RESEARCH PAPER

EXPRESSION OF *SUGARCANE STREAK MOSAIC VIRUS* (SCSMV) COAT PROTEIN IN EXPRESSION VECTOR AS A FUSION PROTEIN WITH MALTOSE BINDING PROTEIN

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Abstract

The occurrence of Sugarcane streak mosaic virus (SCSMV), characterized as 'Susmovirus', a Potyviridae member, was recently reported in India. The virus was identified as one of the causative agents of mosaic in sugarcane along with Sugarcane mosaic virus (SCMV) either alone or in combination. Molecular diagnostic techniques were developed to detect both the viruses together in sugarcane and further studies were carried out to express the coat protein (CP) gene of SCMSV in E. coli system to produce recombinant antiserum. The complete SCSMVcp gene from mosaic infected sugarcane cv. Co 86032 was cloned using the primers SCSMV-F690 and SCSMV-R with BamHI and HindIII restriction sites. The viral protein was expressed as maltose binding protein (MBP)-SCSMVcp fusion protein in the expression vector pMAL c4x. The expressed fusion protein was purified using amylose resin column and eluted. Separation of purified fusion protein in 12% SDS-PAGE and subsequent Western blot with anti-MBP antiserum confirmed the authenticity of the expressed protein. Conventional virus purification is cumbersome, requires sophisticated instrumentation and does not eliminate other viruses and host protein contamination. The new recombinant expression of viral protein in in-vitro system has set a new platform to get large quantities of viral protein devoid of host contaminants. Further studies on serum production against the fusion protein and its validation are in progress.

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Introduction

Sugarcane mosaic caused by *Sugarcane mosaic virus* (SCMV) is nearly a couple of centuries old and one of the most important viral diseases of sugarcane (Koike and Gillaspie, 1989). It occurs worldwide in 70 countries (Grisham, 2000), including India where its presence was first noticed by Barber (Barber 1921). However, in the late 1990s, another *Potyviridae* member called as *Sugarcane streak mosaic virus* (SCSMV) was identified from a quarantine sample imported from Pakistan to USA that caused mosaic in sugarcane and the virus isolate was named as SCSMV-PAK (Hall et al. 1998). Earlier the virus associated in this sample was reported as a distinct strain of SCMV and named as SCMV-F (Gillaspie et al. 1978). Subsequently, the virus was reported from India (Hema et al. 1999). We have carried out detailed studies on the occurrence of SCMV and SCSMV in India and established that the latter belongs to a new genus 'Susmovirus' and the mosaic disease is caused by either SCMV or SCSMV or both (Viswanathan et al. 2007; 2008a). Reports from other countries in Asia, except China, too revealed that mosaic of sugarcane is effected either by SCMV or SCSMV or by both (Chatenet et al. 2005; Gaur et al. 2003; James et al. 2006; Rao et al. 2006; Thomson et al. 2010). However, recently SCSMV was also detected from germplasm accession imported from India to China. Outside the Asian continent, SCSMV was also reported to occur in germplasm collections in Colombia (Cardona et al. 2006).

Being a clonally propagated crop, sugarcane is prone to viral infection which is inherently carried over to the

next generation. Therefore, viral screening technique plays an important role in preventing the entry or spread of the pathogen through germplasm exchange and infected seed cane. At this juncture, it is valuable to establish a rapid, convenient and reliable method of viral detection. A reverse transcription (RT)-PCR protocol was standardized to detect SCSMV (Hall *et al*., 1998) which was subsequently used in India and other countries (Hema et al. 1999, 2002; Chatenet et al. 2005; Viswanathan et al. 2007; Singh et al. 2009). Viswanathan et al. (2008b) developed a duplex-RT-PCR to detect SCMV and SCSMV from sugarcane.

Currently, serological and RT-PCR based techniques are being used to detect the viruses associated with sugarcane germplasm, quarantine and seedlings. Though RT-PCR based detection system is superior to its serological counterparts, sole dependence on the expensive RT-PCR technique for routine screening is not feasible in all the laboratories. Therefore, it is advisable to carry out the initial screening by serological techniques and follow RT-PCR technique for confirmation of doubtful, negative and valuable samples. Screening by serological techniques needs specific and quality antiserum which relies on the quality of purified virions or protein.

Since both SCSMV and SCMV produce similar type of symptoms and causative viruses can not be distinguished based on visible symptoms, purified virion preparation from field samples is not suitable to produce specific antisera against the target virus. Also, virus purification from the host favours contamination from host proteins which leads to lack of specificity and often gives variable background reactions, thus limiting their use in ELISA for diagnosis of virus infection. Thus, the quality of the polyclonal antisera produced against a virus depends on the purity of virus preparation used for immunization. Therefore, production of antibodies to recombinant coat protein, with the potential to work in all serological tests with no background reaction, will be useful in diagnosis. Such high quality antisera would be useful for routine screening by ELISA and developing antibody based PCR assays. Hence, an attempt was made to express the coat protein (CP) gene of SCSMV in *E. coli* system to produce recombinant antiserum against the target protein and the same is reported in this paper.

Materials and Methods

Cloning of SCSMVcp in to expression vector, pMAL c4x

The cDNA fragment encoding the full length CP gene (849bp) of SCSMV was amplified by RT-PCR using gene specific primers *viz*., the sense primer, SCSMV-F690(5'-GGATCCGGACAAGGAA CGCAGCCAC -3') with an *EcoR*I restriction site (underlined) and the antisense primer, SCSMV-R (5'-AAGCTTTCAGTGCTGAGCACGCCCAAACTG-3') with a *Hind*III restriction site (underlined). The PCR reaction was performed in a total volume of 25 µl containing 2 µl cDNA, 2.5 µl of 10x PCR buffer containing 15mM $MgCl_2$, 0.5 µl of 10mM dNTP mix, 20 pmol each of the primers, 1.25 units of *Taq* polymerase (Intron, South Korea), and sterile milliQ water to a final volume. The PCR reaction was performed with initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 1 min and a final extension of 72°C for 10 min in a PCR machine. A 10 µl aliquot of each amplified product was analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide.

The amplicon was purified from the low melting agarose gel (Sigma, USA) using GenElute Gel Extraction Kit (Sigma, USA). The purified amplicon was ligated with pTZ57R/T and then transformed in to the competent cells of *E. coli* K12 strain using InsTA clone™ PCR Cloning Kit (MBI Fermentas, USA). The colony containing recombinant plasmid was confirmed by performing colony PCR and the recombinant plasmid pTZ57R/T-SCSMVcp was purified using GenElute™ Plasmid Miniprep Kit (Sigma, USA). Both the recombinant plasmid i.e. pTZ57R/T-SCSMVcp and expression vector pMAL c4x (New England Biolabs, UK) were double digested with *EcoR*I and *Hind*III restriction enzymes. The digested pMAL c4x and SCSMVcp were purified from low melting agarose gel, quantified, mixed in 1:2 ratio and then ligated using T4 DNA ligase to yield recombinant pMAL c4x-SCSMVcp plasmid. The recombinant plasmid was then transformed into *E. coli* K12. The transformed colonies were screened by colony PCR and restriction digestion. About 20 PCR positive colonies were randomly chosen and back streaked. The recombinant plasmid extracted from three randomly

chosen colonies was checked for its reading frame by sequencing the 5′ end of the insert using *mal*E primer and the 3′ junction using pUC/M13 primers.

Expression and purification of MBP-SCSMVcp fusion protein in *E. coli*

The protocol recommended for pMAL Protein Fusion and Purification System (New England Biolabs, UK) was followed with certain modifications. In brief, 10 ml of an overnight culture of cells containing the pMAL c4x-SCSMVcp fusion plasmid was inoculated in to 1000 ml of rich broth (10g tryptone, 5g yeast extract, 5g NaCl, 2g glucose and ampicillin to a final concentration of 100 ìg/ml). The culture was grown at 37°C with good aeration until the OD reaches ~0.5 at A_{600}. Later, IPTG was added to the culture to a final concentration of 0.3mM and incubated under constant shaking at 37°C for 5 h. After induction, 1 ml aliquots of the culture were withdrawn till 5 h, at hourly intervals. The aliquot taken before adding IPTG served as uninduced control. Additionally 1 ml of non-transformed *E. coli* grown on the same medium without antibiotics was also taken as an untransformed control. All the aliquots were centrifuged for 2 min and the cell pellet was re-suspended in 50 ml SDS-PAGE sample buffer, mixed gently by pipette tip and stored at -20°C.

After 5 h of incubation, the cells from the remaining culture were harvested by centrifugation at 4000*g* for 10 min at 4°C. The supernatant was discarded and the cell pellets were resuspended in 100 ml of Column buffer (20 mM Tris-HCl, 200mM NaCl, 1mM EDTA, 1mM sodium azide, 10 mM â- mercaptoethanol) and frozen overnight at –20°C. The cells were then thawed in cold water and mixed briefly by pipetting up and down to enhance the cells to lyse and release the proteins, taking care to avoid foaming while mixing. The cell lysate was centrifuged at 9,000*g* and 4°C for 20 min. After centrifugation, the supernatant, termed as crude extract hereafter, was transferred to a fresh tube.

In a 2 ml microfuge tube, ~400 ìl of amylose resin (New England Biolabs, UK) was taken and spun briefly in a refrigerated centrifuge and the pellet was washed in 1 ml column buffer thrice by re-suspension and pelleting. After the third wash, the resin was resuspended in 400 ìl column buffer and to that 400 ìl of crude extract was added, mixed, incubated for 15 min on ice with gentle mixing once in 5 min and centrifuged at 5000g for 1 min at 4°C. After removing the supernatant, the resin was washed with 1.0 ml of column buffer 10 times by resuspending and centrifugation as in the above step. After washing, the resin was resuspended in 400µl of column buffer and loaded in 1.5 ml filter column and the column buffer decanted by centrifugation. Finally, the bound MBP-SCSMVcp protein was eluted three times with 100µl of ice cold maltose elution buffer (20 mM Tris-HCl, 200mM NaCl, 1 mM EDTA, 1mM sodium azide, 10 mM â-mercaptoethanol and 5mM Maltose). Fifteen µl aliquots of each eluate was analysed on a 12% SDS-PAGE along with 20 µl of crude extracts from uninduced, induced and untransformed control samples. Authenticity of the recombinant protein was later confirmed by Western blot analysis with anti-MBP antiserum (New England Biolabs, UK). The protein resolved on 12% SDS-PAGE was transferred to PVDF membrane using semidry transblot apparatus (Bio-Rad, USA). The proteins on the membrane were probed with antibodies raised against MBP-SCSMVcp fusion protein followed by detection with alkaline phosphatase conjugated goat anti-rabbit antibody at 1:50,000 dilutions (Sigma, USA). The membrane blots were developed using the BCIP (Bromo chloro indolyl phosphate)/NBT (Nitroblue tetrazolium) colorimetric system (Bangalore Genei, India).

Results and Discussion

The SCSMVcp gene from mosaic infected sugarcane cv. Co 86032 was cloned in pTZ57R/T vector using the primers SCSMV-F690 and SCSMV-R. Subsequently, the SCSMVcp gene was released by digesting the pTZ57R/T-SCSMVcp plasmid construct with *BamH*I and *Hind*III and the released fragment was sub-cloned in to similarly digested expression vector pMAL-c2x downstream to the malE gene, which encodes maltose binding protein. The resulting plasmid construct was designated as pMAL-c2x-SCSMVcp. Colony PCR confirmed the presence of SCSMVcp in the transformed colonies. After induction in the expression medium, the optimum cell density of 0.5 – 0.6 at OD_{600} was reached in 45-60 min at 37°C. The induction of expression study revealed that the fusion protein expression level has increased after 1 h and reached the maximum by 5 h of post-induction with 1 mM IPTG (Fig. 1). Subsequently, large scale experiment was carried out by growing cells

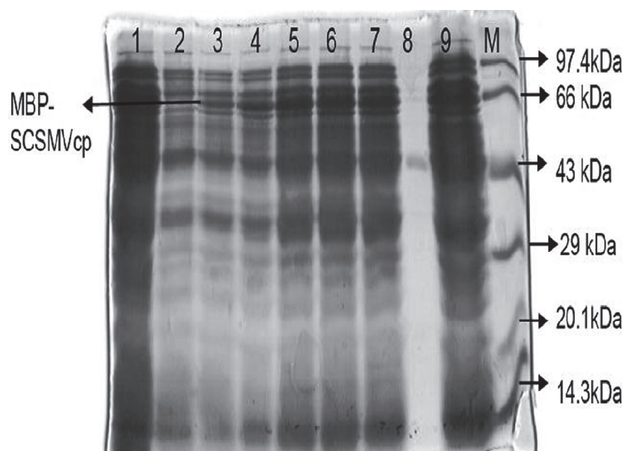

Fig. 1 SDS-PAGE showing the separation of E. coli total cell lysate expressing MBP-SCSMVcp fusion protein. Lanes 1. Total protein of nontransformant; 2.Total protein of transformant before induction; 3-6. Total protein of transformant at 1, 2, 3 and 4 hr after induction, respectively; 7&9. Total protein of transformant at 5 hr after induction; 8. Maltose binding protein; M. Standard molecular weight marker

in 1 liter of LB with IPTG induction and the cells were harvested after 5 h. The MBP-SCSMVcp fusion protein was purified using amylose resin and eluted in four fractions, of which maximum protein was eluted in the

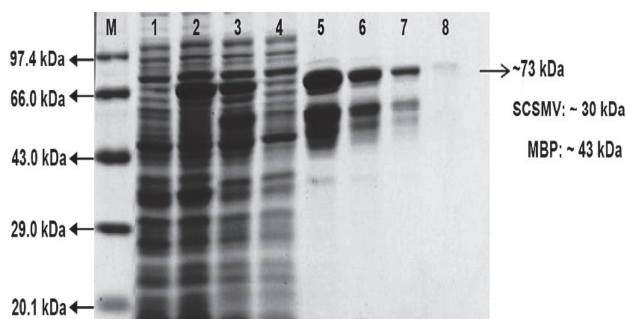

Fig. 2. SDS-PAGE of fractions from the purification of MBP-SCSMVcp fusion protein. Lanes M. Standard molecular weight marker; 1.Total cell lysate from uninduced cells; 2.MBP-SCSMVcp fragment in total cell lysate from induced cells (3 hr after induction); 3. MBP-SCSMVcp fragment in crude extracts from induced cells (3 hr after induction); 4. MBP-SCSMVcp fragment in flow through from amylase column; 5-8. purified protein eluted from amylose column with maltose, elution 1, 2, 3 and 4 respectively.

first two fractions (Fig. 2). Separation of purified fusion protein in 12% SDS-PAGE and subsequent Western blot with anti-MBP antiserum confirmed the authenticity of the expressed protein (Fig. 3). Separation of ~73 kDa indicated the induction of MBP-SCSMVcp fusion protein (MBP: ~43 kDa; SCSMVcp: ~30 kDa). Further,

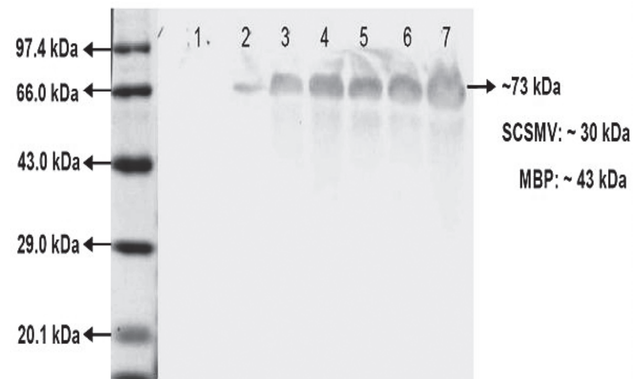

Fig. 3. Western blot showing the confirmation of MBP-SCSMVcp fusion protein immobilized using anti-MBP antibody. Lanes 1. Total protein from nontransformant; 2. Total protein from uninduced transformant; 3-7. Purified MBP-SCSMVcp fusion protein from transformant, 1, 2, 3, 4 and 5 hr after induction, respectively.

the presence of the SCSMVcp in the fusion protein was confirmed by Factor Xa cleavage (Results not shown).

Incidence of sugarcane mosaic is almost 100% in India and considering the vast area under sugarcane cultivation it results in significant yield losses (Agnihotri 1996; Singh et al. 2003; Viswanathan and Balamuralikrishnan 2005). The yield loss due to mosaic is more pronounced as the virus/viral strains persist in infected cane generation after generation which leads to decline in cane yield and sucrose content (Koike and Gillaspie, 1989); ultimately the affected cultivars are removed from commercial cultivation (Singh et al. 1997). According to Viswanathan and Balamuralikrishnan (2005), varietal degeneration due to mosaic is a serious concern that results in near extinction of elite cultivars from cultivation as witnessed recently with the erstwhile ruling varieties such as Co 740 and CoC 671 in the tropical region. The phenomenon was also reported in the subtropical regions of the country in varieties CoLk 8102, CoPant 90223 and CoS 767 (Singh et al. 2003). The impact of mosaic on sugarcane was not established under Indian conditions probably due to more severe constraints like red rot, smut or wilt affecting sugarcane production in different periods of time. Also, precise diagnostic techniques were not available to researchers a few decades earlier. Viswanathan (1997) reported diagnosis of SCMV by ELISA and subsequently studies on SCMV detection and characterization of the viruses associated with mosaic were continued (Balamuralikrishnan et al. 2004; Viswanathan et al. 2007; 2008a,b; 2009).

Generally, viruses are not amenable for management by conventional methods. However, they can be best managed by planting disease free seed cane or tissue culture raised plants whose disease-free status can be ensured by employing precise diagnostic techniques. RT-PCR is cost prohibitive for mass screening as compared to ELISA, which is relatively inexpensive but requires large quantities of antisera. Conventional virus purification is also tedious, requires high-cost instrumentation and suffers from host protein contamination. Nowadays, recombinant expression of virus CP in *E. coli* is the most preferred method to get large quantities of viral protein devoid of host contaminants. In the present study, the amplified SCSMVcp gene was cloned in to the pMAL-c4x expression vector and expressed as a fusion protein containing maltose binding protein in *E. coli*. The MBP-SCSMVcp fusion protein was purified using amylase resin column. The western blot analysis with antiMBP antiserum confirmed the expression of MBP-SCSMVcp fusion protein with a molecular weight of 73 kDa. ~20 ml of high titre polyclonal antiserum was produced against 4 mg of fusion protein in further studies (Viswanathan, unpublished). Although, RT-PCR was found to be more efficient than ELISA, it is not affordable for large scale screening, considering the cost of the testing procedure. Certainly ELISA can be used for large scale screening and those samples found negative and doubtful in this assay may be tested using RT-PCR. Earlier, this virus has been detected using DAC-ELISA, DAS-ELISA, DBIA and IC-RT-PCR (Hema et al. 2003a, b; Bharathi and Reddy 2007). Thus the expression of SCSMVcp and polyclonal antiserum production will serve as an important resource for efficient SCSMV indexing of sugarcane tissue culture and mother plants, and also for routine screening of germplasm and quarantine materials. The large collection of germplasm material maintained at our Institute requires quarantining and indexing for viruses. Also, Plant Pathology lab at this institute is an accredited test lab for virus indexing of sugarcane tissue culture seedlings. Expression of coat protein and production of specific antiserum against the expressed virus proteins further strengthen virus diagnosis work in the country. Further work is in progress to validate the serum against the antigen from field infected samples and its suitability for immuno capture-RT-PCR.

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