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Development of an intra-molecularly shuffled efficient chimeric plant promoter from plant infecting *Mirabilis mosaic virus* promoter sequence

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ABSTRACT

We developed an efficient chimeric promoter, MUASMSCP, with enhanced activity and salicylic acid (SA)/abscisic acid (ABA) inducibility, incorporating the upstream activation sequence (UAS) of *Mirabilis mosaic virus* full-length transcript (MUAS, −297 to −38) to the 5′ end of *Mirabilis mosaic virus* sub-genomic transcript (MSCP, −306 to −125) promoter-fragment containing the TATA element. We compared the transient activity of the MUASMSCP promoter in tobacco/*Arabidopsis* protoplasts and in whole plant (*Petunia hybrida*) with the same that obtained from CaMV35S and MUAS35SCP promoters individually. The MUASMSCP promoter showed 1.1 and 1.5 times stronger GUS-activities over that obtained from MUAS35SCP and CaMV35S promoters respectively, in tobacco (*Xanthi* Brad) protoplasts. In transgenic tobacco (*Nicotiana tabacum*, var. Samsun NN), the MUASMSCP promoter showed 1.1 and 2.2 times stronger activities than MUAS35SCP and CaMV35S[2] promoters respectively. We observed a fair correlation between MUASMSCP-, MUAS35SCP- and CaMV35S[2]-driven GUS activities with the corresponding *uidA-mRNA* level in transgenic plants. X-gluc staining of transgenic germinating seed-sections and whole seedlings also support above findings. Protein-extracts made from tobacco protoplasts expressing *GFP* and human-*IL-24* genes driven individually by the MUASMSCP promoter showed enhanced expression of the reporters compared to that obtained from the CaMV35S promoter. Furthermore, MUASMSCP-driven protoplast-derived human IL-24 showed enhanced cell inhibitory activity in DU-145 prostate cancer cells compared to that obtained from the CaMV35S promoter. We propose chimeric MUASMSCP promoter developed in the study could be useful for strong constitutive expression of transgenes in both plant/animal cells and it may become an efficient substitute for CaMV35S/CaMV35S[2] promoter.

1. Introduction

Plants complete a successful life assimilating multiple environmental stress stimuli; adapting both inducible and spatio-temporal expression patterns of genes (Kaufmann and Alice, 2010). In eukaryotes, at transcriptional level, the pattern of gene expression usually convenes by the genetic regulator 'promoter' in association with RNA Pol II and eventually this association regulates the successful synthesis of mRNA. Generally, plant promoter comprises of a distal (upstream activation sequence; UAS) and a proximal region (core promoter) containing TATA element (Goldberg–Hogness box) located about 30–40 base pairs upstream of TSS (transcription start site) where RNA Pol II, TATA-binding proteins (TBPs) and TBP-associated factors (TAFs) interact to turn on transcription. 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 promoters and sets the 'polarity' and functional anatomy of the promoter. The combinatorial interactions between specific trans-protein factors with respective cis-elements in association with other transcriptional accessory protein factors usually determine the functionality of the promoter and spatio-temporal expression of the gene coupled to the promoter (Fessele et al., 2002; Tjian and Maniatis, 1994). 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).

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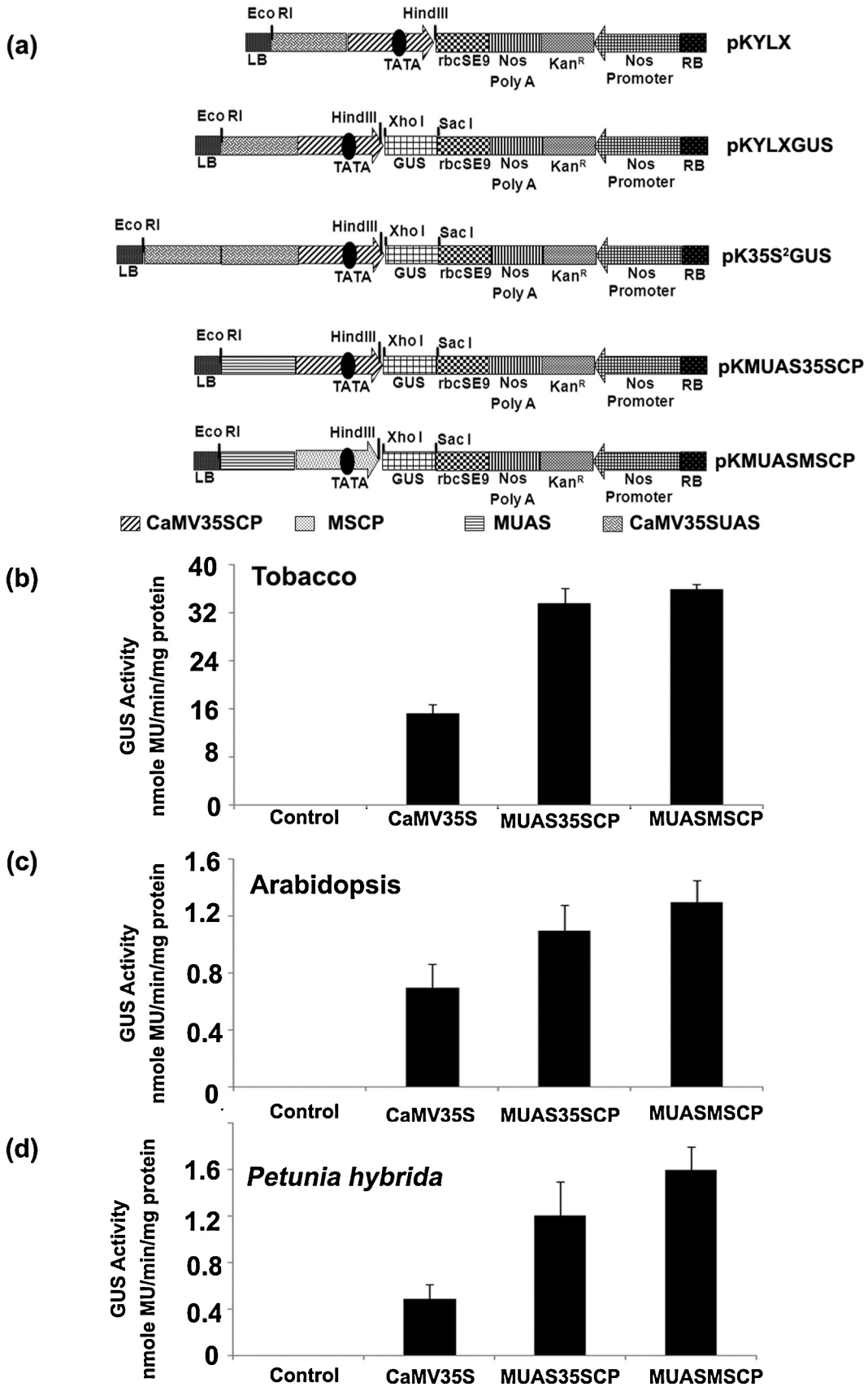

Fig. 1. Transient activity analysis of CaMV35S, MUAS35SCP and MUASMSCP. (a) Graphical representation of plant expression vectors containing recombinant promoters namely; pKYLX (containing 35S), pKYLXGUS, pK35S^{2}GUS, pKMUAS35SCP and pKMUASMSCP coupled to *GUS* reporter gene with respective cloning sites. Position 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), promoter from Nopaline synthase gene (Nos promoter) were shown. Arrow indicates the direction of transcription. (b) Healthy tobacco protoplasts were electroporated with above promoter constructs coupled to the *GUS* reporter gene as described in Section 2. Average transient activity

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Moreover, the structure of plant promoter is modular and contains several interacting functional domains; viz., the CaMV35S promoter comprising of two main domains; domain A and domain B. Domain A was further sub-divided into domain A1 and the minimal promoter (mp) while domain B was subdivided into domains B1, B2, B3, B4 and B5 (Benfey et al., 1989; Bhullar et al., 2003; Fang et al., 1989). The 'genetic manipulation' of plant promoter function could be possible based on its structural modularity. In recent past, many distinctive recombinant transcriptional module (chimeric promoter) with altered 'cis-clustering' were developed by swapping/exchanging/shuffling between different functional part of one promoter with homologous/heterologous counterpart from other promoter and such fusion-promoter demonstrate unique properties combining the properties of both donating and receiving promoters (Comai et al., 1990; deBoer et al., 1983; Kumar et al., 2012; Lee et al., 2007; Ni et al., 1995; Patro et al., 2012b; Ranjan and Dey, 2012). Additionally, adopting the approach of in situ site-directed mutagenesis we can manipulate the number/s and position/s of the prime cis-elements present in the promoter sequence for developing modified promoter with enhanced strength, stress-inducibility and tissue-specificity; ideal for efficient gene expression in plant even under sustainable agronomic conditions (Rushton et al., 2002; Venter, 2007). Use of such novel cis-shuffled promoter/s with heterologous sequences could minimize the chances of promoter-DNA homology based genetic recombination particularly in cases of gene stacking/pyramiding in plant cell when used singly or in combination. Thus, they become valued tools in plant metabolic engineering. However, such efficient chimeric promoters with specific attributes are scanty in plant molecular biology, at present time.

In the present study, with an aim for developing new efficient chimeric plant promoters with enhanced activity and substantial cis-distributions, we developed the MUASMSCP promoter by fusing the *Mirabilis mosaic virus* full-length transcript promoter fragment (MUAS, −297 to −38) upstream of TATA containing 145 bp long *Mirabilis mosaic virus* sub-genomic transcript promoter (MSCP, −306 to −125). We carried out a comparative transient expression activity analysis of this inter-molecularly shuffled recombinant promoter (MUASMSCP) with the transient activities obtained from MUAS35SCP (Patro et al., 2012a) and CaMV35S coupled to the *GUS* reporter using tobacco and *Arabidopsis* protoplasts; along with in whole plant namely *Petunia hybrida* by *Agrobacterium* infiltration assay (Imogen et al., 2006). Furthermore, we performed an in-depth expression analysis of this hybrid promoter, MUASMSCP, coupled to the *GUS* reporter gene in transgenic tobacco plants and compared its activity with the activities obtained from the MUAS35SCP and CaMV35S^2 promoters. To support the above, we correlated (1) the levels of GUS-accumulation with the *uidA-mRNA* in transgenic plants expressing MUASMSCP, MUAS35SCP and CaMV35S^2 promoters individually and (2) X-gluc-staining of transgenic germinating seed cross-sections and whole seedlings expressing *GUS* under the control of MUASMSCP, MUSA35SCP and CaMV35S^2 promoters individually. In addition, we performed a comparative study of the activities of MUASMSCP and CaMV35S^2 promoters (Kay et al., 1987) in presence/absence of 150 μM exogenous SA and ABA. Additionally, we evaluated the activity of the MUASMSCP promoter employing non-*GUS* reporters like GFP and IL-24 (Bhutia et al., 2012; Dash et al., 2010; Dent et al., 2010) in tobacco protoplast. Finally, we tested biological activity of the protoplast-derived IL-24 driven by each of the promoters using human prostate cancer cells (DU-145 prostate cancer cell line).

The MUASMSCP developed in this study could become potential tool for efficient expression of transgene/s in a wide variety of plant/animal cells for promoting agricultural/animal biotechnology. It has prospective to become an efficient substitute for both CaMV35S and CaMV35S^2 {a modified variant of CaMV35S promoter that was constructed by tandem duplication of 253 bp (−343 to −90) of the upstream activation sequences with 10-fold higher transcriptional activity, compared to CaMV35S} promoter in plant biotechnology.

2. Materials and methods

2.1. Materials

General reagents including 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), cellulase (C0615) and pectinase (P4300) were purchased from Sigma–Aldrich (St. Louis, USA). Platinum high fidelity Taq DNA polymerase was procured from Invitrogen (CA, USA). Restriction and modifying enzymes were purchased from Promega (Madison, WI, USA). *IL-24* clone in pCDNA vector was received from Dr. Rupesh Dash, Institute of Life Sciences as a kind gift. DU-145 human prostate cancer cells were obtained from the American Type Culture Collection. RPMI (A 10491), FBS (10270-106) and Penicillin–Streptomycin (15140-122) were obtained from Invitrogen.

2.2. Construction of MUASMSCP recombinant promoter

The up-stream activation (distal) fragment of the *Mirabilis mosaic virus* (MUAS, 335 bp, −297 to −38) and the TATA box containing core-promoter (proximal) domain of the sub-genomic transcript promoter of the *Mirabilis mosaic virus* (MSCP, 145 bp, −118 to +27) were PCR amplified using synthetic primers (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; amplified fragments (5′-*Eco*RI–*Hinc*II-promoter distal/proximal fragment-*Sma*I–*Hind*III-3′) were digested by *Eco*RI and *Hind*III individually, gel-eluted and cloned into the corresponding sites of pBSK+ to generate pBSMUAS and pBSMSCP clones. The MSCP promoter fragment was isolated from the pBSMSCP as *Hinc*II–*Hind*III fragment; and inserted into the *Sma*I and *Hind*III sites of the pBSMUAS to generate pBSMUASMSCP clone. The resulting plasmid clone pBSMUASMSCP thus obtained was subjected to sequencing to check the integrity of the clone.

2.3. Construction of protoplast and plant expression vectors

The MUASMSCP promoter fragment from the pBSMUASMSCP clone was isolated as *Eco*RI-promoter-*Hind*III fragment and subcloned into the corresponding sites of protoplast expressing vector coupled to *GUS* reporter, pUCPMAGUS (Dey and Maiti, 1999),

of each construct from four independent experiments were presented with respective standard deviation. Transformed protoplast by vector pUCPMA with no *GUS* gene was used as control. Statistical (one-way analysis of variance, ANOVA) analysis of the data set showed an extremely significant *P* value of <0.001. (c) Average GUS activities (nmole MU/min/mg protein) of four independent experiments obtained from transformed *Arabidopsis* protoplast expressing above promoter constructs individually were presented with corresponding standard deviations. The statistical ANOVA analysis of the data showed a *P* value <0.05 that indicated extreme significance. (d) Transient GUS activities assay of CaMV35S, MUAS35SCP and MUASMSCP promoter constructs in whole *Petunia hybrida* plant were evaluated. The average GUS activity of each of the above promoter constructs was determined from three-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.

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Table 1

Serial No.	Construct name	Primer sequence in 5′–3′ direction
1	MUASMSCP	Fp: CCCGAATTCGTCGACTTCGTCCACAGACATCAA Rp: ACTAAGCTTCCCGGGTTTGTGTTTCCTGTTTTTGTATTAATT
2	MUAS35SCP	Fp: CCCGAATTCGTCGACTTCGTCCACAGACATCAA Rp: GGGAAGCTTCCCGGGTCCTCTCCAAATGAAATGAAC
3	IL-24	Fp: GCGGGCTCGAGAACCATGGGTAATTTTCAACAGAGGCTGCAA Rp: ATGCAGGAGCTCTCAGTGATGGTGATGGTGATGGAGCTTGTAGAAATTCTGCAT
4	GUS (real time)	Fp: GATCGCGAAAACTGTGGAAT Rp: TAATGAGTGACCGCATCGAA
5	18S	Fp: GCAAATTACCCAATCCTGAC Rp: CTATTGGAGCTGGAATTACC

replacing the CaMV35S promoter to generate the pPMUASMSCP-GUS clone.

MUASMSCP, MUAS35SCP (Patro et al., 2012b) and CaMV35S[2] promoter fragments were inserted into the plant-expression vector/cassette (with GUS reporter) pKYLXGUS (Schardl et al., 1987) following protocol described earlier (Patro et al., 2012b) to obtain pKMUASMSCPGUS, pKMUAS35SCPGUS and pK35S[2]GUS respectively. Structural details (including cloning sites of elementary components) of these plant expression vectors along with (GUS reporter) pKYLXGUS containing 35S promoter and pKYLX (control, without *GUS*) were given in Fig. 1a

2.4. Transient assay of MUASMSCP, MUAS35SCP and CaMV35S promoter clones

Viable protoplasts were isolated from *Nicotiana tabacum* cv. *Xanthi* Brad cell culture following standard protocols (Kumar et al., 2012). Protoplasts (approx. 2×10^6) were electroporated with approximately 10 μg plasmid DNA extracted individually from pPMUASMSCPGUS, pPMUAS35SCPGUS (Patro et al., 2012b), pUCPMAGUS (containing 35S) and pUCPMA (vector control), constructs following protocol described earlier (Kumar et al., 2012). The electroporated protoplasts were incubated for 20 h at 28 °C in dark. Fluorometric GUS assay was carried out following standard protocols (Bradford, 1976; Jefferson et al., 1987).

We isolated *Arabidopsis* protoplast as follows: young leaves of *Arabidopsis* plants were chopped and placed aseptically on MS agar media containing Naphthalene acetic acid (NAA, 1 mg/L), Indole-3-acetic acid (IAA, 1 mg/L), 2,4-Dichlorophenoxyacetic acid (2,4-D, 1 mg/L) and 6-Benzylaminopurine (BAP, 0.5 mg/L) and were kept in dark at 24 °C for callus induction. Subsequently, *Arabidopsis* callus were digested in presence of enzyme solution containing cellulase (2%) and pectinase (2%). Digested cells were centrifuged at $200 \times g$ for 5 min and the pellet was suspended in 1 ml 0.6 M mannitol containing 0.2% $CaCl_2$. Finally, the protoplasts were purified on a 20% sucrose cushion. An aliquot of 10^5 *Arabidopsis* protoplasts were electroporated with 10 μg plasmid DNA extracted individually from pPMUASMSCPGUS, pPMUAS35SCPGUS, pUCPMAGUS and pUCPMA constructs following protocol described earlier and fluorometric GUS assay was carried out following standard protocols (Bradford, 1976; Jefferson et al., 1987).

We performed agro-infiltration assay as follows: *Agrobacterium tumefaciens* strain C58C1:pGV3850 was transformed with pKMUASMSCPGUS, pKMUAS35SCPGUS, pKYLXGUS and pKYLX promoter constructs individually following the freeze–thaw method as described earlier (Chen et al., 1994). Leaves of *P. hybrida* were mechanically infused with *Agrobacterium* culture transformed with pKMUASMSCPGUS, pKMUAS35SCPGUS, pKYLXGUS, and pKYLX individually as described earlier (Imogen et al., 2006). Quantitative measurements of GUS activity for each promoter construct were performed 3–4 days post inoculation following standard protocols (Bradford, 1976; Jefferson et al., 1987).

2.5. Transient assay of recombinant promoters coupled to GFP and IL-24 using tobacco protoplasts

Recombinant promoters MUASMSCP and MUAS35SCP were isolated from respective pBSK-based clones as *Eco*RI-promoter-*Hind*III fragments and sub-cloned into the corresponding sites of pUCPMAGFP (Kumar et al., 2011) promoter to obtain following protoplast expressing promoter clones; pMUASMSCPGFP and pMUAS35SCPGFP respectively, replacing the CaMV35S promoter.

The IL-24 gene was PCR amplified with forward and reverse primers of IL-24 (Table 1) to incorporate *Xho*I and *Sac*I site at 5′ and 3′ end in presence of pCDNA based *IL-24* plasmid. PCR amplified *IL-24* cDNA was cloned into following plasmid; pPMUASMSCPGUS, pPMUAS35SCPGUS and pUCPMAGUS individually replacing *GUS* to obtain pMUASMSCPIL-24, pMUAS35SCPIL-24 and pUCPMAIL-24 respectively. pUCPMA vector without IL-24 was used as a control.

Tobacco protoplasts (approx. 2×10^6) were electroporated with 10 μg plasmid DNA extracted individually from pMUASMSCPGFP, pMUAS35SCPGFP, pUCPMAGFP and pUCPMA (vector control) constructs following protocol described earlier. The intensity of GFP-fluorescence from electroporated protoplasts were measured following earlier protocol (Sahoo et al., 2009).

As above, in another independent transient assay, protoplasts were electroporated with 10 μg plasmid DNA extracted individually from pMUASMSCPIL-24, pMUAS35SCPIL-24 and pUCPMA along with pUCPMAIL-24. Transient accumulations of the IL-24 protein in the crude protoplast extract were measured by an indirect ELISA method as described below.

Total soluble protein from transformed protoplast expressing above promoter construct were extracted individually in a buffer containing 50 mM Tris–HCl, 5 mM EDTA and protease inhibitor cocktail (Sigma, USA), approximately 20 h post incubation. An aliquote of 100 μL PBS (Phosphate Buffer Saline) containing 10 μg of respective promoter-driven protoplast-derived IL-24 protein were coated into a 96 well plate and the concentration of protoplast derived IL-24 was estimated using anti-IL-24 antibody following standard ELISA protocol (Vazquez et al., 1996).

We performed MTT assay for conducting a comparative analysis of accumulation level of protoplast derived IL-24 protein under each promoter construct. For the same, DU-145 human prostate cancer cells (3×10^3 cells) were loaded per well in 96-well microtiter plates in 200 μL RPMI containing 10% FBS and 1% Penicillin–Streptomycin and incubated for 24 h; the medium was removed and fresh growth medium containing 50 ng of protoplast derived IL-24 protein/extract was added. After 48 h, the growth medium was removed and MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, (0.5 mg/mL)] was added and plates were incubated for 4 h at 37 °C. Finally, the medium was discarded and the intracellular formazan product was dissolved in 200 μL of DMSO. The absorbency of each well was measured at 540 nm using an ELISA reader (Synergy HT, BioTek Instruments Inc., Winooski, VT), and cell-viability was expressed as percentage

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of control cells. Average cell-viability presented as a mean of three independent experiments with respective standard deviation.

2.6. Transgenic characterization of MUAS35SCP and MUASMSCP promoters in tobacco

Promoter constructs pKMUASMSCPGUS, pKMUAS35SCPGUS, pK35S^{2}GUS, pKYLXGUS and pKYLX (Control) were used individually for *A. tumefaciens* mediated plant transformation (Chen et al., 1994). On an average, 8–11 independent plant lines were generated for each construct and maintained under standard greenhouse conditions till setting of seeds. Seeds were collected from each plant line under each construct and germinated on MS plate supplemented with 300 mg Kan/L; segregation analysis for each line was determined (with Kan^R:Kan^S = 3:1). Kanamycin-resistant seedlings (T_1 generation) under each construct with appropriate segregation ratio were used for further analysis. Total leaf protein of transgenic seedling (21 days old) expressing above promoter constructs individually were extracted and the average GUS activity was measured according to the standard protocols (Bradford, 1976; Jefferson et al., 1987).

Total RNA was extracted using Trizol reagent (Invitrogen) from selective transgenic tobacco seedlings (T_1 generation) expressing pKYLX35S^{2}GUS, pKMUAS35SCPGUS and pKMUASMSCPGUS promoters individually. Respective RNA (*DNase*I treated) was used for cDNA synthesis employing Kit (Fermentas, USA). The respective qRT-PCR using the corresponding cDNA template (1:15 dilution) in 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. Gene specific primers for *GUS* and *18S* (Table 1) were used and absence of genomic DNA contamination was confirmed using minus-reverse-transcriptase control. The Ct value for each reaction was evaluated using software attached with the Opticon-2 Real-time PCR system and fold changes in the *GUS* transcript level under control of each promoter construct were determined.

Longitudinal cross-section (30 μM) of germinating seeds were obtained using a Cryostat (Modell CM1850-1-1, Leica) at different time point of germination during 0, 2nd, 4th and 8th day. Longitudinal sections of germinating seeds and whole transgenic seedling (21 days old) expressing the *GUS* gene under control of pKMUASMSCPGUS, pKMUAS35SCPGUS, pK35S^{2}GUS, and pKYLX (Control) constructs were immersed 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-b-D-glucuronide (X-gluc)], vacuum infiltrated under pressure for 10 min followed by incubation at 37 °C for overnight; subsequently washed and kept in fixing solutions (50% ethanol, 7% acetic acid). The intensities of blue colour development in different tissues were recorded using Leica DM LS2 microscope (Inverted) at 10× magnification. The intensity of blue colour in developing seed particularly micropylar (mi) and non-micropylar (nmi) were recorded in 10× magnification.

2.7. SA and ABA treatment

Transgenic tobacco seeds expressing pKMUASMSCPGUS, pKMUAS35SCPGUS, pK35S^{2}GUS, pKYLXGUS and pKYLX (Control) constructs were germinated on half MS plate (containing 300 mg/L Kanamycin) and allowed to grow under tissue culture conditions as described earlier. 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 and 0–48 h.

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

GUS activities from 21 days old seedling expressing pKMUASMSCPGUS, pKMUAS35SCPGUS, pK35S^{2}GUS, pKYLXGUS and pKYLX (Control) under 150 μM ABA was carried out as described above.

2.8. Statistical analysis

All the data obtained in the present study were subjected to Statistical analysis employing one way ANOVA analysis using GraphPad Prism version 5.01 and reported as a mean of 3 or 4 independent experiments. *P* value less than 0.05 was considered significant.

3. Results

3.1. Comparative transient activity analysis of MUASMSCP, MUAS35SCP and CaMV35S promoters in protoplast (tobacco and Arabidopsis) and whole plant

We have constructed a chimeric promoter MUASMSCP as described in Section 2 and compared its activity with a recently reported efficient chimeric promoter MUAS35SCP (Patro et al., 2012a) along with the most widely used the CaMV35S promoter. The expression cassettes of plant transformation vectors harbouring these promoters were schematically represented in Fig. 1a.

Upon transient activity analysis of MUASMSCP, MUAS35SCP and CaMV35S promoters in tobacco protoplast (*Xanthi* Brad), we detected that the activity of the MUASMSCP was almost equivalent to the activity of the MUAS35SCP promoter; while the MUASMSCP promoter showed approximately 1.5 times stronger activity compared to the activity of the CaMV35S promoter (Fig. 1b).

Similarly, we observed the MUASMSCP promoter showed approximately 1.8 times stronger activity than the activity obtained from the CaMV35S promoter in *Arabidopsis* protoplast (Fig. 1c).

Additionally, we performed the transient activity analysis of MUASMSCP, MUAS35SCP and CaMV35S promoters in whole *P. hybrida* plant adapting agro-infiltration approach as described in Section 2 and observed the activity MUASMSCP promoter was 3.2 and 1.3 times stronger compared to the activity obtained from the CaMV35S and MUAS35SCP promoter respectively (Fig. 1d).

In all above cases, we presented the GUS activity of each construct as a mean of four independent experiments with respective standard deviation.

3.2. Comparative expression analysis of MUASMSCP, MUAS35SCP and CaMV35S promoter coupled to GFP and IL-24

Using *GFP* as a reporter, we observed that the MUASMSCP promoter was 2.1 and 1.3 times stronger in comparison to the activities obtained from CaMV35S and MUAS35SCP. The mean GFP fluorescence activity (grey scale) obtained from four independent experiments were presented in Fig. 2a with respective standard deviation.

In a separate study, we detected the average accumulation level (from four independent experiments) of MUASMSCP-driven protoplast-derived IL-24 protein was 1.7 and 1.1 times more in comparison to that obtained from CaMV35S- and MUAS35SCP-driven protoplast-derived IL-24 protein by ELISA (Fig. 2b).

We observed that the mean biological activity (DU-145 cell inhibitory) of the MUASMSCP-driven protoplast-derived IL-24 protein was 1.34 times stronger than DU-145 cell inhibitory activity obtained from the CaMV35S-driven protoplast-derived IL-24 protein. Alongside, we observed that MUASMSCP-driven protoplast-derived IL-24 protein was 1.33 times stronger than corresponding protein obtained under control of the MUAS35SCP

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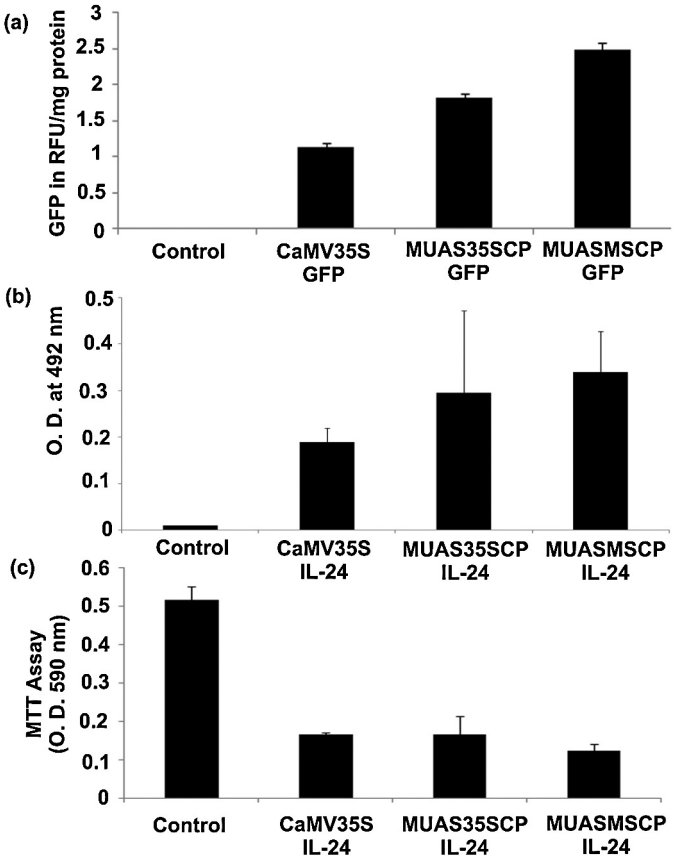

Fig. 2. Transient activity analysis of CaMV35S, MUAS35SCP and MUASMSCP coupled to *GFP* and *IL-24*. (a) Crude protein extract from transformed tobacco protoplast expressing recombinant promoter constructs coupled to *GFP* was extracted individually as described in Section 2. Transformed tobacco protoplast with empty vector (pUCPMA) was taken as control. Average relative fluorescence intensity from four independent experiments for each promoter construct was determined and presented with corresponding standard deviation. The statistical analysis of the data revealed a P value <0.05 implying highly significant. (b) Relative accumulation of protoplast derived IL-24 protein were measured employing indirect ELISA using respective polyclonal antibodies as described in Section 2. Average accumulation of protoplast derived IL-24 under control of each of the recombinant promoter was presented. Crude protein obtained from protoplast transformed with empty vector was taken as control. (c) DU-145 prostate cancer cell viability in presence of respective promoter-driven protoplast-derived IL-24 was determined by standard MTT assay as described in Section 2. The absorbency of each well was measured at 540 nm using an ELISA reader and viability was expressed as percentage of control cells. Data presented are the mean of three independent experiments with their corresponding standard deviation.

promoter. Fig. 2c represents the mean viable cell counts obtained from three independent experiments using promoter-driven protoplast-derived protein on DU-145 cell line with respective standard deviation.

3.3. Comparative transgenic activity analysis of MUASMSCP, MUAS35SCP and CaMV35S^2 promoter

We evaluated the stable GUS activity of tobacco transgenic seedling (21 days old) expressing MUASMSCP, MUAS35SCP and CaMV35S^2 construct individually. The mean GUS activity obtained from three independent experiments with their respective standard deviation was presented in Fig. 3a. The data were statistically significant at a P value of 0.05. Upon comparison, we observed that the activity of MUASMSCP was 1.2 and 1.5 times stronger than the activity obtained from MUAS35SCP and CaMV35S^2 promoter respectively (Fig. 3a).

Real time analysis of the accumulation level of *uidA-mRNA* level in transgenic plant expressing MUASMSCP, MUAS35SCP and CaMV35S^2 promoter construct individually confirmed 1.1 and 1.5-fold more accumulation of *GUS* transcripts driven by the MUASMSCP promoter in comparison to the same under control of MUAS35SCP and CaMV35S^2 promoter respectively (Fig. 3b). Data presented as a mean of three independent experiments with corresponding standard deviation.

Furthermore, we studied in detail the differential accumulation of GUS localization in different regions of the longitudinal cross-sections (30 μm) of transgenic seeds expressing MUASMSCP, MUAS35SCP and CaMV35S^2 promoter individually (Fig. 3c). We detected intense GUS localization particularly in endosperm (both micropylar and non-micropylar) regions of the seed during 2–8 days post germination. From 0 to 2 days we found GUS localization occurs mostly in radical portion of the germinating seeds in all cases (Fig. 3c).

Histochemical (X-gluc) staining of 21 days old transgenic tobacco seedling expressing MUASMSCP, MUAS35SCP and CaMV35S^2 promoter constructs also confirmed above observation (Fig. 3c).

3.4. Spatial distribution of the activities of the MUASMSCP, MUAS35SCP and CaMV35S^2 promoter

We performed spatial distribution analysis of GUS-expression in transgenic root, leaf, stem expressing above promoter constructs individually and observed the MUASMSCP promoter showed 1.7 and 6.4 times stronger root expression compared to that obtained from MUAS35SCP and CaMV35S^2 promoter. We observed the root expression of the MUASMSCP promoter which was 2.3 and 15.3 times stronger than its leaf and stem expression (Fig. 4). While, the MUAS35SCP promoter showed 1.4 and 1.2 time stronger expression in root as compared to its leaf and stem respectively. In contrast, we noted the spatial expression of the CaMV35S^2 promoter follow the following pattern; leaf > stem > root. Data were presented as a mean of four independent experiments with corresponding standard deviation.

3.5. Comparative analysis of MUASMSCP and CaMV35S^2 under SA and ABA stress

We performed a comparative activity analysis of above promoter constructs under both 150 μM SA and ABA stress individually for a period of 48 h as described. We observed that MUASMSCP and CaMV35S^2 promoters showed identical activity under both 24 and 48 h SA treatment. Whereas, the MUASMSCP promoter showed 2.0 and 2.1 times enhanced activity than that of CaMV35S^2 for 24 and 48 h of 150 μM ABA treatment respectively (Fig. 5). Data presented as a mean of four independent experiments with corresponding standard deviation.

4. Discussion

Plant expression vector coupled to efficient promoter with enhanced activity is one of the pre-requisites for gene technology based plant modification. Stacking/pyramiding of multiple numbers of genes in a plant cell and their successful expression is crucial for developing a modified plant with distinct attribute/trait. Multiple use of single promoter for simultaneous expression of multiple genes may lead to retarded plant development with altered morphology, reduced productivity, genetic rearrangement/transgene silencing, crop with altered food composition and unpredictable genetic recombination in plants (Cai et al., 2007; Gago et al., 2011). To avoid such unwanted genetic recombination,

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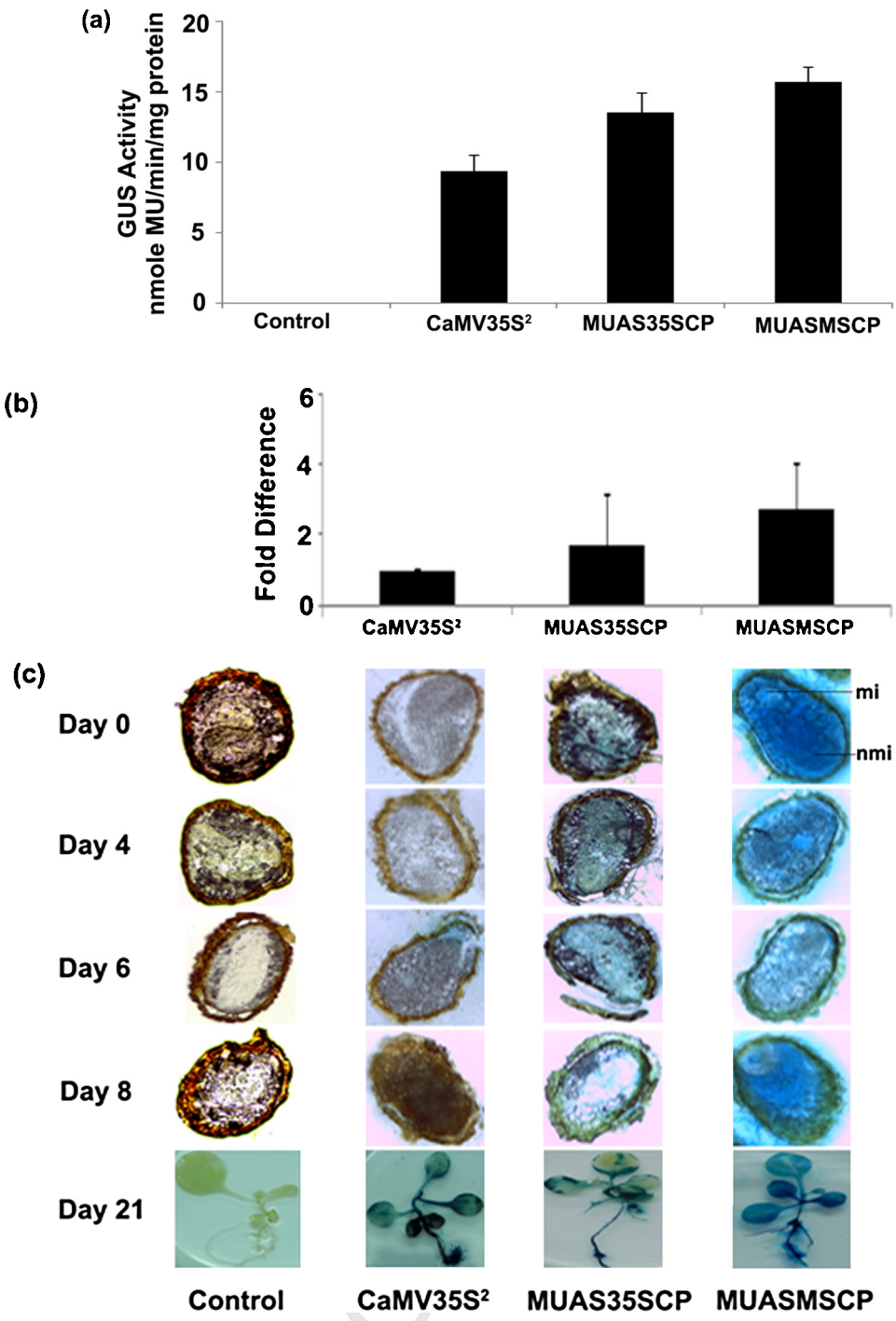

Fig. 3. Transgenic activity analysis of CaMV35S^2, MUAS35SCP and MUASMSCP. (a) Stable transgenic activity analysis of the above promoter construct individually in 21 days old seedlings were performed as described in Section 2. Average GUS activity obtained from four independent experiments for each construct was presented with their corresponding standard deviation. The statistical analysis of the data obtained revealed a *P* value <0.001 implying 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, MUAS35SCP and MUASMSCP promoter considering the accumulation of *GUS*-transcript level under the CaMV35S^2 promoter as 1.0. Average accumulation levels of the GUS transcript detected from three independent experiments for each of the construct with were presented respective standard deviation. (c) Differential expression of the *GUS* reporter gene under control of CaMV35S^2, MUAS35SCP and MUASMSCP promoter constructs during different time-point of seed germination. X-gluc stained 30 μm thick cross-sections of transgenic seeds during 0, 4th, 6th and 8th day post-germination were presented. Light microscopy images of X-gluc treated whole seedlings (21 days) of untransformed control and transgenic tobacco plant expressing CaMV35S^2, MUAS35SCP and MUASMSCP promoters coupled to *GUS* reporter gene were presented at the bottom panel. All promoters showed near constitutive type of expression. mi: micropylar; nmi: non-micropylar regions of endosperm.

use of recombinant promoter/s with altered cis-profiles/cis-arrangements is of high choice. Such 'tailor made promoter' holds better promise for developing modified plant of interest in comparison to the native promoters. Approaches employing exchanging/swapping/shuffling between the proximal and distal domains of native promoter for developing useful recombinant promoters become very popular (Bhullar et al., 2003; Venter, 2007), the basic idea behind developing such approaches lies in the concept that the exchange of specific promoter-domain with respective cis-clustering that bind specific trans-factor/s from one

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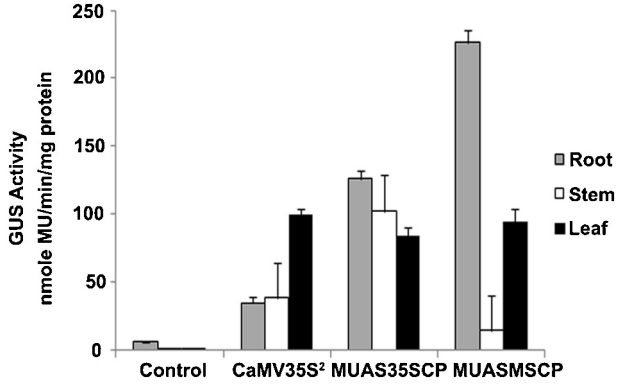

Fig. 4. Spatial distributions of GUS activities under CaMV35S^2, MUAS35SCP and MUASMSCP promoter. GUS activities (in nmole MU/min/mg protein) from the root, leaf and stem of transgenic tobacco seedlings (21 days old) expressing *GUS* under the control of the above promoter constructs were measured presented. Average GUS activity from four independent experiments for each of the promoter construct were presented with corresponding standard deviation. The statistical ANOVA analysis of the data showed a *P* value <0.001 that indicated extremely significant.

promoter into a different promoter containing the TATA sequence might result in a novel regulatory or transcription model. Based on this concept several engineered/modified promoters were developed in recent past (Comai et al., 1990; Lee et al., 2007; Ni et al., 1995; Rushton et al., 2002; Venter, 2007). Our group also developed several useful recombinant plant promoters using both full-length transcript (Flt-) and sub-genomic transcript (Sgt-) promoters from different members of pararetrovirus (Kumar et al., 2011, 2012; Patro et al., 2012b; Ranjan and Dey, 2012). In continuation of our activities towards development of novel chimeric promoter, in the present study we have constructed a inter-molecularly hybridized chimeric 'MUASMSCP' promoter and compared its efficacy with a recently reported chimeric promoter MUAS35SCP from our group (Patro et al., 2012a) and most widely used CaMV35S/CaMV35S^2.

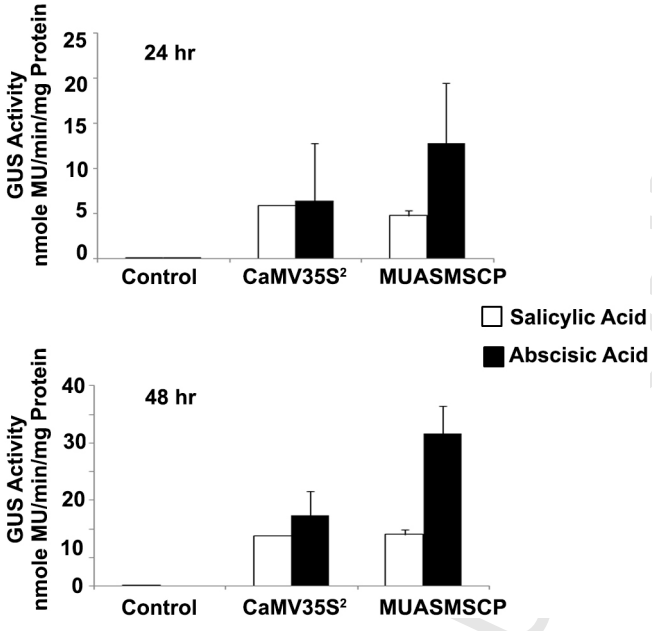

Fig. 5. Expression analysis of GUS activities of CaMV35S^2, MUAS35SCP and MUASMSCP promoter under SA and ABA treatment. GUS activities (in nmole MU/min/mg protein) from whole transgenic tobacco seedlings (21 days old) expressing above promoter constructs under exogenous treatment of 150 μM SA and ABA were measured. Average GUS activity of each promoter construct obtained from four independent experiments were presented along with their respective standard deviation. Data presented showed *P* value less than 0.05 inferring high significance.

Overall, we observed that the MUASMSCP promoter showed stronger activity compared to the activity obtained from CaMV35S promoter while it showed equivalent activity in comparison to the activity of MUAS35SCP and CaMV35S^2. Also, we tested the efficacy of the MUASMSCP promoter coupled to *GFP* and *IL-24*. We have chosen GFP reporter (Chalfie et al., 1994) based on its wide acceptance while we have selected IL-24 as an important pharmaceutical gene of interest that inhibits the growth of a broad-spectrum of cancer cells (Huang et al., 2001; Wolk et al., 2002). We observed, that expression level of protoplast-derived GFP and protoplast-derived IL-24 was enhanced under control of the MUASMSCP promoter compared to the CaMV35S promoter. Likewise, we noted higher accumulation of protoplast derived IL-24 under control of MUASMSCP in comparison to that obtained from MUAS35SCP promoter. Interestingly, the biological activity (cell inhibitory property) of recombinant promoter-driven protoplast-derived IL-24 was confirmed by MTT assay using DU-145 prostate cancer cell line. Our result using 50 ng of protoplast derived IL-24 protein for the assay against cancer cells demonstrates a higher percentage of total soluble IL-24 protein obtained under control of MUASMSCP compared to that obtained from the CaMV35S promoter. This significant result could be due to the unique clustering of important plant specific cis-motifs/elements, viz., TGACG, as-1, DOF, GATA, WRKY, CAAT box in the recombinant MUASMSCP promoter sequence. This salient cis-clustering may have some definite role for enhanced accumulation and biological activity of the MUASMSCP-driven protoplast-derived IL-24 protein. However, an in depth study is required to elucidate on how the MUASMSCP promoter could alter the effectiveness of the protein product.

In transgenic tobacco plant, the MUASMSCP promoter showed equivalent activity compared to the activities from MUAS35SCP and CaMV35S^2 promoter. We observed a good match between the level of GUS accumulation and expression level of corresponding *uidA-mRNA* in transgenic plant. Furthermore, histochemical staining of longitudinal cross-section of germinating seed at different time point (0, 2, 4 and 8 days) and 21 days old seedlings MUASMSCP, MUAS35SCP and CaMV35S^2 are also in accord to our throughout observation. Spatial distribution of GUS accumulation revealed that the MUASMSCP promoter can drive GUS near constitutively in different parts of the plant following the order root > leaf > stem. The higher root expression of MUASMSCP promoter could be due to the presence of multiple copies (at least 3) of TGACG motif (as-1 elements) that confers higher root expression (Benfey et al., 1989). Salicylic acid usually appears as abiotic stress elicitor and differentially stimulated the specific activity of the as-1 elements in root of transgenic plants (Jupin and Chua, 1996; Krawczyk et al., 2002; Niggeweg et al., 2000; Qin et al., 1994). Furthermore, during 24–48 h of SA/ABA treatment we observed 1.8- and 2.1-fold increase in the activity of the MUASMSCP promoter in presence of 150 μM SA and ABA respectively. The influence of exogenous SA on the activity of the MUAS35SCP promoter was studied earlier in detail and reported in the supplementary section of our earlier publication (Patro et al., 2012a).

Taken altogether, we propose that the newly develop the MUASMSCP promoter could be a useful additional tool for gene based modification of plant and has potential to promote sustainable agriculture. It also can serve as an efficient substitute for both CaMV35S and CaMV35S^2 promoter in plant biology.

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