Faculty of Resource Science and Technology

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

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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. Agou1 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® Top10 chemically competent *Escherichia coli* cells using pCR-XL TOPO® cloning plasmids. The virus DNA was later retrieved, digested using *Eco*RI restriction enzyme, and the targeted ORF3 gene fragment isolated through gel electrophoresis and incorporated into pBAD-TOPO® 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 supressed 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® TOP10 yang berkompeten menggunakan plasmid pengklon pCR-XL TOPO®. DNA virus kemudian dipulih, dicerna enzim pencerna *Eco*RI, dan serpihan gen ORF3 diasingkan menggunakan teknik gel elektroforesis. Ia kemudiannya diklon ke dalam plasmid penyataan pBAD-TOPO®, 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 mekanisma pertahanan semulajadi tumbuhan. Ujian keberkesanan menunjukkan VMBB berjaya membenteras 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, perlindugan-merentas

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LIST OF ABBREVIATIONS

µF	microfarad
ARU	Attack rate – unvaccinated
ARV	Attach rate - vaccinate
bp	Base pairs
BREV	Biotic Response Elicitation Vaccine
CFU	Colony forming unit
CSSD	*Cacao Swollen Shoot Disease*
CSSV	*Cacao Swollen Shoot Virus*
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-piperazineethanesaulfonic acid
IBA	Indole-3-butyric acid
ICPCR	Immunocapture polymerase chain reaction
IEM	Immunoelectron microscopy
kb	Kilobases
kV	Kilovolt
LB	Luria-Bertani media
miRNA	Micro-ribonucleic acid

MT	Metric tons
NAA	α-napthaleneacetic 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	Trypic soy agar
TSB	Tryptic soy broth
VBA	Virobacterial Agglutination
v/v	Volume per volume

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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 pareretroviruses, 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 (Frizon *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.