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INTEGRATION OF RNAI CONSTRUCT INTO THE GENOME OF COWPEA (*VIGNA UNGUICULATA* L.) FOR RESISTANCE TO *COWPEA APHID BORNE MOSAIC VIRUS* (CAMV) AND *COWPEA SEVERE MOSAIC VIRUS* (CPSMC)

[1*]Abdu, L. S., Usman[2] I. S., Abdulmalik[2] M. M. and Aragao[3] F. J. L.
1. Department of Plant Biology, Bayero University Kano, Nigeria
2. Department of Plant Science, Ahmadu Bello University, Zaria, Nigeria
3. EMBRAPA- CENARGEN, Brasilia DF, Brazil
*Corresponding author: Email: labdul.bot@buk.edu.ng

ABSTRACT
Improvement of cowpea for resistance to major pests and viral diseases is impeded by the absence of resistance genes in its genome. Genetic engineering is a suitable tool using which genes of interest could be introduced into the genome of cowpea. In order to generate cowpea plant resistant to CAMV and CPSMC, RNA interference construct that target the silencing of CAMV coat protein and CPSMC proteinase cofactor genes were successfully inserted and integrated into cowpea using the biolistic gene delivery system.Three transgenic cowpea lines were regenerated from bombardment of 600 embryos with a transformation frequency of 0.5%. Polymerase Chain Reaction (PCR) analysis of all the primary transformed plants (T_0) and T_1 progeny showed the presence of 340bp fragment amplified by primers COMOPTYF389 within the CPSMV sequence and COMOPOTYR729 within the CABMV sequence indicating successful insertion and integration of the interfering cassette.

Keywords: RNA interference, Cowpea, Virus resistance, Biolistic mediated transformation

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp) is an important source of dietary protein in sub-Saharan Africa for thousands of years. The dry grains contain 23-32% protein and other important food components such as carbohydrate, lipid, fibre, minerals and vitamins (Nielson *et al*., 1993). About 7.56 million metric tons of cowpea is produced annually over an estimated area of 12.76 million hectares with over 70% of the dry grain production coming from sub-Saharan Africa (www.iita.org/cms/detail/cowpea_project_details). In sub-Saharan Africa, insect pests and viral diseases are critical yield limiting factors which reduce the overall grain yield of cowpea to 0.495t/ha, which is per below the crop's potential estimated at 3.0t/ha (FAO, 2012). Improvement of cowpea for resistance to major pests and viral diseases using conventional breeding is impeded by the absence of resistance genes in the genome of cultivated cowpea varieties. Genetic engineering is a suitable avenue through which genes of interest could be introduced into the genome of cowpea. The technology could help breeders overcome major production challenges in cowpea, especially when applied along with conventional breeding.

The *cowpea aphid-borne mosaic virus* (CABMV) belongs to the family *Potyviridae*and genus *potyvirus*. The virus is transmitted by seed and several aphid species which belongs to subfamily *Aphidinae*. CABMV infects cowpea resulting in yield reductions and frequent total crop failure in sub-Saharan countries like Nigeria (Thottappilly and Rossel, 1992). The genome of CABMV is composed of a positive-sense single stranded RNA (ssRNA) molecule with a genome-linked protein (VPg) at the 5′end and a 3′ terminal poly(A) tail that contains a single ORF encoding a large precursor polyprotein (Cruz and Aragao, 2014).

This polyprotein is processed into P1, helper component (HC), P3, cylindrical inclusion (CI), nuclear inclusion A (NIa), nuclear inclusion B (NIb) and capsid protein (CP), and two small putative proteins known as 6K1 and 6K2 (Chung et al., 2008). The cowpea severe mosaic virus (CPSMV) belongs to the family *Secoviridae* and genus *comovirus*. CPSMV is the most important virus infecting cowpea in America and is among the seven most damaging viruses attacking cowpea in Africa. It is transmitted either by leaf feeding beetles belonging to the family Chrysomelidae or by seed. The genome of CPSMV consists of two positive-sense single stranded RNA (ssRNA) molecules; RNA1 and 2, which separately encapsulated and expressed by a polyprotein processing strategy (Cruz and Aragao, 2014). RNA1 encodes proteins necessary for replication; the 32K proteinase cofactor, the 58K helicase, the VPg 5'-linked proteins of the genomic RNAs expected set of five mature proteins, 24K proteinase and 87K polymerase, while RNA2 encodes the two capsid proteins and a protein involved in cell-to-cell movement (Chen and Bruening, 1992).

RNA interference (RNAi) is a mechanism that regulates gene expression by activating a sequence-specific RNA degradation process. RNAi has become a valuable biotechnological tool, because double-stranded (ds) RNA generated from the expression of chimerical constructs encoding self-complementary hairpin (hp) RNA can selectively and robustly induce suppression of specific genes of interest (Cruz and Aragao, 2014). This strategy has been used to generate transgenic plants with systemic resistance to viruses confer by sequence specific degradation of the viral RNA (Lopez *et al*., 2010; Vanderschuren *et al*.,2012; Aragao, 2014; Cruz and Aragao, 2014; Ammara *et al*., 2015). This paper reports successful integration of dsRNA for resistance to CABMV and CPSMV into genome of cowpea.

MATERIALS AND METHODS

The Plasmid Vector

A 1063bp interfering cassette containing sequence which corresponds to 502bp fragment from the Coat Protein sequence of *Cowpea Aphid Borne Mosaic Virus* (CABMV) (Gene Bank accession number-KF181277), and 561bp from the Proteinase Cofactor gene (32K protein) of *Cowpea Severe Mosaic Virus* (CPSMV) RNA1 (Gene Bank accession number-M83830), was synthesized at Epoch life Science Inc. USA.Sites for *XbaI*, *SacI*, *ApaI* and *KpnI* were added to allow cloning in sense and antisense orientations in the vector pSIU (Tinoco *et al*., 2010), generating the vector pSIUCAUPI2.The 1063bp sequence interspersed with intron 3 (370bp) obtained from the malate synthase gene *(*ms-i3) of *Arabidopsis thaliana.* The cassette was removed with enzymes *EcoRI* and *BglII*from the pSIUCAUPI2 vector and cloned into site for *EcoRI* and *BglII*in pBSK vector (Gene Bank accession no.GS54365-1). The pBSK contains the gene *ahas* (which confers tolerance for the herbicide imazapyr) from *Arabidopsis thaliana* and the gene *bla* which confers resistance to ampicillin. The plasmid vector GS54365-1pBSK containing the CABMV/CSMV hairpin sequencewas generated and referred to as COMOPOTY.

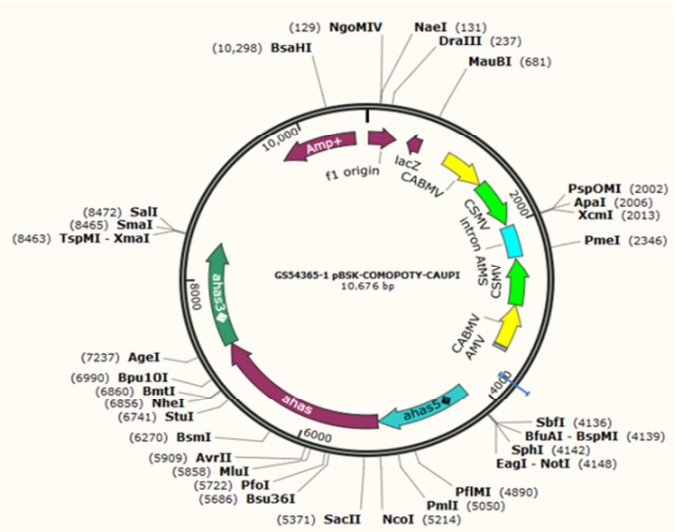

Figure 1:The map of the plasmid vector GS54365-1pBSK-COMOPOTY used to transform the standard cowpea variety, containing the *ahas* gene (*ahas*5′: *ahas* gene promoter; *ahas*: *Arabidopsis thaliana* AHAS coding sequence; ahas3′: *ahas* gene terminator), the ΔCABMVCSMV cassette (AMV: untranslated leader sequence from *Alfalfa mosaic virus*; intron AtMS: intron 3 from the malate synthase gene from *A. thaliana.* The *bla* gene codes for a *β-lactamase* that confers resistance to ampicillin.

Cowpea transformation

The cowpea line (MNCO5-828-3-15) was transformed with the plasmid vector GS54365-1pBSK-COMOPOTY according to Cruz and Aragao, (2008). Mature seeds were washed under running tap water and surface sterilized by dipping in 70% ethanol for 1 minute and subsequent shaking in 2% sodium hypochlorite (w/v) for 20 minutes. The seeds wer rinsed three times in sterile distilled water then soaked for 16–18 h. Embryonic axes were excised from the sterilized seeds and the apical meristems were exposed by removing both the primary and primordial leaves under microscope. The embryonic axes were then placed with the apical region directed upward in bombardment plates containing basal Murashige and Skoog (MS) medium supplemented with 30g/L sucrose and 8g/L agar. The bombardment was conducted using a high-pressure, helium-driven particle acceleration biolistic machine based on the procedure described by Aragao *et al.* (2005) and Rech *et al.* (2008). After the bombardment, embryonic axes were immediately transferred to culture bottles containing 80ml of cowpea selection media containing MS medium fortified with 22.2M BAP and 300nM imazapyr. Elongated shoots developed from the bombarded apical meristems after 3 weeks of culture on the selection medium. Regenerated shoots were allowed to reach 4-5 cm in length before they were transferred to hormone free media, without selection pressure for rooting. The transgenic plant lines (T_0 generation) were transferred to plastic cups containing soil:vermiculite (1:1) and covered with transparent polyethylene bags for two weeks for acclimatization. After two weeks of hardening in a small plastic cups, plants were kept for one week before they were transferred to large plastic pots and allowed to grow to maturity.

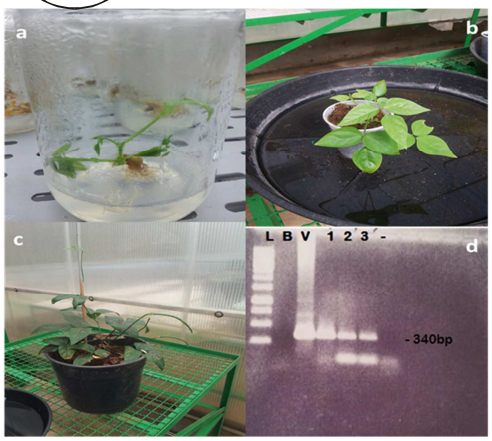

Figure 2: Regeneration of transgenic plant lines and detection of 340bp fragment from COMOPOTY using PCR analyses. (**a**) Transgenic plant line regenerated on selection media. (**b**) Acclimatized transgenic seedling (**c**) Matured and fertile transgenic plant in greenhouse (**d**) Polymerase chain reaction (PCR) amplification of 340bp fragment (1, 2 and 3), B and C-represent three primary transformed plants,blank (water) and negative control (untransformed cowpea plant).

Detection of the Transgenes in Primary Transformed (T_0) Plants

After one week of hardening, leaf discs were collected from the T_0 and T_1 lines for detection of the interfering cassette. DNA was isolated from leaf discs according to Edwards *et al*, (1991). PCR was carried out according Bonfim *et al*, (2007). The primers COMOPTYF389 within the CPSMV sequence and COMOPOTYR729 within the CABMV sequence were used to amplify a 340bp fragment from the interfering cassette-ΔCABMVCSMV.

RESULTS

In order to generate transgenic cowpea lines resistant to *Cowpea Aphid Borne Mosaic Virus* (CABMV) and *Cowpea Severe Mosaic Virus* (CPSMV), the plasmid vector GS54365-1pBSK-COMOPOTY was used to deliver the chimeric fragment (ΔCSMVCABMV) containing a sequence from the Coat Protein sequence of CABMV and Proteinase Cofactor gene (32K protein) of CPSMV- RNA1 (Figure 1).

The results showed that siRNA interfering cassette was successfully delivered into genome of cowpea following bombardment of the apical regions of embryonic axes with the plasmid GS54365-1pBSK-COMOPOTY. A total of three primary transgenic plant lines (T0) harbouring the chimeric fragment containing dsRNA of Coat Protein gene of CABMV and Proteinase Cofactor gene of CSMV were regenerated from 10 transformation events each containing 60 embryos which corresponds to the transformation frequency of 0.5%. After 3 weeks in culture under selection in the presence of 300 nM imazapyr, shoot buds were observed. Microscopic examination of the apical region showed that numerous shoot buds were induced, but only about 1.0% of shoot buds elongated (Figure 2a).

All the primary transformed plants (T0) presented normal phenotype (Figure 2c).

PCR analysis of genomic DNA isolated from the primary transformed plants (T0) showed a successful insertion of the RNAi cassettes (ΔCSMVCABMV). This is confirmed by the presence of 340bp fragment amplified byprimers COMOPTYF389 within the CPSMV sequence and COMOPOTYR729 within the CABMV sequence (Figure 2d). The primary transformed plants (T0) transferred the transgene to T1 progeny. When T_1 plants were cultivated in the greenhouse, the plants presented morphologically normal growth and were fertile (Figure 2c). The plants presented normal plant height, site of insertion of first pod, number of branches, internode length, foliar area, flowers, pods and seeds per pod. The PCR analysis of the T1 progeny also showed the presence of 340bp fragment amplified by primers COMOPTYF389 within the CPSMV sequence and COMOPOTYR729 within the CABMV sequence indicating successful transfer of the interfering cassette (Figure 3). The results showed that the siRNA cassette inserted in the primary transformed (T0) plant has been successfully integrated into genome of cowpea and transmitted to the T1 generation.

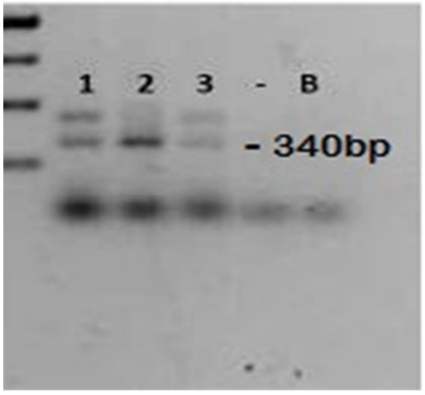

Plate VII: PCR amplification of the 340bp fragment amplifies by the primer (COMOPTYF389 and COMOPOTYR729) from the T_1 progeny. Three T_1 transgenic lines (1, 2 and 3) obtained from the first primary transformed (T_0) plant, B and C- represent blank and negative control (untransformed cowpea plant).

DICUSSION

The diseases coused by CABMV and CPSMV constitute important constraints for the production of cowpea especially in America and Africa (Aliyu et al., 2012; Abreu et al., 2012). In this work, we developed a reproducible system in which integration of dsRNA chimeric construct comprising a fragment of the coat protein gene of CABMV and a fragment from the proteinase cofactor gene of CPSMV(DCSMVCABMV) is routinely achieved. The system is based on the bombardment of pre-exposed apical meristem of cowpea embryonic axes followed by selection of transgenic line using herbicide molecule – Imazapyr. The herbicide molecule belongs to the imidazolinone class (imazapyr), which is capable of systemically translocating and concentrating in the apical meristematic region of the plant (Ivo *et al*, 2008). Earlier reports (Citadin *et al.*, 1013 ; Cruz and Aragao, 2014) have shown that this system could be used to introduce genes of agronomic importance into genome of cowpea.

The system target multiple shoots induction in the presence of BAP as means of achieving significant levels of regeneration of transgenic plants. A relatively high transformation frequency (0.5%) was achieved compared to 0.05 - 0.15% reported by Popelk *et al*, (2006) and Chaudhury *et al*, (2007) using *Agrobacterium*-mediated transformation system. On the other hand, transformation frequency of 1.41% was reported by Ivo *et al*, (2008), Cruz and Aragao (2014) using a similar approach. In addition to relatively higher transformation frequency, this system is much simpler because it utilizes embryonic axes and is devoid of multiple cultivation steps employed in the *Agrobacterium* mediated transformation of cowpea.

A strategy involving the of RNAi techonology to generate genetically modified plants resistant to RNA viruses have earlier been reported in cowpea (Cruz and Aragao, 2014), sweet potato (Kreuze et al, 2008) and Mexican lime (Lopez *et al*, 2010). Transgenic plants expressed considerable resistance following mechanical inoculation with the viruses and resistance was found to correspond with the presence of transgene-derived siRNA in the leaves of all transgenic lines. Earliar reports (Cruz and Aragao, 2014) indicated that the presence of the silencing construct in the transgenic cowpea lines induced the silencing of the viral genes by reducing or preventing viral RNA accumulation through sequence specific degradation of the viral RNA there by rendering the transgenic plants to be resistant to the targeted viruses. The results of this work further confirmed that integrating a vector containing contructs confering resistance multiple viruses into plant genome is possible. Therefore, the system could be used to generate transgenic lines with resistance to different viral or bacterial diseases.

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