

Preparation of TA-cloned positive control for diagnostic PCR detection of phytoplasma associated with Cassava phytoplasma disease

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Abstract— *Cassava is one of the main foods in the country with more than 15 million Filipinos consuming this crop as staple or supplemental food. It is also a raw material used for feeds, alcohol and other industrial products. However, the Philippine cassava industry faces an emerging disease called Cassava Phytoplasma Disease (CPD). CPD is caused by a bacteria-like organism called phytoplasma. Here, we wanted to confirm phytoplasma infection in cassava showing witches' broom symptoms typical of CPD. We carried out molecular analysis using polymerase chain reaction (PCR), cloning, restriction enzyme digestion and sequencing methods to identify a cassava phytoplasma strain from an infected cassava plant showing symptoms of CPD. Plant DNA was extracted and used as a template in PCR reaction using universal primers amplifying the 16S rRNA region of phytoplasma. A nested PCR using primer pair R16mF2/R16mR1 in the first amplification followed by R16F2n/R16R2n in the second amplification was performed. PCR products were purified and transformed in a TA cloning vector. Following restriction enzyme digestion for insert verification, plasmid DNA about 1.5 kb in size was sequenced. Blast n search revealed homology with a phytoplasma strain causing sugarcane grassy shoot disease. Plasmid DNA with established sequence identity based on homology to 16S rRNA phytoplasma will serve as our positive control template for future PCR diagnostics of plant or insect samples associated with cassava phytoplasma disease.*

Keywords— *phytoplasma, 16S rRNA, nested PCR, insect vector, TA cloning*

I. INTRODUCTION

Cassava (*Manihot esculenta* crantz) is a woody shrub that has tuberous roots. It is cultivated annually throughout the world for its edible and starchy roots. It is the staple food in most of the countries in the developing world. In the Philippines, Northern Mindanao is the top cassava producer with 331.83 thousand metric tons sharing 41.0 percent of the total production for the quarter starting April 2018 to June 2018. The other leading producers were Autonomous Region in Muslim Mindanao (ARRM) with 27.5 percent and Cagayan Valley, 9.1 percent (PSA, 2018). The tuber has many uses, it can be eaten boiled, made into dessert like pichi pichi or cassava pudding. Its roots can also be used as animal feed, its stalks can be used for making particle boards, it can also be used as an adhesive and can be fermented to produce alcohol. However, the production of cassava has been reduced due to diseases like cassava phytoplasma disease (CPD). The disease is caused by a phytoplasma which is a wall-less bacteria limited to the phloem, it is insect transmitted but it cannot be cultured. The disease causes reduction in tuber yield and starch content in affected plants. Its symptoms also include shortening of the internodes and proliferation of small leaves along the stem. This study was done to develop a

positive control template for PCR diagnosis of cassava plants for CPD and make this readily available for our routine diagnosis of the pathogen.

II. MATERIALS AND METHODS

A. Amplification of 16S rRNA

Leaves and stems of cassava witches' broom infected cassava (*Manihot esculenta* Crantz) plants were collected from Carmen, Bohol. The DNA extraction protocol of Montano (2000) was used in plant DNA extraction but with some modifications. Primary polymerase chain reaction (PCR) was done using the universal primer pair, R16mF2/R16mR1. Each reaction was performed in 0.5ml PCR tubes in a final volume of 25ul with 12.5 ul of 2x PCR Mix by Vivantis 0.5 ul each of the primer (R16mF2/R1) and 3.0 ul of the DNA template. Thermocycling conditions were as follows; initial denaturation at 94 °C for 1 min, 35 cycles using the following conditions: denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, extension at 72 °C for 3 min and final extension at 72 °C for 7 min. Nested PCR reaction was done using the primary PCR product as template. Conditions for nested PCR reactions were the same except for the annealing temperature which was 55 °C and primers used, R16F2n/R16R2. The DNA fragment size standard was 1 kb ladder. Put here PCR reaction run, parameters, primers, amount DNA template

B. Gel purification of insert

Analysis of PCR products were done by electrophoresis using 0.75% agarose gel at 100 volts. Nested PCR products of the same band sizes were pooled and ran through 0.75% agarose gel by electrophoresis. The DNA fragment was excised using a scalpel blade under UV light. The gel was purified using the Zymoclean Gel DNA Recovery Kit of Zymo research. The excised DNA fragment was transferred into a pre-weighed 1.5 ml microcentrifuge tube. The size of each gel was determined, and 3 volumes of agarose dissolving buffer was added to each volume of agarose excised from the gel. This was incubated at 55°C for 10 min until the gel slice was fully dissolved. The melted agarose solution was transferred to a Zymo-Spin Column in a collection tube. The solution was centrifuged for 60 seconds and the flow-through discarded. Two hundred microliters of DNA wash buffer was added to the column and centrifuged at 10,000 rpm for 30 seconds and then wash through discarded. The wash step was repeated once. After discarding the flow through in the previous step, 6ul of DNA elution buffer was directly added to the column matrix. The column was placed in a 1.5 ml tube stood, for 3 min then centrifuged for 60 seconds to eluted the DNA. The eluted DNA was stored in -20°C.

C. TA cloning and Restriction Enzyme digestion

The pGEM-T Easy vector was used for TA cloning. A 10 uL ligation reaction was performed. The 10 uL ligation reaction was set up as follows: 2X Rapid ligation buffer, T4 DNA ligase, 5 uL, pGEM®TEasy Vector (50ng) 1 uL, PCR product 3uL, T4 DNA ligase 1 uL. The reactions were mixed by pipetting and incubated overnight at 4°C. One ul from this reaction was transformed into 50 ul JM109 competent cells through heat shock transformation. Positive transformants were picked and miniprep of plasmid DNA was performed. About 500 ng of plasmid DNA was used for restriction enzyme digestion. Both vector and competent cells were from Promega (Madison, WI, USA).

D. Transformation of JM109 High – Efficiency Competent Cells

Luria-Bertani medium with ampicillin, IPTG and X-GL plates were prepared beforehand. JM109 High Efficiency Competent Cells were placed in an ice bath until just thawed. Cells were mixed by flicking the tubes. The ligation reactions were briefly centrifuged and 2 ul of each reaction was added to a sterile 1.5 microcentrifuge tube on ice. Fifty microliters of JM109 High Efficiency Competent cells previously thawed in an ice bath was added to each ligation reaction. The tubes were flicked gently and incubated on ice for 20 min. The cells were heat shocked for 50 seconds in a water bath at 42°C and immediately returned to ice for 2 min. Nine hundred fifty microliters of room temperature SOC medium was added to the ligation reaction transformations and incubated for 1.5 hours at 37°C with shaking. One hundred microliters of each transformation culture was surface plated onto duplicate LB/Ampicillin/IPTG/X-Gal plates. The plates were incubated overnight at 37°C. White colonies which are assumed to contain successful ligations were picked and were individually inoculated onto three ml of LB medium and was incubated overnight at 37°C with shaking. The plasmid DNAs were purified using the protocol in the PureLink Quick Plasmid Miniprep Kit (Invitrogen). After purifying the DNA, restriction enzyme digestion of the purified DNA was done using EcoRI (Vivantis). The 50 ml reaction digestion was composed of 1 ul EcoRI, 2 uL plasmid DNA, 10x reaction buffer and 42 uL molecular grade buffer. The digestion reaction was done at 37°C for one hour. The digestion reaction was analysed through electrophoresis on an 0.75% agarose gel. Two bands, one the size of your vector and one the size of your new insert should be observed.

E. Sequencing and phylogenetic analysis

Sequence and contig assembly were done using BioEdit Sequence Alignment Editor v.7.0.4. Phylogenetic analysis was done through evolutionary history using the Maximum Parsimony method through MEGA7. The analysis involved six nucleotide sequences. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 0 in which the initial trees were obtained by the random addition of sequences (10 replicates). There was a total of 25 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [1].

III. RESULTS AND DISCUSSION

Cassava is one of the main agricultural crops in the country and the thriving cassava industry contributes well to the Philippine economy. In the last decade, we have seen a dramatic increase of production of cassava. However, a disease of cassava called Cassava phytoplasma disease (CPD) has been reported in the country in 2007 and has been an important major disease of this root crop until now. Phytoplasmas are phytopathogens which are wall-less, unculturable bacteria of the class Mollicutes that are associated with diseases, collectively referred to as yellows diseases in more than 1000 plant species worldwide [2]. In the Philippines, at least seven phytoplasma strains were identified (VSU, PhilRootCrops) in phytoplasma-infected cassava plants. Here, we aimed to produce positive control DNA templates for PCR detection of phytoplasma associated with CPD by cloning a specific gene fragment from a previously known CPD positive cassava plant. For detection and diagnostic of phytoplasma in cassava, we needed to have positive control to be used for routine PCR diagnostics.

We used cassava plants with symptoms of witches' broom and its leaves and/or stem were our sources of DNA for PCR amplification of the 16S rRNA region of phytoplasma. We followed established protocols using CTAB for DNA extraction because we knew from experience that it is very good for extraction of highly polymerized DNA from plant material. Using CTAB and then ethanol purification, we expect the DNA is not hydrolyzed during the

isolation process and high-quality genomic DNA can be obtained. At least three primer pairs were compared and used for nested PCR reactions. Primary PCR products showed different band patterns ranging from 500 bp to 10 kb (Figure 1). We used undiluted PCR products as templates for the second PCR (Figure 2). The amplified 16Sr RNA region was cloned into pGEM- T easy cloning vector. We selected the strongest DNA band signal of about 1.3 to 1.5 kb and purified it. This purified product was then used for cloning into the vector. The vector has 3'-T overhangs on both ends which allowed direct, high-efficiency cloning of our PCR products. The reaction was facilitated by complementarity between the PCR product's 3'-A overhangs and vector 3'-T overhangs. Heat shock transformation on JM109 E.coli strain was used and selection of positive transformants was done using blue-white screening. Included with the transformation reaction is one treatment using a DNA positive control provided by the company to check for transformation efficiency. Colonies that were picked were grown in liquid media and its plasmid DNA extracted using a commercial miniprep kit. Plasmid DNA concentration ranges from 500 ng to 1000 ng/ul (data not shown). To check for the insert, restriction enzyme digestion was performed (Figure 3) using 1 ug of plasmid DNA. All six colonies selected showed an insert size corresponding to ~1.5 kb. The insert was gel purified and sequenced.

Sequencing of the plasmid DNA showed significant similarity with different phytoplasma strains such as Sugarcane grassy shoot phytoplasma and Leafhopper aster yellows phytoplasma (Table 1). Sugarcane grassy shoot (SCGS) is one of the most destructive diseases of sugarcane. It is characterized by white leaves with partial or complete loss of chlorophyll and proliferation of tillers, giving the plant a grass appearance hence the name 'Grassy Shoot'. This sugarcane disease is economically important in India and is present in parts of Southeast Asia like Malaysia [3]. SCGS found in India belonged to 16Sr XI-F phytoplasma group [4]. In the Philippines, there is no report of the disease yet although grassy shoot symptoms in sugarcane was observed in quarantined planting materials [5]. Also, a sugarcane white leaf phytoplasma has been reported to be present from sugarcane planting materials imported from the Philippines [6] which is under the 16SrXI clade of Rice yellow dwarf [7]. Another similarity of the cassava 16S rRNA sample is with leafhopper aster yellows phytoplasma. Aster yellows (AY) is an important disease of Brassica crops [8] transmitted by the insect vector, Aster leafhopper (*Macrostelus quadrilineatus*). It is interesting that we found sequence similarity with an insect vector since published reports of any vector for cassava phytoplasma is scarce. Leafhoppers are potential vector candidates for transmission of cassava phytoplasma [2] and cassava mealybugs (*Phenacoccus manihoti*) may be potential vectors as well. Indeed, our preliminary PCR reactions with mealybugs using the same primer sets we used for plant samples showed the same band size corresponding to the 16S rRNA region of phytoplasma (data now shown). The bacterial transformant which was positive for the 16S rRNA insert was maintained as glycerol stocks and its plasmid DNA used as positive control for routine PCR diagnostic runs (Figure 4). The red arrow pointing to the band corresponds to the amplified target region of the template.

Based on the PCR reactions we performed, titre of phytoplasma differs based on the parts of the plant. Although we did not measure the titre of the pathogen present, we observed these amount differences on the samples from stems, leaves or petioles semi-quantitatively based on the strength of the DNA bands when we run agarose electrophoresis. At this point, the source and parts of the plants for DNA extraction must be considered very carefully as the low copy number of initial target DNA can affect the secondary PCR reactions which we thought are very necessary to amplify gene target.

TABLE I

BLASTn similarity search of PCR product from infected cassava plant sample

| Description | E-value | Identity | Accession |
|---|----------------|-----------------|------------------|
| Leafhopper (Deltocephalinae) aster yellows phytoplasma partial 16S rRNA gene, isolate Colombia isolate L12 | 3.45E-10 | 95.238 | HG764367.1 |
| Sugarcane grassy shoot phytoplasma partial 16S rRNA gene, isolate BV2 | 3.45E-10 | 95.238 | AM261832.1 |
| Sugarcane grassy shoot phytoplasma partial 16S rRNA gene, isolate VD7 | 3.45E-10 | 95.238 | AM261831.1 |
| Leafhopper (Deltocephalinae) aster yellows phytoplasma partial 16S rRNA gene, isolate Colombia isolate L11 | 1.60E-08 | 100 | HG764366.1 |
| Phytoplasma sp. Guerrero LY-P6 16S ribosomal RNA gene, partial sequence | 2.07E-07 | 100 | DQ073334.1 |
| Leafhopper (Deltocephalinae) aster yellows phytoplasma partial 16S rRNA gene, isolate Colombia isolate L12 | 3.45E-10 | 95.238 | HG764367.1 |

Figures

Figure 1. Representative gel of DNA products after primary PCR using DNA templates from infected cassava plants.

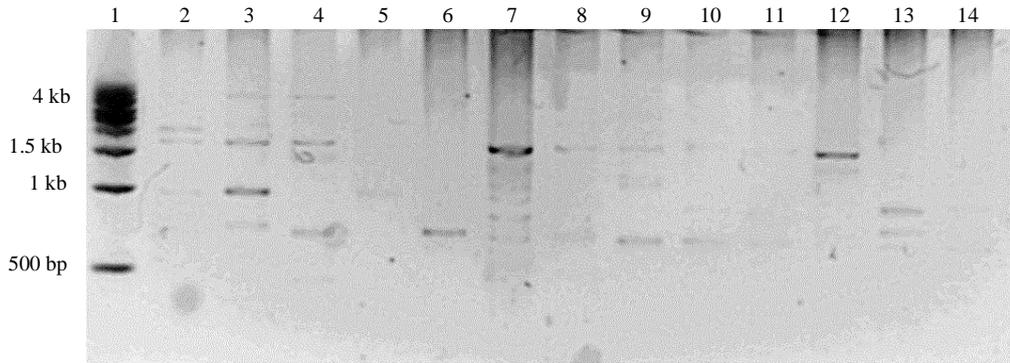


Figure 2. Representative gel of nested PCR products with templates coming from the primary PCR products.

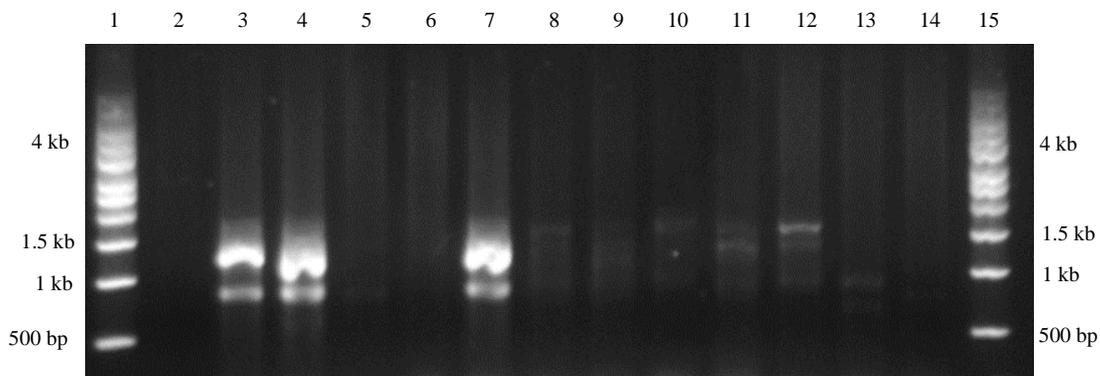


Figure 3. Agarose gel image of digested plasmid DNA (1 ug) using *EcoRI* restriction enzyme.

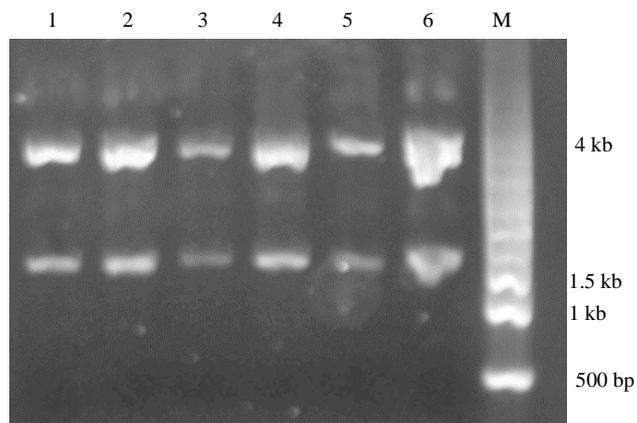
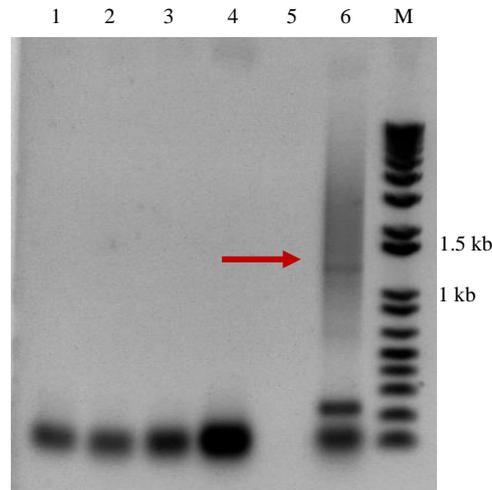


Figure 4. Representative agarose gel of nested PCR products using universal primers for detecting partial 16S rRNA region of phytoplasma in plant samples. Plasmid DNA from our TA-cloned recombinant bacteria on well 6 served as positive control.



IV. CONCLUSIONS

Cassava phytoplasma disease is an economically important disease of cassava and management of the disease continues to be a challenge since this bacteria can not be cultured. However, control of its spread can be possible if we have available diagnostics or identification tools in detecting the disease even before the onset of symptoms. The development of a PCR protocol on CPD diagnosis has been established and a source of positive control developed from this study can be very useful to efficiently identify presence of the pathogen in cassava. The recombinant DNA transformed in bacteria is an invaluable source as positive control template for routine PCR work. We recommend the use of this recombinant DNA as control plasmid which should enable distinction between presence or absence of CPD in cassava plants using qualitative PCR.

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