific oligonucleotides pairs (Table 1) containing the appropriate sequence to generate *Eco*RI at the 5′-end and *Hind*III site at the 3′-end. PCR-amplified products were restriction digested in the presence of *Eco*RI and *Hind*III endonucleases. The relative positions of these deletion promoter fragments with respect to PClSV genome (across gene VI and VII) are diagrammatically presented in Fig. 1a.

Dropped out promoter fragments were gel-purified and cloned into the corresponding sites of the pBSK+ vector and resulting plasmids were designated as pBS-P1, pBS-P2, pBS-P3, pBS-P4, pBS-P5, pBS-P6, pBS-P7, pBS-P8, pBS-P9, pBS-P10, pBS-P11, pBS-P12, pBS-P13, pBS-P14, pBS-P15, pBS-P16, pBS-P17, pBS-P18, pBS-P19, pBS-P20, pBS-P21, pBS-P22, pBS-P23, pBS-P24, pBS-P25, pBS-P26 and pBS-P27. All these clones were subjected to sequencing to confirm the sequence integrity.

Construction of protoplast expression vectors with 5′, 3′ and 3′–5′deletion promoter fragments

All 5′, 3′ and 3′–5′deletion PClSV-Flt promoter fragments (P1 to P27) from respective pBSK-based clones were isolated as *Eco*RI-promoter-*Hind*III fragment individually and sub-cloned into the corresponding sites of protoplast expressing vector, pUCPMAGUS coupled to *GUS* reporter (Dey and Maiti 1999), replacing the CaMV35S promoter (present in the pUCPMAGUS vector at *Eco*RI and *Hind*III sites) to generate following clones: pP1GUS, pP2GUS, pP3GUS, pP4GUS, pP5GUS, pP6GUS, pP7GUS, pP8GUS, pP9GUS, pP10GUS, pP11GUS, pP12GUS, pP13GUS, pP14GUS, pP15GUS, pP16GUS, pP17GUS, pP18GUS, pP19GUS, pP20GUS, pP21GUS, pP22GUS, pP23GUS, pP24GUS, pP25GUS, pP26GUS and pP27GUS (set A).

Transient assay of 5′, 3′ and 3′–5′deletion PClSV-Flt promoter fragments using tobacco protoplasts

Viable protoplasts were isolated from *Nicotiana tabacum* cv. Xanthi Brad cell culture following standard protocol (Kumar et al. 2012). Protoplasts (approx. 2×10^6) were electroporated with approximately 10 µg plasmid DNA extracted individually from pUCPMA (vector control), plasmids under set A (pP1GUS to pP27GUS) constructs following protocol described earlier (Kumar et al. 2012). The electroporated protoplasts were incubated for 20 h at 28 °C in dark. Fluorimetric GUS assay was carried out following standard protocols (Bradford 1976; Jefferson et al. 1987).

Construction of plant expression vectors with 5′, 3′ and 3′–5′ PClSV-deletion promoter fragments

All twenty-seven (27) 5′–3′ and 3′–5′ PClSV promoter deletion constructs (P1–P27) as *Eco*RI-promoter-*Hind*III fragments were isolated from respective pBSK-based clone and sub-cloned into the corresponding sites of plant expressing pKYLXGUS vector (Schardl et al. 1987) coupled to GUS reporter gene, to obtain the following clones: pKP1GUS, pKP2GUS, pKP3GUS, pKP4GUS, pKP5GUS, pKP6GUS, pKP7GUS, pKP8GUS, pKP9GUS, pKP10GUS, pKP11GUS, pKP12GUS, pKP13GUS, pKP14GUS, pKP15GUS, pKP-16GUS, pKP17GUS, pKP18GUS, pKP19GUS, pKP20GUS, pKP21GUS, pKP22GUS, pKP23GUS, pKP24GUS, pKP-25GUS, pKP26GUS, and pKP27GUS (set B). The native pKYLXGUS vector contains a CaMV35S promoter at *Eco*RI and *Hind*III sites.

Transgenic assay of 5′, 3′ and 3′–5′ PClSV-deletion promoter fragments in tobacco plants

All twenty-seven 5′, 3′ and 3′–5′ PClSV promoter deletion constructs under set B (pKP1GUS–pKP27GUS) were used individually for *Agrobacterium tumefaciens* mediated plant transformation following standard protocol (Chen et al. 1994). On an average, 10–12 independent plant lines were generated under each construct and maintained under standard green house conditions till the setting of seeds. Total leaf proteins of transgenic plant expressing the above promoter constructs (pKP1–pKP27) were extracted individually and average GUS activity was measured according

Table 1 Sequences of oligo primers used for PCR amplification of promoters and genes

Serial number	Promoter	Primer sequence in 5′–3′ direction
1	P1	Fp: GCGGGCGAATTCGTCAACGCAAACAAGCATCTACCC Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
2	P2	Fp: GCGGGCGAATTCGTCAACGGATTTATCCATGCCATT Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
3	P3	Fp: GCGGGCGAATTCGTCAACGAAAAAGGTATGCGTGAA Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
4	P4	Fp: GCGGGCGAATTCGTCAACCCTGAGTTCGATGAAAGT Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
5	P5	Fp: GCGGGCGAATTCGTCAACTTGGCGGAAGTAACTCCA Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
6	P6	Fp: GCGGGCGAATTCGTCAACGCTAGAGGCATTTCTTCA Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
7	P7	Fp: GCGGGCGAATTCGTCAACGTGTTCAAGGAAGAATTA Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
8	P8	Fp: GCGGGCGAATTCGTCAACAGCCAACAAGCTAGGAAG Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
9	P9	Fp: GCGGGCGAATTCGTCAACGAGTTTTTACTTCGGACA Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
10	P10	Fp: GCGGGCGAATTCGTCAACAAAACAAGAAATATGCTT Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
11	P11	Fp: GCGGGCGAATTCGTCAACTTTGCCAGCTATGCGAAC Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
12	P12	Fp: GCGGGCGAATTCGTCAACGAGATCTTGAGCCAATCA Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
13	P13	Fp: GCGGGCGAATTCGTCAACGAGCCATGACGTAAGGGC Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
14	P14	Fp: GCGGGCGAATTCGTCAACTGTGACCTGTCGGTCTCT Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
15	P15	Fp: GCGGGCGAATTCGTCAACTTTAAATTTCCACGGCAA Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
16	P16	Fp: GCGGGCGAATTCGTCAACGCTTTGCCTATAAATAAG Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
17	P17	Fp: GCGGGCGAATTCGTCAACTTTCTCCGTATCTACTCA Rp: ATGCAGAAGCTTCTTCTGTAGGATATAAGT
18	P18	Fp: GCGGGCGAATTCGTCAACTTTCTCCGTATCTACTCA Rp: ATGCAGAAGCTTTGTAAGGATCTGAATATC
19	P19	Fp: GCGGGCGAATTCGTCAACTTTCTCCGTATCTACTCA Rp: ATGCAGAAGCTTTTAAACTCATTTTTGACT
20	P20	Fp: GCGGGCGAATTCGTCAACTTTCTCCGTATCTACTCA Rp: ATGCAGAAGCTTTTCTTGTTTTACCTCGGC
21	P21	Fp: GCGGGCGAATTCGTCAACTTTCTCCGTATCTACTCA Rp: ATGCAGAAGCTTTTCGTAATGGCGTAAGCC
22	P22	Fp: GCGGGCGAATTCGTCAACTTTCTCCGTATCTACTCA Rp: ATGCAGAAGCTTCGCCAAATATAAAAAGTA
23	P23	Fp: GCGGGCGAATTCGTCAACTTTCTCCGTATCTACTCA Rp: ATGCAGAAGCTTTGGAAATTTAAAAATACA
24	P24	Fp: GCGGGCGAATTCGTCAACTTTCTCCGTATCTACTCA Rp: ATGCAGAAGCTTTATTACAAGACTCGTTCT
25	P25	Fp: GCGGGCGAATTCGTCAACTTTCTCCGTATCTACTCA Rp: ATGCAGAAGCTTGTGACTCATAGAATAACT
26	P26	Fp: GCGGGCGAATTCGTCAACTTTCTCCGTATCTACTCA Rp: ATGCAGAAGCTTTTCTATAACAGTATACCT
27	P27	Fp: GCGGGCGAATTCGTCAACTTTCTCCGTATCTACTCA Rp: ATGCAGAAGCTTTCAGTCGTGCGAGTTCCT
28	PFlt-UAS-1X	Fp: GCGGGCGAATTCGTCAACGAGTTTTTACTTCGGACA Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA

Table 1 continued

Serial number	Promoter	Primer sequence in 5′–3′ direction
29	PFlt-UAS-2X	Fp: GCGGGCGAATTCGTCAACGAGTTTTTACTTCGGACA Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
30	FSgt-PFlt	Fp: CCCGAATTCGTCGACTTTACAGTAAGAACTGATAACA Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
31	MSgt-PFlt	Fp: ACTGAATTCGTCGACAGCGGTAAAACAGGTGATTACT Rp: ATGCAGAAGCTTATGGCCGTGTCTTCTCGA
32	*GUS* (real time)	Fp: GATCGCGAAAACTGTGGAAT Rp: TAATGAGTGACCGCATCGAA

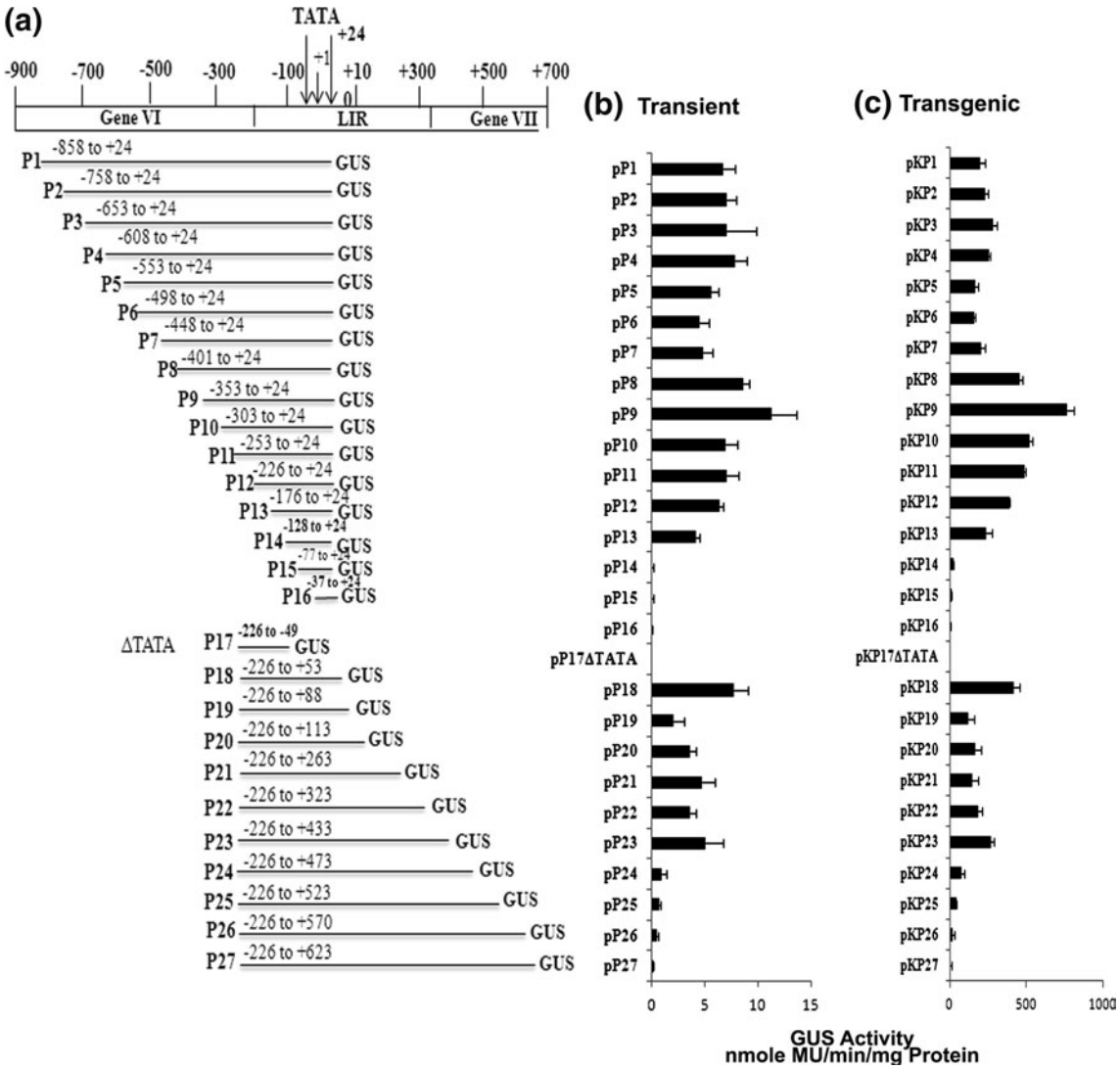

Fig. 1 5′, 3′ and 3′–5′ deletion analysis of PClSV-Flt (−900 to +700) The relative position of the TATA box and the transcription start site (TSS, +1) were shown at the *top*. **a** A schematic map of the deletion constructs (27 Nos.) coupled to *GUS* reporter gene was presented; 5′- and 3′-end coordinates of the relative deletion fragments were given. **b** Transient GUS expression analysis of PClSV-Flt promoter deletion constructs (27 Nos.) coupled to *GUS* reporter in tobacco protoplast (*Xanthi* brad). **c** Stable GUS expression analysis of the above promoter constructs (27 Nos.) in transgenic tobacco. Each construct was assayed in four independent experiments for both transient and transgenic analyses. The average GUS activity (nmole MU/min/mg protein) with respective standard deviation was presented. Statistical (one-way analysis of variance, ANOVA) analysis of the data set showed an extremely significant *P* value of <0.001

to the standard protocol (Bradford 1976; Jefferson et al. 1987) .

Characterization of recombinant promoter clones

Construction of recombinant promoter clones (PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt)

The up-stream activation (distal) fragments of *Peanut Chlorotic Streak Virus* (PFlt-UAS, 305 bp, −353 to −49), *Figwort Mosaic Virus* (FSgt-, 210 bp, −270 to −60), *Mirabilis Mosaic Virus* (MSgt-, 231 bp, −356 to −125) and the TATA box containing core promoter domain of *Peanut Chlorotic Streak Virus*, (PFlt-, 377 bp, −353 to +24) were PCR amplified using synthetic pair of primer (Table 1) containing the appropriate restriction sites to generate *Eco*RI and *Hinc*II overhangs at the 5′-end and *Sma*I and *Hind*III overhangs at the 3′-end. These amplified fragments (5′-*Eco*RI − *Hinc*II- promoter fragment −*Sma*I −*Hind*III-3′) were digested by *EcoR*I and *Hind*III individually, gel-eluted and cloned into the corresponding sites of pBSK+ to generate pBS-PFlt-UAS, pBS-FSgt, pBS-MSgt and pBS-PFlt clones. The PFlt promoter fragment was isolated from pBS-PFlt as *Hinc*II-*Hind*III fragment; and inserted into the *Sma*I and *Hind*III sites of pBS-PFlt-UAS, pBS-FSgt and pBS-MSgt individually to generate pBS-PFlt-UAS-2X, pBS-FSgt-PFlt and pBS-MSgt-PFlt clones. All plasmid clones thus obtained were subjected to nucleotide sequencing to check the integrity of the clone.

Construction of protoplast expression vectors with recombinant promoters coupled to GUS and GFP

Recombinant promoters; Viz. PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt were isolated from respective pBSK-based clones as *Eco*RI-promoter-*Hind*III fragments and sub-cloned into the corresponding sites of the pUCPMA-GUS vector to obtain following protoplast expressing promoter clones namely pPFltGUS, pPFlt-UAS-2XGUS, pFSgt-PFltGUS, pMSgt-PFltGUS (set C).

Likewise, these promoters as *Eco*RI-promoter-*Hind*III fragments were cloned into the corresponding sites of the pUCPMAGFP (Kumar et al. 2012) to obtain following protoplast expressing promoter clones namely pPFltGFP, pPFlt-UAS-2XGFP, pFSgt-PFltGFP and pMSgt-PFltGFP (set D), respectively, by replacing the CaMV35S promoter.

Transient assay of recombinant promoters coupled to GUS and GFP using tobacco protoplasts

An aliquot of 10^6 viable protoplasts was electroporated with 10 µg plasmid DNA extracted individually from pUCPMA (vector control), pUCPMAGUS (carrying CaMV35S promoter), pPFltGUS, pPFlt-UAS-2XGUS, pFSgt-PFltGUS and pMSgtPFltGUS constructs (Set C) following protocol described earlier, and fluorimetric GUS assay was carried out following standard protocols.

In another independent transient assay, protoplasts were electroporated with 10 µg plasmid DNA extracted individually from pUCPMA (vector control), pUCPMAGFP, pPFltGFP, pPFlt-UAS-2XGFP, pFSgt-PFltGFP and pMSgt-PFltGFP constructs (set D) following protocol described earlier. The GFP fluorescence from electroporated protoplasts was measured following earlier protocol (Sahoo et al. 2009).

Transient assay of recombinant promoter in Arabidopsis protoplast

Arabidopsis protoplast was isolated as described below. Sterile young leaves of *Arabidopsis* plants were chopped under sterile condition and placed in MS agar media containing the following supplements: 1 mg/l of Naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l of 6-benzylaminopurine (BAP). The plates were kept in dark at temperature 24 °C for 1 month for callus development. *Arabidopsis* callus were shred from plates to 30 ml enzyme solution containing 2 % cellulose and 2 % pectinase and incubated in dark at 50 rpm for 4 h. After 4 h, digested *Arabidopsis* cells were passed through a sieve, the flow through was collected and centrifuged at 200 g for 5 min. The pellet was suspended in 1 ml 0.6 M mannitol and 0.2 % $CaCl_2$. Finally, the protoplasts were purified over 20 % sucrose cushion and resuspended in a fresh medium containing 0.6 M mannitol and 0.2 % $CaCl_2$. The isolated protoplast was counted with the help of hemocytometer.

An aliquot of 10^5 *Arabidopsis* protoplasts was electroporated with 10 µg plasmid DNA extracted individually from pUCPMA (vector control), pUCPMAGUS, PFltGUS, PFlt-UAS-2XGUS, FSgt-PFltGUS and MSgt-PFltGUS constructs following protocol described earlier, and fluorimetric GUS assay was carried out following standard protocols (Bradford 1976; Jefferson et al. 1987).

Construction of plant expression vector with recombinant promoter coupled to GUS

All recombinant promoters Viz. PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt were isolated from respective pBSK-based clones as *Eco*RI-promoter-*Hind*III fragments and cloned into the corresponding sites of pKYLXGUS vectors to generate the following clones: pKPFltGUS, pKPFlt-UAS-2XGUS, pKFSgt-PFltGUS and pKMSgt-PFltGUS.

Transient agro-infiltration assay of chimeric promoters in Solanum lycopersicum, Petunia hybrida and Spinacia oleracea

Agrobacterium tumefaciens strain C58C1:pGV3850 was transformed with pKPFltGUS, pKPFlt-UAS-2XGUS, pKFSgt-PFltGUS and pKMSgt-PFltGUS constructs (pKYLXGUS based) individually following the freeze–thaw method as described earlier (Chen et al. 1994). *Agrobacteria* lines were grown as individual culture as described earlier. Leaves of *Spinacea oleracea*, *Petunia hybrida* and *Solanum lycopersicum* (var. Samsun NN) were mechanically infused with each of the *Agrobacterium* constructs individually as described (Imogen et al. 2006). Quantitative measurements of the GUS activity were performed 3–4 days post-infusion following standard protocol.

Development of transgenic plants using recombinant promoters

On an average, 10–12 independent plant lines were generated for each construct (pKPFltGUS, pKPFlt-UAS-2XGUS, pKFSgt-PFltGUS and pKMSgt-PFltGUS) using *A. tumefaciens*-mediated plant transformation and maintained under standard greenhouse conditions till setting of seeds. Seeds were collected from each plant line under each constructs and germinated on MS plate supplemented with 300 mg Kan/litre. Segregation analysis for each line was determined. Kanamycin-resistant plants (T_1 generation) were used for further analysis including GUS activity analysis. GUS activity in 21 days old seedlings (with Kan^R: $Kan^S = 3:1$) was measured according to the protocol described earlier.

Quantitative real-time PCR

Total RNA was extracted from transgenic tobacco plants expressing pKPFltGUS, pKPFlt-UAS-2XGUS, pKFSgt-PFltGUS and pKMSgt-PFltGUS promoters individually using Trizol reagent (Invitrogen, USA). DNaseI treated RNA was used for cDNA synthesis employing Kit (Fermentas, USA). The qRT-PCR using the corresponding cDNA template of *GUS* and *18S* (1:15 dilution) in the presence of SYBR Premix Ex TaqTM II (Perfect Real Time, Takara Bio Inc., Japan) employing Opticon-2 Real-time PCR machine (MJ Research, Bio-Rad; Model; CFD-3220) was performed as described earlier (Lee et al. 2007). Gene-specific primers for *GUS* and *18S* are presented in Table 1. The absence of genomic DNA contamination was confirmed using minus-reverse-transcriptase controls. The Ct value for each reaction was evaluated using software attached with the Opticon-2 Real-time PCR system. Fold changes in the transcript level of the *GUS* under control of each promoter were determined (Pfaffl 2001).

Histochemical staining of germinating seeds, whole seedlings and Fluorescent imaging of floral organs

Longitudinal cross sections of germinating seeds expressing pKYLXGUS, pKPFltGUS, pKPFlt-UAS-2XGUS, pKFSgt-PFltGUS and pKMSgt-PFltGUS promoter constructs individually were performed at different time-points Viz. 0, 2nd, 4th and 8th day of post-germination using a Cryostat (Model CM1850-1-1, Leica). Longitudinal sections of transgenic seed and whole seedlings of transgenic plant (21 days old) expressing pKYLXGUS, pKPFltGUS, pKPFlt-UAS-2XGUS, pKFSgt-PFltGUS and pKMSgt-PFltGUS constructs were immersed separately into histochemical GUS staining buffer [100 mM $NaPO_4$, 0.5 mM $K_3[Fe(CN)_6$, 0.5 mM $K_4[Fe(CN)_6]$, 10 mM EDTA, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc)] and vacuum infiltrated for 10 min followed by incubation at 37 °C for overnight. Treated samples were subsequently washed and kept in fixing solutions (50 % ethanol, 7 % acetic acid). The intensities of blue color development in different tissues were recorded using Leica DM LS2 microscope (Inverted) at 10× magnification.

Detailed histochemical expression analysis of the reporter gene (*GUS*) in different sections of floral organs/parts of transgenic plant expressing pKYLXGUS, pKPFltGUS, pKPFlt-UAS-2XGUS, pKFSgt-PFltGUS and pKMSgt-PFltGUS constructs was carried out individually in the presence of 55 mM ImaGene Green™ C12FDGlcU substrate (ImaGene Green™ GUS Gene Expression Kit; Invitrogen, Oregon, USA,) as per kit's instructions. Subsequently, treated samples were kept in dark after vacuum infiltration for 10 min. Fluorescence images of the ImaGene Green™ treated floral sections were captured using a CLSM (TCSSP5; Leica, D-68165 Mannheim, Germany) and GUS localizations at cellular/tissue level were detected. Intensities of Green fluorescence obtained from different 50/60 ROIs (regions of interest) of transgenic floral organs for individual promoter construct were recorded using 'LAS-AF' analytical software, and average intensity obtained from different floral organ/tissue was measured.

SA and ABA treatment

Transgenic tobacco seeds expressing pKYLX (control), pKYLX, pKYLXGUS, pKYLXGUS35S[2], pKPFltGUS, pKPFlt-UAS-2XGUS, pKFSgt-PFltGUS and pKMSgt-PFltGUS constructs were germinated on half MS plate (containing 300 mg/l kanamycin) and allowed to grow under tissue culture conditions as described earlier (Kumar et al. 2012). The whole transgenic seedlings (21 days old)

under each constructs were treated individually in the presence of 150 μM SA (pH 6.8) and ABA (pH 6.8) for a period of 0–24 h, respectively.

After treatments, GUS activity from SA- and ABA-treated root, leaf, stem portion of seedlings under each of the above-mentioned promoter constructs were measured (Bradford 1976; Jefferson et al. 1987).

Statistical analysis

All the data obtained in the present study were subjected to statistical analysis employing one-way ANOVA analysis using GraphPad Prism version 6 and reported as a mean of 3 or 4 independent experiments. Data were statistically significant at a *P* value of 0.05.

Results

Finer deletion analysis of *Peanut Chlorotic Streak Virus* PClSV-Flt promoter

We have evaluated the transient activity of each of the pUCPMA-based promoter construct (under set A) coupled to the *GUS* reporter in tobacco protoplast (Xanthi Brad). Figure 1b presented the average GUS activity of each construct obtained from four independent experiments with respective standard deviation. Data obtained revealed that the P9 promoter fragment (−353 to +24) showed strongest activity among the other promoters. The P9 promoter was 1.3, 1.6, 1.5 and 1.4 times stronger than P8 (−401 to +24), P10 (−303 to +24), P18 (−226 to +53) and P11 (−253 to +24) promoters, respectively. On the contrary, TATA less P17 (−226 to −49) promoter showed minimum activity (Fig. 1b).

We evaluated the stable transgenic GUS expression activity of each of the pKYLXGUS-based promoters (under set B) as described in "Materials and methods". Figure 1c presented the average GUS activity of each promoter construct obtained from four independent experiments with respective standard deviation. Data analysis revealed that the P9 promoter was 1.62, 1.51, 1.80, 1.44 times stronger than P8, P18, P11 and P10 promoter constructs. Again TATA less P17 promoter failed to drive GUS expression in plant tissue.

Comparative analysis of transient activity of CaMV35S, PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoter coupled to *GUS* and *GFP*

Figure 2 represents the schematic maps with constitutive component elements of these chimeric promoter constructs. The transient GUS expression activity obtained in transformed protoplast by the above promoter construct individually was measured as described in "Materials and methods". Figure 3a presented the average GUS activity of each of the promoter constructs from four independent experiments with respective standard deviation. Using *GUS* as reporter gene, we observed that the FSgt-PFlt promoter construct showed 2.81, 3.11, 1.25 and 1.41 times stronger activity than that obtained from CaMV35S, PFlt, PFlt-UAS-2X and MSgt-PFlt promoter constructs, respectively, in tobacco protoplast. Alongside, we evaluated the activity of above promoter constructs coupled to *GFP* as described in "Materials and methods". Figure 3b presented the mean GFP fluorescence intensity from four independent experiments for each of the promoter with respective standard deviation. Using *GFP* as reporter gene, we detected that the FSgt-PFlt promoter was 2.2, 2.3, 2.0 and 2.1 times stronger than CaMV35S, PFlt, PFlt-UAS-2X and MSgt-PFlt promoter constructs in tobacco protoplast.

Moreover, we carried out a comparative analysis of promoter activity of above promoter constructs in *Arabidopsis* protoplast as described in "Materials and methods". Figure 3c presented the mean GUS activity obtained from four independent experiments for each of the above promoter constructs and confirmed that the FSgt-PFlt promoter was 3.25, 3.91, 1.95 and 3.01 times stronger than CaMV35S, PFlt, PFlt-UAS-2X and MSgt-PFlt promoters, respectively.

Again, we performed independent in vivo transient experiments for measuring the promoter activity of each of the above promoter constructs coupled to *GUS* reporter gene in different plants like petunia (*P. hybrida*), spinach (*S. oleracia*) and tomato (*S. lycopersicum*). Figure 4 presented the mean GUS activity (obtained from four independent experiments) for each of the promoter constructs in above-mentioned plant with respective standard deviations. On analysis of the result obtained, we found that the FSgt-PFlt promoter showed 2.83, 1.62 and 2.31 times stronger activity than the CaMV35S promoter in petunia, spinach and tomato plant systems, respectively.

Comparison among promoter activities of CaMV35S, CaMV35S^2, PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoter in transgenic tobacco

We developed transgenic plants expressing the above-mentioned promoter construct individually as described in "Materials and methods". Seedlings from selected lines under each construct showing appropriate segregation ratio (Kan^R: Kan^S = 3: 1) were considered for GUS analysis and other relevant expression. We conducted four independent experiments for evaluating the mean GUS activity for each of the above-mentioned promoter constructs coupled to *GUS* reporter as described in "Materials and methods". Figure 5a presented the mean GUS activities for each

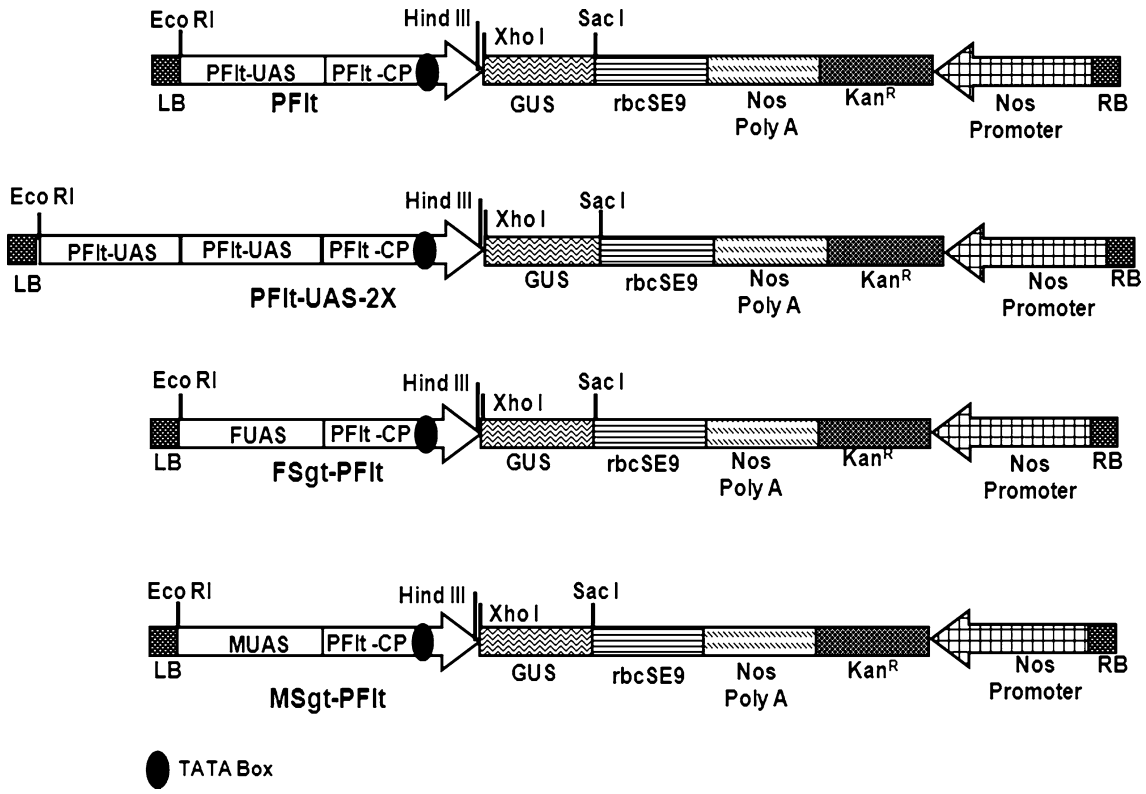

Fig. 2 Schematic map of recombinant promoter constructs graphical representation of plant expression cassettes containing recombinant promoters namely, PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt coupled to *GUS* reporter gene with respective cloning sites. Positions of upstream activation sequence (UAS), core promoter sequences with TATA element of different promoters, were illustrated. The relative position of the rbcSE9, polyA 3′ region, Nos Poly A region (Nos poly A), Kanamycin resistance gene (Kan^R), and promoter from Nopaline synthase gene (Nos promoter) were shown. *Arrow* indicates the direction of transcription

promoter construct with respective standard deviation. Result obtained clearly demonstrated that the FSgt-PFlt promoter was 3.47, 2.33, 3.29, 1.84 and 2.0 times stronger than CaMV35S, $CaMV35S^2$, PFlt, PFlt-UAS-2X and MSgt-PFlt promoters, respectively.

Total RNA was extracted from 21-day-old seedling expressing the above-mentioned promoter constructs individually and real-time analysis of *GUS* transcript was performed as described in "Materials and methods". Figure 5b represented the mean relative fold differences obtained from three independent experiments for each of the promoter constructs with respective standard deviation. We observed 3.91- and 3.0-fold enhanced expression of *GUS* transcripts in transgenic plants expressing the FSgt-PFlt than corresponding GUS expression obtained from CaMV35S and $CaMV35S^2$ promoter, respectively (Fig. 5b).

X-gluc staining of germinating seeds expressing chimeric promoters

We studied the GUS localizations in 30 μm longitudinal section of transgenic seed expressing CaMV35S, $CaMV35S^2$, PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoters individually (Fig. 6) and identified regions of intense GUS localization in micropylar and non-micropylar endosperm sections of the seed during 2–4 days post-germination. From 0 to 2 days, we found GUS localization occurs mostly in radical portion of the germinating seeds. On 8th day disruption of seed coat 'testa' occurred, root formation initiated and root tip showed intense blue coloration.

We performed histochemical (X-gluc) staining of 21 days old transgenic tobacco seedlings expressing vector control, CaMV35S, $CaMV35S^2$, PFlt, PFlt-UAS-2X and MSgt-PFlt promoter constructs individually and observed constitutive pattern of GUS localizations in almost all parts of the seedling with highest expression in the root for all the above promoters (Fig. 6).

ImaGene Green™ staining of different flower parts expressing chimeric promoters individually

We performed histochemical staining of different reproductive organs namely style, ovary and filament of mature flower of tobacco (T_1 generation) expressing CaMV35S,

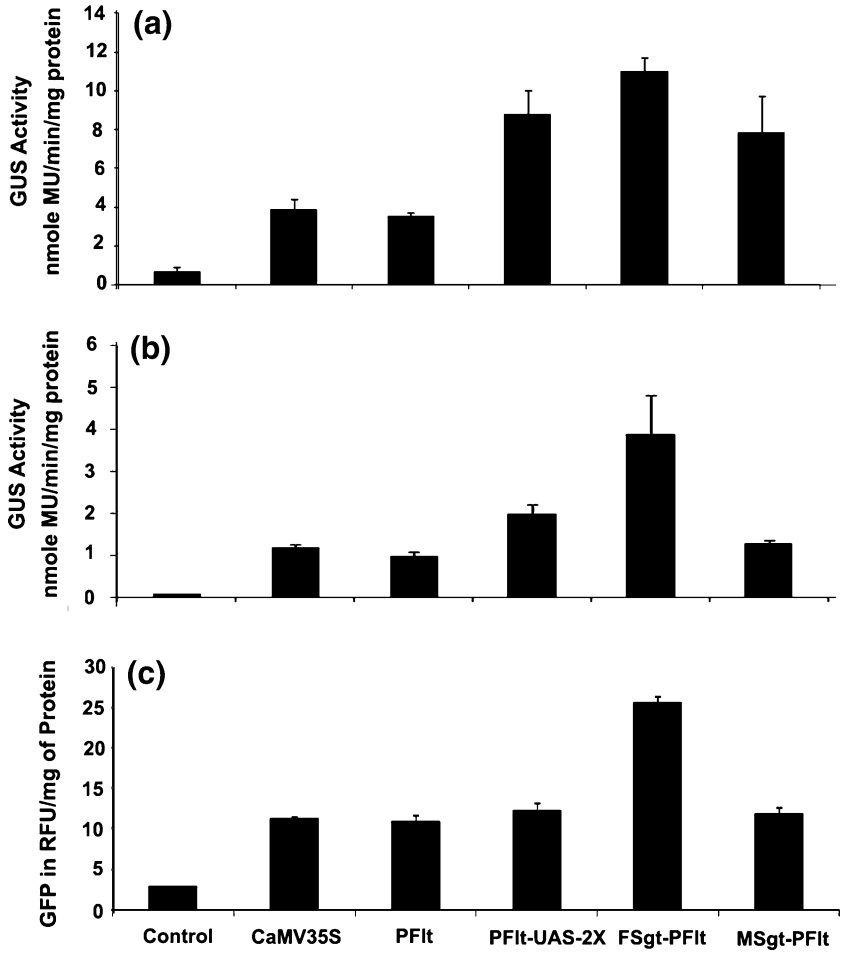

Fig. 3 Transient activity analysis of CaMV35S, PFlt, PFLt-UAS-2X, FSgt-PFlt and MSgt-PFlt. **a** Healthy tobacco protoplasts (approx 10^6) were electroporated with the above promoter constructs coupled to the *GUS* reporter gene individually as described in "Materials and methods". Transient activity of each construct from four independent experiments in tobacco protoplast individually was presented. Transformed protoplast by vector pUCPMA with no *GUS* gene was used as control. The statistical ANOVA analysis of the data set showed a *P* value <0.05 revealing high significance. **b** Average GUS activities (nmole MU/min/mg protein) of four independent experiments obtained from transformed *Arabidopsis* protoplast expressing the above promoter constructs individually were presented with corresponding standard deviations. The statistical analysis of the data obtained revealed a *P* value <0.001 implying highly significant. **c** Average relative fluorescence intensities from the protoplast-derived GFP were determined and presented in the lower panel. Fluorescence of four independent experiments was presented with corresponding standard deviation. The statistical ANOVA analysis of the data set indicated a *P* value <0.001 showing extreme significance

CaMV35S[2], PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoters individually using ImaGene Green™ dye. We analyzed the dye-treated employing confocal laser scanning microscopy as mentioned in "Materials and methods". We observed near constitutive type GUS of expression under control of these promoters developed in the present study with the highest expression in ovary particularly in ovules (Fig. 7a).

We measured the intensity of green fluorescence obtained from treated sample using LAS-AF analytical software attached to the confocal system as described earlier (Ranjan et al. 2012). Figure 7b presented the average green fluorescence intensities obtained from 50/60 ROIs of transgenic floral organs expressing the above-mentioned promoter constructs individually with respective standard deviation. We observed that PFlt-UAS-2X and FSgt-PFlt promoter showed almost equivalent activity in different floral organs.

Spatial distribution of GUS activities obtained from CaMV35S, CaMV35S[2], PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoters

We conducted four independent experiments to assay the GUS activity in different parts of plant (root, leaf and stem) expressing the above promoter constructs individually. Figure 8 presented the mean promoter activity for each of the promoter constructs with respective standard deviation. The FSgt-PFlt promoter showed 3.76 and 2.42 times stronger

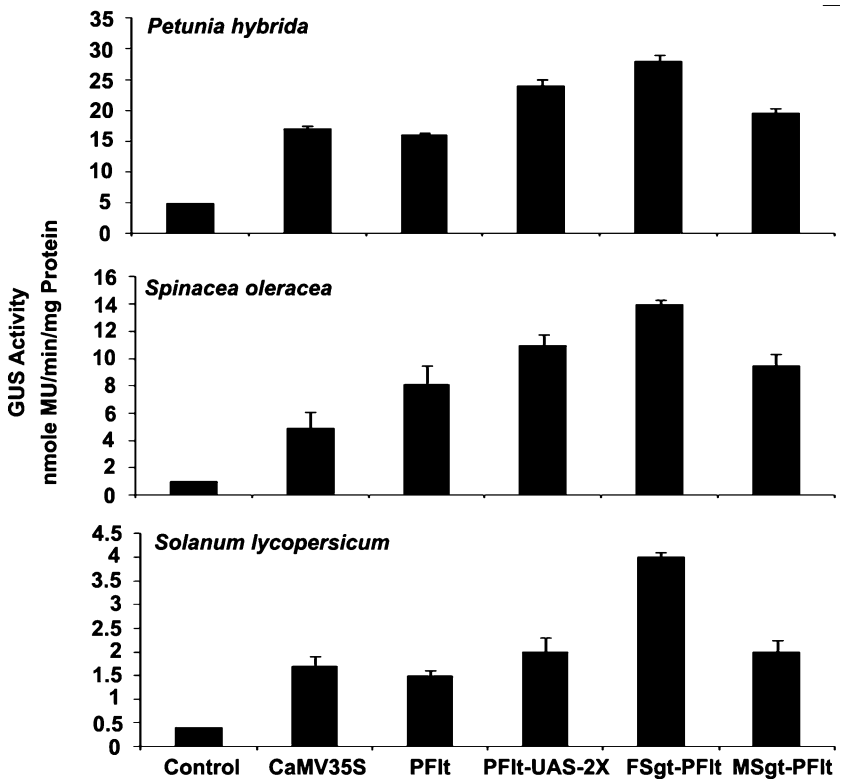

Fig. 4 Transient activity analysis of CaMV35S, PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt in different plants. Transient GUS activities' assay of CaMV35S, PFlt, PFLt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoter constructs in three different plants system namely *Petunia hybrida, Spinacea oleracia* and *Solanum lycopersicum* was evaluated. The average GUS activity of each of the above promoter constructs was determined from four independent experiments and presented with respective standard deviation. GUS activities obtained from respective wild plants were treated as control. Statistical analysis of the data sets showed a *P* value of <0.01, indicating highly significant

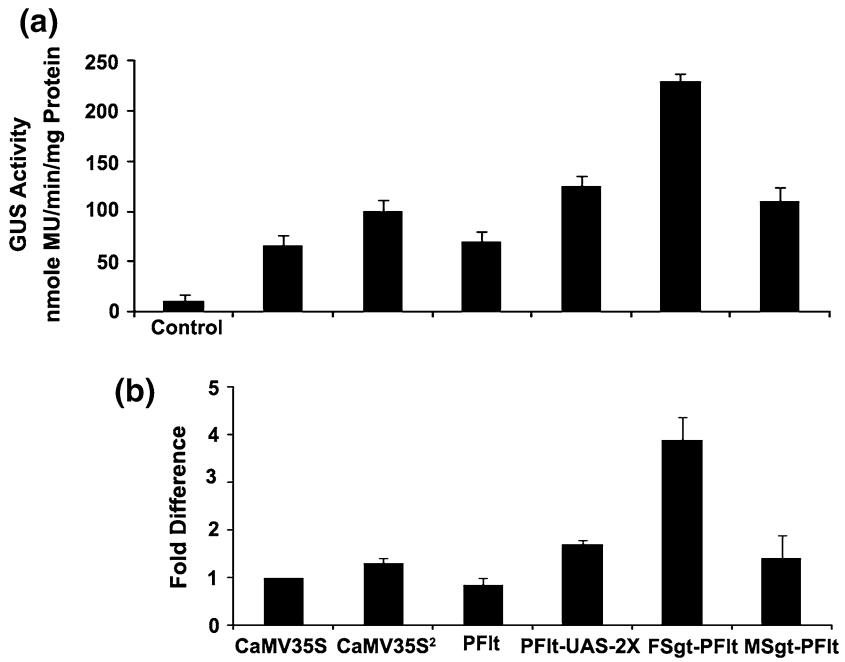

Fig. 5 Transgenic activity analysis of CaMV35S, PFlt, PFLt-UAS-2X, FSgt-PFlt and MSgt-PFlt stable transgenic GUS activity analysis of the above promoter constructs in 21-day-old transgenic seedlings expressing the above promoter construct individually were performed as described in "Materials and methods". **a** Average GUS activities obtained from four independent experiments for each of these constructs were presented with corresponding standard deviation. The statistical analysis of the data revealed a *P* value <0.05 indicating highly significant. **b** *Each bar* represents the relative fold difference of *GUS* transcript levels in transgenic seedlings (21 days old) under control of CaMV35S[2], PFlt, PFlt-UAS-2X, FSgt-PFlt, and MSgt-PFlt promoter considering the accumulation of GUS transcript level under the CaMV35S promoter as 1.0

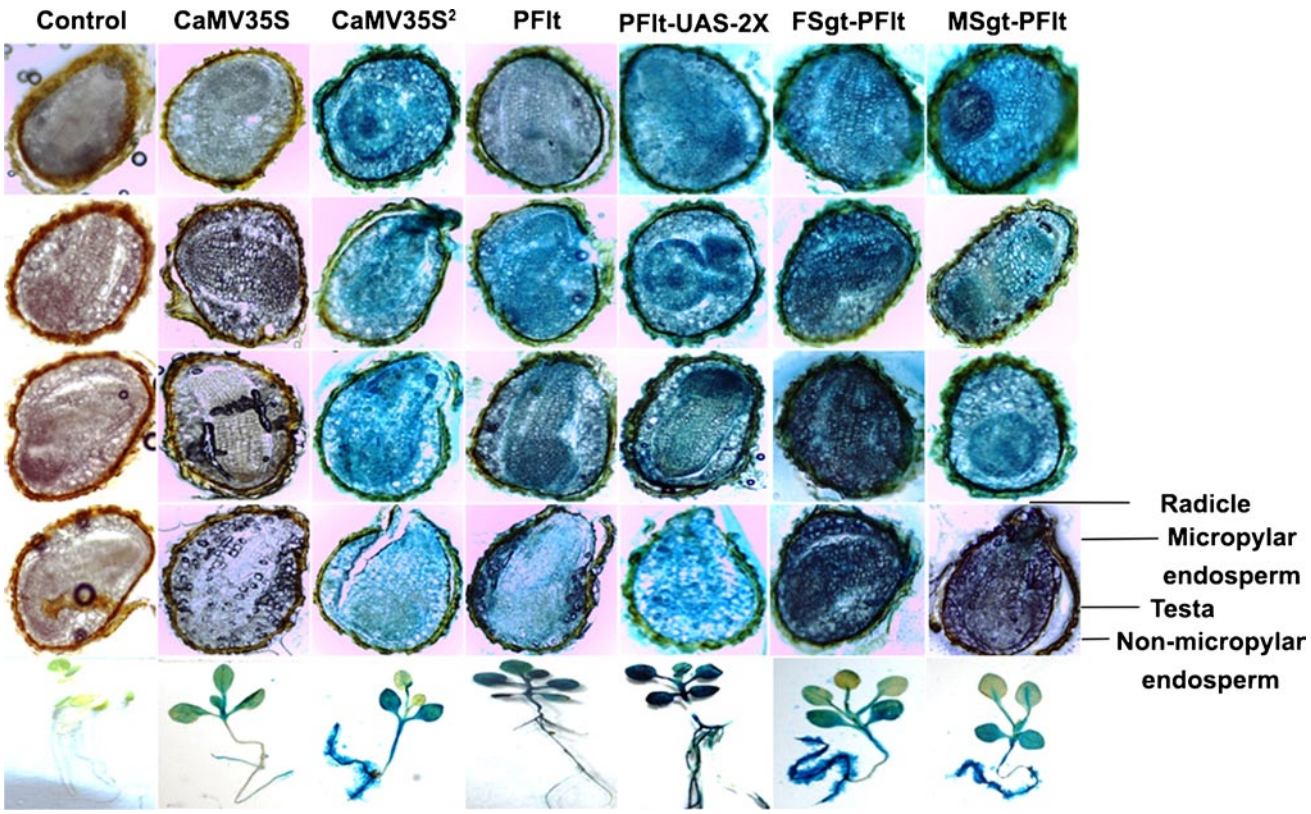

Fig. 6 Histochemical staining of germinating transgenic seeds at various time-point expressing recombinant promoters individually. Differential expression of the *GUS* reporter gene under control of CaMV35S, CaMV35S^2, PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoter constructs during different time points of seed germination. X-gluc stained 30 µm thick cross sections of transgenic seeds at different time points viz. 0, 4th, 6th and 8th day post-germination were presented. All promoters showed near constitutive type of expression. Micropylar region of seed endosperm under control of pPFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoter constructs showed intense blue coloration. Light microscopy images of X-gluc treated whole seedlings (21 days) of untransformed control and transgenic tobacco plant expressing CaMV35S, CaMV35S^2, PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoters coupled to *GUS* reporter gene

root expression compared to that obtained from CaMV35S and CaMV35S^2 promoter, respectively. We observed that the root expression of the FSgt-PFlt promoter was almost 2.22 and 2.95 times stronger than its leaf and stem expression, respectively (Fig. 8). Furthermore, we observed that the root activities of PFLt-UAS-2X and MSgt-PFlt were equivalent. The root activity of MSgt-PFlt promoter was 1.3 times stronger than that of the CaMV35S^2 promoter.

Comparative analysis of chimeric promoter activity CaMV35S, CaMV35S^2, PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoters under exogenous SA and ABA stress

We performed comparative activity analysis of the above promoters under SA and ABA stress individually. For SA treatment, we separated root, leaf, stem of 21 days old transgenic seedling and treated them in the presence of 150 µM SA as described in "Materials and methods". The GUS activities of SA-treated transgenic root, leaf and stem expressing the above promoter constructs were evaluated individually. Figure 9 represented the mean GUS activities (obtained from four independent experiments) for SA treated root, leaf and stem expressing the above promoter constructs individually with respective standard deviation. Data obtained clearly revealed that the root activity of the FSgt-PFlt promoter was 10.91, 5.18, 4.0, 2.0 and 2.35 times more in comparison to that obtained under CaMV35S, CaMV35S^2, PFlt, PFlt-UAS-2X and MSgt-PFlt promoter, respectively, in the presence of 150 µM SA .

Likewise, we evaluated the GUS activities of transgenic root, leaf and stem expressing the above promoter constructs under 150 µM ABA treatment. We presented the data obtained as a mean of four independent experiments in Fig. 9 with respective standard deviation. The FSgt-PFlt promoter showed 13.13, 7.37, 6.52, 2.38 and 4.32 stronger activities in root compared to that of CaMV35S CaMV35S^2, PFlt, PFlt-UAS-2X and MSgt-PFlt promoter, respectively.

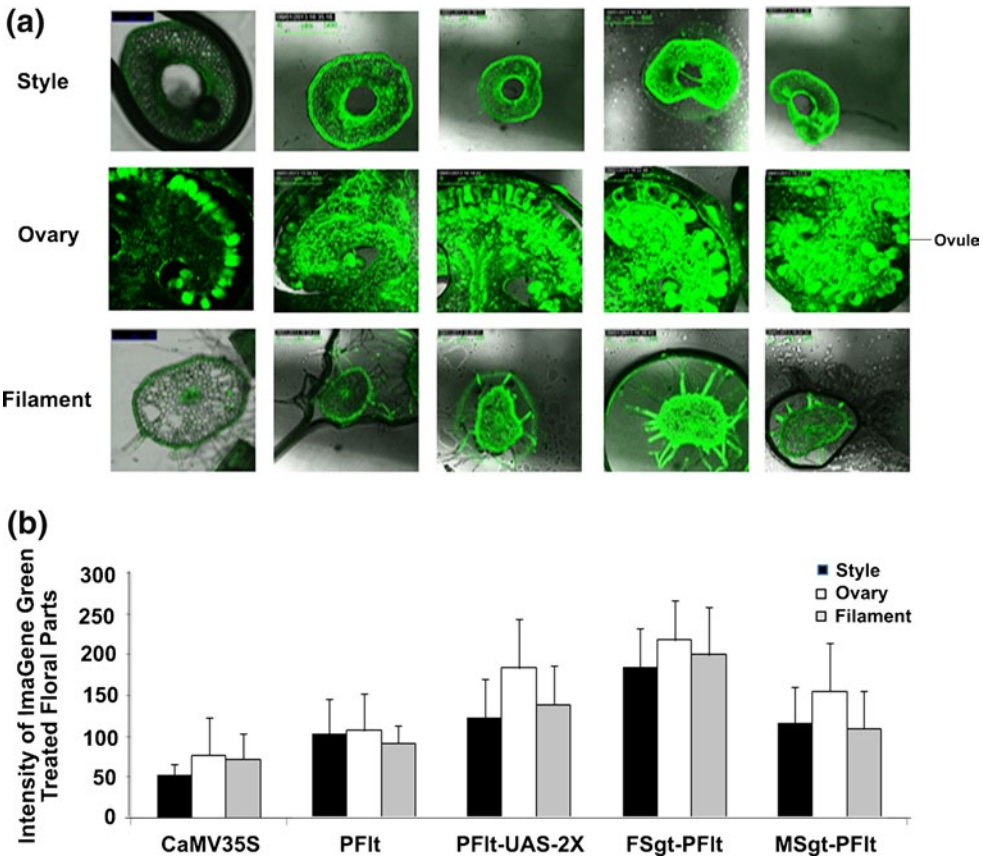

Fig. 7 ImaGene Green™ staining of different floral organ/tissue. **a** CLSM-based comparative analysis of the relative GUS expression under control of CaMV35S, PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoter in transgenic floral organs. Superimposed (bright field and fluorescent) images of ImaGene Green™ treated cross sections of transgenic floral organ (style, ovary and filament) expressing the *GUS* gene under respective promoter construct were presented. **b** Intensities of green fluorescence obtained from 50/60 regions of interest (ROIs) of transgenic floral organs expressing the above promoter construct individually were recorded using LAS-AF software. The average intensity was determined for respective floral organs/tissues and presented with corresponding standard deviation. The statistical analysis of the data obtained revealed a P value <0.05 implying highly significant

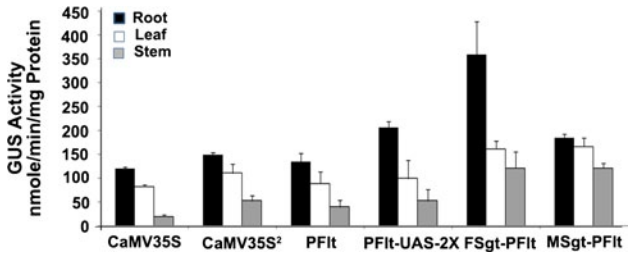

Fig. 8 Spatial distributions of GUS activities under CaMV35S, PFlt, PFLt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoter. Average GUS activities (in nmole MU/min/mg protein) from the root, leaf and stem of transgenic tobacco seedling (21 days old) expressing *GUS* under the control of the above promoter constructs individually were measured from four independent experiments and presented with corresponding standard deviation. The statistical analysis of the data obtained revealed a P value <0.001 implying extremely significant

Discussion

Promoters are an important component in plant biotechnology. Plant expression vector coupled to promoter with enhanced activity and sequence heterogeneity is one of the prime needs in gene-based approach for plant modification. In context to the above, several novel native plant promoters were identified from different members under *Caulimoviruses* group to boost the plant biotechnology-based application (Bhattacharyya et al. 1993; Maiti et al. 1997; Medberry et al. 1992; Odell et al. 1985; Verdaguer et al. 1996). At present time, worldwide attempts are being made to improve efficacy of pararetrovirus-based promoters for enhancing their strength and tissue specificity. Initially, approaches involving duplication of enhancer were employed to increase the promoter activity and it is observed that the modified version of promoter with duplicated enhancer domain usually demonstrates two- to five-fold or more enhanced activity compared to the activity of native promoter with single enhancer. During last decade, numbers of modified promoter with duplicate enhancer were constructed and applied in plant biotechnology-based program (Dey and Maiti 1999; Kay et al. 1987; Maiti et al. 1997; Maiti and Shepherd 1998a). Gradually, the concept of developing recombinant promoter by exchanging/swapping important domains of two or more homologous/heterologous promoter becomes well accepted and such domain-shuffled recombinant promoters appear as a "better" choice for expressing transgene in plant. Furthermore, it is possible to redesign the promoter architecture employing either site-directed mutagenesis or promoter-DNA shuffling approaches (Ranjan et al. 2011; Venter 2007). Such *cis*-shuffled/*cis*-rearranged efficient promoters are certainly

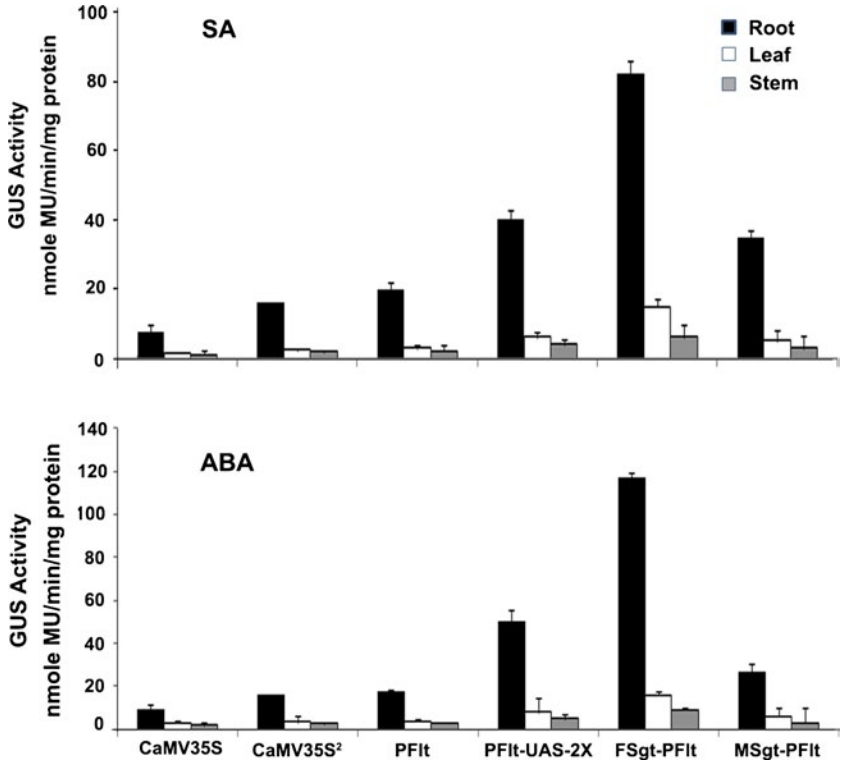

Fig. 9 Spatial distributions of GUS activities of recombinant promoter under SA and ABA treatment. Spatial distribution of GUS expression driven by CaMV35S, CaMV35S^2, PFlt, PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt promoter constructs under 150 μM salicylic acid and abscisic acid treatment. Average GUS activities (in nmole MU/min/mg protein) from the root, leaf and stem of 21-day-old transgenic tobacco seedlings were measured from four independent experiments for both SA and ABA treatments as discussed in "Materials and methods" and presented along with their respective standard deviation. The statistical ANOVA analysis of the data showed a P value <0.05 indicating high significance

'better' choices for ectopic expression of transgene in plant when one wants to introduce a transgene into plant cell avoiding genetic rearrangement.

With an object for searching efficient core promoter for developing chimeric promoters, we have performed a finer deletion analysis of a 1,600 bp long of the PClSV-Flt promoter (−900 to +700) encompassing gene VI, LIR and gene VII based on 50 bp deletion from both 5′, 3′ and 5′–3′ ends and identified a strong core promoter P9 (−353 to +24) which is about 1.4 times stronger than the previously reported pKP6 (−253 to +24; genomic coordinate: 5,825 and 6,101) promoter by our group (Maiti and Shepherd 1998b). Transcriptional start site of PClSV-Flt promoter is a "T" residue reported by Richard Richins in his PhD dissertation thesis submitted to university of Kentucky (Richins 1993).

Subsequently, we developed three modified recombinant promoters viz., PFlt-UAS-2X, FSgt-PFlt and MSgt-PFlt employing newly derived core promoter PFlt (P9, −353 to +24) and tested their efficacy in driving the reporter genes in different plant species. We observed that they are able to express transgene efficiently at three- to fourfold elevated level compared to the level of reporter gene expression under control of most widely used CaMV35S promoter. As these recombinant promoters contain either homologous (in case of PFlt-UAS-2X) or heterologous (in case of FSgt-PFlt and MSgt-PFlt) enhancer domains, we further compared their activity with the activity of the CaMV35S^2 promoter developed by duplicating the '−343 to −90' enhancer domain of CaMV35S promoter (Kay et al. 1987) in transgenic plant. We observed that the activities of recently developed chimeric promoter were equivalent to the activity obtained from the CaMV35S^2 promoter in transgenic plants. These observations clearly demonstrated that newly developed promoter could become efficient substitute for CaMV35S^2 in plant biotechnology.

Furthermore, we confirmed that these recombinant promoters are efficient to drive reporter gene in different plant species. This observation clearly demonstrated that they are active in broad range of plant species. Also, we established that these promoters are capable of driving both *GUS* and *GFP* reporters in transformed protoplast indicating their capability of driving different/multiple gene of interest in plant cell.

Among these three newly developed chimeric promoters, the FSgt-PFlt promoter showed maximum activity compared to the other in both transient and transgenic systems. Our observation was confirmed by biochemical GUS-assays, histochemical assays and analysis of *uidA-mRNA* level in transgenic plants expressing recombinant promoter individually. Furthermore, we observed a fair co-relation among the accumulation level of *GUS* transcripts and corresponding GUS activity in transgenic plant expressing individual recombinant promoter. This indicates that these chimeric promoters positively involve in transcription and subsequent translation of *GUS* transcript in

plant cell. Moreover, spatial expression analysis of recombinant promoter in different plant parts demonstrates that these chimeric promoters show the following order of GUS accumulation in plant cell: root > leaf > stem.

Salicylic acid (SA) acts usually as an abiotic stress elicitor and differentially stimulates the specific activity of as-1 element in root of transgenic plant (Jupin and Chua 1996; Krawczyk et al. 2002; Niggeweg et al. 2000; Qin et al. 1994). Based on these, we further carried out our investigation to assay the effect of SA on the activities of the promoter constructs developed in the present study. Overall, upon induction by SA (150 μM) for period of 24 h, we observed that these promoters were inducible by SA and they showed enhanced expression in plant particularly in root tissue. Also, we observed that newly developed hybrid promoter FSgt-PFlt showed enhanced activity under exogenous ABA treatment compared to the activities of both CaMV35S and CaMV35S^2 promoters suggesting that these recombinant promoters can effectively be used for developing genetically modified plant under abiotic stresses.

Our previous study (Bhattacharyya et al. 2002) indicated that the activities of pararetroviruses in monocot plant were usually less compared to their activity in dicot plants; based on this report, we assume that activities of recombinant promoters developed in this study could be less in monocot plants compared to their activities in dicot plants.

Taken altogether, the use of such newly derived hybrid promoter with enhanced activity and abiotic stress (SA and ABA) inducibility could be of immense importance in future engineering of broad range of agronomically important plants for developing gene-shuffled modified plants resistant to abiotic stresses. Also the FSgt-PFlt promoter along with PFlt-UAS-2X and MSgt-PFlt may be proposed as efficient tools for promoting agricultural biotechnology for boosting plant productivity and can safely be used in combination with both CaMV35S and CaMV35S^2 regulating plant gene expression in a complex metabolic pathway/s.

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