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An alternative method of promoter assessment by confocal laser scanning microscopy

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A B S T R A C T

A rapid and useful method of promoter activity analysis using techniques of confocal laser scanning microscopy (CLSM) is described in the present study. The activities of some pararetroviral promoters such as CaMV35S (*Cauliflower mosaic virus*), FMVSgt3 (*Figwort mosaic virus* sub-genomic transcript) and MMVFLt12 (*Mirabilis mosaic virus* full-length transcript) coupled to GFP (green fluorescent protein) and GUS (β-glucuronidase) reporter genes were determined simultaneously by the CLSM technique and other available conventional methods for reporter gene assay based on relevant biochemical and molecular approaches. Consistent and comparable results obtained by CLSM as well as by other conventional assay methods confirm the effectiveness of the CLSM approach for assessment of promoter activity. Hence the CLSM method can be suggested as an alternative way for promoter analysis on the basis of high throughput.

1. Introduction

Several important promoters have been characterized from viruses belonging to the family *Caulimoviridae* (Medberry et al., 1992; Bhattacharyya-Pakrashi et al., 1993). In general there are two major types of transcriptional promoters present in *Caulimovirus* genome: a full-length transcript promoter (FLt-promoter, equivalent to 35S) and the other one is a sub-genomic transcript promoter (Sgt-promoter; equivalent to 19S). Some *Caulimoviridae* genomes have been fully sequenced and their promoters have been characterized; these include *Cauliflower mosaic virus* (CaMV 35S; Odell et al., 1985; Ow et al., 1987; CaMV 19S; Lawton et al., 1987), *Figwort mosaic virus* (FMVFLt; Maiti et al., 1997; FMV-Sgt; Bhattacharyya et al., 2002), *Peanut chlorotic streak virus* (PClSV; Maiti et al., 1998), *Strawberry vein banding virus* (SVBV; Petrzik et al., 1998; Pattanaik et al., 2004), *Petunia vein clearing virus* (PVCV; Richert-Poggeler and Shepherd, 1997) and *Mirabilis mosaic virus* (MMVFLt; Dey and Maiti, 1999a,b; MMV-Sgt; Dey and Maiti, 2003). Full-length transcript (FLt) promoter of *Cauliflower mosaic virus* (CaMV 35S) is the most widely used promoter in biotechnology. The availability of efficient promoters is still lacking in molecular biology. Several attempts were already made for identifying better promoters using unique approaches (Vaucheret and Fagard, 2001; Bhullar et al., 2003; Rance et al., 2002; Xie et al., 2001; Chaturvedi et al., 2006) and the search for efficient promoters is still continuing to meet the demand for ideal promoters for biotechnological applications.

The expression analysis of promoters is usually carried out by measuring the expression levels of the reporter genes like GUS, GFP, etc., that attached to it, using relevant fluorometric, biochemical and molecular assays. Sometimes, these conventional methods do not appear suitable for analysis of promoter activity in terms of their rapidness and specifically for the accuracy as they are unable to detect the expression level of reporter gene in individual transformed cells. Presently, an alternative substitute for these conventional methods is required for accurate and rapid estimation of GFP and GUS expression levels for analysis of promoter activity.

The present study describes a reproducible laser technology based promoter activity assay with details of image capturing and analysis using confocal laser scanning microscopy (CLSM). CLSM has become a valuable tool in the field of plant biological research during the past decade which is evident from the increasing number of research studies employing confocal microscopy in plant biology (Hepler and Gunning, 1998; Wymer et al., 1999). The confocal microscope functions by exciting the sample with a highly focused beam of laser light and allowing the emitted fluorescent light to enter to a photomultiplier tube (PMT) detector system

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through an adjustable pinhole aperture, and finally the light signal passes to a computer to generate a sharp image with high resolution and minimum background noise. Image processing, and analysis of fluorescence intensity of the captured image(s) can be assessed efficiently using different sophisticated software programs such as the Zeiss LSM 510 Meta Software Package (available in Zeiss LSM 510 Meta Confocal Laser Scanning Microscope), Olympus FluoView FV1000 Softwares Packages (for Olympus FluoView Confocal Laser Scanning Microscope-FV1000) and Leica Application Suit Advanced Fluorescence; Version 1.8.1 (LAS AF; for Leica TCS SP5 Confocal Laser Scanning Microscopy). Precise estimation of fluorescence is possible using CLSM where a fluorescence reporter gene is attached to a promoter. By comparing the emission of fluorescence between different promoters it is also possible to evaluate the promoter's efficiency. CLSM based methods have several advantages over biochemical and molecular approaches: (i) it is simple, rapid and accurate; (ii) it is non-hazardous; (iii) it is independent of chemicals such as substrates and co-factors; and (iv) also independent of physical factors such as temperature, pH.

In the present study, the activities of the 35S promoter of the *Cauliflower mosaic virus* (CaMV 35S; Fang et al., 1989; Benfey et al., 1990; Lam, 1994; Wu et al., 2003), *Figwort mosaic virus* sub-genomic transcript promoter (FMVSgt3; Bhattacharyya et al., 2002) and *Mirabilis mosaic virus* full-length transcript promoter (MMVFLt12; Dey and Maiti, 1999a) were evaluated by the CLSM method, followed by conventional methods using biochemical and molecular approaches in both prokaryotic (bacteria) and eukaryotic (protoplast) systems to establish the efficacy of the CLSM method.

2. Materials and methods

2.1. Materials

Restriction enzymes, RNA isolation kits (Cat# KT79) were purchased from Bangalore Genei (Bangalore, India) and were used according to the manufacturers' instructions. Antibodies for GFP (Cat# IMG-5126A) and β-Actin (Cat# IMG-5142A) were procured from Imgenex (San Diego, CA 92121, USA). Oligonucleotides were synthesized from Ocimum Biosolutions Inc. (Indianapolis, USA). Pre-stained Protein Marker (Cat# P7708S, broad range 6–175 kDa) was obtained from Promega (Madison, WI 53711-5399, USA).

2.2. Construction of expression vectors

The respective promoter fragments CaMV35S (−901 to +1), FMVSgt3 (−270 to +31) and MMVFLt12 (−297 to +63) were PCR amplified using corresponding templates (pBSCaMV 35S, pBSFMVSgt3 and pBSMMVFLt12) and appropriately designed primers to tailor the EcoRI site at 5′- and HindIII site at 3′-ends of the amplified promoter fragments, respectively. Subsequently, each of these promoter fragments was gel-purified and cloned into the protoplast expression vector pUCPMA (Dey and Maiti, 1999a) at EcoRI and HindIII sites to develop pPCaMV35S, pPFMVSgt3 and pPMMVFLt12 plasmids. The GFP gene from pBSGFP as a 5′-XhoI-GFP-SacI-3′ insert was incorporated into the above pUCPMA expression vectors and the resulting plasmids were designated as pPCaMV35SGFP, pPFSgt3GFP and pPMMVFLt12GFP (Set 1, GFP constructs). The GUS reporter gene from pBSGUS was purified as a 5′-XhoI-GUS-SacI-3′ insert and introduced at XhoI and SacI sites of these promoter constructs to generate pPCaMV35SGUS, pPFMVSgt3GUS and pPMMVFLt12GUS (Set 2, GUS constructs).

2.3. Bacterial transformation and slide preparation

E. coli (strain TB-1) was transformed with pPCaMV35SGFP, pPFMVSgt3GFP, pPMMVFLt12GFP (Set 1) and pPCaMV35SGUS, pPFMVSgt3GUS pPMMVFLt12GUS (Set 2) promoter constructs. An aliquot of 2 ml overnight grown transformed bacterial cells from each of the constructs under Set 1 (having GFP as reporter gene) were centrifuged at 3000 × *g* for 5 min and the pellet from individual construct was washed 3 times with PBS, finally suspended in 200 μl of low melting agarose (1% w/v) in PBS at 45 °C and then applied quickly on the slide. The slides were kept in the dark at room temperature for 5 min for cooling before taken for image acquisition under CLSM. Transformed bacteria obtained from each of the constructs under Set 2 (having GUS reporter) were harvested for biochemical determination of GUS activity.

2.4. Protoplast isolation and electroporation

Protoplasts from tobacco cell suspension cultures (Xanthi 'Brad') were purified on 20% sucrose gradient after digestion by cellulose (Sigma Cat# C0615-1 G) and pectinase (Sigma Cat# P4300-5 KU). Protoplasts were electroporated with supercoiled DNA containing GUS and GFP reporter genes by using the GenePulser II Apparatus (BioRad, California, USA) with the Capacitance Extender II (Model 165-2107) as described earlier (Maiti et al., 1998). Briefly, an aliquot of 750 μl containing 2×10^6 protoplasts in an electroporation cuvette (0.4 cm electrode gap) was electroporated (200 V used for charging 965 μF capacitance for 40–50 ms) with 5 μg of each of the following promoter constructs: pPCaMV35SGFP, pPFMVSgt3GFP and pPMMVFLt12GFP (Set 1) and pPCaMV35SGUS, pPFMVSgt3GUS and pPMMVFLt12GUS (Set 2).

After 20 h of incubation, transformed protoplasts obtained by each of the constructs under Set 1 (having GFP reporter gene) were used for estimation of GFP by CLSM, northern analysis and Western blot analysis.

An aliquot (1/10 volume) of transformed protoplasts obtained from constructs under Set 2 (coupled to the GUS reporter) was incubated in 1 mM MUG (4-methyl-umbelliferyl-D-glucuronide) at 37 °C for 30 min to produce MU (7-hydroxy-4-methylcoumarin) for detection of the GUS localization (by blue fluorescence) in protoplasts by CLSM and the rest (9/10 volume) fraction of transformed protoplast was treated for biochemical GUS estimation (Jefferson et al., 1987).

2.5. Image capture and analysis by CLSM method

The fluorescence images of transformed tobacco (Xanthi 'Brad') protoplasts or bacteria were captured with a confocal laser scanning microscope (TCS SP5; Leica Microsystems CMS GmbH, D-68165 Mannheim, Germany) using LAS AF (Leica Application Suite Advanced Fluorescence) 1.8.1 build 1390 software under HCX PL APO lambda blue oil-immersion objective (63.0X/N.A.1.40) with confocal pinhole set at Airy 2 and 2× zoom factor for improved resolution with eight bits.

For exciting the expressed GFP in protoplasts/bacteria, argon laser (40%) with AOTF for 488 nm (at 30%) was used (Jakobs et al., 2000; Zimmer, 2002; Bongaerts et al., 2002) and the fluorescence emissions were collected between 501 and 598 nm with photomultiplier tube (PMT) detector gain set at 1150 V. Fluorescent images were captured after passage through double dichroite DD 488/561 and to optimize the image quality, the offset was adjusted for a maximum range of fluorescence from 0 to 255 (50% green pixels). After image acquisition, GFP fluorescence intensity was quantified by using LAS AF Software, according to the instructions provided by Leica Microsystems (Mannheim, Germany).

For estimating GUS, after MUG incubation the protoplasts (electroporated with Set 2 constructs) were excited with 405 diode laser and fluorescence emissions were collected between 440 and 480 nm with detector (PMT) gain set at 1200 V. After image acquisition, MU fluorescence intensity (for GUS) was quantified by using