A DIAGNOSTIC TEST FOR CHLOROTIC STREAK DISEASE

By

KS BRAITHWAITE¹, BJ CROFT²

BSES Limited, Indooroopilly¹ and Woodford²
kbraithwaite@bses.com.au

KEYWORDS: Chlorotic Streak, PCR Diagnostic Assay, Actin Gene, Causal Agent.

Abstract

CHLOROTIC STREAK IS A soil-borne disease of sugarcane widespread throughout the cane growing countries of the world. It is one of the major diseases of the Australian sugar industry, causing yield losses of up to 40% in susceptible varieties. The disease infects the crop through the root system and is spread through flood and drainage water and via infected planting material. Despite chlorotic streak being recognised as a disease since 1929, the identity of the causal agent remains elusive. Successful disease management is hampered by a lack of understanding of the causal agent and a lack of a diagnostic assay. Research carried out by BSES has identified a DNA fragment which is highly correlated with chlorotic streak and has the potential to be developed into a diagnostic test. A diagnostic assay for chlorotic streak could be used for screening cane, soil and water. It would assist in delivering disease-free seedcane, in carrying out disease surveys and in understanding disease transmission and epidemiology. This in turn would greatly improve our ability to manage the disease. This paper describes initial testing of a potential diagnostic assay based on DNA sequences of the actin gene.

Introduction

Chlorotic streak is one of the most serious and widespread diseases of the Australian sugar industry. The disease was first recognised in Java, Hawaii and Australia, around 1929. The disease can cause yield losses of up to 40% in susceptible varieties, leading to annual losses in Australia of $8–10m. It reduces germination, ratooning, stalk number and stalk weight. Disease incidence surveys have shown chlorotic streak disease to be the most widely recorded sugarcane disease in Queensland over the past 40 years (Magarey, 2005). The worst affected areas are the high rainfall wet tropics and low lying, poorly drained areas in other regions.

Chlorotic streak is spread through wet soil, flood and drainage water and via infected planting material. The symptoms are characterised by yellow to creamy-white leaf streaks with irregular wavy margins. Internal stalk symptoms include a reddening of vascular bundles at the nodes. The disease infects the crop through the root system and root temperature affects streak production, with 30 °C found to be optimal for symptom development (Sturgess, 1962). In the field, leaf symptoms are often transient, probably due to environmental conditions, including soil temperature.

Despite its importance and distribution, the causal agent of the disease is still unknown. A diverse range of organisms has been proposed as the causal agent. Histological evidence for the presence of a chytrid fungus in roots was presented by Carpenter (1940). Louisiana researchers claimed that a leafhopper, Draeculacephala portola, was the vector responsible for transmitting the disease (Abbott and Sass, 1945). However, later research clearly established the role of soil and water in disease transmission, rather than an insect (Antoine, 1957). Transmission experiments carried out in Australia demonstrated that the causal agent could pass through a 0.5 μM filter,
implicating a virus (Sturgess, 1963) but by using modern virology techniques Rogers et al. (2001) could find no evidence of viral particles by electron microscopy and no viral-associated RNA or DNA could be isolated.

In recent years, BSES recommenced research into identifying the causal agent, this time using molecular techniques. Generic virological and bacterial approaches were initially tried. Then, while testing a wide range of ‘universal’ fungal PCR primers, a primer set was identified that generated a DNA fragment highly correlated with chlorotic streak which has the potential to be developed into a diagnostic test. This paper will describe the discovery of the DNA and the applications for a diagnostic assay.

Materials and methods

Plant material

Disease-free or ‘healthy’ (H) and chlorotic streak (CS)-infected sugarcane was maintained in the field or glasshouse at BSES Woodford Experiment Station to provide material for research. Various types of samples (leaves, stalks, roots) were taken for molecular analysis during the course of this work, with all analyses done at the BSES laboratory at Indooroopilly. Sett roots were prepared by germinating surface-sterilised single-eye setts on 1% water agar for two weeks at 28 °C in the dark. Vascular extracts (xylem) were taken by blowing the juice from stalk pieces in the same manner used for detection of ratoon stunting disease.

A glasshouse yield trial was established in September 2011 using three varieties susceptible to chlorotic streak: Q155, Q170A and Empire. Single-eye setts were planted into pots of soil, with four setts per pot and six replicate pots. The disease-free control setts were hot water treated using the standard industry practice for chlorotic streak of 50 °C for 30 minutes, prior to being planted (Croft et al., 2011). Pots were arranged randomly into long troughs of water with diseased and disease-free treatments in separate but adjacent glasshouse chambers. The plant crop was harvested after three months (December 2011). Stalk height (from base to top visible dewlap), number of nodes, number of leaves and number of symptomatic leaves were recorded and leaves and stalks were sampled for molecular analysis.

The ratoon crop was harvested two months later (February 2012). The number of germinated stools and stalks was recorded and the stalks were sampled for molecular analysis. The trial data were analysed using SAS version 9.3. A linear mixed model was fitted with Type, Variety and Type*Variety as fixed effects and replicates (Type) as a random effect. Mean comparisons for the significant effects were calculated using the Tukey-Kramer multiple comparison test. The mean comparison letter groupings were determined by the PDMIX800.SAS macro written by Arnold M. Saxton (2000).

Molecular analysis

DNA was extracted using Qiagen DNeasy Plant mini or maxi kits depending on the size of the sample. Leaf samples consisted of 10 small disks punched from symptomatic areas of leaves using a paper hole-punch and were ground in a MP FastPrep-24 machine. The larger stalk, root and ratoon shoot samples were first processed into smaller pieces in a Blixer Robot-Coupe before being ground in the FastPrep-24 machine with the TeenPrep adaptor for 15 mL tubes. Vascular extracts were untreated.

A wide range of universal PCR (polymerase chain reaction) primers were tested for their potential to amplify fungal or fungal-like organisms from CS-infected samples. These include the widely used ribosomal ITS (internal transcribed spacer) primers of White et al. (1990), as well as IGS (intergenic spacer), large subunit and small subunit primers. Oomycete-specific primers targeted the ITS, cytochrome oxidase II, elongation factor, actin, β-tubulin and 60S ribosomal protein L10. PCR products were visualised by agarose gel electrophoresis. Some PCR products were cloned, sequenced and the putative identity of the sequences determined from BlastN searches of the GenBank database.
Results and discussion

Glasshouse trial

The glasshouse trial clearly showed the serious effects of chlorotic streak on plant growth and ratooning. Average stalk height, total number of nodes, and average number of leaves and percentage of leaves expressing symptoms were compared for the plant crop. The number of nodes was not significantly affected by the disease (data not shown) but there were significant differences in stalk height and leaf production (Figures 1 and 2). Leaf symptoms were seen in 37% of leaves in Q155, 65% in Q170\(^\circ\) and 70% in Empire in the chlorotic streak treatments.

![Graph showing average stalk height](image1)

Fig. 1—Average stalk height in the plant crop. The Tukey-Kramer multiple comparison test showed significant differences between H and CS treatments for stalk height when results were combined for varieties.

![Graph showing average number of leaves](image2)

Fig. 2—Average number of leaves in the plant crop. Solid bars represent average total number of leaves per stalk, shaded bars represent average number of leaves showing symptoms of chlorotic streak. The Tukey-Kramer multiple comparison test for logit percent leaves with symptoms of chlorotic streak grouped Q170\(^\circ\) + Empire (A) > Q155 (B).
The effects of the disease were more evident in the ratoon crop. Many chlorotic streak-infected plants did not germinate and those that did were stunted. So, for this time point, only the number of surviving stools (out of a maximum of 24) and total number of stalks were recorded (Figure 3), both showing significant differences. No Empire plant in the chlorotic streak treatment survived ratooning. Empire is the third most widely planted variety in NSW and chlorotic streak has caused serious losses in wet seasons in this variety.

![Fig. 3—Number of surviving stools (solid bars) and total number of stalks (shaded bars) in the ratoon crop. The Tukey-Kramer multiple comparison test showed significant differences between H and CS treatments for both surviving stools and total number of stalks when results were combined for varieties.](image)

**PCR using universal primers**

A range of universal fungal primers and Oomycete-specific primers were tested for their ability to generate a PCR product that was specific to chlorotic streak-infected plants. Most primer pairs generated non-specific PCR amplification, seen as a PCR band in both disease-free and chlorotic streak-infected samples. One primer pair, designed to target the Oomycete actin gene, did generate a CS-specific PCR band from DNA extracted from infected stalks. This primer pair was selected for further characterisation.

The Oomycete actin primer pair F1-R1, designed by Clint Magill (Texas A&M University, USA) and supplied by Nicole Thompson (BSES Limited), generates a 750 bp product from DNA extracted from chlorotic streak-infected stalks and leaves (Figure 4). The sequence of the DNA fragment has now been determined from infected stalks of Q170, Q155, KQ228, Empire and Q232.

The sequence contains a 200 bp intron which shows considerable sequence diversity between the infected sugarcane varieties. Unfortunately because the actin gene is so highly conserved, the GenBank database matches produced by a BlastN search revealed sequence similarity to a very diverse range of organisms including mammals and insects (Table 1). Encouragingly, two plant pathogens, *Phytophthora cinnamomi* and *Plasmodiophora brassicae* rank highly, however both of these organisms lack the intron found in the chlorotic streak PCR product. By aligning the chlorotic streak sequences with a number of *Phytophthora* and *Pythium* actin sequences, further primers targeting the intron and the region near the stop codon could be designed. The full sequence obtained so far is now 1300 bp.

When the PCR test was applied to roots grown in soil at Woodford, a 550 bp PCR product was generated from both CS-free and CS-infected samples (Figure 4, lanes 15 to 18). This product has been sequenced and found to have 100% homology to *Phytophthora* and *Pythium*, and is believed to be *Pythium arrhenomanes*, a common soil and water contaminant at Woodford. When the PCR test is applied to sett roots prepared by germinating surface-sterilised chlorotic stalk-infected setts in a contained system, the CS-specific 750 bp PCR product was generated.
Fig. 4—Agarose gel showing the 750 bp chlorotic streak-specific PCR product generated by the actin primers F1-R1. The 750 bp band is visible in chlorotic streak (CS)-infected stalk samples (Lanes 4, 6, 8, 10, 12 and 14) but not in disease-free (H) stalks (lanes 3, 5, 7, 9, 11 and 13). The band can be produced by infected leaves (lane 20), but not reliably. A 550 bp non-specific band is produced in both healthy and chlorotic streak-infected roots (lanes 15 to 18). Lane 1 is the no DNA control and lane 2 is the 100 bp marker.

Table 1—Top 10 BlastN database matches for the 750 bp F1-R1 PCR product amplified from Q170A chlorotic streak-infected stalks. The Expect value (E) estimates the significance of the match.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>BlastN database match</th>
<th>Common name</th>
<th>Genbank accession</th>
<th>E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oryctolagus cuniculus</td>
<td>Rabbit</td>
<td>XR085276</td>
<td>4 x 10^{-145}</td>
</tr>
<tr>
<td>2</td>
<td>Physcomitrella patens</td>
<td>Moss</td>
<td>XM001783607</td>
<td>2 x 10^{-142}</td>
</tr>
<tr>
<td>3</td>
<td>Gryllus bimaculatus</td>
<td>Cricket</td>
<td>AK283278</td>
<td>5 x 10^{-138}</td>
</tr>
<tr>
<td>4</td>
<td>Helicoverpa armigera</td>
<td>Bollworm</td>
<td>JF417981</td>
<td>6 x 10^{-137}</td>
</tr>
<tr>
<td>5</td>
<td>Phytophthora cinamomi</td>
<td>Oomycete</td>
<td>AM412176</td>
<td>9 x 10^{-135}</td>
</tr>
<tr>
<td>6</td>
<td>Plasmopara brassicae</td>
<td>Club Root</td>
<td>AM411663</td>
<td>3 x 10^{-134}</td>
</tr>
<tr>
<td>7</td>
<td>Oryctolagus cuniculus</td>
<td>Rabbit</td>
<td>NM001101683</td>
<td>3 x 10^{-134}</td>
</tr>
<tr>
<td>8</td>
<td>Micromonas pusilla</td>
<td>Algae</td>
<td>XM003061058</td>
<td>4 x 10^{-133}</td>
</tr>
<tr>
<td>9</td>
<td>Auxenochlorella protothecoides</td>
<td>Algae</td>
<td>JN714258</td>
<td>1 x 10^{-132}</td>
</tr>
<tr>
<td>10</td>
<td>Anopheles gambiae</td>
<td>Mosquito</td>
<td>XM003437002</td>
<td>2 x 10^{-131}</td>
</tr>
</tbody>
</table>

Development of a diagnostic test

We were interested to see if the actin F1-R1 primers would serve as a diagnostic test for chlorotic streak. The material generated during the glasshouse yield trial was used as the initial screening material (Table 2). Replicate material from each pot was pooled prior to DNA extractions. Unfortunately, the PCR test was not specific to chlorotic streak-infected leaves, because healthy leaf samples could generate a PCR product.

Table 2—PCR screening results from a glasshouse yield trial. DNA extracted from pooled chlorotic streak-infected material (CS) or disease-free (H) material was screened with the actin F1-R1 primers. The number of samples giving a 750 bp PCR product out of the number tested is shown.

<table>
<thead>
<tr>
<th></th>
<th>Plant crop leaves</th>
<th>Plant crop stalks</th>
<th>Ratoon crop shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>CS</td>
<td>H</td>
</tr>
<tr>
<td>Q155</td>
<td>3/3</td>
<td>2/3</td>
<td>1/6</td>
</tr>
<tr>
<td>Q170A</td>
<td>3/3</td>
<td>3/3</td>
<td>0/6</td>
</tr>
<tr>
<td>Empire</td>
<td>3/3</td>
<td>3/3</td>
<td>0/6</td>
</tr>
</tbody>
</table>
In contrast, the PCR test appeared to be much more specific to chlorotic streak-infected stalks, with all except one of the CS stalk treatments giving a positive PCR result and all except one of the healthy stalk treatments testing negative. For the ratoon crop, of the surviving plants in the CS-treatment that were screened, only one tested positive. No healthy ratoons gave the PCR product.

Because of a concern that surface organisms (such as insects, mites and fungi) were contaminating the sugarcane DNA and causing non-specific amplification, fresh samples were taken in June 2012. For this trial, there was greater emphasis on sample preparation prior to grinding. Chlorotic streak-infected stalks from five varieties, Q170\(^b\), Q124, Q155, Empire and Q238\(^b\), were cut from the field at Woodford.

To provide ‘healthy’ material, stalks of symptom-free cane of Q170\(^b\), Q238\(^b\) and Empire were also cut from the field, although these cannot be guaranteed to be disease-free. For Q124 and Q155, stalks were taken from the BSES export collection held in the quarantine glasshouse at Indooroopilly, which can reliably be considered disease-free. All stalks were washed with soap and water, and sprayed with 70% ethanol and then processed into five sample types: single-eye setts were cut and separated into pith, rind and buds; vascular extracts (xylem) were taken from the lower part of the stalks; and sett roots were prepared by germinating surface-sterilised single-eye setts on water agar. DNA was extracted from the five tissue types and screened with actin F1-R1. Results are shown in Table 3. For all tissue types, the disease-free material tested negative with the diagnostic test. Most CS-infected tissues gave a positive test result, except for Q170\(^b\) and Q238\(^b\) xylem and root samples.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Buds</th>
<th>Rind</th>
<th>Pith</th>
<th>Xylem</th>
<th>Sett roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>CS</td>
<td>H</td>
<td>CS</td>
<td>H</td>
</tr>
<tr>
<td>Q124</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Q155</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Q170(^b)</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Q238(^b)</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Empire</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

Universal PCR primers are a useful molecular technique when working with an unknown or new organism. These primers are designed to target conserved areas in the genome that are shared between known, closely related organisms. Many of the organisms that cause serious diseases in plants are commonly but incorrectly referred to as fungi. One such group are the Oomycetes, which include many well known pathogens such as *Pythium*, *Phytophthora* and *Pachymetra*. These organisms are not in the fungal kingdom but are more closely related to algae. Another important group are the Plasmodiophorans, which group with protozoa and slime moulds. Along with a group of true fungi, known as the Chytrids, these three groups of organisms all have a number of features in common that make them potential candidates for the causal agent of chlorotic streak. They have motile zoospores that swim in water and they infect plants through the roots, causing serious plant diseases. Many exist in an amoebic life form and cannot be grown on artificial media. These features could explain why the causal agent has not yet been isolated in pure culture, or unequivocally observed by microscopy.

Our initial choice of universal primers was targeted towards the Oomycetes because they are well studied with many primers publicly available. The Plasmodiophorans and Chytrids are less well studied. From 15 primer combinations tested, only one pair, which amplified the actin gene, appeared to have potential.
Actin is a protein found in all eukaryotic cells and participates in many important cellular processes including muscle contraction, cell motility, cell division and organelle movement. The protein is one of the most highly conserved throughout evolution, with 80.2% sequence conservation at the gene level between humans and yeast. Unfortunately this high degree of conservation means that the common approach of identifying DNA sequences through database searching has not allowed us to determine the origin of the DNA fragment at present.

Encouragingly, the DNA fragment does not have homology to sugarcane actin or actin from other closely related grasses such as maize, sorghum or rice. It is our intention to extend the sequence, allowing a more accurate identification to be made.

For the PCR reaction to be successfully implemented as a diagnostic test for the industry, aspects such as specificity and reliability combined with a practical sampling strategy need to be established. Leaves have the advantage of being easily accessible, easy to sample with a paper hole-punch and easy to extract DNA from. However leaves do not appear to be a reliable screening material for this test, possibly due to commonly occurring surface organisms such as fungi or mites being amplified non-specifically.

Roots grown in soil also generated non-specific PCR products. Surprisingly, young infected ratoon plants did not generate many positive PCR results. It is possible that the two-month-old ratoons were too immature for full expression of the causal agent, or the glasshouse temperatures over summer affected disease expression. This is not unexpected because chlorotic streak development can be transient and temperature dependant (Sturgess, 1962).

The most consistent and specific assay results were obtained from stalks. By taking more effort to clean the outside of the stalk in the second trial, we managed to eliminate much of the non-specific amplification observed in the first screening trial. The stalks were scrubbed clean to remove surface contaminants such as sooty mould.

The pith was separated from the rind and buds in the hope of further reducing surface contaminants, however all three tissues gave similar results. No disease-free material gave a positive PCR result.

Unfortunately testing stalks is a destructive sampling method and time consuming. The stalks had to be processed through an industrial-style food processor to reduce the material into smaller pieces suitable for DNA extraction. However, this assay is the first ever reported that could be used to screen planting material for freedom from chlorotic streak and could be used in disease-free seed plots, for material used to establish tissue cultures and in quarantine.

An easier sampling approach could be the use of vascular fluids blown from stalk pieces. In the second trial, the PCR assay of vascular fluids failed to detect chlorotic streak in some samples so it is yet to be determined how the causal agent is distributed throughout the length of the stalk. A major advantage of having a diagnostic test for vascular fluid is that the same sample could be screened for both ratoon stunting disease and chlorotic streak.

Conclusion

While the causal agent of chlorotic streak is still unknown at this stage, we have identified a DNA fragment that is highly correlated with the disease and has the potential to be developed into a diagnostic test for the industry that could be applied to screening sugarcane, soil and water.

A number of aspects still remain to be optimised before validation is complete. Further research will also concentrate on extending the current DNA fragment and determining the identity of the causal agent.

Acknowledgements

We acknowledge the scientific advice of Scott Hermann, the statistical support of Joanne Stringer and the hands-on assistance from many members of the BSES pathology group during the glasshouse trial. We acknowledge BSES and SRDC (BSS346) for funding.
REFERENCES