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RT-PCR and quantitative real-time RT-PCR detection of *Sugarcane Yellow Leaf Virus* (SCYLV) in symptomatic and asymptomatic plants of Hawaiian sugarcane cultivars and the correlation of SCYLV titre to yield

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Abstract *Sugarcane yellow leaf virus* (SCYLV) has been reported worldwide to infect sugarcane and to cause significant yield losses. Current detection methods include tissue blot immunoassay (TBIA), reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR assay (qRT-PCR). In this paper, we report the use and comparison of these detection methods for the study of SCYLV in Hawaiian cultivars. We observed positive RT-PCR and qRT-PCR reactions in cultivars previously thought (based on TBIA) to be immune to virus infection. The semi-quantitative virus titre in these cultivars was however at least 10^6-fold lower than in the cultivars which were known to be SCYLV-susceptible. The RT-PCR methods also revealed that plants of the cultivar H65-7052, which were previously shown to vary strongly between TBIA-positive and TBIA-negative, indeed exhibited fluctuating SCYLV-titres in a range of 10^3–10^4-fold. The virus titre was carried through to the next vegetative generation, i. e. plants grown from seed pieces with low virus titre had low virus titre and plants grown from seed pieces with high virus titre contained high virus titre. A small field trial comparing plants of cv. H65-7052 of low and high SCYLV-titre showed that the field plots with plants of high virus titre developed Yellow Leaf symptoms and yielded only 54–60% of cane and sugar tonnage compared to plots with plants of low virus titre.

Keywords *Luteoviridae* · qRT-PCR · *Saccharum spec.* hybrid · Sugar yield · Tissue blot immunoassay · Virus titre · Yellow leaf symptom

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Introduction

Sugarcane yellow leaf virus (SCYLV) is a *Polerovirus* (Moonan et al. 2000) (family *Luteoviridae*) with a single stranded positive sense RNA genome, that has been reported to infect sugarcane worldwide (Vega et al. 1997; Comstock et al. 1998; Moutia and Saumtally 1999; Abu Ahmad et al. 2006). Like others in this group, SCYLV is phloem-limited (Vega et al. 1997; Schenck et al. 1997; Lehrer et al. 2007). Symptoms in sugarcane include yellowing of the leaf midribs, short terminal internodes, reduced biomass and reduced plant vigour (Grisham et al. 2002; Rassaby et al. 2003). Growth reduction was also reported to occur without visible yellowing symptoms (Lehrer et al. 2001; Lehrer et al. 2009). Plants of cultivars with yellowing

symptoms were reported to show an increase in the sucrose content of their leaves (Izaguirre-Mayoral et al. 2002; Lehrer and Komor 2008), which was interpreted as the inhibition of assimilate translocation due to the disruption of phloem integrity.

An initial survey using TBIA of the main sugarcane cultivars grown in Hawaii found the virus to be much more widespread than was expected, based on leaf visual symptoms (Schenck et al. 1997; Schenck and Lehrer 2000). Most plants of susceptible cultivars in Hawaiian plantation fields were shown to be infected, although most infected plants were asymptomatic (Lehrer and Komor 2008). Symptom expression tends to appear when plants are placed under conditions of stress, such as drought or cold temperatures (Rassaby et al. 2003), but the effect varies greatly between cultivars. Hawaiian commercial cultivar H65-7052 frequently shows yellowing symptoms, but continues to yield well. On the other hand, cultivar H73-6110, rarely showing yellowing symptoms, was also known to be infected using TBIA, but showed significant yield loss when exposed to drought stress. The TBIA assay currently in use is fast, efficient and can be used to screen large numbers of plants in field trials (Schenck et al. 1997). It is thought that there is a threshold level of virus titre (virus particle concentration in the plant) below which the TBIA cannot detect the virus (Schenck et al. 1997). The RT-PCR method has also been used and gave similar results. The threshold is similar to TBIA (Comstock et al. 1998; Korimbocus et al. 2002). For H65-7052, TBIA gave positive results for 30 to 60% of leaves tested (Schenck et al. 1997). Some other Hawaiian cultivars almost always gave positive reactions, but very rarely show yellowing. In several cases, (H78-7750, H78-3567, H78-4153) positive TBIA reactions have hardly ever been obtained even when the canes were inoculated with SCYLV via aphid vectors. These cultivars were thought to be resistant to the virus (Schenck and Lehrer 2000). Lehrer and Komor (2007) reported similar results in a detailed study on YL-symptom development and SCYLV-presence over the growing season and they also found that some plants with YL symptoms tested negative for SCYLV by TBIA. They concluded that there was no close correlation between SCYLV and YL symptoms. The question was whether these apparently immune cultivars were really immune or whether the virus titre was merely below the detection threshold of TBIA. Cultivar H65-7052 was especially intriguing as the TBIA results showed large fluctuations. At some points during the growing season up to 60% of leaves were positive for SCYLV, while at others almost none of the leaves were positive. This "titre fluctuation" correlated with the extent of visible YL-symptoms (Lehrer and Komor 2008), but the question still remained whether the plants of this cultivar gained the virus in some growth periods and reduced it in others, or whether it was a fluctuation of virus titre of permanently-infected plants.

Conventional RT-PCR assays are thought to be better predictors of virus presence *in planta* than TBIA. Quantitative RT-PCR (qRT-PCR) would give more accurate relative quantitative information on the ratios of virus titre in different cultivars and was expected to be a sensitive diagnostic tool for breeders. It should identify sugarcane cultivars which are likely to be severely affected by adverse environmental conditions because of SCYLV-infection. In this paper, we report the use of RT-PCR and qRT-PCR methods to test several SCYLV-susceptible and –resistant Hawaiian sugarcane cultivars and comparison of the results with TBIA. In addition these methods were used to correlate symptom expression and yield of the high-yielding cultivar H65-7052.

Materials and methods

Plant materials

Leaves from ten Hawaiian sugarcane cultivars (*Saccharum* spp. hybrids H65-7052, H77-4643, H78-3567, H78-4153, H78-7750, H87-4094, H87-4319, H87-5794, H93-4068 and H95-4655) all grown on Hawaii were used in the three assays (TBIA, conventional RT-PCR and qRT-PCR) to evaluate the degree of infection with SCYLV in terms of percentage of infected plants based on TBIA and virus titres based on RT-PCR and qRT-PCR. Mature leaves were collected from field-grown plants at Hawaii Agriculure Research Center (HARC) Experiment Station on the island of Oahu and at HC&S sugar plantation on the island of Maui in Hawaii. Midribs of freshly-collected leaves were used fresh for TBIA. TBIA was carried on individual leaves. Leaf midribs from the same leaves were stored at −80°C until required for RNA extraction and RT-PCR testing. For RT-PCR, a minimum of

twelve leaves per cultivar from both symptomatic and asymptomatic plants were randomly grouped and individual leaves were combined into 2–3 replicates. The experiments were repeated at least twice.

Field plot trial

In a small field trial, stalk seed pieces from symptomatic and asymptomatic plants of cultivar H65-7052 were planted in four replicate plots and grown using standard cultural practices. The replicate plots for the two treatments (symptomatic and asymptomatic seed pieces) sized 3 m×10 m, were each planted with 40 seed pieces. Each plot had a center drip tubing line and 2 cane rows spaced 1 m apart. A 2 m inter-row space was left between adjacent plots. At 11.5 months of age whole stalks were cut at the base and compiled from each plot. Total above-ground biomass (leaves and stalks) of each plot was weighed with a mechanical Cameco harvester crane, fitted with a hydraulic load cell accurate to +/− 2.5 kg. After recording the weight, a random subsample of ~50 kg of above ground biomass was chopped using a John Deere forage chopper (model no. 34-1980). The chopped subsample was thoroughly mixed and 2.5 kg of the mixed sample was collected for pol ratio analysis, a measure of sucrose content based on the method of Payne (1968).

General analysis of variance was carried out for total cane biomass (t/ha) and estimated sugar yield (t/ha) to determine significance of differences in values between the treatment and field plots (Statistix 7.0, 2000).

Tissue blot immunoassay

Tissue blot immunoassay (TBIA) followed the method of Schenck et al. (1997). TBIA was carried on individual leaves with a minimum 12 leaves per sample. The percentage of positive leaf midrib blots from one set of samples was recorded.

RNA extraction

Sugarcane midribs were ground to a fine powder in liquid nitrogen. An aliquot (75 mg) of ground power was used for RNA isolation. RNA was extracted using the RNeasy kit (Qiagen) as per manufacturer's protocol and then stored at -80°C until further analysis. To eliminate DNA contamination of RNA, DNase treatment was performed using RQ1 RNase-free DNase (Promega, Madison, WI). RNA purity and yield was determined spectrophotometrically using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). A minimum of twelve individual leaves per cultivar from both symptomatic and asymptomatic plants were randomly grouped and combined into 2–3 replicates.

Reverse-transcription and PCR (RT-PCR)

Reverse transcription (RT) was performed on 100 ng of DNase-treated RNA using ImProm-II Reverse Transcriptase (Promega, Madison, WI). PCR was carried out using GoTaq Flexi DNA Polymerase (Promega, Madison, WI) as per manufacturer's protocol in a Biorad iCycler (Biorad Laboratories, Hercules, CA). Conserved primers were designed to the ORF0 (P0) sequences to cover all known SCYLV strains available in the GenBank data library (accession numbers AM072750.1, AM072751.1, AF157029 AY236971.1, AM072752.1, AM072753.1, AJ249447.1, AM072754.1, AM072755.1 and AM072756.1). *Sugarcane yellow leaf virus* and triosephosphate isomerase (TPI) primers (as internal control) were as follows: SCYLV forward, 5'-AGA TCT ATG CTT TTC AAC GAA TTC-3'; SCYLV reverse, 5'-GTC GAC CCA GTT GTA AAC GGG AGT G-3' (201 bp product); TPI forward, 5'-CAA TGA CTG GAG CAA CGT AG-3'; TPI reverse, 5'-GTA ACA GAG CCT CCG TAG AT-3' (200 bp product). In addition, the conserved region was also verified by sequencing Hawaiian SCYLV strains isolated from three different cultivars (ElSayed et al. unpublished). The phylogenetic analysis of our sequences from SCYLV of 3 Hawaiian cultivars indicates that these Hawaiian strains are closest to the published PER strain, derived from originally Hawaiian cultivars (ElSayed, unpublished). Therefore, P0 primers targeted to a conserved region.

All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Each 50 µL PCR reaction contained 1 µL of cDNA with a final primer concentration of 400 nM. Cycling conditions were as follows: 95°C for 30 s; 35 amplification cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s; 72°C for 45 s for both sets of primers. PCR products were visualized on a 2% agarose gel stained with ethidium bromide.

Real-time qRT-PCR assay

Reverse transcription was performed on 100 ng of DNase-treated RNA using ImProm-II Reverse Transcriptase (Promega, Madison, WI). Quantitative real-time PCR was performed using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) in a DNA Engine Opticon 2 (MJ Research, Inc). PCR was performed in white 96-well PCR plates (MJ Research) with 5 µL of cDNA template, 12.5 µL of SYBR Green 1 qPCR mix and 500 nM of each primer in a 25 µL reaction volume. Samples were subjected to the following conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s, 55°C for 15 s, 72°C for 20 s. Melting curve analysis was performed with software package Opticon2 Continuous Fluorescence Detection System for denaturation for melting curve analysis (MJ Research) to assure that a homogenous amplification product had been produced. The PCR was optimized with low initial concentration of primers in order to minimise primer dimer formation. It was demonstrated in the melting curve that the dimer peak was much smaller than the PCR product.

Leaf material from a virus-free sugarcane plant in tissue culture (H62-4671) (Fitch et al. 2001) was used as a negative control. The pooled leaf midribs from symptomatic leaves of H87-4094 were ground to a fine powder and stored at -80^0 C and an aliquot of the powder was used as a positive control. A dilution series was performed with the positive control to verify the quantitative response of the range used in qRT-PCR (Fig. 1).

Data analysis

The relative amounts of virus RNA detected by the qRT-PCR method were compared as the means of two or three biological replicates and 3 technical (i.e., PCR amplification) replicates based on the "Comparative C_T" method as described (Applied Biosystems 2001). Briefly, for SCYLV determination, ΔC_T was derived by subtracting C_T (TPI) from C_T (SCYLV). The ΔC_T values of the dilution series of the positive control (H87-4094) ranging from 1:1, 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000 were determined and the intercept and slope was calculated based on the linear regression (Fig. 1b). $\Delta\Delta C_T$ was derived by subtracting ΔC_T (negative control) from ΔC_T of samples. $\Delta\Delta C_T$ values of samples were determined and normalized to the positive control (H87-4094) using the intercept and slope. The fold differences of test samples were calculated and plotted on a log scale of 10 normalized to the positive control (H87-4094).

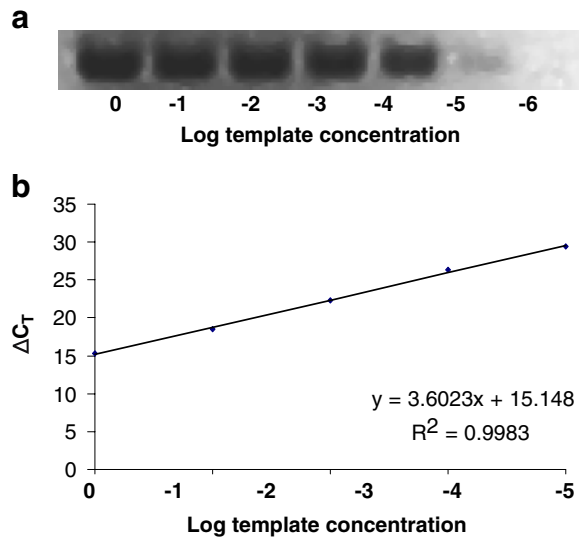

Fig. 1 RT-PCR (**a**) and qRT-PCR (**b**) performed using a dilution series made using a standard template sample. Leaf midribs from infected H87-4094 plants were powdered in liquid nitrogen and stored at -80°C. RNA was extracted and transcribed to cDNA as described in methods section. The concentration 10^0 corresponds to 5 µL of cDNA template. The dilution series was performed in increments of 10. The linear correlation is y=3.60x+15.15 (r^2=.998)

Results

Test range of RT-PCR and qRT-PCR

A standard sample of template was prepared from powdered midribs of the highly -susceptible cultivar H87-4094. A dilution series of the standard template was used to explore the range of the linear relationship between template concentration and PCR-product formation. RT-PCR product was observed until a dilution factor of 10^5, whereby no differences in product quantity could be identified above 10^3-fold dilution (Fig. 1a). Quantitative real-time RT-PCR covered the range until a dilution factor 10^5 (at least) by a linear relationship (Fig. 1b). The slope of correlation indicated that 3.6 cycles were needed to increase the PCR-product by a factor of 10, i. e. each

qPCR-cycle multiplied the product by a factor of 1.90.

Test of cultivars by TBIA, RT-PCR and qRT-PCR

The difference in sensitivity between TBIA and RT-PCR is clearly illustrated by the lack of detection of SCYLV in 4 out of the 10 cultivars using TBIA (Fig. 2a). In this study, none of the plants of cultivars H78-4153, H78-3567, H87-5794 and H78-7750 gave positive results for SCYLV with the TBIA method. Other cultivars (H77-4643, H93-4068, and H87-4094) gave 85–99% positive test blots or gave variable positive results of 30–35% (H65-7052, H87-4319 and H95-4655). RT-PCR was able to detect the presence of SCYLV in all of the above cultivars (Fig. 2b). Plants of cultivars H78-7750, H78-3567 and H78-4153, which were almost always negative for SCYLV with TBIA (sometimes 1–4% of blots were previously found positive (Lehrer and Komor 2008)), were positive using RT-PCR (Fig. 2b). qRT-PCR was employed to get a quantitative comparison across all cultivars. The results of qRT-PCR assay showed a variation in the viral template over a range of 10^5–10^6 folds (Fig. 2c).

Based on the previous results from TBIA (Schenck and Lehrer 2000) and the results here, the commercial cultivars were arranged into three groups (Fig. 2); high (H87-4094 H93-4068 and H77-4643, shown in dark grey), intermediate (H95-4655, H87-4319 and H65-7052, shown in medium grey), and low virus incidence (H78-7750, H87-5794, H78-3567 and H78-4153, shown in light grey). Previously these cultivar groups were called SCYLV-susceptible, intermediately-susceptible and resistant. The group giving low qRT-PCR product had on average a 1,000-fold lower virus titre than the intermediate group, which in turn had on average a 10~100-fold lower virus titre than the high group. We assume that the amount of RT-PCR product detected in these analyses correlates with the actual SCYLV particle titre in the leaves because of the linear correlation of qRT-PCR product with the dilution factor of a standard sample (Fig. 1).

QRT-PCR (Fig. 2c) showed a 10–100 fold variation in virus titre within the same cultivar depending on the sample, especially in cultivars of the previously-labeled intermediately-susceptible group (H95-4655, H65-7052). It appears that the virus titre fluctuated in that group over a range which was sometimes below the detection limit of TBIA. Only 20–60% of leaves (or plants) of these cultivars seem infected, from TBIA, although according to RT-PCR results all samples contained SCYLV (Fig. 2b). Because the high-yielding, often "yellowing" cultivar H65-7052 was among these intermediately-susceptible cultivars, further experiments on SCYV-infection and yield focused on this particular cultivar.

Virus detection in symptomatic and asymptomatic plants of cv. H65-7052

Cultivar H65-7052 was selected for further detailed analysis because this cultivar has high fluctuation of TBIA results and frequently shows typical YL symptoms in the field so that symptomatic and asymptomatic leaves could be compared. Three methods were used for virus detection: TBIA, conventional RT-PCR and qRT-PCR.

YL symptomatic and asymptomatic leaves (Fig. 3a) were sampled over a six-month period in test plots on the island of Maui and TBIA was performed with the midribs of these samples. From a total of 110 leaves analyzed, 67% of plants tested positive for SCYLV in leaves showing symptoms, while only 34% of asymptomatic leaves were positive (Fig. 3c). In addition, the quantity of product obtained with conventional RT-PCR (Fig. 3b), was greater in yellowed leaves than in asymptomatic leaves. Thus the data obtained from TBIA and conventional RT-PCR on mature H65-7052 sugarcane showed a strong correlation between YL-symptoms and the amount of RT-PCR products (Fig. 3). Repeated studies using samples from the island of Oahu gave similar results (data not shown). This was supported with all tested leaf parts of cultivar H65-7052, mature lamina, midribs (normally used for TBIA) and new leaves (Fig. 4a, b). Based on the qRT-PCR data, symptomatic mature sugarcane lamina and midribs exhibited a difference of approximately 10^3 and 10^4-fold, respectively, compared to asymptomatic. New sugarcane leaves contained less virus, but it was still about 30-fold higher in symptomatic than asymptomatic leaves. In general, the virus titres in samples from symptomatic leaves were in the same range as those from so-called highly susceptible cultivars (see Fig. 2c), whereas the samples from asymptomatic leaves were in the range of so-called resistant cultivars. Cultivar H65-7052 obviously exhibits a wide range of virus titre, which might make comparison of high-titre plants and low-titre plants possible.

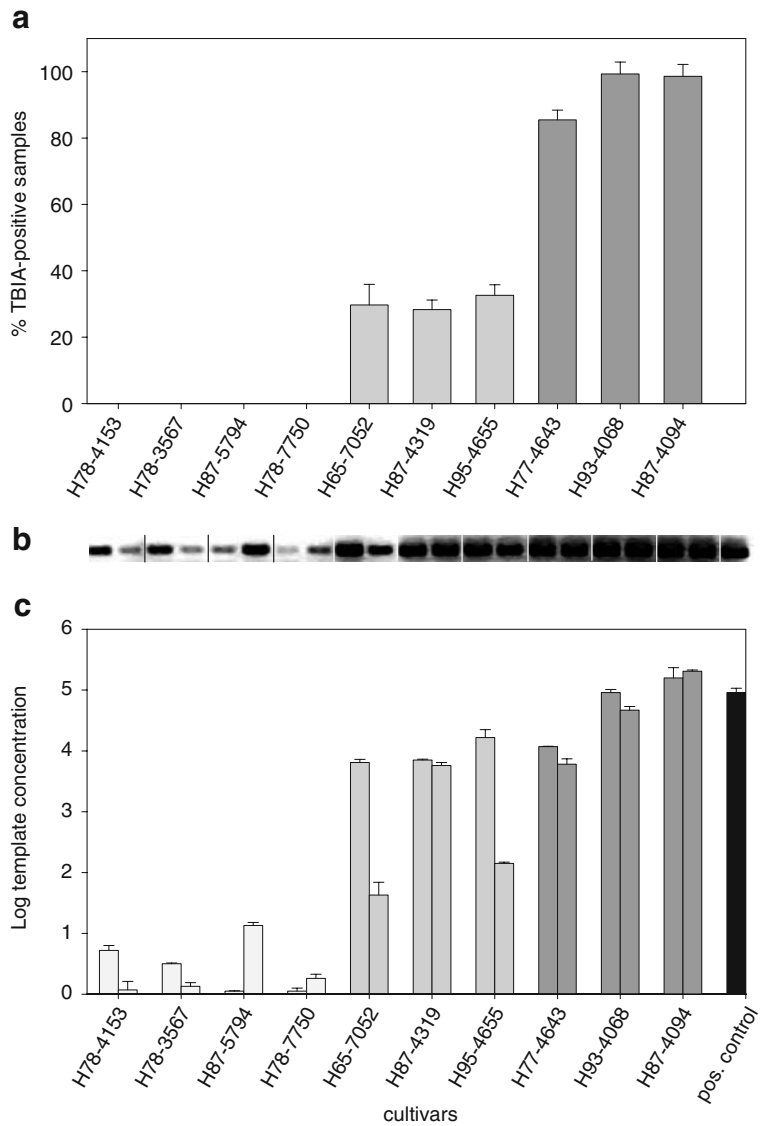

Fig. 2 SCYLV detection in Hawaiian commercial cultivars. **a** percentage of SCYLV-positive leaves based on TBIA method (result from at least 12 leaves per cultivar), **b** gel electrophoresis of RT-PCR product from SCYLV using primers based on ORF0 sequence of SCYLV (two biological replicates, same as for c), **c** relative quantification of RT-PCR product from SCYLV-RNA by qRT-PCR. The positive control was powdered leaf sample from H87-4094, the negative control was somatic embryogenic callus derived from H62-4671 (not shown). The quantification is calculated using the 'Comparative C_T' method. Data presented are the means of two biological replicates and 3 technical (i.e., PCR amplification) replicates each with standard error bars. The same leaf midribs were used for TBIA (**a**) and for RNA extraction to perform RT-PCR (**b**) and qRT-PCR (**c**). Cultivars were divided into three groups based on the previously-reported susceptibility groups (Schenck and Lehrer, 2000): resistant cultivars (*very light gray*), intermediately-susceptible cultivars (*medium grey*) and highly susceptible cultivars (*dark grey*), the positive control in black

Correlation of SCYLV-titre and YL symptoms with biomass and sugar yield

A greater number of symptomatic plants was present in plots which were derived from seed pieces of YL-symptomatic plants of cultivar H65-7052 than in plots grown from asymptomatic plants. Using TBIA (Fig. 5b), the percentage of leaves giving positive results for SCYLV ranged from 66 to 100% in plots planted with seed derived from symptomatic plants.

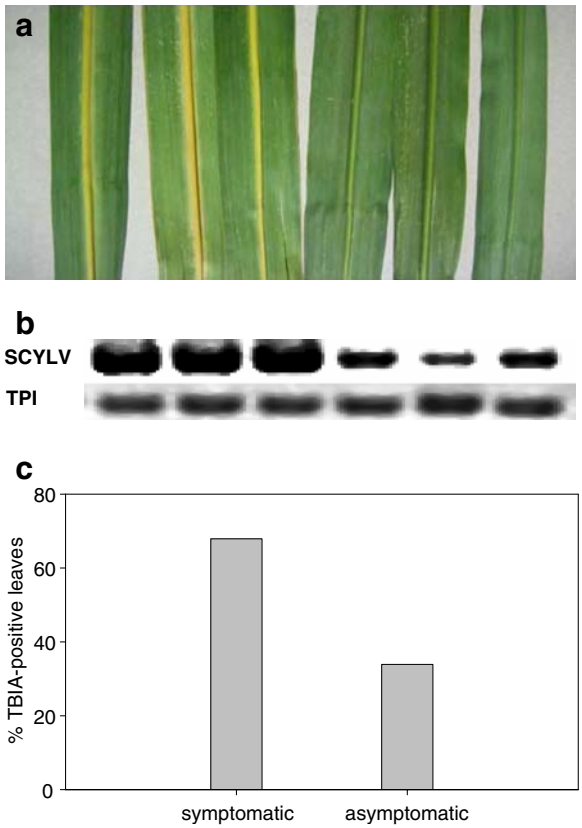

Fig. 3 Detection of SCYLV in symptomatic vs asymptomatic leaves of cv. H65-7052, using TBIA and RT-PCR. **a** Photograph of six sugarcane leaves. Three leaves on the left show yellow midrib symptoms while the three on the right show no symptoms **b** Gel electrophoresis of RT-PCR products amplified from RNA of symptomatic and asymptomatic leaves. SCYLV: PCR using the SCYLV-ORF0 primers, TPI: PCR using TPI-primers as an internal control **c** Percentage of leaves positive for SCYLV using TBIA (result from 110 leaves)

None of the leaves were positive in plots derived from asymptomatic plants. Likewise the correlation of symptom expression in the field plots with RT-PCR results was largely confirmed (Fig. 5a), since there were intense bands detected in samples from plots derived from symptomatic seed pieces and very weak bands detected in samples from plots derived from asymptomatic seed pieces. Thus the prevalence of symptoms was carried through from one field planting to the next. Samples of these plants had been used for qRT-PCR of leaf parts (Fig. 4b), a 10^3–10^4 -fold difference in virus titre existed between the plots.

The plots with low and high virus titre were harvested after 11.5 months to obtain yield comparisons. Biomass and sugar yields were lower in symptomatic plots, at only 60% and 54%, respectively, of those of asymptomatic plots (Table 1).

Discussion

Soon after SCYLV was identified as the causal agent of sugarcane yellow leaf symptoms (Vega et al. 1997), RT-PCR was adopted as the virus detection method (Comstock et al. 1998). Later, quantitative real-time RT-PCR was utilized (Korimbocus et al. 2002; Gonçalves et al. 2002). Four genetically different strains of SCYLV were identified and loosely correlated to geographical sites (Abu Ahmad et al. 2006). SCYLV found in Hawaii has not yet been sequenced. However, it was found that plants of a La Réunion cultivar (R570) obtained from the Hawaiian breeding station and also the Hawaiian sugarcane cultivars H32-8560 and H50-7209 which were exported to Peru, contained solely the BRA-PER type of SCYLV (Abu Ahmad et al. 2006). It is highly likely that all sugarcane plants originating from the Hawaii sugarcane breeding station on Oahu would have been exposed to the same strain of the virus.

All sugarcane samples used in this study were collected from commercial fields (HC&S) on Maui, and plants used at the Experiment Station field were derived from seed pieces from this plantation. Plant samples therefore had a very narrow geographical origin. Primers for SCYLV used in RT-PCR and qRT-PCR, (based on ORF0) were selected as they have been shown to be conserved for all strains published in GenBank. Gel electrophoresis, melting curve analysis, and a dilution series of a standard sample all confirmed that the amplicon obtained was the SCYLV fragment. The dilution series covered a template range of five orders of magnitude. The virus titre of some RNA samples was one order of magnitude below the lowest standard sample. Based on the linear relationship of the template versus ΔC_T (Fig. 1), the extrapolation is probably justified.

The development of a tissue-blot immunoassay (TBIA) for detection of SCYLV has made it possible to quickly screen large numbers of plants (Schenck et al. 1997). However, as this method is of a qualitative nature it was expected that there would be a minimum threshold level for detection. Comparative measurements with RT-PCR allowed an estimation of TBIA sensitivity to be made. TBIA was shown to be at least

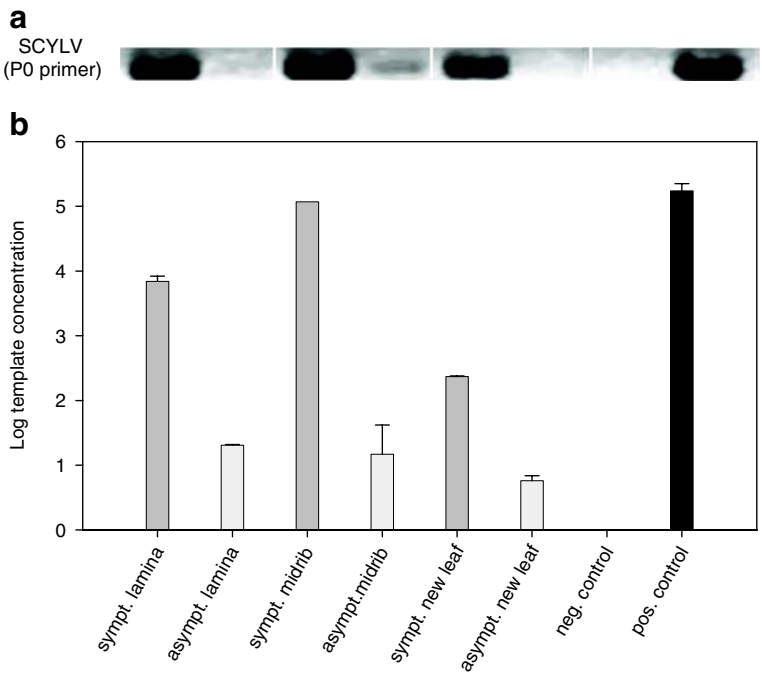

Fig. 4 SCYLV detection in leaf tissues by conventional RT-PCR **a** gel electrophoresis of RT-PCR product amplified from leaf samples using primers of SCYLV-P0(ORF0). **b** relative quantification of RT-PCR products (qRT-PCR) using RNA derived from leaf tissues of symptomatic or asymptomatic plants of cv. H65-7052. The same experimental procedures were used as described for Fig. 2b and c

10^3 to 10^4 fold less sensitive than RT-PCR. Using RT-PCR with Hawaiian sugarcane plants has shown that previous designations of "susceptible" and "resistant" (meaning virus-free) were incorrect. Several cultivars which were always negative in TBIA experiments produced positive RT-PCR results indicating that they do, in fact, contain the virus. The only truly virus-free plants were found to be those derived from tissue culture (Fitch et al. 2001; Chatenet et al. 2001) and that therefore had never been exposed to viruliferous

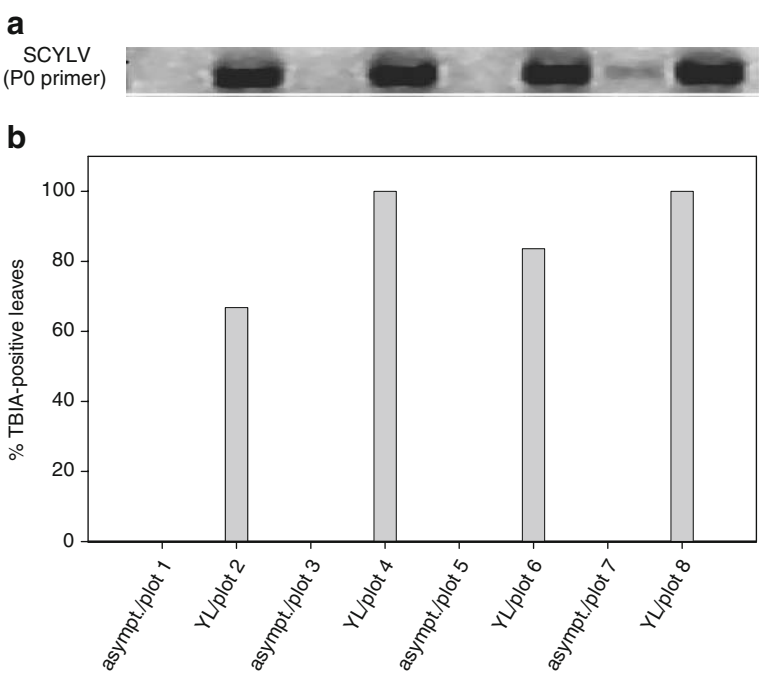

Fig. 5 SCYLV detection in sugarcane plants from field plots. Seed pieces which had been derived from symptomatic or asymptomatic plants were planted and these produced symptomatic (YL) or asymptomatic plants, respectively. **a** RT-PCR using the SCYLV ORF0 primers. **b** Percentage of plants positive for SCYLV based on TBIA results

Table 1 Comparison of plant growth (biomass) and sugar yield from sugarcane plants symptomatic or asymptomatic for SCYLV. Above-ground biomass and sugar yield of plants of cv. H65-7052 grown in experimental field plots (mean±SD). At 11.5 months of age above-ground biomass (leaves and stalks) of 4 plots each were harvested and analyzed

	symptomatic	asymptomatic
Biomass (t/ha)	135.6±28.2	224.6±16.6 *
Raw sugar yield (t/ha)	14.9±4.84	27.8±4.42 **

*,**indicates that the results are significantly different between the groups ($P<0.05$)

aphids. This highlights the importance of selecting virus-free plants from tissue culture in order to study the effect of SCYLV on plant growth and yield.

Our results with RT-PCR showed that four Hawaiian cultivars, previously thought to be uninfected and "resistant" to infection (Schenck and Lehrer 2000), were, in fact, infected with SCYLV (Fig. 2), albeit at a very low level. The differences in qRT-PCR amplification between these "resistant" cultivars and cultivars thought to be highly-susceptible was in the order of $10^{5\sim6}$. That result explains some earlier observations which were not previously understood:

- Infection experiments with *Melanaphis sacchari* showed that, in one case, virus-free aphids feeding on the "resistant" H87-4319 plants became viruliferous, and could subsequently transmit the virus. Previously, that result was considered erroneous (Schenck, unpublished).
- An extended survey of more than a hundred leaf samples of a "resistant" cultivar yielded a small number (4%) of weak positive TBIA reactions (Lehrer and Komor 2008). These "resistant" cultivars only rarely exhibited yellowing symptoms and it was not clear if these symptoms were due to viral infection or caused by physical damage such as broken midribs or severed stalks (Lehrer and Komor 2008)

The designation of a cultivar as "resistant" or "susceptible" may still be justified, especially if the terms can be correlated with the virus titre calculated by RT-PCR. Assuming the amount of RT-PCR product directly correlates with the amount of virus present, the results here are consistent and repeatable. It would appear that each cultivar supports a certain virus titre relative to other cultivars, although this may vary with environmental conditions and presence/absence of symptoms.

Previous studies have failed to closely correlate SCYLV-infection with visible symptoms (Lehrer and Komor 2008). An explanation for this might be the fluctuation in virus titre. For example, in this study, a resistant cultivar such as H87-5794, exhibited a 10-fold variation in virus titre. A fluctuation above and below the minimum threshold for TBIA might explain why results for a given cultivar might vary.

The reason for this variation in virus titre is not known. Low-titre and high-titre conditions of cv. H65-7052 were carried through by seed piece plantings (Fig. 5), which points to a plant and/or viral genetic basis of variation. One possibility is that the two plant populations carry differently virulent SCYLV strains (Abu Ahmad et al. 2007), both of which would be detected by PCR analysis. Another possibility is that of somaclonal variation, which has been shown to be high in sugarcane (Heinz et al. 1969) and which may affect virus susceptibility (Oropeza and de García 1996). Small genetic variation may suppress viral multiplication in one plant population more than in another. The commercial Hawaiian sugarcane cultivars tested, although having a relatively narrow genetic parentage, showed a SCYLV-sensitivity range of up to 10^5-fold between "resistant" and highly susceptible plants.

Although it is normally around the leaf midrib that symptoms appear, RT-PCR revealed that for a given symptomatic or asymptomatic plant, the virus titre was similar in both the lamina and the midrib area. If SCYLV was directly causing the leaf yellowing, for example by interaction with cellular components, the extent of yellowing would be the same for midribs and lamina. However, yellowing is predominantly confined to the midribs. It is therefore thought that SCYLV, which is a phloem-limited virus, causes a blockage in the flow of carbohydrates and thus assimilates build up. A field trial indicated a positive correlation between SCYLV titre and SCYLV symptoms (Figs. 4 and 5) and an inverse correlation to cane yield (Table 1). Our results are consistent with previous reports that SCYLV affected sugarcane yields by reducing stalk diameter, tillering and tonnage even when plants remained asymptomatic (Vega et al. 1997; Grisham et al. 2002; Rassaby et al. 2003; Lehrer et al. 2009). Of particular note is the fact that cv. H65-7052 is consistently high-yielding

despite being visibly infected with SCYLV. One explanation for this might be that the relatively long harvest cycle in Hawaiian plantations (18–24 months) allows the infected plants to "grow out" and recover from the effects of adverse seasonal conditions. In a previous field study, the yield of virus-free and infected plants of cv. H87-4094 was measured and it was found that there was a 30% yield loss for infected plants harvested after 11 months, however, there was no yield loss, when these plants were harvested after 18 or 24 months (Lehrer et al. 2009).

In conclusion, while TBIA is still a useful method for quickly screening a large number of samples, its sensitivity is at least 10^3–10^4-fold below the sensitivity of RT-PCR. This lack of sensitivity should be taken into consideration as samples which give negative TBIA results may still be infected with a low titre of SCYLV. This study has shown RT-PCR to be a more sensitive and reliable detection technique. Also, it has shown that sugarcane previously thought not to be infected with SCYLV does in fact contain the virus but at too low a level to be detected by TBIA.

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