is also valid as most pathogenic bacteria control the expression of their virulence factors rather tightly.

The data presented here reveal a novel aspect. The TlyA that is devoid of rRNA methylation activity (contributed to evolution of second-line antibiotic resistance) can still reach the cell wall of the bacterium and aid the survival strategies. This indirectly suggests that the cell-wall association is not dependent on the rRNA methylation activity. Based on these observations, we have a valid speculation that TlyA could be a virulence factor for the bacterium. For example, infection of THP-1 macrophages with of *Mycobacterium avium* subsp. *paratuberculosis* has resulted in 5-fold upregulation of its *tlyA* gene (protein ID: MAP_1401), which has over 80% identity with the *Mtb* protein studied here. We have predicted the possibility of TlyA acting as a virulence factor post-infection. *Mycobacterium avium* subsp. *paratuberculosis* is a gut-associated pathogen of humans and linked to Crohn's disease. This observation also suggests that regulation of gene expression of mycobacterial species may be linked to their environment. Future studies that focus on the cellular location and quantitation of the membrane proteins under a variety of environments might reveal new clues to the establishment of tuberculosis disease. We also believe that the *E. coli* model employed by us may not be perfect, but is reasonable enough to understand the function of TlyA *per se*, and also such proteins of other bacteria in the absence of the host bacterium's milieu.

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Quantification of *Sugarcane yellow leaf virus* in *in vitro* plantlets and asymptomatic plants of sugarcane by RT-qPCR

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Yellow leaf caused by *Sugarcane yellow leaf virus* (SCYLV) is a serious viral disease affecting production and productivity in many ruling sugarcane varieties in India. Usually the characteristic disease symptoms appear during maturity phases of the crop; also many of the infected varieties do not exhibit disease symptoms and disease expression is influenced by virus titre and other factors, including the prevailing climate. The present study was taken up to quantify and

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compare SCYLV in meristem-derived tissue culture raised *in vitro* plantlets and asymptomatic sugarcane plants in RT-qPCR by relative standard curve method. The standard curve was prepared with serial dilutions of plasmid standards from 3×10^7 to 3×10^3 copy numbers of virus target gene. In this assay, copy number of virus population in *in vitro* plantlets and asymptomatic plants was estimated from 20,314.58 to 4,330.87 and from 8.96 to 0.27 million copies of viruses respectively. Relative expression level of the virus between *in vitro* plantlets and asymptomatic plants was in the ratio 73.7 : 243,393.1 based on $2^{-(\Delta\Delta Ct)}$. The results clearly established that meristem-derived tissue culture significantly reduced SCYLV population and it is concluded that the relative standard curve method efficiently detects the copy numbers of the target virus in different sugarcane samples.

Keywords: Asymptomatic plants, *in vitro* plantlets, *Sugarcane yellow leaf virus*, viral titre.

YELLOW leaf (YL) of sugarcane caused by the *Sugarcane yellow leaf virus* (SCYLV) belonging to the genus *Polerovirus*, family Luteoviridae was first reported in Hawaii in the late 1980s (refs 1, 2). In India it was reported during 1999 (ref. 3). SCYLV is an emerging virus evolved from recombination of the ancestors in the three genera *Luteovirus, Polerovirus* and *Enamovirus*[4–6]. SCYLV has a positive-sense ss RNA genome containing six overlapping open reading frames (ORFs) (ORF 0–ORF 5) and three untranslated regions (UTRs) consisting of ~5.8 kb nucleotides[6,7]. The characteristic symptoms of the disease include intense yellowing of midribs on the abaxial surface, lateral spread of yellow discolouration to the leaf lamina followed by tissue necrosis from the leaf tip spreading downwards along the midrib and a bushy appearance of the top of the plant due to internode shortening in maturing plants. However, there are reports that midrib yellowing may be related to other biotic or abiotic factors such as stress conditions, insect damage, waterlogging and cool winters[8–10]. Long-range SCYLV transmission is achieved through infected seed canes and secondary spread in the field is mediated through aphids *Melanaphis sacchari*, *Rhopalosiphum maidis* and *R. rufiabdominalis*[11–14]. Commercial fields planted with susceptible cultivars have resulted in 100% YL incidence in different countries. The disease causes loss to cane production and sugar yield. In India, a serious threat to sugarcane cultivation is caused by this disease and it adversely affects some of the ruling varieties[15–18].

Diagnosis of SCYLV has been reported with serological methods like tissue blot immune assay (TBIA), DAS-ELISA[19], RT-PCR[20] and recently with real-time PCR[21]. Conventional RT-PCR assays have proved to be predictors for presence of the virus in plants than serological methods due to certain drawbacks such as limited availability of antisera and lack of sensitivity in serological methods[22,23]. Earlier, we had established the occurrence of three genotypes of SCYLV in India[17]. Recently, we have established complete genome of SCYLV-IND genotype from India[24]. Further, the disease has attained epidemic status in the country and the situation warranted management approaches to sustain sugarcane productivity. Hence establishing disease-free healthy nurseries is suggested to the sugar industries to manage the disease and the strategy has found success in different states (R. Viswanathan, unpublished). Recently, elimination of the virus through meristem culture combined with molecular diagnosis was demonstrated[18]. We are currently using RT-PCR to diagnose SCYLV in tissue culture-derived plantlets and in germplasm varieties[25]. However, this technique is not appropriate to assess virus load in tissue culture-derived plants and to relate disease severity with virus titre. Hence quantitative RT-PCR (RT-qPCR) was standardized and reported. Also relative standard curve method has been standardized in the present study in India for sugarcane plants in real-time PCR to find the absolute quantity of virus population in asymptomatic and tissue culture-raised *in vitro* plantlets of sugarcane.

In vitro plantlets (meristem-derived) and asymptomatic plants of popular sugarcane cv. Co 86032 in India were used to assess viral titre. *In vitro* plantlets were derived from 0.3 mm apical meristem of Co 86032 infected with SCYLV and grown in MS medium. Twenty days after growth initiation, leaf samples were taken from the *in vitro* plantlets. Field asymptomatic samples were drawn from 200-day-old plants of the same cultivar.

Earlier, all the samples were tested in RT-PCR for the confirmation of viruses. Total RNA was extracted from leaf samples using TRI reagent (Sigma, USA) following the manufacturer's protocol. The extracted RNA was suspended in a final volume of 40 µl sterile milli Q water and stored at –20°C. After checking the quality of RNA in 1.5% agarose gel, concentration of RNA was measured in NanoDrop 2000C (Thermo Scientific, USA). RNA (1 µg) was reverse transcribed to double-stranded cDNA using RevertAid H minus First Strand cDNA Synthesis Kit (MBI Fermentas, USA), primed with 50 pmol SCYLV-EXPR following the manufacturer's protocol (MBI, Fermentas, USA) in an Eppendorf PCR mastercycler gradient (Germany). The primers SCYLV-EXPF (AGAATTCTCTAGAATGAATACGGGCGCTAACCG) and SCYLV-EXPR (GAATTCTCTAGATTAGTGATGGTGATGGTGATGTGATCGGCAGACG AATTGTCCTG) targeting partial ORF3 and ORF4 were used to amplify 580 nt of the genome to diagnose SCYLV in the samples. PCR was performed with 2 µl double-stranded cDNA, 2.5 µl 10X PCR buffer containing 15 mM $MgCl_2$, 0.5 µl 10 mM dNTP mix, 10 pmol each of forward and reverse primers, 1 unit of *Taq* polymerase (MERCK, India), and sterile milliQ water to the final volume of 25 µl. The PCR programme for the diagnosis of SCYLV consisted of an initial denaturation at 94°C for 4 min, 38 cycles of

94°C for 1 min, 65°C for 1 min, 72°C for 1 min with a final 72°C extension for 10 min (Mastercycle gradient, Eppendorf, Germany). Amplicons were visualized by electrophoresis on 1.6% agarose gels stained with ethidium bromide.

For real-time RT-qPCR assay, 300 ng of RNA was measured using NanoDrop 2000C (Thermo Scientific, USA) and reverse transcribed to double-stranded cDNA using Revert Aid H minus First Strand cDNA Synthesis Kit, primed with 40 nmol of SCYLV-R613 (AGATCT-GTGTTGGGGRAGCGTCGCYTACC) following the manufacturer's protocol (MBI, Fermentas, USA) in a thermocycler (Mastercycle gradient, Eppendorf, Germany). Real-time quantitative PCR was performed with three replications of each sample with 700 ng of double-stranded cDNA template measured with NanoDrop 2000C (Thermo Scientific, USA), 12.5 µl SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma, India) and 40 nM YLS RT FOR-N (GGACCGAACCTATCTCAGTAC) and YLS RT REV-N (TAGTAATCTTGGAGCCTGTTG-TTG) primer in a 25 µl reaction volume in StepOnePlus Real-Time PCR System (Applied Biosystems, USA). PCR conditions were 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. 25SrRNA was used as an internal control and amplified using 40 nM of 25SrRNA RT-F (GGCAGCCAAGCGTTCATAGC) and 25SrRNA RT-R (GGGTAAAACTAACCTGTCTCACGAC) primers. Melt curve analysis was performed at 60°C to assure that a homogenous amplification product was produced. Leaf sample from tissue culture-raised virus-free plant of sugarcane cv. Co 86032 was used as a negative control and symptomatic leaf from another cv. Co 86010 was used as a positive control.

pTZ57R/T plasmid vector (2886 nt) ligated with SCYLV coat protein (580 nt) of SCYLV isolate Co 85019 was used to prepare the standards. Plasmid copy numbers were calculated using on-line calculator provided by URI Genomics and Sequencing Centre, whereas the plasmid copy numbers were calculated using the formula – number of copies = (amount(ng) * 6.022×10^{23})/(length(nt) * 1×10^9 * 650). Based on the plasmid copy numbers, five different serial dilutions (1 : 10-fold) were prepared with 3×10^7 to 3×10^3 target copies and an initial plasmid concentration of 700 ng. Based on the cycle threshold (Ct) and log template concentration of the serial dilutions, a standard curve was obtained. Linear correlation and coefficient for the standard curve were estimated. Using slope and intercept, accurate copy numbers of SCYLV present in the sample were assessed for the template concentration of 700 ng. The comparative copies of viral target were assessed as the mean of three replicates based on the relative quantification (RQ) method. ΔCt was derived by subtracting Ct of internal control (25SrRNA) from Ct of test samples (SCYLV). ΔΔCt was derived by subtracting ΔCt (negative control) from ΔCT of samples. The RQ between *in vitro* plantlets and asymptomatic field plants was measured using the formula $2^{-(\Delta\Delta CT)}$.

RT-PCR has shown greater titre in all the four asymptomatic field samples. In case of tissue culture-raised plants, three of them were negative, although the remaining three (TC2, TC3 and TC4) were obtained with a mild positive amplification (Figure 1). In RT-qPCR, Ct ranges were optimized between 15 and 35 with 40 nmol of YLS RT FOR-N and YLS RT REV-N primers. 700 ng of cDNA from samples and 700 ng of initial plasmid concentration in five different (1 : 10-fold) serial dilutions were optimized. In RT-qPCR, three positive *in vitro* plantlets had late Ct ranges of 32.06, 32.49 and 34.19, whereas the remaining negative samples had Ct values higher than 37. All the field samples with high virus titre had different Ct ranges of 20.87, 23.76, 19.54 and 27.54. Positive control had threshold cycle in 21.08, whereas no amplification was observed in negative control (Table 1). ΔCt and ΔΔCt values of all the plant samples were assessed and relative quantification between *in vitro* plantlets and asymptomatic field plants was found to be 73.7 : 243,393.1 using ΔΔCt values (Table 1).

Positive amplification confirmed the presence of SCYLV coat protein in serial dilution of plasmid standards (Figure 2 *a*). Plasmid standards prepared with five different serial dilutions (3×10^7, 3×10^6, 3×10^5, 3×10^4 and 3×10^3 copies) reached their respective cycle thresholds of 18.96, 21.68, 26.54, 31.67 and 34.68 (Table 1) in RT-qPCR. Using log scale of standard template concentration and their Ct ranges, standard curve was plotted. Linear correlation and coefficient were obtained using slope and intercept as $y = 4.123x + 18.5$ and $R^2 = 0.991$ respectively (Figure 2 *b*). Exact numbers of SCYLV copies present in the *in vitro* plantlets and asymptomatic field plants were obtained from the standard curve (Figure 2 *c*). The number of SCYLV copies for the Ct ranges of samples in *in vitro* plants with late positive amplifications in RT-qPCR ranged from 4330.87 to 20,314.58. *In vitro* plantlets with negative amplification

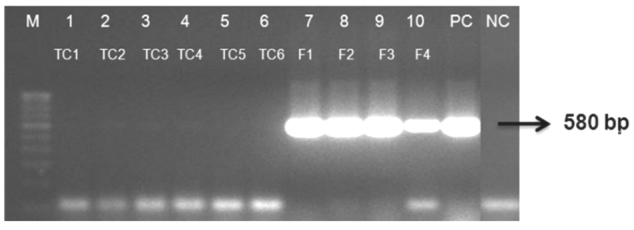

Figure 1. Amplification of *Sugarcane yellow leaf virus* (SCYLV) with SCYLV EXPF and SCYLV EXPR primers from tissue culture-derived plantlets and asymptomatic field plants of sugarcane cv. Co 86032. Three of the tissue culture (TC) raised plantlets (TC2–TC4) showed mild amplification and bright amplification was noticed in all the field samples. M, 100 bp marker; lanes 1–6, Tissue culture plantlets (TC1–TC6); lanes 7–10, Asymptomatic field plants (F1–F4); PC, Positive control (Co 86010); NC, Negative control (Co 86032) plantlet derived from meristem culture.

Table 1. Relative level of *Sugarcane yellow leaf virus* (SCYLV) coat protein expression between *in vitro* plantlets and asymptomatic field samples of sugarcane in qRT-PCR. Ct mean and the corresponding copy numbers of SCYLV target genes obtained in plasmid standards, *in vitro* plantlets and asymptomatic field samples of sugarcane using relative standard curve method of qRT-PCR.

Variety (Co 86032)	Ct mean	Ct mean (25S rRNA)	ΔCt	ΔΔCt	Relative level ($2^{-(\Delta\Delta Ct)}$)	No of target genes
TC1	38.52 ± 0.52	16.32 ± 0.12	22.20	–0.10	1.07	412.05 ± 98
TC2	34.19 ± 0.64	16.54 ± 0.22	17.65	–4.65	25.04	4,330.87 ± 245
TC3	32.06 ± 0.84	16.79 ± 0.14	15.27	–7.03	131.04	20,314.58 ± 945
TC4	32.49 ± 0.19	16.22 ± 0.09	16.27	–6.03	65.22	19,413.58 ± 897
TC5	37.04 ± 0.42	16.98 ± 0.27	20.06	–2.24	4.72	729.69 ± 59
TC6	37.52 ± 0.39	16.09 ± 0.24	21.43	–0.87	1.83	714.81 ± 84
F1	20.87 ± 0.84	16.82 ± 0.12	4.05	–18.25	311,995.35	6,370,414.00 ± 956
F2	23.76 ± 0.29	16.05 ± 0.21	7.71	–14.59	24,696.87	2,229,140.09 ± 1058
F3	19.54 ± 0.74	16.51 ± 0.24	3.03	–19.27	632,639.79	8,961,475.25 ± 1279
F4	27.04 ± 0.92	16.79 ± 0.19	10.25	–12.05	4,240.45	278,086.00 ± 687
PC (Co 86010)	21.08 ± 0.62	16.04 ± 0.14	5.04	–17.26	156,845.24	6,082,227.66 ± 598
NC (Co 86032)	38.59 ± 0.41	16.29 ± 0.20	22.30	0.00	1.00	378.21 ± 28
Plasmid standards (1 : 10-fold dilution)						
1	18.96 ± 0.24	–	–	–	–	3×10^7
2	21.68 ± 0.54	–	–	–	–	3×10^6
3	26.54 ± 0.67	–	–	–	–	3×10^5
4	31.67 ± 0.34	–	–	–	–	3×10^4
5	34.68 ± 0.47	–	–	–	–	3×10^3

TC, Tissue culture raised plants: F, Asymptomatic field plants (200 days): PC, Positive control (180 days): NC, Negative control. Relative level of SCYLV, coat protein in TC and field samples – 73.7 : 243,393.1.

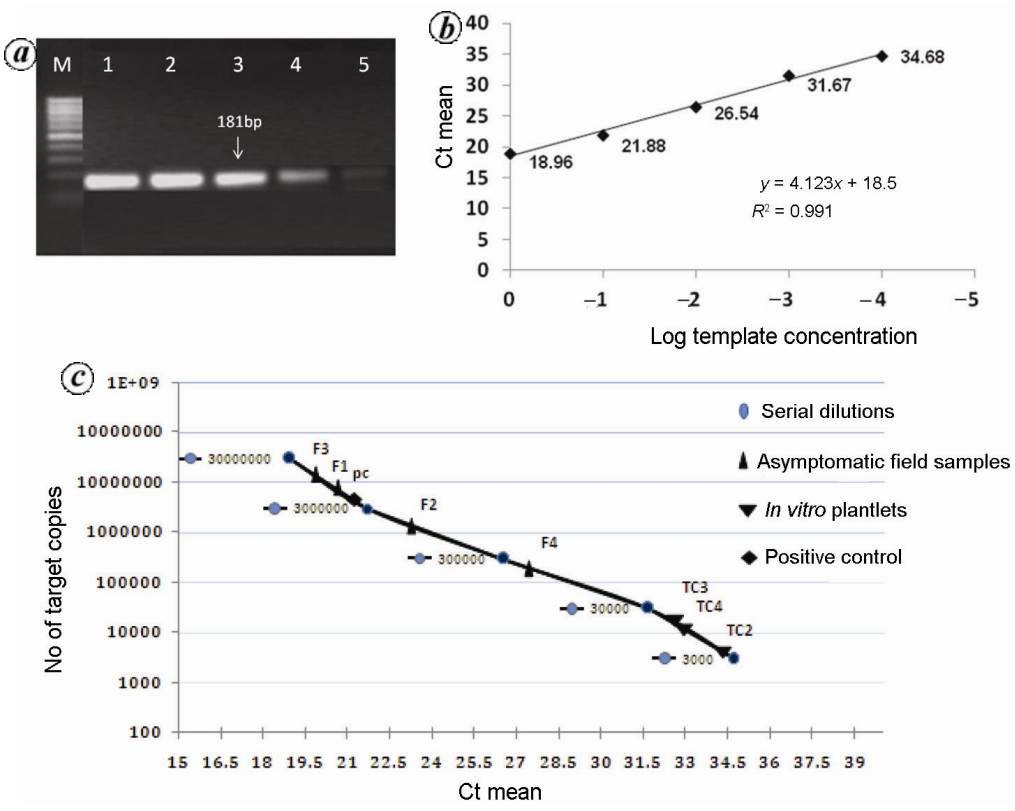

Figure 2. Quantification of SCYLV copy numbers in yellow leaf infected sugarcane plants using RT-qPCR. Serial dilutions in RT-qPCR performed with standard plasmid template of SCYLV-Co 85019. ***a***, PCR amplification to confirm presence of coat protein in serial dilution of plasmid standards. M, 100 bp marker; lanes 1–5; 1 : 10-fold serial dilution of plasmids standards (details given in Table 1). ***b***, Standard curve generated based on five different log template concentrations (1 : 10-fold) and their Ct values. Concentration of 10° corresponds to 700 ng of plasmid DNA with 3×10^7 copies. ***c***, SCYLV copy number of samples plotted in relative standard curve with their corresponding Ct ranges.

had shown copy numbers ranging from 412.05 to 729.69 (Table 1). SCYLV copy numbers in asymptomatic field samples with high titre in RT-PCR ranged from 278,086.0 to 8,961,475.25 (Table 1). Similarly, positive control (Co 86010) had 6,082,227.66 copies of SCYLV. Melt curve analysis at 60°C resulted in a single peak in all the three replications of the positive samples and negative samples were recorded no peaks.

In the present study diagnosis of SCYLV in sugarcane by RT-PCR and RT-qPCR was carried out with coat protein (CP) specific primers. Optimum primer concentration (40 nmol) was standardized for the specific amplification. CP is directly associated with the success of infection, as it is involved in viral transmission, particle packaging and viral accumulation within the plant and a high degree of conservation in CP sequence is expected[22,26,27]. In CP genome, a high level of homogeneity was observed among the different SCYLV genotypes[17]. In the present study, we found mild or no amplification of SCYLV in three *in vitro* plantlets. In meristematic propagation, elimination of virus particles in explant cells is reached within a short time. Probably it is due to the following: in many cases, meristematic cells do not contain virus particles because of non-existing vascular connection with other plant parts. Also, virus distribution is uneven in a plant and is much less in a meristem. Viruses cannot travel quickly enough through plasmodesmata to keep up with the actively growing tip[28]. Our results concluded that in micropropagation, proper meristematic apex without adjacent leaf primordia (size 0.2–1.0 mm) could be used as starting explants. Any irregular sizing of apical meristem with an increased size leads to the presence of limited number of viruses[29]. Positive amplification with greater titre in 200 days in the field samples confirmed the presence of viruses in all the asymptomatic plants. Vegetative propagation through SCYLV infected setts favours transmission of the virus in the field, reoccurrence of the disease and eventually yield loss. Hence virus elimination is a pre-requisite to raise disease-free sugarcane plants. Usually YL symptoms are expressed in 6–8 months in the field[25]. SCYLV often persists in the plants without being noticed by the growers. In fact, this non-symptomatic stage seems to be the most common epidemiological state for this viral pathogen. Screening of SCYLV in Hawaii revealed positive infection in all susceptible sugarcane cultivars, but disease symptoms appeared only occasionally[30]. However, this non-symptomatic stage can still lead to significant (20–30%) yield declines[31]. Izaquirre-Mayoral *et al.*[9] noticed that in all the cultivars of locally grown sugarcane in Venezuela, symptoms are rarely observed before or during the first ratoon, and only become evident after the second ratoon. Zhu *et al.*[21] reported that resistant cultivars thought to be virus-free contain much lower titre of viruses in Hawaii.

In RT-qPCR, the cycle threshold is indirectly proportional to the expression of targets in the samples. We found significant differences in the presence of target virus population between *in vitro* plantlets and field samples (Table 1). *In vitro* plantlets clearly established the occurrence of limited number of viral populations in the micropropagated plants (Table 1). RT-qPCR would give more accurate relative quantitative information on the ratio of SCYLV titre in different cultivars of sugarcane and was expected to be a sensitive diagnostic tool for breeders[21]. Single peak in melt curve analyses showed the presence of target coat protein in positive samples and few of the negative samples showed decreased blunted dimer peaks. The standards with log template concentrations showed greater differences in Ct ranges and an average of 3.9 cycles was taken to reach its threshold in the next template concentration. Low viral templates of 3×10^3 and 3×10^4 reached their threshold only above 30 cycles in the standard curve. Similarly, positive samples of *in vitro* plantlets obtained with mild amplification in RT-PCR reached their threshold between 32 and 34 cycles. It is emphasized that there is a threshold level of virus titre for their diagnosis, where the amplification was very mild with significantly very low titre. Likewise, RT-qPCR resulted in late cycle threshold for the samples with such low viral titre. Copy numbers obtained in the negative results of *in vitro* plantlets are due to the primer–dimer formation in the amplification. Rarely primer molecules hybridized to each other because of strings of complementary bases in the primers due to the absence of targeted gene in the PCR reaction[32]. We observed greater differences in virus population between the *in vitro* plantlets and asymptomatic field plants. Micropropagation through meristem culture ensures elimination of SCYLV in sugarcane; nevertheless, much attention is needed in case of meristamatic tissue selection to remove the virus. Further studies are required to establish the relationship between symptom expressions with virus titre in plants. In conclusion, the relative standard curve method developed will efficiently detect copy numbers of target virus present in different sugarcane samples. Earlier, Parmessur *et al.*[20] and Chatenet *et al.*[33] reported micropropagation for SCYLV elimination in sugarcane. Now the process is being followed routinely to manage the disease in India[34]. Our studies clearly established that micropropagation serves as a tool to eliminate SCYLV in sugarcane. This process would sustain sugarcane productivity and deter varietal degeneration in proven commercial varieties.

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