GENETIC DIVERSITY OF *SUGARCANE YELLOW LEAF VIRUS* IN A SUGARCANE SELECTION PLOT IN GUADELOUPE (FWI)

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KEYWORDS: SCYLV, Genetic Diversity.

Abstract

THE GENETIC diversity of *Sugarcane yellow leaf virus* (SCYLV) was investigated in a sugarcane selection plot in Guadeloupe to determine the incidence of the different known virus genotypes (BRA-PER, CUB and REU) in a breeding progeny. Four F1 leaves were randomly collected from each of 154 sugarcane clones and tested for the presence of SCYLV by tissue blot immunoassay (TBIA). The leaf samples were stored at –80°C until total RNA was extracted from SCYLV-infected leaves. The virus genotypes were determined by RT-PCR and primer pairs specific to each virus genotype. Eighty-two percent of the tested leaves were infected by SCYLV and all known virus genotypes occurred in the selection plot. The majority of plants were infected by genotypes CUB or REU, or by a mixture of these two genotypes. This situation was completely different from the situation observed in commercial fields in Guadeloupe where the incidence of SCYLV is much lower and where most plants are infected by genotype REU. The significance of this striking situation will be further investigated.

Introduction

Yellow leaf of sugarcane is an emerging disease that was first reported as yellow leaf syndrome in Hawaii and Brazil at the end of the 1980s (Rott et al., 2008). It was subsequently found to be associated with a member of the *Luteoviridae* family called *Sugarcane yellow leaf virus* or SCYLV that was assigned to the genus *Polerovirus* (Rott *et al*., 2008; D'Arcy and Domier, 2005).

Sequencing of the almost entire genome of 13 SCYLV isolates revealed that SCYLV was genetically heterogeneous, and four genotypes of the virus were identified: BRA for Brazil, CUB for Cuba, PER for Peru, and REU for Réunion Island. Genotypes BRA and PER, that are closely related, cannot be easily distinguished, and were assigned to a group of genotypes called 'BRA-PER' (Abu Ahmad *et al*., 2006a).

This latter genotype is widely distributed, whereas the other ones are geographically restricted (Abu Ahmad *et al*., 2006b). Recently, a new genotype named IND was reported in India (Viswanathan *et al*., 2008).

Because (i) infected plants are frequently symptomless, and (ii) efficient diagnostic methods were only available by the end of the 1990s, SCYLV was spread all over the world through infected germplasm. So far, the virus has been reported in at least 30 countries (Rott *et al*., 2008).

Consequently, many of the sugarcane clones that are imported into CIRAD's sugarcane quarantine in Montpellier (France) are infected with SCYLV, and have to be cleaned up from the virus before distributing to other countries (Chatenet *et al*., 2001; Girard *et al*., 2007). During the process of SCYLV detection in CIRAD's quarantine, we recently noticed that a high proportion of SCYLV-infected clones received from CIRAD's sugarcane breeding station in Guadeloupe (FWI) were infected by genotype CUB. This situation was unexpected because other data from Guadeloupe indicated that REU was the most common genotype on this island (Daugrois *et al*., 2008).

Leaf samples were therefore collected in the sugarcane selection plot used to distribute sugarcane clones from Guadeloupe to the sugarcane quarantine in Montpellier. These samples were tested to analyse the incidence and the genetic diversity of SCYLV in this selection plot, and to compare this genetic diversity to the one occurring in commercial fields in Guadeloupe.

Materials and methods

Selection of leaf samples

Four F1 (= top visible dewlap) leaves were randomly sampled from each of 154 sugarcane clones planted in a selection plot at CIRAD's research station in Guadeloupe (FWI). These clones were progenies of 14 crosses and they were at third selection stage of a family selection. After tissue blot immunoassay (see below), the leaves were stored at –80°C until further processing. RT-PCR detection and genotyping tests were performed with two successive sets of leaf samples: in the first step, 38 out of the 154 sugarcane clones were randomly selected among the clones, and two leaves per clone were individually analysed.

This sample selection will be thereafter called set A. Later on, 18 sugarcane clones were selected for additional analyses among clones that tested either positive or negative by TBIA using all four sampled leaves. This sample selection will be called set B. Each leaf of set B was individually tested by RT-PCR.

Virus detection by tissue blot immunoassay

Tissue blot immunoassay (TBIA) was performed as described by Schenck *et al*. (1997), except that nitrocellulose membranes and Fast Blue salt (Sigma®) were used. TBIA membranes were analysed with a stereomicroscope (x10) to determine positive reactions.

Virus detection and genotyping by RT-PCR

RNA extraction

One hundred mg of each selected leaf sample was used for RNA extraction with QIAGEN® RNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Total RNA was eluted in DEPC RNase-free water and stored at –20°C for future use.

RT-PCR test with generic primers

A specific SCYLV genome fragment from the coat protein coding region was amplified by RT-PCR using primers ScYLVf1 (GACAGACTCGGCCAGTGGTCGTG) and ScYLVr1 (GTAAGCCATTGTTGAACGCTGCG). The RT-PCR reaction was performed using QIAGEN® OneStep RT-PCR Kit.

The 25 µL RT-PCR reaction mix consisted of 1 µL of eluted RNA, 17.05 µL of RNAse-free water, 5 µL of RT-PCR buffer (5X), 0.5 µL of dNTP mix (10 mM), 0.10 µL of each primer (100 µM), 1 µL of RT-PCR mix and 0.25 µL of RNAse inhibitor (Invitrogen, Carlsbad, CA). The RT-PCR program was as follows: 50°C for 30 min, 95°C for 5 min, 30 cycles at 94°C for 1 min, 71°C for 1 min and 72°C for 30 sec with a final 72°C extension for 10 min. The PCR product was analysed by electrophoresis through a 1.0% agarose gel in TAE buffer, stained with ethidium bromide and visualised under UV light. The SCYLV amplification product had an expected size of 219 bp.

Virus genotyping

Only samples that tested positive with SCYLV generic primers were used to identify the SCYLV genotype(s) by RT-PCR with genotype specific primers. The amplification of a specific genome fragment from each SCYLV genotype was performed as described by Ahmad *et al.* (2006b), except that QIAGEN® OneStep RT-PCR Kit was used. The 25 µL RT-PCR reaction mix consisted of 1 µL of eluted RNA, 12.05 µL of RNAse-free water, 5 µL of Q solution, 5 µL of RT-PCR buffer (5X), 0.5 µL of dNTP mix (10 mM), 0.10 µL of each primer (100 µM), 1 µL of RT-PCR mix and 0.25 µL of RNAse inhibitor (Invitrogen ref.10777-019).

The RT-PCR program was as follows: 50°C for 30 min, 95°C for 5 min, 30 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min with a final 72°C extension for 10 min. The PCR products were analysed by electrophoresis through a 1% agarose gel in TAE buffer, stained with ethidium bromide and visualised under UV light. The SCYLV amplification products of SCYLV genotypes BRA-PER, CUB and REU had expected sizes of 362, 450, and 905 bp, respectively.

Statistical analyses

All statistical analyses were made using SAS software v.9.1. Frequency comparisons between different sugarcane populations of RT-PCR positive and negative samples as detected with generic primers were made using Fisher's exact test under freq procedure. Frequency comparisons of virus genotypes between the same sugarcane populations were made among RT-PCR positive samples as detected with generic primers using Fisher's exact test.

Comparisons of virus genotype frequencies within set A and set B pooled data were made under GLM procedure after arcsin transformation of genotype proportions.

Results

SCYLV detection by TBIA

Five hundred and twenty out of 632 sampled leaves (82%) tested SCYLV positive by TBIA. Additionally, at least one virus-infected leaf was found for 143 out of 154 sugarcane clones (93%). The virus was not detected in 112 plants (18%) and 11 sugarcane clones (7%).

Detection by RT-PCR

Among the 76 leaves of set A (38 sugarcane clones), 65 were positive for SCYLV by RT-PCR. Five leaves that tested negative by TBIA were positive by RT-PCR, and seven leaves that tested positive by TBIA were negative by RT-PCR. Fifty five of 61 leaves of set B were SCYLV-positive by RT-PCR, and eight leaves that were TBIA negative tested positive by RT-PCR. Frequencies of RT-PCR positive leaves were not different between set A and set B ($P = 0.44$, Fisher's exact test).

Genotyping

The three SCYLV genotypes (BRA-PER, CUB and REU) were found in sets A and B, and the observed genotype incidences are detailed in Table1. Because incidences of genotypes BRA-PER, CUB and REU were not different between sample sets A and B, the data from both sets were pooled for further analyses. Virus genotype CUB had the highest incidence, followed by genotype REU and genotype BRA-PER (Table 2).

Most samples were infected by a single virus genotype (47.4%), but patterns of co-infection combining two or three genotypes were also found. Seventeen (12.4%) of the leaves were RT-PCR negative, 8 leaves (5.8%) were infected by BRA-PER only, 16 leaves (11.7%) by REU only, and 41 leaves (29.9%) by CUB only.

Five leaves (3.6%) were infected simultaneously by BRA-PER and REU, 10 leaves (7.3%) by BRA-PER and CUB, and 36 leaves (26.3%) by CUB and REU. Four leaves (2.9%) contained all three virus genotypes.

Table 1—SCYLV genotype incidences in two sets of leaf samples collected in a stage 3 breeding plot.

RT-PCR primers	Test result	Number of leaves in sample set A	Number of leaves in sample set B	P = (Fisher's exact test)
SCYLV generic primers	+	65	55	0.44
	–	11	6	
CUB specific primers	+	51	40	0.52
	–	14	15	
REU specific primers	+	30	31	0.28
	–	35	24	
BRA-PER specific primers	+	18	9	0.19
	–	47	46	

Table 2—Comparison of SCYLV genotype incidences in a stage 3 breeding plot.

SCYLV genotype	% of virus-infected leaves	Homology groups (student's test)
CUB	66.4	a
REU	44.5	b
BRA-PER	19.7	c

Interaction between plant characteristics and virus genotype incidences

The plant genetic background (i.e. crosses) and the characteristics of TBIA positive clones were studied taking into account virus genotype incidences.

The progenies of two crosses (#28 and #160) represented by more than 20 leaf samples were chosen and compared together, but also to the other remaining sampled crosses (Table 3).

RT-PCR positive sample frequencies of these three plant genetic groups varied from 75% to 95%. The plant genetic background had a significant effect on RT-PCR positive leaf frequency (P = 0.037) and also a strong effect on CUB and REU incidences, but not on BRA-PER incidence (Table 3).

Virus genotype incidences between the three groups of clones varied from 56% to 98%, 33% to 71% and 13% to 30% for CUB, REU and BRA-PER, respectively.

Table 3—SCYLV genotype incidences in 3 different plant genetic backgrounds of samples collected in a stage 3 breeding plot.

RT-PCR primers	RT-PCR test result	Cross 28 B85342xCP76-331	Cross 160 B7784xD172	Other crosses	P = (Fisher's exact test)
SCYLV generic primers	+	21	42	57	0.037
	–	7	2	8	
CUB specific primers	+	18	41	32	1.65×10^{-6}
	–	3	1	25	
REU specific primers	+	7	30	24	0.003
	–	14	12	33	
BRA-PER specific primers	+	5	5	17	0.107
	–	16	37	40	

The sugarcane clones were distributed into two groups according to TBIA results. The first group (= group 1) contained all clones with four TBIA-positive leaves and the second group (group 2) contained all clones with at least one TBIA-negative leaf. We may assume that clones belonging to group 1 were more susceptible to SCYLV than clones belonging to group 2.

The frequency of RT-PCR-positive clones was compared within these two groups (Table 4), and it was significantly higher in group 1 that included the most susceptible clones as determined by TBIA. The incidence of each SCYLV genotype within the RT-PCR positive leaves was then compared between groups 1 and 2. Incidence of genotype CUB was not different in group 1 and group 2.

The same result was found for SCYLV genotype BRA-PER. In contrast, the frequency of REU genotype varied between the two groups (P = 0.014): 56% and 25% for groups 1 and 2, respectively. This latter result suggested that SCYLV genotype REU is able to infect the susceptible clones more easily than the other ones.

Table 4—Comparison of SCYLV genotype frequencies of sugarcane clones with no virus-free leaves and clones with at least one virus-free leaf (as determined by TBIA).

RT-PCR primers	Test result	Clones with no virus-free leaves	Clones with at least one virus-free leaf	P = (Fisher's exact test)
SCYLV generic primers	+	100	20	1×10^{-5}
	–	5	12	
CUB specific primers	+	72	14	0.57
	–	28	6	
REU specific primers	+	56	5	0.014
	–	44	15	
BRA-PER specific primers	+	21	6	0.387
	–	79	14	

Interaction between genotypes

Because CUB was the most frequent genotype in the studied population, the incidence of genotypes REU and BRA-PER in CUB positive and CUB negative leaves was investigated. The incidence of genotype REU in CUB positive and CUB negative leaves was 44% and 45%, respectively. The frequency of genotype BRA-PER in CUB positive and CUB negative leaves was 15% and 28%, respectively, but this difference was not significant (P = 0.11).

Discussion and conclusion

A very high percentage of sugarcane clones of the selection plot (93%) were found infected with SCYLV, indicating rapid infection of plants after planting the seedlings a few years ago (assuming that SCYLV cannot be transmitted through true seed or fuzz; Rott *et al*., 2008). This situation is completely different from the situation observed in commercial fields in Guadeloupe where only few plants (0.5–37 %) were found infected by the virus (Daugrois *et al*., 2008). However, high incidence of SCYLV in commercial cultivars was described in Hawaii (Schenck and Lehrer, 2000) and in Réunion Island.

In this latter location, almost all plants of cultivar R575 were infected with the virus (Rassaby *et al*., 2004). The high variation of SCYLV incidence between CIRAD's breeding station in Guadeloupe and commercial fields could be attributed to low resistance to yellow leaf of the sugarcane cultivars used as parents in the crossings. This hypothesis is supported by our data suggesting that the genetic background of the crosses used in the selection plot played a role in the

number of RT-PCR positive leaves (presence of the virus) and the number of leaves harbouring the different virus genotypes. This situation should be taken into account in breeding sugarcane for resistance to SCYLV.

Furthermore, the nature and dynamics of aphid vector populations might also account for the striking differences between the sugarcane selection plot and commercial fields in Guadeloupe (Edon Jock, 2008). SCYLV genotype REU was more frequently detected in the group of sugarcane clones considered as the most susceptible (all four analysed leaves tested positive by TBIA) than in the other group of clones (at least one leaf tested negative by TBIA).

This result is in agreement with previous experiments performed in Guadeloupe in which genotype REU was less aggressive than the other virus genotypes (Abu Ahmad *et al*., 2007).

The most frequent virus genotype in the selection plot was CUB, whereas genotype REU is the most frequent, if not to say the only one, in commercial fields of Guadeloupe. Additionally, a high proportion of leaf samples were co-infected with two different virus genotypes. We even found four single leaves (representing two clones) in which the three genotypes were present together.

The detection of all three SCYLV genotypes in a single plant is most likely related to the set up of the selection plot on a research station where sugarcane clones and genetic resources have been introduced for a long time, especially when sugarcane yellow leaf was unknown and/or when efficient detection tools were not available. It would be interesting to investigate whether the situation observed in Guadeloupe is unique or encountered in other locations where breeding stations are used to produce and select new cultivars.

This study shows that SCYLV genotype CUB has a better fitness than genotypes BRA-PER and REU among the breeding progenies of the selection plot in Guadeloupe. The reasons that account for this better fitness should be further investigated. Interestingly, the detection of genotype CUB remained confidential until very recently in Guadeloupe (Daugrois *et al*., 2008).

Occurrence of genotype CUB may have been underestimated because the detection primers that were used up to recently failed to detect this genotype in several circumstances (Girard *et al*., 2008). Alternatively, its occurrence may be very recent and, therefore, genotype CUB may be an emerging genotype in Guadeloupe.

The high proportion of leaves in which two different virus genotypes (and even three genotypes in four leaves) were detected suggests that the presence of one genotype in a plant does not prevent infection by another genotype in the same plant.

These multiple infections were most likely favoured by the planting of clones near SCYLV-infected sugarcane germplasm and in the presence of large and active aphid populations.

To conclude, the results of this experiment give rise to at least two important questions: 1/ Does this situation, observed on a sugarcane breeding station, dictate the disease situation in sugarcane commercial fields within the next several years?, and 2/ Will the co-existence of two or three different SCYLV virus genotypes in the same plants promote the genesis of new virus genotypes that could be more virulent? Large fields of investigations regarding yellow leaf of sugarcane remain unexplored or insufficiently explored so far.

Acknowledgments

We would like to express our thanks to Dr Monique Royer for designing the new SCYLV primers used in this study.

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DIVERSITE GENETIQUE DU *SUGARCANE YELLOW LEAF VIRUS* DANS UNE PARCELLE DE SELECTION DE CANNE A SUCRE EN GUADELOUPE

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MOTS CLEFS: SCYLV,
Génotypes.

Résumé

LA DIVERSITÉ génétique du *Sugarcane yellow leaf virus* (SCYLV) a été étudiée dans une parcelle de sélection de canne à sucre en Guadeloupe pour déterminer l'incidence des différents génotypes connus du virus (BRA-PER, CUB and REU) dans une descendance. Quatre feuilles F1 ont été collectées au hasard sur chacun des 154 clones de canne à sucre et testées pour la présence de SCYLV par la technique d'immuno-empreintes (TBIA). Les échantillons foliaires ont été stockés à –80°C en attendant l'extraction de l'ARN total des feuilles infectées par le SCYLV. Les génotypes du virus ont été déterminés par RT-PCR en utilisant des paires d'amorces spécifiques de chaque génotype. Quatre-vingt-deux pour cent des feuilles testées étaient infectées par SCYLV et tous les génotypes connus du virus étaient présents dans la parcelle de sélection. La majorité des plantes étaient infectées par les génotypes CUB ou REU, ou par un mélange de ces deux génotypes. Cette situation est totalement différente de celle observée dans des champs commerciaux en Guadeloupe où l'incidence du SCYLV est beaucoup plus faible, et où la plupart des plantes sont infectées par le génotype REU. La signification de cette situation surprenante fera l'objet d'autres études.

DIVERSIDAD GENÉTICA DEL *SUGARCANE YELLOW LEAF VIRUS* EN UNA PARCELA DE SELECCIÓN DE CAÑA DE AZÚCAR EN GUADELOUPE (FWI)

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PALABRAS CLAVES: SCYLV, Genotipos.

Resumen

La diversidad genética del *Sugarcane yellow leaf virus* (SCYLV) fue investigada en una parcela de selección de caña de azúcar en Guadalupe (FWI = Caribe francés) para establecer la incidencia de diferentes genotipos conocidos de este virus (BRA-PER, CUB y REU) en una descendencia. Cuatro hojas F1 (primera hoja totalmente desarrollada) fueron recolectadas al azar sobre cada uno de los 154 clones de caña de azúcar y la presencia de SCYLV fue determinada con la técnica de 'tissue blot immunoassay' o TBIA. Las muestras de hojas fueron almacenadas a –80°C y el ARN total fue extraído de las hojas infectadas por el SCYLV. Los genotipos del virus fueron determinados por RT-PCR utilizando tres pares de cebadores específicos de cada genotipo. Un ochenta y dos por ciento de las hojas analizadas estaban infectadas por el SCYLV y todos los genotipos conocidos del virus estaban presentes en la parcela de selección. La mayor parte de las plantas estaban infectadas por los genotipos CUB o REU, o por una mezcla de estos dos genotipos. Esta situación es totalmente diferente a la observada en los campos comerciales en Guadalupe donde la incidencia del SCYLV es mucho más baja y donde la mayoría de las plantas están infectadas por el genotipo REU. Las causas de esta situación asombrosa serán investigadas más adelante.