Quantification of sugarcane yellow leaf virus in sugarcane following transmission through aphid vector, *Melanaphis sacchari*

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Abstract Yellow leaf caused by Sugarcane yellow leaf virus (SCYLV) is a serious constraint to sugarcane production in India and currently the disease epidemics occur on many of the susceptible varieties under field conditions. Studies were conducted on the virus transmission by sugarcane aphid *Melanaphis sacchari* in sugarcane by inoculating virus-free meristem derived from micro-propagated plants of sugarcane cv Co 86032 with viruliferous aphids. Virus transmission was confirmed through RT-PCR assays and subsequently SCYLV population was established through RT-qPCR. A maximum of 22.3×10^3 , 3.16×10^6 and 4.78×10^6 copies of SCYLV-RNA targets were recorded in the plants after 7, 180 and 300 days, respectively. This study showed that the aphid species *M. sacchari* acts as an effective vector of SCYLV. The relative standard curve method in RT-qPCR efficiently detected the increment in SCYLV copy numbers in sugarcane following transmission through *M. sacchari*.

Keywords Sugarcane aphid · *Melanaphis sacchari* · Yellow leaf · SCYLV · RT-qPCR

Introduction

Yellow leaf (YL) of sugarcane was first reported in the cv H65-0782 in Hawaii in 1989 [16] and later in more than 30 countries [10]. Vega et al. [19] identified the causal agent of YL of sugarcane as sugarcane yellow leaf virus (SCYLV) (genus *Polerovirus*, family *Luteoviridae*). The virions of luteovirids are restricted to the phloem tissue of plants and are not transmitted mechanically [7]. Long range SCYLV transmission in sugarcane is achieved through infected seed canes and secondarily by aphids in a persistent manner [14]. Earlier, Schenck and Lehrer [17] reported three aphid species namely *Melanaphis sacchari* (sugarcane aphid), *Rhopalosiphum maidis* (corn leaf aphid) and *R. rufiabdominalis* (rice root aphid) as vectors of SCYLV in Hawaii. However, later studies revealed that, among them, *M. sacchari* was the only important vector in Hawaii. Transmission of SCYLV in other parts of the world reported *M. sacchari* and *R. maidis* as vectors [5]. In Mauritius, the distribution of *M. sacchari* was limited and its population was low in the commercial plantations and in very few localities [12]. In Guadeloupe, *M. sacchari* is widespread in all areas [6]. However, in Louisiana, the incidence and rate of infection remained low despite the wide occurrence of the potential vectors [11]. In China, the other aphid species, *Ceratovacuna lanigera* was reported as a vector of SCYLV [23].

In India, the occurrences of YL were reported all over the country and susceptible cultivars in commercial fields reach up to 100 % [22]. In our previous study, we have established the occurrence of three SCYLV genotypes in India among the seven genotypes reported based on their genomic diversity [21]. Recently, we have reported complete genome of SCYLV-IND genotype from India [3]. Presently, YL has attained epidemic status in India and the

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situation warranted management approaches to sustain sugarcane productivity [20]. Based on the surveys in the commercial plantations, *M. sacchari* was found as common in both tropical and subtropical regions in India as compared to other two aphid species. Although, transmission of SCYLV through *M. sacchari* was reported in other parts of the world, there are no clear reports in India except Singh and Rao [18] who reported 75 % virus transmission through the aphid. A detailed study on SCYLV transmission by viruliferous *M. sacchari* to meristem derived virus-free tissue culture plants was conducted and successful transmission was confirmed by RT-PCR. After confirmation of SCYLV transmission, the progress of virus titre in virus-free plants was quantified at different periods in RT-qPCR by relative standard curve method.

Materials and methods

Mass multiplication of *M. sacchari*

The aphid species, *M. sacchari*, was collected from sugarcane fields of Sugarcane Breeding Institute, Coimbatore and identity of the aphids was confirmed by National Bureau of Agriculturally Important Insects, Bangalore. SCYLV-infected sugar cane plants cv CoPant 84211 were maintained in an insect-proof shade net house. The aphid colonies of *M. sacchari* along with the leaf collected from the field were clipped on the abaxial surface of the leaves. Usually leaf number 2–5 (totally visible dewlap in the crown is considered as leaf number 1) were clipped with these aphid-infested leaves and developed viruliferous aphid population on SCYLV infected sugarcane.

Developing healthy plants through meristem culture

In vitro plantlets of cv Co 86032 were derived from 0.3 mm apical meristem and grown in Murashige and Skoog medium using standard procedure adopted at the Institute. Following root formation, plantlets were individually planted in polythene bags and hardened. After 20 days of hardening, 12 plants were taken and maintained inside an insect-proof glass house.

Reverse transcription (RT) PCR

Prior to aphid inoculation, the presence of SCYLV was confirmed by RT-PCR in aphids and leaves from the sugarcane cv CoPant 84211. Similarly, meristem derived tissue culture plants of the cv Co 86032 were tested in the RT-PCR assays for absence of SCYLV. Total RNA was extracted from all the samples comprising of aphids (single adult aphid per plant) and leaves using TRI reagent (Sigma,

USA). Diagnoses of SCYLV were carried out with SCYLV-615F (GGATCCATGAATACGGGCGCTAACC GYYCAC) and SCYLV-615R (AGATCTGTGTTGGG GRAGCGTCGCTACC) primers targeting 615 nt partial genome of SCYLV coat protein-ORF3 [21]. The RNA (1 µg) was reverse-transcribed to cDNA using RevertAid H minus First Strand cDNA Synthesis Kit (MBI Fermentas, USA), primed with 50 pmol of SCYLV-615R primer following the manufacturer's protocol in a thermocycler (Eppendorf, Germany). PCR was performed with 2 µl cDNA, 2.5 µl of 10× PCR buffer containing 15 mM MgCl₂, 0.5 µl of 10 mM dNTP mix, 40 nmol each of forward and reverse primers, 1 unit of *Taq* polymerase (Merck, India), and sterile milli-Q water to the final volume of 25 µl. The PCR programme consisted of an initial denaturation at 94 °C for 4 min, following 30 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min with a final extension for 10 min at 72 °C. Amplicons were visualized by electrophoresis on 1.5 % agarose gels stained with ethidium bromide.

SCYLV transmission

The viruliferous aphid colonies of *M. sacchari* on the leaves were disturbed using mild brushing. Five numbers of individual adult aphids were carefully taken using moistened tip of a Camel 'O' hair brush and transferred to the leaves of 12 virus-free tissue cultured plants. After aphid inoculation, all the 12 plants were maintained in an insect-proof cage. After 7 days, all the aphids were killed by spraying malathion (5 ml L⁻¹ of water). Leaf samples from all the aphid-inoculated plants and the control plants were collected on the same day. RT-PCR assays were performed as mentioned above to confirm the presence of SCYLV transmitted by aphids. Similarly, leaf samples were collected after 180 and 300 days of aphid inoculation and RT-PCR assays were performed. The intensity of the amplified PCR products of 7, 180 and 300 day leaf samples was compared in agarose gel electrophoresis.

Real-time RT-qPCR assay

After transmission of SCYLV, the progress of virus titre was quantified in RT-qPCR with relative standard curve method. Leaf samples were taken after 7, 180 and 300 days of viruliferous aphid inoculation from all the 12 plants. RNA (300 ng) was reverse transcribed to cDNA using RevertAid H minus First Strand cDNA Synthesis Kit, primed with 40 nmol of SCYLV-R615 (5'-AGATCTGTG TTGGGGRAGCGTCGCTACC-3') primer by following the manufacturer's protocol (MBI, Fermentas, USA) in a thermocycler (Mastercycle gradient, Eppendorf, Germany). Real-time quantitative PCR was performed with three

replicates of each sample with 700 ng of double stranded cDNA template, 12.5 μ L of SYBR[®] Green JumpStart[™] TaqReadyMix[™] (Sigma, India) in a 25 μ L reaction volume in StepOnePlus Real-Time PCR System (Applied Biosystems, USA). The primer concentration in the range of 20–100 nM of YLS RT FOR-N (5'-GGACCGAACC-TATCTCAGTAC-3') and YLS RT REV-N (5'-TAG-TAATCTTGGAGCCTGTTGTTG-3') primers targeting 181 nts partial genome of SCYLV coat protein (ORF3) were used for the standardization with melting temperature in the range of 50–65 °C. 25SrRNA RT-F (5'-GGCAGC-CAAGCGTTCATAGC-3') and 25SrRNA RT-R (5'-GGGTAAACTAACCTGTCTCACGAC-3') targeting 156 nts of 25S rRNA gene was used as an internal control in all the samples [4]. PCR conditions were optimized at 95 °C for 10 min, 40 cycles of 95 °C for 15 min and 50–60 °C for 1 min. Melt curve analysis was performed to assure that a homogenous amplification product was produced. Leaf sample from tissue cultured virus-free plant of sugarcane cv Co 86032 was used as a negative control and symptomatic leaf from cv Co 86010 was used as a positive control.

Relative standard curve analysis

pTZ57R/T plasmid vector (2886 nts) ligated with SCYLV-coat protein (580 nts) of SCYLV isolate CB 85019 was used to prepare standards. Plasmid copy numbers were calculated using online 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 – 3×10^3 target copies with an initial plasmid concentration of 700 ng. Based on the cycle threshold and log template concentration of the

serial dilutions, a standard curve was created. Linear correlation and coefficient of determination for the standard curve were estimated. Using slope and intercept, accurate copy numbers of SCYLV-RNA targets present in the sample were assessed for the template concentration of 700 ng.

Results

Reverse transcription PCR assay for SCYLV in aphids and sugarcane

Aphid culture was successfully established on the sugarcane cv CoPant 84211 by clip inoculation method and in 1 month, the aphids multiplied and colonized all the mature leaves. RT-PCR assay confirmed the presence of SCYLV in all the samples of aphid and the infested leaves of the cv CoPant 84211 (Fig. 1a). RT-PCR assay also revealed that tissue cultured plants of the cv Co 86032 were free from the virus (Fig. 1b). Subsequent RT-PCR assay with virus inoculated plants, 7 days after inoculation, showed amplification of virus in all the plants (Supplementary Fig. 1a). This assay clearly proved that *M. sacchari* successfully transferred SCYLV from plant to plant in sugarcane. The comparison of amplicons between the samples of 7 and 180 days after virus inoculation in virus-free plants showed mild differences in intensities in agarose gel and subsequently, 7 and 300 days showed greater differences (Supplementary Fig. 1a–b). The concentration of the amplicon in the gel was in the range of 50–240 ng/ μ L and 500–1400 ng/ μ L after 7 and 300 days respectively.

Real time (RT)-qPCR assay

In RT-qPCR, cycle threshold (Ct) ranges were optimized between 15 and 35 with 40 nmol of YLS RT FOR-N and

Fig. 1 Detection of *Sugarcane yellow leaf virus* in viruliferous *Melanaphis sacchari* by RT-PCR. **a** Lane M, 100 bp marker; 1–6, aphid collected from different leaf samples; lane 7–12, leaves corresponding to the aphids of lane 1–6; lane 13–16, leaves of the cv Co Pant 84211 used for the multiplication of aphids. PC-Positive control (Co 86010), NC-Negative control. **b** Confirmation of virus freedom in meristem derived seedling of sugarcane cv Co 86032 by RT-PCR

YLS RT REV-N primers with T_m of 60 °C. 700 ng of cDNA from samples and 700 ng of initial plasmid concentration in five different serial dilutions (1:10 fold) were optimized. Ct values in sugarcane plants ranged from 31.06 to 33.04 after 7 days of virus inoculation and were reduced to 24.63–29.67 after 180 days. By 300 days, Ct values were further reduced from 18.92 to 24.38 in six plants whereas the remaining plants recorded Ct values ranging from 28.16 to 30.54. Positive control had Ct values at 17.94, 18.01 and 17.92 at 7, 180 and 300 days respectively whereas no amplification was observed in negative control (Supplementary Table 2). The internal control (25S rRNA) of all the samples recorded Ct values between 16.09 and 16.92.

Relative standard curve analysis

In RT-PCR, positive amplification confirmed the presence of SCYLV coat protein in serial dilution of plasmid standards. 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 17.76, 20.60, 25.34, 30.00 and 33.68 (Supplementary Table 1) in qPCR. Using log scale of standard template concentration and their Ct ranges, a standard curve was plotted. Using slope and intercept values, linear correlation and coefficient of determination were $y = 4.124x + 17.22$ and $R^2 = 0.994$ respectively (Fig. 2). Accurate copy number of SCYLV-RNA targets present in sugarcane leaves at 7, 180 and 300 days after virus inoculation by viruliferous aphids were obtained from the standard curve.

In RT-qPCR, the copy number of SCYLV-RNA targets ranged from 7.6×10^3 to 2.2×10^4 in the plants 7 days after virus inoculation. At 180 days, it gradually increased to $0.26\text{--}3.16 \times 10^6$ in seven plants and the remaining plants had $1.65 \times 10^4\text{--}5.17 \times 10^4$ copies of SCYLV. By 300 days, copy number of SCYLV-RNA targets increased

Fig. 2 Serial dilutions in RT-qPCR performed with standard plasmid template of SCYLV-Co 85019. Standard curve generated based on five different log template concentrations (1:10 fold) and their Ct values. Concentration of 10^0 corresponds to 700 ng of plasmid DNA with 3×10^7 copies

rapidly in six plants to a maximum of $4.78\text{--}0.78 \times 10^6$. However, the other plants recorded $2.86 \times 10^4\text{--}9.62 \times 10^4$ copies. Positive controls at 7, 180 and 300 days recorded 9.68, 8.26 and 9.58×10^6 of target copies respectively to their Ct value of 17.94, 18.01 and 17.92.

Discussion

SCYLV-free plants became infected rapidly when infested with aphids from nearby infected sugarcane. The virus was first detected in root tips, stem tissue under the apical meristem and in a few bundles of young leaf (–4) only after 3 weeks of infestation by viruliferous *M. sacchari* on the blade of top-visible dewlap leaf-1. It was also found that the infection pressure was very high. 80 % of virus-free plants became infected within 4 months in Hawaii in heavily aphid-infested and SCYLV-infected sites [9]. Due to piercing-sucking mouthparts and their feeding behaviour, aphids are well suited for spreading viruses from one plant to another. Brault et al. [2] studied the transport mechanism of luteovirids through aphids and they found that earlier, ingested virions are acquired in the food canal and passed to the haemolymph through the posterior midgut or hindgut or through both depending on the virus species. Virions then enter the accessory salivary glands and are subsequently released into plant hosts through the salivary duct.

McAllister et al. [11] found greatest temporal increase of virus infection during late spring and early summer. This coincided with the initial infestation and increase of the virus vector, *M. sacchari*, in Louisiana. During 2002 and 2003, the aphid infestations in experimental fields ranged from 1.2 to 33.0 and 1.0 to 4.2 aphids per leaf respectively. It was established that both SCYLV and its aphid vector exhibited a predominantly random spatial distribution with occasional aggregation. The present work showed high fluctuations in the aphid population ranging from trace to 484 per plant in May and up to 643 aphids in October, 2012 (Viswanathan, unpublished). Rassaby et al. [14] reported virus-free susceptible cultivar planted near SCYLV-infected sugarcane resulted in 45 % of infection after 4 months. Schenck and Lehrer [17] reported that *R. maidis* can transmit SCYLV from sugarcane to cereal grasses. Apart from sugarcane, the other crops such as barley, oats and wheat are susceptible to SCYLV. These crops are not likely to be a source of infection for sugarcane infection as these crops are propagated by seeds and these viruses are not known to be seed-borne [6]. However, these cereals could become infected in the field by viruliferous *M. sacchari* or *R. maidis* from neighbouring sugarcane [14].

The present studies successfully demonstrated SCYLV transmission in sugarcane through *M. sacchari* and this was

validated through RT-PCR and RT-qPCR assays. Coat protein specific primers used in RT-PCR and RT-qPCR efficiently detected the presence of SCYLV in all the virus-inoculated plants. In coat protein genome, a high level of homogeneity was observed among the different SCYLV genotypes [21]. Conventional RT-PCR assays are proved to be better to detect the virus in the plants than serological methods due to certain drawbacks such as limited availability of antisera and lack of sensitivity in serological methods [13]. RT-qPCR would give more accurate relative quantitative information on the ratios of SCYLV titre in different cultivars of sugarcane and was expected to be a sensitive diagnostic tool for breeders [24].

In RT-qPCR, melt curve analysis showed a single peak in all the three replicates of the positive samples and this also confirmed the specificity of the primers. The relative standard curve method efficiently detected the copy numbers of the targeted virus whereas the cycle threshold is indirectly proportional to the expression of targets in the samples. Among the 12 plants, seven recorded a rapid increase in target copies of the virus 300 days after virus transmission by *M. sacchari*. Maximum increase of 4.75 and 4.17×10^6 copies were recorded from 7 to 300 days in two different plants. However, the plants with maximum SCYLV copy number of 4.78×10^6 after 300 days did not express disease symptoms. In our previous studies, $0.27\text{--}8.96 \times 10^6$ copy number of SCYLV population was detected in SCYLV-infected asymptomatic plants using RT-qPCR [4]. Izaguirre-Mayoral et al. [8] rarely observed symptoms in all cultivars locally grown in Venezuela before or during the first ratoon, these becoming evident after the second ratoon. Also, they found about 25 % of the plants within a plot were asymptomatic. Screening of SCYLV in Hawaii revealed positive infection in all susceptible sugarcane cultivars but disease symptoms appeared only occasionally [14]. However, this non-symptomatic stage can still lead to significant (20–30 %) yield decline [15]. These studies clearly indicated that non-symptomatic plants still harbour very high titre for SCYLV.

Yellow leaf management in sugarcane has been achieved by planting virus-free materials derived through meristem culture and certification standards for micro propagated sugarcane require freedom from SCYLV in Louisiana [1] and India [20]. Presently in India, yellow leaf has attained epidemic status with 100 % disease incidence in commercial fields where susceptible cultivars are grown [20, 22]. Epidemic nature of the disease may also be attributed to the prevalence of large populations of the vector in the field. This study also revealed new information on the rapid increase in virus titre within a crop season in sugarcane after virus transmission. The regular stripping of mature leaves from sugarcane may help to reduce the

aphid population from the plant since, *M. sacchari* preferring the most mature leaves for their colonization and multiplication. Since *M. sacchari* was found as the only sugarcane aphid species spreading SCYLV in India, developing cultivars resistant to the vector can be a good management tactic to reduce the spread and incidence of the virus and its aphid vector.

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