



**Faculty of Resource Science and Technology**

**Development of Recombinant Plant Vaccine for  
Cacao Swollen Shoot Virus (CSSV) Infection**

**Joel Michael Ponniah**

**Master of Science  
(Biotechnology)  
2015**

**Development of Recombinant Plant Vaccine for  
Cacao Swollen Shoot Virus (CSSV) Infection**

**Joel Michael Ponniah**

A thesis submitted  
In fulfillment of the requirements for the degree of Master of Science

Faculty of Resource Science and Technology  
UNIVERSITI MALAYSIA SARAWAK  
2015

UNIVERSITI MALAYSIA SARAWAK

Grade: \_\_\_\_\_

Please tick (✓)

Final Year Project Report

Masters

PhD

DECLARATION OF ORIGINAL WORK

This declaration is made on the 12<sup>th</sup> day of OCTOBER 2015.

Student's Declaration:

I JOEL MICHAEL PONNIAH (13020067), FACULTY OF RESOURCE SCIENCE & TECHNOLOGY (PLEASE INDICATE STUDENT'S NAME, MATRIC NO. AND FACULTY) hereby declare that the work entitled, DEVELOPMENT OF RECOMBINANT PLANT VACCINE FOR CACAO SWOLLEN SHOOT VIRUS INFECTION is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

12<sup>th</sup> OCTOBER

Date submitted

JOEL MICHAEL PONNIAH (13020067)

Name of the student (Matric No.)

Supervisor's Declaration:

I Rebecca Edward (SUPERVISOR'S NAME) hereby certifies that the work entitled, DEVELOPMENT OF RECOMBINANT PLANT VACCINE FOR CACAO SWOLLEN SHOOT VIRUS INFECTION (TITLE) was prepared by the above named student, and was submitted to the "FACULTY" as a \* ~~partial~~ full fulfillment for the conferment of MASTER OF SCIENCE (BIOTECHNOLOGY) (PLEASE INDICATE THE DEGREE), and the aforementioned work, to the best of my knowledge, is the said student's work

Received for examination by: Rebecca Edward  
(Name of the supervisor)

Date: 30/9/2015

I declare this Project/Thesis is classified as (Please tick (√)):

- CONFIDENTIAL** (Contains confidential information under the Official Secret Act 1972)\*  
 **RESTRICTED** (Contains restricted information as specified by the organisation where research was done)\*  
 **OPEN ACCESS**

### Validation of Project/Thesis

I therefore duly affirmed with free consent and willingness declared that this said Project/Thesis shall be placed officially in the Centre for Academic Information Services with the abide interest and rights as follows:

- This Project/Thesis is the sole legal property of Universiti Malaysia Sarawak (UNIMAS).
- The Centre for Academic Information Services has the lawful right to make copies for the purpose of academic and research only and not for other purpose.
- The Centre for Academic Information Services has the lawful right to digitise the content to for the Local Content Database.
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis for academic exchange between Higher Learning Institute.
- No dispute or any claim shall arise from the student itself neither third party on this Project/Thesis once it becomes sole property of UNIMAS.
- This Project/Thesis or any material, data and information related to it shall not be distributed, published or disclosed to any party by the student except with UNIMAS permission.

Student's signature \_\_\_\_\_  
(Date)  
12 OCTOBER 2015

Supervisor's signature: \_\_\_\_\_  
(Date)  
20/10/2015

Current Address: No.9, JALAN TASIK BERINGIN 3, PANTAI SEPANG PUTRA,  
SUNGAI PELEK, 43950 SEPANG, SELANGOR

Notes: \* If the Project/Thesis is **CONFIDENTIAL** or **RESTRICTED**, please attach together as annexure a letter from the organisation with the period and reasons of confidentiality and restriction.

[The instrument was duly prepared by The Centre for Academic Information Services]

## **ACKNOWLEDGEMENTS**

My deepest thanks and gratitude are directed towards my advisors, Dr. Rebecca Edward and Dr. Samuel Lihan, for their tireless follow-ups, critical reading of manuscripts, comments, suggestions and upgrading my awareness about my chosen field of research. I am also grateful to Dr. George A. Ameyaw, Research Scientist (Virologist) of Cocoa Research Institute of Ghana, for providing the necessary gene samples that enabled this project to move forwards.

I am also indebted to Prof. Dr. Sepiah Muid of UNIMAS for availing her research laboratories and materials during the course of my research, without which, this project would not have progressed.

I accord my thanks to Mr. Haya bin Ramba, the station manager of Malaysian Cocoa Board, Kuching (Sarawak) for his assistance in providing the necessary planting materials for this project.

I extend my deepest thanks for the organizations: University of Malaysia Sarawak (UNIMAS) for funding (grant no. RAGS/SG/06(1)/930/3012(31)), access to Internet, Library, Laboratory and other administrative facilities.

Last but not least, I thank God and my family for all the support given, be it in spiritual or emotional form, without which I would not have been able to press-on with the challenge of completing my research.

# **Development of Recombinant Plant Vaccine for Cacao Swollen Shoot Virus (CSSV) Infection**

**Joel Michael Ponniah**

## **ABSTRACT**

Cacao Swollen Shoot Disease (CSSD) is a persistent, incurable viral infection that kills infected cacao plants within 2-years after symptom development. Initially limited to West Africa, the disease has spread to other cacao growing areas, and poses a serious threat to the US\$107 billion global chocolate industry. A prospective vaccine against CSSD was developed in this study using recombinant gene technology. A cacao swollen shoot virus genome was isolated from total plant DNA using an established PCR-based isolation methodology, and the resultant full-length linear virus DNA amplified in OneShot<sup>®</sup> Top10 chemically competent *Escherichia coli* cells using pCR-XL TOPO<sup>®</sup> cloning plasmids. The virus DNA was later retrieved, digested using *EcoRI* restriction enzyme, and the targeted ORF3 gene fragment isolated through gel electrophoresis and incorporated into pBAD-TOPO<sup>®</sup> expression plasmids. This were then transformed into domesticated *Paenibacillus polymyxa* cells through electroporation, which acted as vaccine carrier. The resultant Biotic Response Elicitor Vaccine (BREV) produced a fragment of the virus particle, which was hypothesized to induce augmented systemic expression of the natural plant defense mechanism. When tested, the BREV suppressed CSSD symptoms in 74% of the test population at 68.3% efficacy rate, indicating a large impact against the disease (effect size of 1.172). PCR-based assessment further showed that 36% of vaccine-treated plants had no detectable levels of active virus particles (vaccine efficacy rate of 27.3%), indicating a moderate impact against active virus particle proliferation (effect size of 0.730). Overall, it was concluded that limited cross-protection effect against CSSD was attained using the current method. BREV had successfully abated symptoms of CSSD, and may extend the economic lifespan of CSSD affected cacao trees. Further studies and improvement of the BREV technology is thus highly recommended. The present development is the first known vaccine of its kind to be reported for a plant species.

**Keywords:** Cacao Swollen Shoot Disease, plant vaccination, recombinant vaccine, cross-protection

# **Penghasilan Vaksin Rekombinan untuk Tumbuhan bagi Jangkitan Penyakit Pembengkakan Pucuk Koko (PPPK)**

**Joel Michael Ponniah**

## **ABSTRAK**

Penyakit Pembengkakan Pucuk Koko (PPPK) merupakan sejenis jangkitan virus berterusan yang tidak boleh diubati, dan berupaya untuk memusnahkan tanaman koko dalam tempoh masa 2-tahun. Penyakit yang mulanya terhad kepada kawasan penanaman koko di Afrika Barat ini kini merebak ke kawasan penanaman lain, dan menjadi ancaman serius bagi industri coklat global yang bernilai sebanyak US\$107 billion. Kajian ini telah menghasilkan satu vaksin berasaskan teknologi penggabungan semula genetik yang berupaya untuk mengawal PPPK. Genom virus pembengkak pucuk koko Agou1 yang diasingkan daripada campuran DNA tumbuhan menggunakan kaedah pengasingan berasaskan PCR diamplifikasi dalam sel-sel *Escherichia coli* OneShot<sup>®</sup> TOP10 yang berkompeten menggunakan plasmid pengklon pCR-XL TOPO<sup>®</sup>. DNA virus kemudian dipulih, dicerna enzim pencerna *EcoRI*, dan serpihan gen ORF3 diasingkan menggunakan teknik gel elektroforesis. Ia kemudiannya diklon ke dalam plasmid pernyataan pBAD-TOPO<sup>®</sup>, dan diubah ke dalam sel-sel *Paenibacillus polymyxa* melalui teknik elektroporasi. Vaksin Maklum-Balas Biotik (VMBB) yang dicipta menghasilkan serpihan-serpihan virus yang mendorong peningkatan mekanisme pertahanan semulajadi tumbuhan. Ujian keberkesanan menunjukkan VMBB berjaya membentasi gejala PPPK dalam 74.0% populasi kajian pada kadar keberkesanan 68.3%, iaitu kesan vaksin yang amat ketara (saiz impact 1.172). Penilaian berasaskan PCR turut menunjukkan 36% daripada tumbuhan yang diberi vaksin tiada entiti virus aktif (kadar keberkesanan vaksin 27.3%), iaitu kesan vaksin yang sederhana terhadap entiti virus aktif (saiz impact 0.730). Kajian menunjukkan perlindungan-merentas seumpama VMBB boleh dicapai menggunakan kaedah yang telah dikaji. VMBB telah berjaya mengurangkan gejala dan kesan PPPK, dan berupaya untuk memanjangkan jangka hayat ekonomi pokok koko yang dijangkiti PPPK. Kajian lanjutan amat disarankan. Ini merupakan kajian vaksin seumpama yang pertama bagi tumbuhan.

**Kata-kata kunci:** Penyakit Pembengkakan Pucuk Koko, vaksinasi tumbuhan, vaksin rekombinan, perlindungan-merentas

## TABLE OF CONTENTS

|                                | <b>PAGE</b> |
|--------------------------------|-------------|
| <b>DECLARATION</b>             | <b>ii</b>   |
| <b>ACKNOWLEDGEMENT</b>         | <b>iii</b>  |
| <b>ABSTRACT</b>                | <b>iv</b>   |
| <b>ABSTRAK</b>                 | <b>v</b>    |
| <b>TABLE OF CONTENTS</b>       | <b>vi</b>   |
| <b>LIST OF ABBREVIATIONS</b>   | <b>xi</b>   |
| <b>LIST OF TABLES</b>          | <b>xiii</b> |
| <b>LIST OF FIGURES</b>         | <b>xv</b>   |
| <b>CHAPTER 1: INTRODUCTION</b> | <b>18</b>   |
| 1.1 Background Information     | 18          |
| 1.2 Problem Statement          | 22          |
| 1.3 Research Objectives        | 23          |
| 1.3.1 General objectives       | 23          |
| 1.3.2 Specific objectives      | 23          |

|   | <b>PAGE</b> |
|---|-------------|
| <b>CHAPTER 2: LITERATURE REVIEW</b>   | <b>24</b>   |
| 2.1 <i>Theobroma cacao</i> L. and the Global Chocolate Trade                          | 24          |
| 2.2 Diseases Affecting Cacao Production   | 27          |
| 2.3 Cacao Swollen Shoot Disease   | 30          |
| 2.3.1 Virus description   | 31          |
| 2.3.2 Transmission and symptoms of infection  | 34          |
| 2.3.3 Current research on Cacao Swollen Shoot Disease (CSSD)                          | 37          |
| 2.3.4 Challenges in controlling Cacao Swollen Shoot Disease (CSSD)                    | 39          |
| 2.4 Engineered Plant Resistance to Viruses  | 43          |
| 2.4.1 Existing methods of engineered plant resistance to viruses and<br>their vectors | 43          |
| 2.4.2 Induced resistance  | 47          |

|   | <b>PAGE</b> |
|---|-------------|
| <b>CHAPTER 3: MATERIALS &amp; METHODS</b>   | <b>50</b>   |
| <b>Experiment: Stage 1</b>  |             |
| 3.1 Selection of Suitable Native Cacao Bacteria with Plasmid Carrying Competency                                      | 51          |
| 3.1.1 Isolation and identification of heat tolerant, endospore-forming<br>endophytic bacteria from cacao inner tissue | 51          |
| 3.1.2 Cacao foliage colonization test using selected isolated bacterial<br>endophytes                                 | 54          |
| 3.1.3 Domestication of selected bacteria and plasmid competency test  | 55          |
| <b>Experiment: Stage 2</b>  |             |
| 3.2 Acquisition of High Grade Cacao Swollen Shoot Virus (CSSV) DNA  | 59          |
| 3.2.1 Primer design, PCR amplification of CSSV DNA, and isolation and<br>purification of amplified virus DNA          | 59          |
| 3.2.2 Cloning PCR product into TOPO <sup>®</sup> XL plasmids  | 61          |
| 3.3 Development of Recombinant Biotic Response Elicitation Vaccine  | 62          |
| 3.3.1 Recovery of virus DNA using restriction enzymes   | 62          |
| <b>Experiment: Stage 3</b>  |             |
| 3.4 Controlled Vaccine Test   | 65          |
| 3.4.1 Preparation of cacao rooted cuttings  | 66          |

|  | PAGE      |
|--|-----------|
| 3.4.2 Deployment of Biotic Response Elicitation Vaccine (BREV) using selected bacterial plasmid carrier            | 67        |
| 3.4.3 Inoculation of cacao rooted cuttings with virus-expressing pBAD-ULTR   | 69        |
| 3.4.4 Observation, evaluation and data analysis  | 70        |
| <br>   |           |
| <b>CHAPTER 4: RESULTS &amp; DISCUSSION</b>   | <b>71</b> |
| <br>   |           |
| <b>Experiment: Stage 1</b>   |           |
| 4.1 Selection of Suitable Native Cacao Bacteria with Plasmid Carrying Competency                                   | 71        |
| 4.1.1 Isolation and identification of heat tolerant, endospore-forming endophytic bacteria from cacao inner tissue | 71        |
| 4.1.2 Cacao foliage colonization test using selected isolated bacterial endophytes                                 | 77        |
| 4.1.3 Domestication of selected bacteria and plasmid competency test   | 82        |
| <br>   |           |
| <b>Experiment: Stage 2</b>   |           |
| 4.2 Acquisition of High Grade Cacao Swollen Shoot Virus (CSSV) DNA   | 90        |
| 4.2.1 Primer design, PCR amplification of CSSV DNA, & isolation and purification of amplified virus DNA            | 90        |
| 4.2.2 Cloning PCR product into TOPO <sup>®</sup> XL plasmids   | 93        |

|   | <b>PAGE</b> |
|---|-------------|
| 4.3 Development of Recombinant Biotic Response Elicitation Vaccine  | 95          |
| 4.3.1 Recovery of virus DNA using restriction enzymes   | 95          |
| <br><b>Experiment: Stage 3</b>  |             |
| 4.4 Controlled Vaccine Test   | 102         |
| 4.4.1 Preparation of cacao rooted cuttings  | 103         |
| 4.4.2 Deployment of Biotic Response Elicitation Vaccine (BREV) using<br><i>Paenibacillus polymyxa</i> plasmid carrier | 105         |
| 4.4.3 Inoculation of cacao rooted cuttings with virus-expressing pBAD-ULTR  | 107         |
| 4.4.4 Observation, evaluation and data analysis   | 108         |
| 4.4.5 Overall discussion  | 116         |
| <br><b>CHAPTER 5: CONCLUSION &amp; RECOMMENDATIONS</b>  |             |
| 5.1 Conclusion  | 129         |
| 5.2 Recommendations   | 131         |
| 5.3 Final Thoughts and Reflections  | 131         |
| <b>References</b>   | <b>138</b>  |
| <b>Appendices</b>   | <b>154</b>  |

## LIST OF ABBREVIATIONS

|               |  |
|---------------|--|
| $\mu\text{F}$ | microfarad   |
| ARU           | Attack rate – unvaccinated                           |
| ARV           | Attach rate - vaccinate                              |
| bp            | Base pairs   |
| BREV          | Biotic Response Elicitation Vaccine                  |
| CFU           | Colony forming unit                                  |
| CSSD          | <i>Cacao Swollen Shoot Disease</i>                   |
| CSSV          | <i>Cacao Swollen Shoot Virus</i>                     |
| DNA           | Deoxyribonucleic acid                                |
| dNTP          | Deoxynucleotide (nucleotide triphosphate)            |
| dsDNA         | Double stranded deoxyribonucleic acid                |
| ELISA         | Enzyme-Linked Immunosorbent-Assay                    |
| GB            | Glucose-beef broth                                   |
| HEB           | HEPES electroporation buffer                         |
| HEPES         | (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) |
| IBA           | Indole-3-butyric acid                                |
| ICPCR         | Immunocapture polymerase chain reaction              |
| IEM           | Immunolectron microscopy                             |
| kb            | Kilobases  |
| kV            | Kilovolt   |
| LB            | Luria-Bertani media                                  |
| miRNA         | Micro-ribonucleic acid                               |

|       |                                  |
|-------|----------------------------------|
| MT    | Metric tons                      |
| NAA   | $\alpha$ -naphthaleneacetic acid |
| NC    | Standard nutrient content        |
| OD    | Optical density                  |
| ORF   | Open reading frame               |
| PCR   | Polymerase chain reaction        |
| RBD   | Randomized block design          |
| RH    | Relative humidity                |
| RNA   | Ribonucleic acid                 |
| rpm   | Revolutions per minute           |
| scFv  | Single chain variable fragment   |
| shRNA | Short hairpin ribonucleic acid   |
| TSA   | Tryptic soy agar                 |
| TSB   | Tryptic soy broth                |
| VBA   | Virobacterial Agglutination      |
| v/v   | Volume per volume                |

## LIST OF TABLES

|          |  | <b>PAGE</b> |
|----------|--|-------------|
| Table 1  | Cacao production figures for the top 12 producing countries  | 26          |
| Table 2  | Major diseases of <i>Theobroma cacao</i> L.  | 29          |
| Table 3  | Five putative Open Reading Frames (ORFs) of CSSV   | 32          |
| Table 4  | Geographical spread of Cacao Swollen Shoot Disease   | 40          |
| Table 5  | Simulation of selected biotic stressor for domestication of wild-sourced endophytic endospore-forming bacteria | 56          |
| Table 6  | Visual scoring of CSSV Agou1 infection in cacao rooted cuttings  | 70          |
| Table 7  | Endophytic endospore-forming bacteria isolated from cacao inner tissue   | 72          |
| Table 8  | Selected isolates for colonization test on clean BR25 cacao leaves   | 77          |
| Table 9  | Variation in colonization population among isolates  | 78          |
| Table 10 | Isolate identity of persistent applied endophyte in cacao leaf tissue  | 81          |
| Table 11 | Mean growth of <i>Paenibacillus polymyxa</i> under simulated biotic stress                                     | 83          |
| Table 12 | Mean growth of <i>Paenibacillus polymyxa</i> by individual biotic stress parameters                            | 87          |
| Table 13 | Result of plasmid competency test on <i>Paenibacillus polymyxa</i>   | 88          |
| Table 14 | Quantitation of recovered amplified CSSV DNA from total plant DNA  | 92          |
| Table 15 | Mean transformation efficiency of pCSSV-XL compared to control   | 94          |
| Table 16 | Mean transformation efficiency of pBAD-AVGR compared to control  | 97          |
| Table 17 | Mean transformation efficiency of pBAD-ULTR compared to control  | 100         |
| Table 18 | Propagation efficiency of BR25 cacao cuttings  | 103         |

|          |  | <b>PAGE</b> |
|----------|--|-------------|
| Table 19 | Isolation of BREV on ampicillin-infused media                              | 105         |
| Table 20 | PCR-based detection of CSSV DNA  | 107         |
| Table 21 | Mean incidences of overall CSSV infection (visual assessment)              | 109         |
| Table 22 | Statistical analysis of vaccine efficacy                                   | 110         |
| Table 23 | Correlation of coefficient (visual vs. PCR assessment)                     | 111         |
| Table 24 | Statistical analysis of mean incidences of CSSV infection (PCR assessment) | 114         |
| Table 25 | Statistical analysis of vaccine efficacy                                   | 115         |

## LIST OF FIGURES

|  | <b>PAGE</b> |
|--|-------------|
| Figure 1: Principal cacao production countries and key export destinations   | 26          |
| Figure 2: Purified preparation of CSSV obtained after sucrose density gradient centrifugation. Bar represents 200nm  | 31          |
| Figure 3: Genome map of CSSV Agou1. ORF locations are indicated by numbers and alphabets   | 33          |
| Figure 4: Example of adult <i>Dysmicoccus sp.</i> mealybug, measuring around 2mm   | 34          |
| Figure 5: Visual symptoms of CSSV infection (a) red vein banding in young leaves, (b) chlorosis in major leaf veins, (c) stem swelling in adult trees, and (d) stem swelling in seedlings  | 36          |
| Figure 6: Geographical representation of cacao growing countries with reported incidence of <i>Cacao Swollen Shoot Disease</i>   | 39          |
| Figure 7: Example of young leaf and branch segment sampled   | 50          |
| Figure 8: Example of Interflush I-2 soft-wooded orthotropic shoot  | 66          |
| Figure 9: Mean endophytic colonization by endospore-bearing bacteria in mature cacao leaves at 7, 14 and 21 days after inoculation with cacao bacterial endophytes. Bars extending around the means indicate the standard error of that mean. Inoculation occurred at day 0, when the plants were sprayed with 0.25% Silwet L-77 (vol/vol) and Log <sub>10</sub> 8.0 CFU/ml bacterial suspensions. The horizontal dashed line indicates the minimum detection level for the experiment | 79          |
| Figure 10: Isolate BI-6, <i>Paenibacillus polymyxa</i>   | 81          |
| Figure 11: Cluster graphing on the effects of biotic stress treatment on <i>Paenibacillus polymyxa</i> colony growth. Treatments are pair-grouped in order to create each point on the graph, and are only indicative of the likely influencers of bacteria growth   | 85          |
| Figure 12: Histogram of BI-6 ( <i>Paenibacillus polymyxa</i> ) colony growth after being subjected to domestication regiment. Area under the curve equals the total number of colonies evaluated. Histogram is skewed to the left, with highest frequency being centered around the 7cm growth rate  | 86          |

|   | <b>PAGE</b> |
|---|-------------|
| Figure 13: Example of successfully transformed <i>Paenibacillus polymyxa</i> colony containing plasmid conferring kanamycin resistance grown on kanamycin infused media   | 89          |
| Figure 14: Gel electrophoresis showing isolated and amplified CSSV DNA. Lane 1 and 8 are the $\lambda$ DNA <i>Hind</i> III ladder used as molecular size markers (numbers indicate marker DNA size in kbp). Lane 2 is untreated control total plant DNA with added 7k PCR control fragment; Lane 3 and 4 contains total plant DNA with amplified CSSV DNA, and can be seen as a clear band around the 6.6kb marker (highlighted by red boxes). Band(s) seen above the 23.1kb marker are part of the total plant DNA | 91          |
| Figure 15: Blue-white colony formation of transformed <i>E. coli</i> cells. Red arrows indicate example of transformed colonies on the selective media plate (highlighted by red boxes)   | 94          |
| Figure 16: Gel electrophoresis showing <i>Eco</i> RI digested CSSV genome (Lane 5). Lane 1 and Lane 8 are $\lambda$ DNA <i>Hind</i> III ladder and $\lambda$ DNA <i>Hind</i> III + <i>Eco</i> RI fragment used as molecular size markers (numbers indicate marker DNA size in kbp). Three bands can be seen in the digest around the 1.4kb, 1.6kb and 2.0kb mark. Arrow indicates band corresponding to ORF3 gene fragment (in red box), located around the 1.6kb mark  | 96          |
| Figure 17: Successfully transformed and sub-cultured <i>Paenibacillus polymyxa</i> colonies containing the pBAD-AVGR plasmid-vaccine  | 98          |
| Figure 18: Gel electrophoresis showing isolated and amplified CSSV genome. Lane 1 is $\lambda$ DNA <i>Hind</i> III ladder used as molecular size markers (numbers indicate marker DNA size in kbp). Lane 5 contains the <i>Pst</i> I digested CSSV genome (in red box), and can be seen as a clear band around the 6.6kb marker   | 99          |
| Figure 19: Example of (a) Successful rooted cutting after five weeks of root induction in sand tray. NAA and IBA (dissolved in 50% alcohol) at a ratio of 1:1 were used to induce root formation in the cuttings (b) Successful bud-break within five weeks after transplanting into polybags containing autoclaved coarse silica sand  | 104         |
| Figure 20: Gel electrophoresis showing band corresponding to ORF3 gene fragment of <i>Eco</i> RI digested pBAD-AVGR plasmid recovered from treated plants (Lane 4). Lane 1 contains $\lambda$ - <i>Hind</i> III + <i>Eco</i> RI ladder (numbers indicate marker DNA size in kbp). Arrow indicates the excised ORF3 fragment (in red box); band around the 5.0kb marker is the remnant of the pBAD-AVGR plasmid  | 106         |

|            |  |     |
|------------|--|-----|
| Figure 21: | Bar graph showing the mean recorded incidence of CSSV infected plants according to visual assessment of symptoms   | 108 |
| Figure 22: | Bar graph comparing the mean recorded incidence of CSSV infected plants according to visual assessment of symptoms against PCR-based detection of active virus particles in new leaf growth  | 112 |
| Figure 23: | Close-up comparison of (a) leaf chlorosis pattern in CSSV infected plant with those of (b) iron deficiency. Notice the stark similarities  | 119 |
| Figure 24: | (a) Light microscope image of nucleic-acid rich spherical inclusion bodies after CSSV infection (b) TEM micrograph of CSSV infected plant stained with uranyl acetate, showing spherical inclusion bodies. Bar corresponds to 600nm. | 120 |

## CHAPTER 1

### INTRODUCTION

#### 1.1 Background Information

Cacao, or better known among botanists as *Theobroma cacao* L., is considered among the most important edible industrial crop in the world. First cultivated at least 2,600 years ago in Mesoamerica (Bartley, 2005; Motomayor *et al.*, 2002), cacao is now grown on roughly 17 million acres of land and directly supports between 5 to 6 million cacao farmers worldwide (CacaoNet Strategy, 2012; Paoletti *et al.*, 2012). As a significant globally-traded commodity, numerous major downstream and inter-related industries have been developed around the cacao trade, where a further 40 to 50 million people depend upon it for their livelihood (CacaoNet Strategy, 2012).

The sheer size and economic importance of cacao and its related industries should not be underestimated. In the year 2012 alone, approximately 4.1 million metric tons of cacao beans worth US\$10 billion were produced; the global chocolate industry, which heavily depend upon cacao production for its raw ingredient, carried a value of US\$107 billion in that same year (Anga, 2014). However, cacao production has been negatively impacted by a sleuth of biotic constraints, the chief among them being pest and diseases that have become rife within cacao growing regions globally (Aime and Phillips-Mora, 2005; Bowers *et al.*, 2001; Entwistle, 1972; Wood and Lass, 1985).

The emergence of Cacao Swollen Shoot Virus (CSSV) and the resultant Cacao Swollen Shoot Disease (CSSD) in particular has presented itself as a major constraint and concern for cacao production worldwide, as unlike most other pest and diseases affecting the crop, there is no known cure for CSSD (World Cocoa Foundation, 2012; Griffith, 2000). Although the disease was initially thought to be limited to cacao growing regions in West Africa, the lack of adequate quarantine measures together with unrestricted movement of cacao germplasm and infected plant materials has enabled CSSD to spread elsewhere (Griffith, 2000; Dongo and Orisajo, 2010; Lokchart and Sachey, 2001). Presently, incidences of CSSD have been reported in Ghana, Benin, Ivory Coast, the Sumatran island of Indonesia, Liberia, the state of Sabah in Malaysia, Nigeria, Papua New Guinea, Sierra Leone, Sri Lanka, and Togo (Brunt *et al.*, 1996; Lockhart and Sachey, 2001; Olunloyo, 2004; Liu and Liew, 1975; Liu, 1979, CacaoNet, 2012).

Infection by Cacao Swollen Shoot Virus (CSSV) (Family: *Caulimoviridae*; Genus: *Badnavirus*) is the root cause for the disease. The virus consists of numerous strains, with at least 16 strains being mentioned in literature (Posnette, 1947; Frison *et al.*, 1999; Lockhart and Sachey, 2001; Posnette *et al.*, 1950; Posnette, 1950; Posnette and Todd, 1955; Thresh and Tinsley, 1959; Olunloyo, 2004; Kay, 1961). The virus comprises a non-enveloped bacilliform particle that is 28nm thick and 130nm long, which contains a circular double-stranded DNA genome that is roughly 7.16kb in size (Lot *et al.*, 1991; Muller *et al.*, 2001). Five putative open reading frames (ORFs) have been indentified thus far, with some of these ORFs being successfully studied and described (Muller and Sackey, 2005; Yang *et al.*, 2003; Huang and Hartung, 2001). Like other pararetroviruses, the CSSV

is known to incorporate its genome into its host upon infection, where it begins expressing its RNA silencing genes to disable the host plant's defense (Bowick and McAuley, 2011).

The primary mode and mechanism of CSSV transmission is through the feeding activity of mealybugs, of which at least 14 species of mealybugs in the genera *Planococcus*, *Planoccocoides*, *Pseudococcus*, *Dysmicoccus*, and *Ferrisia* are involved (Frison *et al.*, 1999; Jeger, 2001). The movement of vegetative material has also helped to spread the virus, where it can latently infect cuttings that are used for clonal propagation for up to 20 months, even though it is not sap transmissible nor seed-transmitted (Frison *et al.*, 1999). Common indications of the disease include leaf pattern symptoms, chlorotic mosaic effects and red banding along the veins of the cacao leaves (Posnette, 1947; Olunloyo, 2004). Very often, stem swellings and malformation of pods may be seen (Posnette, 1947; Thresh and Tinsley, 1959; Adegbola, 1971; Olunloyo, 2004). At the onset of the disease symptoms, early senescence and leaf shedding are noticeable, which eventually leads to defoliation and death of outer branches and twigs, followed by the gradual dying back of the main branches (Posnette, 1947; Olunloyo, 2004). In the most severe strain of the virus, death occurs within two years of onset of symptoms (Posnette, 1947; Olunloyo, 2004).

Thus far, the management of Cacao Swollen Shoot Disease (CSSD) has depended upon preventive strategies, including identifying and eradicating infected cacao trees, since there are no known cures for the viral infections (World Cocoa Foundation, 2012; Griffith, 2000). Various detection protocols have been developed for CSSV, including ELISA, immunoelectron microscopy and PCR methods. However the serological and genomic variability of CSSV has made its detection complicated, whereas identification difficult

and unreliable (Sagemann *et al.*, 1985; Griffith, 2000; Lockhart and Sachey, 2001; Hughes *et al.*, 1995; Ploetz, 2007). Furthermore, initial symptoms of infection have often been confused for other diseases, including agronomic deficiencies, thereby making early detection and prevention tricky (Griffith, 2000; Dongo and Orisajo, 2010).

Other methods of control have also been attempted, including the management of mealybug vectors, none of which have proven to be practical or effective (Olunloyo, 2004; Posnette and Strickland, 1948; Olunloya, 2004). Concerted efforts were therefore directed towards breeding CSSV tolerant/resistant plants, but even purportedly Cacao Swollen Shoot tolerant clones are beginning to fail against the onslaught of the disease (Olunloya, 2004; Mars Sustainable Cocoa Initiative, 2012; Nestlé Cocoa Plan, 2012; Quainoo *et al.*, 2008a). A potential disease management method utilizing attenuated or mild strain CSSV was investigated as a means of providing cross-protection effect against the more severe strains of virus, but despite favorable protective effect against virulent CSSV isolates had been reported, deep caution was made against its application (Griffith, 2000; Posnette and Todd, 1945; Hughes and Ollennu, 1993). The general concern with such strategy was that the use of intact mild strain viruses as a means of cross-protection control represented a huge risk, since there is a danger of the possibility that the mild strains of viruses may mutate into an aggressive, virulent strain (Griffith, 2000). Thus, a reliable solution for the imminent Cacao Swollen Shoot problem is not only elusive, but urgently required to stem the spread of this threatening disease (Olunloya, 2004; Ollennu and Owusu, 2002).

## **1.2 Problem Statement**

Cacao Swollen Shoot Virus (CSSV) is found in many cacao growing regions of the world, where it has resulted in severe losses. Current detection methods have thus far been unreliable, making the identification and elimination of diseased trees difficult. Control and eradication of mealybug vectors have also been ineffective, and in many cases, even considered impractical. Even though the development of CSSV resistant planting materials is considered the best way forward in the fight against CSSD, the development program has met with many challenges and is a long way away from attaining a permanent solution. A method that had previously shown promise was by inoculating trees using attenuated or mild virus strains as a form of cross-protection against infection by CSSV. However, this strategy was scuttled due to the concern with that the use of intact mild strain viruses as a means of cross-protection control represented a huge risk, based on the possibility that the mild strains of viruses may mutate into an aggressive, virulent strain. However, in light of the ineffectiveness of other control strategies, a strategy of applying modern molecular methods in developing a potential vaccine against CSSD is therefore proposed to present a less risky option as part of a long term solution of immunizing cacao trees against infection by CSSV. The hypothesis that recombinant gene technology may be used to produce an engineered vaccine capable of expressing inert particles of CSSV to elicit cross-protection effect is explored in this research.