

A geminivirus-induced gene silencing system for gene function validation in cassava

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Abstract

We have constructed an African cassava mosaic virus (ACMV) based gene-silencing vector as a reverse genetics tool for gene function analysis in cassava. The vector carrying a fragment from the *Nicotiana tabacum* sulfur gene (*su*), encoding one unit of the chloroplast enzyme magnesium chelatase, was used to induce the silencing of the cassava orthologous gene resulting in yellow–white spots characteristic of the inhibition of *su* expression. This result suggests that well developed sequence databases from model plants like *Arabidopsis thaliana*, *Nicotiana benthamiana*, *N. tabacum*, *Lycopersicon esculentum* and others could be used as a major source of information and sequences for functional genomics in cassava. Furthermore, a fragment of the cassava *CYP79D2* endogenous gene, sharing 89% homology with *CYP79D1* endogenous gene was inserted into the ACMV vector. The resultant vector was inducing the down regulation of the expression of these two genes which catalyze the first-dedicated step in the synthesis of linamarin, the major cyanogenic glycoside in cassava. At 21 days post-inoculation (dpi), a 76% reduction of linamarin content was observed in silenced leaves. Using transgenic plants expressing *antisense* RNA of *CYP79D1* and *CYP79D2*, Siritunga and Sayre (2003) obtained several lines with a reduction level varying from 60% to 94%. This result provides the first example of direct comparison of the efficiency of a virus-induced gene silencing (VIGS) system and the transgenic approach for suppression of a biosynthetic pathway. The ACMV VIGS system will certainly be a complement and in some cases an alternative to the transgenic approach, for gene discovery and gene function analysis in cassava.

Abbreviations: bp, base pair; dpi, days post-inoculation; μ g, microgram; μ l, microliter; nts, nucleotides

Introduction

Cassava, *Manihot esculenta* Crantz, is a euphorbiaceous crop mainly cultivated in the lowland tropics for its starchy tuberous roots (Nweke *et al.*, 1994). Food security and cash crop for small-scale

farmers, the development of cassava as a sustainable culture is threatened to the greatest degree by cassava mosaic disease (CMD). Yield losses due to CMD can be as high as 95% (Fauquet and Fargette, 1990). The development of CMD resistant cassava cultivars started in the 1930s by Storey

and Nichols (1938), and although highly resistant genotypes have been identified the control of CMD remains problematic because of the difficulty to combine CMD resistance with other agronomical and organoleptic properties. Furthermore, the genetic basis for this resistance has not been completely resolved and CMD resistance genes have not yet been cloned. Moreover, the improvement of the nutritional value of cassava is of great importance because it is a starch crop with almost no protein and very little amount of vitamins in the roots.

For the last decade, the increasing availability of a number of genetic markers and expressed sequence tags (EST) developed from cassava have raised the hope for identification of resistance genes as well as the characterization of different biosynthetic pathways. For instance, these advances concern genes of resistance to CMD (Akano *et al.*, 2002), to cassava bacterial blight (Lopez *et al.*, 2004), to *Phytophthora* species causing cassava root rot (Llano *et al.*, 2004) and to a complex of whitefly species acting as pests or virus vectors (Bellotti *et al.*, 2004). Similarly, progress has also been made towards the discovery of starch biosynthesis genes (Lopez *et al.*, 2004) and carotenoid synthesis genes (Salcedo *et al.*, 2004). However, the validation of the function of candidate genes relies on a cassava genetic transformation system that is tedious, time consuming and cannot be used in a high throughput manner, in contrast to that used for *Arabidopsis thaliana*.

Virus-induced gene silencing (VIGS) constitutes a rapid and interesting alternative to genetic transformation. VIGS is an excellent reverse genetics tool that can be used to generate mutant phenotypes for assigning function to unknown genes. In VIGS systems, viruses are engineered as a vector to carry a partial sequence of known or candidate genes in order to correlate their function to the mutant phenotype. Upon infection, the recombinant virus will induce the degradation of the targeted endogenous gene via post-transcriptional gene silencing (PTGS) (Baulcombe, 1999). In plants, most of the DNA and RNA VIGS systems have been described for plants belonging to the family *Solanaceae* (Kumagai *et al.*, 1995; Ruiz *et al.*, 1998; Jones *et al.*, 1999; Ratcliff *et al.*, 1999; Turnage *et al.*, 2002; Liu *et al.*, 2002a,b).

In the present paper we have constructed an African cassava mosaic virus (ACMV)-based vec-

tor for silencing in cassava. The permissive plant *N. benthamiana* that can be infected with ACMV-[CM] was used as a positive control for phytoene desaturase (*PDS*) and sulfur gene (*su*) silencing. When using a fragment of *N. benthamiana* phytoene desaturase (*PDS*) gene as an inducer of silencing, we observed in *N. benthamiana*, but not in cassava, photo-bleached spots characteristic of *PDS* silencing. By using a fragment of the *N. tabacum su* gene, we were able to induce the silencing of the orthologous gene in cassava. Finally, a cassava *CYP79D2* gene fragment sharing 89% identity with the corresponding *CYP79D1* fragment was inserted in the VIGS vector. The resultant vector induced the down-regulation of the expression of the two cassava endogenous genes *CYP79D1* and *CYP79D2*. These two genes catalyze the first-dedicated step in synthesis of linamarin, the major cyanogenic glycoside in cassava and consequently we recorded a substantial decrease of linamarin in the cassava leaves.

Material and methods

Plasmid construction

Infectious clones of ACMV isolate Cameroon ACMV-[CM] consisting of DNA-A (GenBank accession number: AF112352) and DNA-B (GenBank accession number: AF126806) were described by Fondong *et al.* (2000). Partial repeat of the same ACMV-[CM] DNA-A was re-constructed (pVF1.4AA) by cloning a full-length copy into the 1.2 kb *Bam*HI/*Eco*RI subclone previously cloned into pBluescript II KS (+) (Fondong, unpublished data).

Insertion of a multiple cloning site (MCS) in replacement of 459 bp capsid protein (CP) fragment: ACMVΔCP

ACMVΔCP was constructed from ACMV-[CM] DNA-A by insertion of a MCS in replacement of a part of the CP open reading frame (ORF). MCS composed of the five restriction enzymes (*Kpn*I, *Sac*I, *Xma*I, *Avr*II and *Xho*I) was inserted into the full-length part of ACMV-[CM] partial repeat infectious clone using the polymerase chain reaction (PCR) (Figure 1). This cloning site was inserted in frame with the CP carboxy-terminal in replacement of the segment starting at 16 nucleotides (nts) after AV2 stop codon to 15 nts before

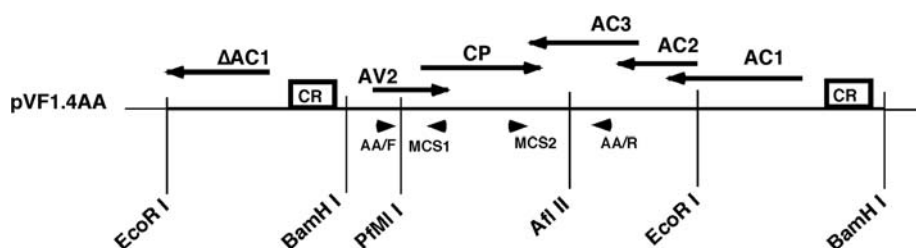


Figure 1. Restriction map of ACMV-[CM] DNA-A used as a vector in this study. Partial repeat of ACMV-[CM] DNA-A (pVF1.4AA) was constructed by cloning a full-length copy into the 1.2 kb *Bam*HI/*Eco*RI subclone previously cloned into pBluescript II KS (+) (Fondong, unpublished data); Arrows (←) indicate ORF in virion sense and (→) indicate ORF in complementary sense; (▶;◀) indicate position and orientation of primers, CR: common region, position of cloning sites *Pfl*MI and *Afl*II are indicated, Map is not to scale.

the *CP* stop codon. In a first step, primer AA/F (5'-ACTGGTGAATGAGTTTCCAGACTCGGT-3') and primer MCS1 (5'-CTCGAG GAGCTC CCCGGG CCTAGG GGTACC CCTAGGG-TCTGGGC TTCTATACATCCTGTACAT-3') were used to amplify a fragment of 383 bp corresponding to N-terminal region of the *CP* and *AV2* ORF. MCS1 contains the five restriction sites of MCS followed by 33 nts of the *CP* N-terminal region. Another PCR fragment of 739 bp was obtained using primer AA/R (5'-TCCAA-CACGAAATACGGGAAACCC-3') and MCS2 (5'-GGTACC CCTAGG CCCGGG GAGCTC CTCGAG GACAGTATTGGCAATTAATAA-ACATTGAATTGTATTTCA-3'). MCS2 contains the MCS followed by the 15 nts located before the *CP* stop codon, the stop codon plus an additional 21 nts. Sharing the MCS in common, the two PCR fragments (383 and 739 bp) are connected as a single fragment during *in vitro* PCR amplification. One microliter of both PCR products were mixed and used as template to generate another PCR product of 1122 bp covering the region from primer AA/F (49 nts upstream of the *CP*) to primer AA/R (691 nts downstream of the *CP*). The AA/F-AA/R fragment was digested with *Pfl*MI and *Afl*II and the resulting 449 *Pfl*MI/*Afl*II fragment was cloned into the ACMV-[CM] DNA-A infectious clone by replacement of the original 977 bp *Pfl*MI/*Afl*II fragment.

Cloning of *N. benthamiana* PDS fragment into ACMVΔCP: AC-PDS

PCR primers were designed from *Lycopersicon esculentum* PDS genomic sequence (Gene bank accession number: X71023) and amplified using the forward primer *PDS1* (5'-CGGGGTACC-

TAAAATGCCTCAA ATTGGACTTG-3') containing a *Kpn*I site and the reverse primer *PDS2* (5'-TCCCCCGGGCTGCACCAGCAATAACA ATCTC-3') with a *Xma*I site. These primers cover the region from bases 1005 to 1455 of the *L. esculentum* PDS gene sequence. The 450 bp PCR amplification product of the *N. benthamiana* endogenous PDS gene was cloned into ACMVΔCP via a *Kpn*I/*Xho*I digestion.

Cloning of *N. tabacum* su fragment into ACMVΔCP: AC-su

Transgenic *N. tabacum* expressing a double-stranded RNA (dsRNA) derived from *N. tabacum* su fragment (Gene bank accession number: NTU67064; nts 437–913) were recently obtained (C. Taylor, Danforth Plant Science Center; unpublished data). The 476 bp fragment was digested from the dsDNA plasmid used for genetic transformation and was cloned in *antisense* in ACMVΔCP via a *Kpn*I/*Xho*I digestion.

Cloning of cassava CYP79D2 fragment into ACMVΔCP: AC-CYP79D2

Primers were designed to amplify a region of 89% homology between *CYP79D1* (Gene bank accession number: AF140613) and *CYP79D2* (Gene bank accession number: AF140614). This region contains two stretches (27 and 31 bp) of 100% identity between *CYP79D1* and *CYP79D2*. The forward primer *CYP79D2/F* (5'-CGGGGTACCCTCCATGGCCACTCATCG GAAAC-3') containing a *Kpn*I site and the reverse primer *CYP79D2/R* (5'-CCGCTCGAGGC-

TCTCTTATCATGGAGCCATTTGTG-3') with a *XhoI* allow the amplification of a PCR product of 345 bp from both *CYP72D1* and *CYP79D2* endogenous genes (corresponding to nts 208–586 and 235–555 respectively). This product was cloned in ACMV Δ CP via a *KpnI/XhoI* digestion. After sequencing, plasmids containing *CYP79D1* and *CYP79D2* were respectively designated AC-*CYP79D1* and AC-*CYP79D2*.

Plant preparation, virus inoculation, symptoms and silencing effect assessment

Three-week old cassava cultivar TMS60444 plantlets and 30-day old *N. benthamiana* seedlings were inoculated with infectious viral clones using the Bio-Rad biolistic device as previously described by Pita *et al.* (2001b). The wild type ACMV-[CM] DNA-A infectious clone or the engineered clone were inoculated along with wild type ACMV-[CM] DNA-B. After inoculation plants were kept in a greenhouse at 24 °C with a 14 h photoperiod and 70% relative humidity. Each plant was assessed visually for development of disease symptoms or silencing effect. Symptom severity was scored as described by Fauquet *et al.* (1988). The inhibition of expression of *PDS*, essential for production of the carotenoid pigments resulted in photo-bleached spots. The *su* gene is a member of the magnesium chelatase complex required for chlorophyll production. The inhibition of *su* expression resulted in yellow–white spots.

Plant DNA extraction and Southern blot analysis

The second most upper leaf was collected at 21 days post-inoculation (dpi) from plants infected with ACMV-[CM] DNA-A and ACMV Δ CP, each inoculated along with ACMV-[CM] DNA-B. Plant total DNA was extracted as described by Dellaporta *et al.* (1983).

Viral DNA accumulation was determined by Southern blot analysis as described by Pita *et al.* (2001a,b). A total of 4 μ g of total DNA was separated on a 1% agarose gel. Specific radio-labeled probes were used to detect DNA-A or DNA-B of ACMV-[CM] and ACMV Δ CP. DNA-A of ACMV-[CM] and ACMV Δ CP were detected using a 799 bp *EcoRI*-fragment digested from full length DNA-A of ACMV from Uganda (ACMV-[UG]) (Pita *et al.*, 2001a). It should be noted that the

799 bp fragment does not correspond to the *CP/AV2* region so that the detection is not affected by the *CP* deletion effected in ACMV Δ CP. DNA-B of ACMV-[CM] was detected using a 1060 bp *EcoRV/HindIII* fragment digested from DNA-B of ACMV-[CM].

Total RNA extraction and reverse-transcription PCR (RT-PCR)

These analyses were performed only for experiments concerning the silencing of *CYP79D1* and *CYP79D2*.

Total RNA was extracted at 21 dpi from the second most systemic leaves using Qiagen Rneasy Plant Mini Kit (Qiagen, Valencia, California, USA). Complementary DNA (cDNA) was obtained with 6.0 μ g of total RNA by following instructions from Qiagen Omniscript Reverse Transcriptase Kit. RT-PCR was performed as described by Siritunga and Sayre (2003). The primers used were specific to the 3' end of *CYP79D1* (D1F1: 5'-GCTAAATCAACCAGAA ATCCTGAAG-3' and D1R4: 5'-TGCAAGAG AAACAAGATAACCCC-3') and *CYP79D2* (D2-F1: 5'-CTGATAAATCAACCAGAA CTTCTG GCA-3' and D2-R5: 5'-CTAACAACTCACATT CATCCCTTCCC-3'). These primers amplify a region with no homology with the *CYP79D2* insert so that they report only the level of expression of endogenous *CYP79D1* and *CYP79D2* respectively. In our experiment, cassava ribulose 1-5-bisphosphate carboxylase oxygenase (rubisco) small subunit precursor was used as a control and was amplified as previously described using rubisco specific primers (rbc1: 5'-CTAC-TATGGTGGCTCCGTTC-3' and rbc2: 5'-CCGTTTCAGTCGGAGAAACTC-3') (Mak and Ho, 1995; Monger *et al.*, 2001a,b). These primers generate a 619 bp PCR product with cassava cDNA. Contaminating genomic DNA can be distinguished because it produces a larger PCR product of approximately 800 bp.

Leaf linamarin quantification

Leaf linamarin content was detected using the picrate paper method (Egan *et al.*, 1998; Bradbury *et al.*, 1999). The protocol previously established for cassava roots and flour was adapted to leaves (www.anu.edu.au/Bozo/CCDN/protocols/

ProtE.pdf). The second most upper leaf was collected at 21 dpi, from 10 different ACMV Δ CP, ACMV-[CM] and mock-inoculated plants.

Results

Infectivity of ACMV-[CM] construct: ACMV Δ CP

Three-week old cassava plantlets of cultivar TMS60444 were inoculated with wild-type ACMV-[CM] DNA-A and ACMV Δ CP along with cognate wild-type ACMV-[CM] DNA-B. Symptom severity was scored as described by Fauquet *et al.* (1988).

For the three different experiments, CMD-symptoms were observed on 15/15 plants inocu-

lated with wild-type ACMV-[CM] DNA-A. Only 9/15 plants showed symptoms in the case of ACMV Δ CP inoculation. At 7 dpi, symptoms were visible on plants inoculated with ACMV-[CM]. In the case of ACMV Δ CP symptoms were observed around 11–14 dpi. By 11 dpi, symptoms were systemic in ACMV-[CM] inoculated plants while there were systemic around 14–21 dpi with ACMV Δ CP. The severity of symptoms observed with ACMV-[CM] inoculation was maximum with a score of 5 from 14–21 dpi (Figure 2A). From 21 to 35 dpi, symptoms decreased to a score of 4–3. After 35 dpi, symptom severity was maintained at a level of 4–3 even 3 months after inoculation. A severity score of 2 was observed at 21 dpi with ACMV Δ CP

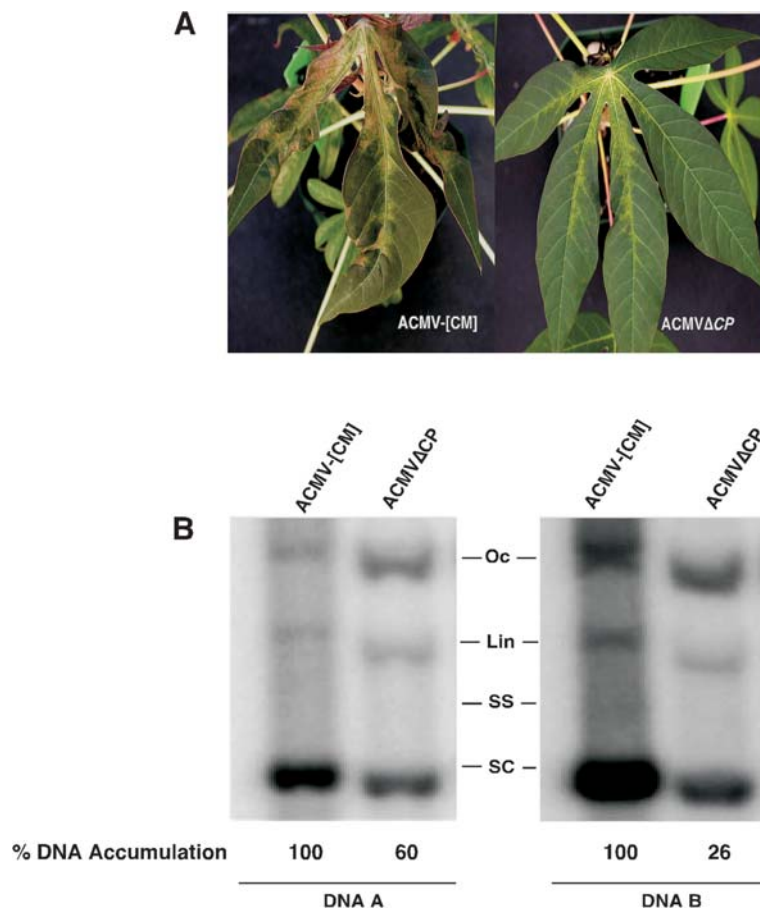


Figure 2. Infectivity of ACMV-[CM] modified vectors in cassava cultivar TMS60444. Wild-type ACMV-[CM] DNA-A and ACMV Δ CP were inoculated along with complementary wild-type ACMV-[CM] DNA-B. (A) CMD-symptoms at 21 dpi; (B) Accumulation of viral DNA in systemic leaves at 21 dpi; Southern blots show detection of ACMV-[CM] DNA-A and -B using an ACMV specific probe; DNA accumulation was quantified and the values indicated under each lane for each blot refer to the percentage of viral DNA amount using wild type ACMV-[CM] as a reference; The positions of open-circular (OC), linear (Lin), single-stranded (SS) and super-coiled (SC) forms of the viral DNA are indicated.

(Figure 2A). A maximum severity score of 3 was obtained around 35 dpi and was maintained at that level even 3 months after inoculation.

ACMV Δ CP presented less viral DNA accumulation on Southern blots for either the DNA-A (60%) or DNA-B (26%) at 21 dpi (Figure 2B).

The attenuation of CMD symptoms with the CP deletion constructs ACMV Δ CP is a beneficial effect for VIGS, as wild-type ACMV-[CM] induces very severe symptoms that could interfere with the observation of the silencing effect. Moreover, the AC4 gene located on the A-component of ACMV-[CM] is a strong suppressor of PTGS particularly during the first 3 weeks after inoculation (Vanitharani *et al.*, 2004). The activity of AC4 in a context of non-attenuation could therefore drastically reduce the silencing effect induced by the ACMV vector if the severity was not affected.

Silencing of cassava endogenous genes using orthologous solanaceous gene

PDS fragment from *N. benthamiana* does not induce silencing of the orthologous gene in cassava

PDS constitutes a good visual marker for endogenous gene silencing (Ruiz *et al.*, 1998). Since there is no published sequence information of cassava *PDS* gene, we designed PCR primers in a region of homology comparing sequence from other plants. As reported by Ruiz *et al.* (1998), it appears that *PDS* is highly conserved among plants of the family *Solanaceae*. In addition, ACMV-[CM] can infect *N. benthamiana* which can therefore serve as a positive control of gene silencing.

The primers were designed from *L. esculentum* *PDS* gene sequence. These primers allowed for the amplification of 450 bp of the *N. benthamiana* endogenous *PDS* gene but despite several attempts, did not give any amplification product from cassava. The *PDS* fragment was cloned into the ACMV vector (ACMV Δ CP), in *sense* orientation with respect to the CP reading frame. The resulting silencing vector AC-*PDS* was assayed for silencing effect onto *N. benthamiana* and cassava.

In *N. benthamiana*, photo-bleaching spots indicating silencing of the *PDS* endogenous gene were observed 2 weeks after inoculation. At 21 dpi, the bleaching was systemic but limited to the veins. At 35 dpi the bleaching continued to spread to invade entire leaves (Figure 3). The

silencing effect does not decrease with time and is maintained even 2 months after inoculation.

In cassava, no photo-bleaching spot was observed during the experiment (Figure 3) indicating the possibility that the *PDS* sequence from cassava does not share enough homology with *PDS* from *N. benthamiana* and other solanaceous plants. The pattern of AC-*PDS* symptoms (Figure 3) was overlapping with the pattern of CMD-symptoms obtained with ACMV-[CM] and ACMV Δ CP (Figure 2).

N. tabacum su fragment induces silencing of an orthologous gene in cassava

The *su* gene has been used as a visual marker for endogenous gene silencing (Kjemtrup *et al.*, 1998; Peele *et al.*, 2001). *N. tabacum su* gene fragment was cloned in ACMV Δ CP in *antisense* with the CP reading frame to generate a silencing vector AC-*su*. Inoculation of AC-*su* in *N. benthamiana* and cassava resulted in the appearance of yellow–white spots characteristic of inhibition of *su* expression. The silencing effect was observed at 2 dpi in *N. benthamiana* and 7 dpi in cassava.

Before 21 dpi, the cassava leaf area covered by the photo-bleached spots was similar to the area covered by CMD-symptoms obtained with ACMV-[CM]. From around 21 dpi, the pattern of AC-*su* photo-bleaching spots (Figure 3) was not overlapping with the pattern of CMD-symptoms obtained with ACMV-[CM], ACMV Δ CP (Figure 2) and AC-*PDS* (Figure 3). At 21 dpi, the entire surface of the young leaf was photo-bleached. The photo-bleached surface was reduced by 20% as the leaf was aging (Figure 3). A photo-bleached leaf never reverted to an entire green leaf. The surface coverage was maintained at 80% of the silenced leaf for at least 2 months after inoculation. At 6 months after inoculation, photo-bleached spots were still observed on cassava leaves but the surface coverage was greatly reduced (20–40%). In the case of *N. benthamiana*, the photo-bleached spots were mostly limited to the veins at 21 dpi (Figure 3). The maximum leaf coverage was observed between 35 and 45 dpi (Figure 3) and was maintained until 3 months after inoculation. The *N. benthamiana* were not kept more than 3 months because they were beginning to senesce.

The *N. tabacum su* gene constitutes a valuable marker of gene silencing in cassava. The silencing effect can be observed from the first week until

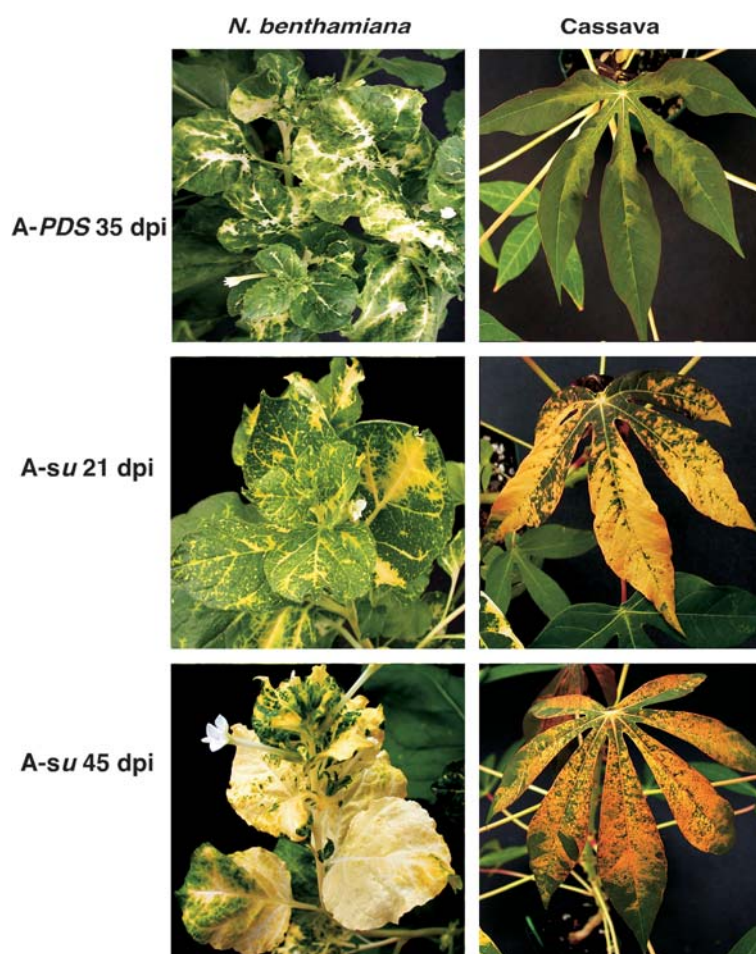


Figure 3. Phenotypic effect of silencing ACMV vectors bearing *Nicotiana benthamiana* PDS fragment (AC-PDS) and *N. tabacum su* fragment (AC-su). AC-PDS 35 dpi: effect of AC-PDS at 35 dpi; AC-Su 21 dpi and AC-Su 45 dpi: effect of AC-su at 21 and 45 dpi.

3 months after inoculation. The ACMV-based vector provides a comparative model of the effect of the VIGS-system between two different plant families (*Solanaceae* and *Euphorbiaceae*) but also between a model plant (*N. benthamiana*) and a crop plant (cassava).

A CYP79D2 fragment with 89% homology to CYP79D1 induces silencing of both cassava endogenous CYP79D1 and CYP79D2 genes

A 345 bp fragment of *CYP79D2* sharing 89% homology with correspondent *CYP79D2* was cloned into ACMV Δ CP to generate A-CYP79D2. The same region contains two segments (27 and 31 bp) of 100% identity between *CYP79D1* and *CYP79D2*.

Seven days after inoculation CMD-symptoms were observed on inoculated leaves. These symp-

toms were somehow different from ACMV-[CM], ACMV Δ CP (Figure 2), and AC-PDS induced symptoms (Figure 3). Unlike symptoms induced by wild-type ACMV-[CM], the CMD-symptoms were evenly distributed on the entire leaf surface at 21 dpi (Figure 4). After 21 dpi, CMD-symptoms were not covering the entire leaf but were still different from the pattern observed with the wild-type ACMV-[CM], ACMV Δ CP, and AC-PDS or even AC-su (Figure 3). The CMD-symptoms were covering about half of the total leaf surface for at least 2 months. Three months after inoculation CMD-symptoms were covering 20–40% of the total leaf surface.

The *CYP79D1* and *CYP79D2* transcript levels were analyzed by RT-PCR using specific primers. The rubisco gene was used as a control for cDNA expression.

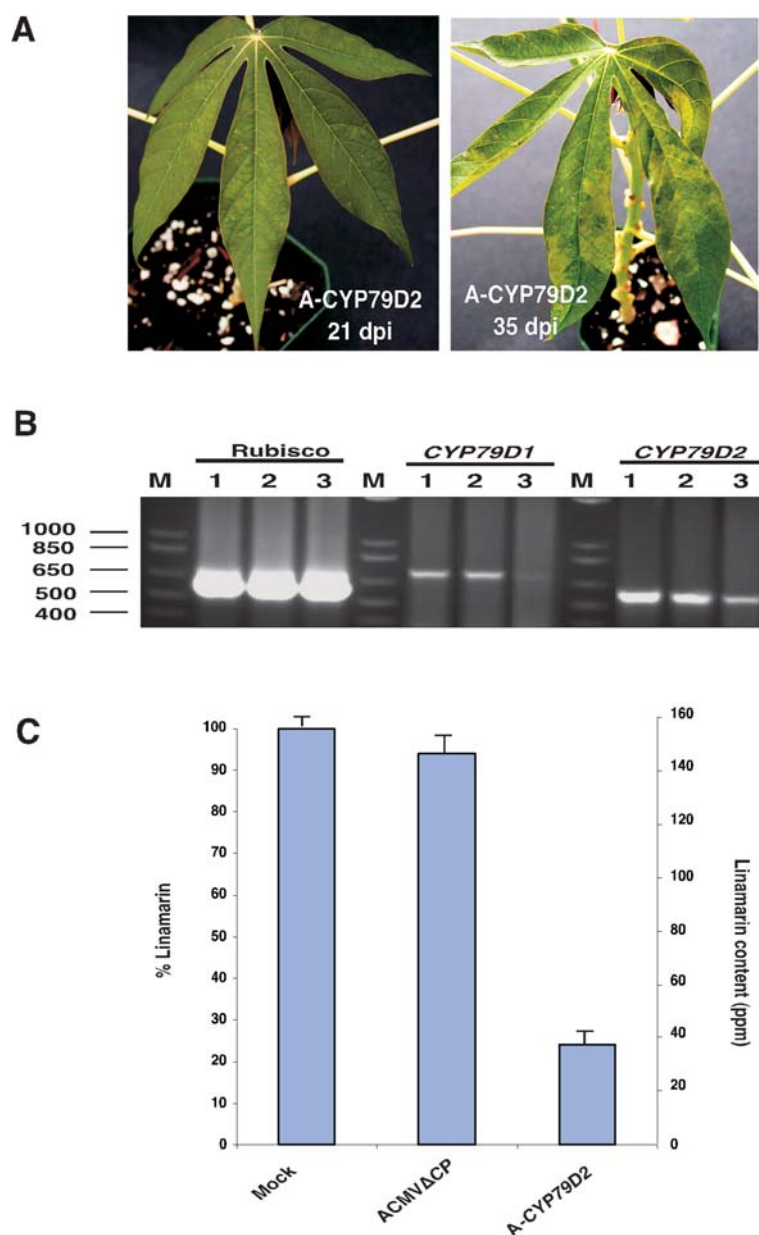


Figure 4. Silencing of cassava endogenous genes *CYP79D1* and *CYP79D2* using the silencing vector bearing fragment of *CYP79D2* gene (AC-*CYP79D2*). The *CYP79D2* fragment has an 89% overall homology and two stretches (27 and 31 bp) of 100% identity with the corresponding *CYP79D1* fragment. (A) leaves of A-*CYP79D2*-inoculated plants at 21 and 35 dpi; (B) RT-PCR amplification of the *CYP79D1* and *CYP79D2* transcripts from cassava leaves at 21 dpi. Specific primers were used as described by Siritunga and Sayre (2003). A cassava ribulose 1-5-bisphosphate-carboxylase (rubisco) small subunit precursor control was included as previously described (Mak and Ho, 1995; Monger *et al.*, 2001a,b); M: marker 1 kb + DNA Ladder; 1, 2, 3: mock, empty vector ACMVΔCP and AC-*CYP79D2*-inoculated plants respectively, numbers on the left indicate corresponding size of the marker in bp. (C) Linamarin content in mock, empty vector ACMVΔCP and AC-*CYP79D2*-inoculated plants. The picrate paper method was used for linamarin content quantification. The method includes a blank and standard linamarin controls. The percentage of linamarin was determined using the mock-inoculated plants as reference. 1 ppm = 1 mg hydrogen cyanide (HCN) per kilogram cassava.

The transcript level of the control rubisco was similar in the control plants and the plants inoc-

ulated with ACMVΔCP or AC-*CYP79D2* (Figure 4B). As previously described (Mak and Ho,

1995; Monger *et al.*, 2001a,b) the rubisco primers used in this study generate a PCR amplification product of 619 bp or approximately 800 bp when using cDNA or genomic DNA as template, respectively. The observation of a 619 bp rubisco fragment confirmed the effective synthesis of cDNA for all the samples. The absence of 800 bp fragment indicated that no genomic DNA was contaminating the total RNA extracted from the different samples.

The *CYP79D1* and *CYP79D2* fragments, of 700 and 550 bp respectively, were obtained as expected (Siritunga and Sayre, 2003). Both *CYP79D1* and *CYP79D2* transcript levels were similar in leaves of mock and ACMV Δ CP-inoculated plants, but were severely reduced in AC-*CYP79D2*-inoculated plants (Figure 4B).

The leaf linamarin content was determined at 21 dpi using the picrate paper method (Egan *et al.*, 1998; Bradbury *et al.*, 1999). A blank sample and standard linamarin were used as control of the method. Picrate papers were used to determine the linamarin content by absorbance using a spectrophotometer at 510 nm on 10 plants from four different experiments. The reduction of linamarin in AC-*CYP79D2*-inoculated plants was 76% compared to mock inoculated (Figure 4C). No significant difference was observed between the negative control (100%) and empty vector ACMV Δ CP (94%) inoculations, indicating that the *CYP79D2* insert is responsible for the inhibition of both *CYP79D1* and *CYP79D2* endogenous genes.

The ability of a *CYP79D2*-fragment to silence *CYP79D1* and *CYP79D2* endogenous genes could be explained by the presence of the two stretches of 27 and 31 nts of 100% identity in this fragment. The presence of stretches of 23 nts of 100% identity between the inducer and target fragments is probably more responsible for the induction of silencing of both genes than the average (percentage) homology between the two fragments (Thomas *et al.*, 2001).

ACMV Δ CP-based gene silencing vector appears as a powerful tool for the study of many other biosynthetic pathways in cassava.

Discussion

Several RNA viruses have been successfully used in a VIGS system, to target transgenes as well as

endogenous plant genes. Using a Tomato mosaic virus (ToMV) vector carrying a partial cDNA fragment of the tomato *PDS* gene, Kumagai *et al.* (1995) were able to manipulate the carotenoid biosynthetic pathway in *N. benthamiana*. The leaves of plants infected with ToMV vector carrying the partial *PDS* cDNA in the *antisense* presented a photo-bleaching white phenotype approximately one week after inoculation. The photo-bleaching white phenotype was delayed of one week with the vector carrying the *PDS* fragment in *sense*. Ruiz *et al.* (1998) demonstrated the ability of a Potato virus X (PVX)-silencing vector to interfere with the expression of a *GFP* transgene as well as the endogenous *N. benthamiana PDS* gene. These authors demonstrated that the *sense* and *antisense PDS* cDNA fragment were inducing the silencing at the same extent and during the same time course. The same PVX vector was used to down regulate the expression of the endogenous ribulose-1,5-biphosphate carboxylase oxygenase (Rubisco) in *N. benthamiana* (Jones *et al.*, 1999). Burton *et al.* (2000) were successful in establishing the function of putative cellulose synthase genes (*CesA*) in *N. benthamiana* by using a PVX vector carrying specific cDNA fragments corresponding to these genes. Some cDNAs induced shorter internode lengths, smaller leaves and dwarf phenotype resulting from the loss of cell wall cellulose. They also observed that the two sequences *NtCesA-1* and *NtCesA-2*, although 80% identical were inducing dramatically different effects. The plants in which *NtCesA1* was used as inducer displayed a dwarf phenotype while the plants inoculated with PVX-*NtCesA-2* were as normal as the plants inoculated with the PVX control. The latter result was strengthened by the observation of Thomas *et al.* (2001) when studying the size constraint for inducing PTGS of a *GFP* transgene as well as the endogenous *PDS* in *N. benthamiana*. They concluded that stretches of 23 nts of 100% identity between the inducer and target fragments were more responsible for induction of PTGS rather than the overall percentage homology between the two fragments. A tobacco rattle virus (TRV)-based VIGS system that has the advantage of inducing PTGS, of *GFP* transgene as well as endogenous *PDS* and rubisco, in the absence of virus symptoms was described in *N. benthamiana* (Ratcliff *et al.*, 2001). Liu *et al.* (2002a,b) used a TRV-based vector to investigate the role of

candidate genes in the *N*-mediated response. Using transgenic *N. benthamiana* plants that express the *N* gene conferring resistance to Tobacco mosaic virus (TMV), they were able to switch the transgenic plants from the resistance phenotype to susceptible. The TRV carrying fragment of candidate genes was suppressing the correspondent genes and subsequently the *N*-mediated response so that the resistant transgenic plants were becoming susceptible to TMV infection. All previous VIGS systems were described in *N. benthamiana*. Liu *et al.* (2002a,b) used a TRV based vector to induce the silencing of tomato endogenous genes like *PDS*, rubisco and *CTR1*. The suppression of *CTR1* induces an ethylene response phenotype along with the up-regulation of *CHITINASE B*, an ethylene response gene. Gosselé *et al.* (2002) described the first example of VIGS system in tobacco, using a satellite of Tobacco mosaic virus (STMV) silencing vector. The system was called satellite virus-induced silencing system (SVISS) and was used to down regulate transgene and endogenous genes (*PDS*, rubisco, transketolase, chalcone synthase, glutamine synthase, acetolactate synthase, cellulose synthase and RNA polymerase II) involved in various biochemical pathways and expressed in different plant tissues. Holzberg *et al.* (2002) illustrated the first example of VIGS system in monocot plant by using the Barley stripe mosaic virus (BSMV) as vector. Using a partial cDNA from rice and maize *PDS* genes that were 90% and 88% identical to the corresponding barley cDNA fragment, they were successful in inducing the silencing of the endogenous *PDS* in barley. Conversely, the *N. benthamiana PDS* fragment that was 74% identical to the corresponding barley cDNA fragment was unable to induce photo-bleached spots or accumulate phytoene.

The ability of DNA viruses, more precisely geminiviruses, to induce PTGS was shown by Kjemtrup *et al.* (1998). These authors used a tomato golden mosaic virus (TGMV) vector to down-regulate the expression of a luciferase (*luc*) transgene as well as the endogenous *su* of the magnesium chelatase complex in *N. benthamiana*. This silencing vector was based on the Tomato golden mosaic virus (TGMV) DNA-A component. Peele *et al.* (2001) demonstrated that TGMV DNA-B component could also be used as a VIGS vector. Turnage *et al.* (2002) showed that the ge-

minivirus Cabbage leaf curl virus (CbLCV) was suitable for gene silencing, and therefore for gene function validation, in *A. thaliana*.

In the present study, we have demonstrated that the geminivirus ACMV-based VIGS system is suitable for gene function validation in the model plant *N. benthamiana* and the crop plant cassava. Using a *PDS* fragment from *N. benthamiana*, we were able in *N. benthamiana* but unable in cassava, to suppress the expression of the corresponding endogenous gene. A similar observation was previously made by Holzberg *et al.* (2002), unable to suppress the expression of the barley endogenous *PDS* gene with a BSMV vector carrying a *N. benthamiana PDS* fragment. Seemingly, *N. benthamiana PDS* gene is not a good candidate for targeting corresponding gene in plants belonging to a different family. In contrast, the *N. tabacum su* gene can induce the silencing of the orthologous gene in cassava. The *su* gene might constitute a universal visual marker for demonstrating the validity of silencing system. Because the attempts to PCR-amplify the cassava *su* gene were unsuccessful, we were unable to compare the sequence to correspondent *N. tabacum su* gene. At this point, we do not know the level of homology between the *su* genes of the two plants and we are constructing cassava cDNA libraries for the identification of the *su* gene. It has to be noted that the virus vector Ac-*su* was detected by PCR in photo-bleached leaves (data not shown) indicating a correlation between the bleaching and the silencing vector. The possibility of existence of sufficient sequence homology between *N. tabacum* and cassava *su* genes indicates that sequences from model plants like *A. thaliana*, *N. benthamiana* and *N. tabacum*, *L. esculentum* and others, all of which possess robust genomic and post-genomic programs, could be great support for studies in an important crop like cassava. This deduction can have limitations as observed with the *N. benthamiana PDS* fragment. Recently, Salcedo *et al.* (2004) have amplified *PDS* and phytoene synthase orthologous fragment from cassava cultivars CM 523-7 and Mper 297 by using consensus primers. According to these authors, fragments amplified from genomic DNA and cDNA present high homology to known sequence of other plants. In order to understand why the *PDS* silencing using a DNA fragment from *N. benthamiana* did not work in cassava, it will be interesting to determine the

percentage of homology between the *PDS* fragment used in this study and the corresponding fragment from cassava CM 523-7 and Mper 297 but also TMS 60444 when the sequence will be available.

Using the ACMV VIGS vector carrying a fragment the cassava *CYP79D2* sharing 89% homology with *CYP79D1*, we obtained the down-regulation of the expression of both endogenous *CYP79D1* and *CYP79D2*. In our system, a 70% reduction of linamarin was observed in silenced leaves at 21 dpi. Transgenic plants expressing *antisense* RNA of part of both *CYP79D1* and *CYP79D2* showed a decrease of 60–94% of leaf linamarin content depending on the line (Siritunga and Sayre, 2003). Therefore, the results obtained with a transient system like the VIGS system seems to provide data of the same level and quality as compared with transgenic plants. This is the first example of a direct comparison of the effect of VIGS and a transgenic approach for the suppression of a biosynthetic process. We will further strengthen the comparison with more data and particularly we will verify a linamarin decrease in the roots as observed in the transgenic study. The *su* silencing experiment shows maintenance of the silencing for more than 3 months, it is consequently possible to envisage transient VIGS assays with enough effect in time to be comparable to transgenic effect, but in a few months time instead of a few years time.

A difference was observed on the pattern (distribution) of CMD-symptoms/silencing effect obtained with AC-*CYP79D2* and AC-*su* compared to ACMV-[CM], ACMV Δ CP and ACMV-*PDS*. A correlation was observed between the PCR-detection of silencing vectors (data not shown) and the observation of the silencing effect. As the PCR cannot provide an explanation of this change of pattern in the silenced leaves, we are conducting virus immuno-localization experiments to further investigate the matter.

In cassava, classical genetic analysis and molecular genetic mapping have demonstrated that a major dominant gene/locus controls the new source of resistance to CMD (Akano *et al.*, 2002). Bellotti *et al.* (2004) have identified micro satellite markers associated with resistance to whitefly species that cause important yield losses on cassava in Latin America. Quantitative trait loci (QTL) associated with resistance to *Phytophthora*

tropicalis, *P. melonis* and *P. palmivora* causing cassava root rot were identified (Llano *et al.*, 2004). Finally, Lopez *et al.* (2004) by developing a catalogue of 6000 expressed genes have identified a family of genes putatively involved in the resistance to cassava bacterial blight as well as several other genes involved in cassava starch biosynthesis. For all these examples, we believe that our ACMV-based silencing vector has the potential to provide quick answers for the role or involvement of these sequences in the designated resistance phenotypes or biosynthetic processes. This system provides an assay to determine the function of candidate genes. In the case of resistance genes, it should be possible to knock down a virus, bacterial, fungi or insect resistance, provided that the biological test is robust and that the partial silencing of gene(s) involved is sufficient to alter the phenotype. Considering that several years of hard work are required to express transgene sequences in cassava, this effort can only be dedicated to gene/sequences that have been shown to be responsible for the phenotypes. In consequence, we believe that the ACMV-based VIGS in cassava will be a very useful tool to address and answer these questions.

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References

- Akano, O., Dixon, O., Mba, C., Barrera, E. and Fregene, M. 2002. Genetic mapping of a dominant gene conferring resistance to cassava mosaic disease. *Theor. Appl. Genet.* 105: 521–525.
- Baulcombe, D.C. 1999. Fast forward genetics based on virus-induced gene silencing. *Curr. Opin. Plant Biol.* 2: 109–113.
- Bellotti, A.C., Bohrquez, A., Arias, B., Vargas, J., Vargas, H.L., Mba, C., Duque, M.C. and Tohme, J. 2004. Recent advances in the identification of genes conveying whitefly (*Aleurotrachelus socialis* Bondar; Homoptera: *Aleyrodidae*) in cassava (*Manihot esculenta* Crantz). In: International Scientific Meeting of the Cassava Biotechnology Network. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia, p. 119.
- Bradbury, M.G., Egan, S.V. and Bradbury, J.H. 1999. Picrate paper kits for determination of total cyanogens in cassava roots and all forms of cyanogens in cassava products. *J. Sci. Food Agric.* 79: 595–601.

- Burton, R.A., Gibeaut, D.M., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe, D.C. and Fincher, G.B. 2000. Virus-induced silencing of a plant cellulose synthase gene. *Plant Cell* 12: 691–706.
- Dellaporta, S.L., Wood, J. and Hicks, J.B. 1983. A plant DNA miniprep: version II. *Plant Mol. Biol. Rep.* 1: 19–21.
- Egan, S.V., Yeoh, H.H. and Bradbury, J.H. 1998. Simple picrate paper kit for determination of the cyanogenic potential of cassava flour. *J. Sci. Food Agric.* 76: 39–48.
- Fauquet, C. and Fargette, D. 1990. African cassava mosaic virus: etiology epidemiology, and control. *Plant Dis.* 74: 404–411.
- Fauquet, C., Fargette, D. and Thouvenel, J.C. 1988. Some aspects of the epidemiology of African cassava mosaic virus in Ivory Coast. *Trop. Pest Manage.* 34: 92–96.
- Fondong, V.N., Pita, J.S., Rey, M.E., de Kochko, A., Beachy, R.N. and Fauquet, C.M. 2000. Evidence of synergism between African cassava mosaic virus and a new double-recombinant geminivirus infecting cassava in Cameroon. *J. Gen. Virol.* 81: 287–297.
- Gosselé, V., Faché, I., Meulewaeter, F., Cornelissen, M. and Metzclaff, M. 2002. SVISS – a novel transient gene silencing system for gene function discovery and validation in tobacco plants. *Plant J.* 32: 859–866.
- Holzberg, S., Brosio, P., Gross, C. and Pogue, G.P. 2002. Barley stripe mosaic virus-induced gene silencing in a monocot plant. *Plant J. Cell Mol. Biol.* 30: 315–327.
- Jones, L., Hamilton, A.J., Voinnet, O., Thomas, C.L., Maule, A.J. and Baulcombe, D.C. 1999. RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell* 11: 2291–2301.
- Kjemtrup, S., Sampson, K.S., Peele, C.G., Nguyen, L.V., Conkling, M.A., Thompson, W.F. and Robertson, D. 1998. Gene silencing from plant DNA carried by a geminivirus. *Plant J. Cell Mol. Biol.* 14: 91–100.
- Kumagai, M.H., Donson, J., Della-Cioppa, G., Harvey, D., Hanley, K. and Grill, L.K. 1995. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc. Natl. Acad. Sci. USA* 92: 1679–1683.
- Liu, Y., Schiff, M. and Dinesh-Kumar, S.P. 2002a. Virus-induced gene silencing in tomato. *Plant J.* 31: 777–786.
- Liu, Y.L., Schiff, M., Marathe, R. and Dinesh-Kumar, S.P. 2002b. Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. *Plant J.* 30: 415–429.
- Llano, G.A., Alvarez, E., Fregene, M. and Muñoz, J.E. 2004. Identification of resistance-gene analogs in cassava (*Manihot esculenta* Crantz), and their relationship to three *Phytophthora* species. In: International Scientific Meeting of the Cassava Biotechnology Network. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia, p. 121.
- Lopez, C., Jorge, V., Mba, C., Cortes, D., Soto, M., Restrepo, S., Piégu, B., Cooke, R., Delseny, M., Tohme, J. and Verdier, V. 2004. A catalogue of 6000 expressed genes in cassava: identification of genes implicated in cassava bacterial blight resistance and starch biosynthesis. In: International Scientific Meeting of the Cassava Biotechnology Network. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia, p. 120.
- Mak, Y.M. and Ho, K.K. 1995. Sequence of cassava ribulose-1,5-bisphosphate carboxylase small subunit precursor cDNA. *DNA Seq* 5: 229–232.
- Monger, W.A., Seal, S., Cotton, S. and Foster, G.D. 2001a. Identification of different isolates of cassava brown streak virus and development of a diagnostic test. *Plant Pathol.* 50: 768–775.
- Monger, W.A., Spence, N.J. and Foster, G.D. 2001b. Molecular evidence that the aphid-transmitted Tomato mild mottle virus belongs to the Potyviridae family but not the Potyvirus genus. *Arch. Virol.* 146: 2435–2441.
- Nweke, F., Dixon, A.G.O., Asiedu, R. and Folayan, S.A. 1994. Cassava varietal needs of farmers and the potential for production growth in Africa. COSCA working paper No. 10. Collaborative study of cassava in Africa. In: International Institute of Tropical Agriculture, Ibadan, Nigeria.
- Peele, C., Jordan, C.V., Muangsan, N., Turnage, M., Egelk-rout, E., Eagle, P., Hanley-Bowdoin, L. and Robertson, D. 2001. Silencing of a meristematic gene using geminivirus-derived vectors. *Plant J. Cell Mol. Biol.* 27: 357–366.
- Pita, J.S., Fondong, V.N., Sangaré, A., Kokora, R.N.N. and Fauquet, C.M. 2001a. Genomic and biological diversity of the African cassava geminiviruses. *Euphytica: Netherlands J. Plant Breed.* 120: 115–125.
- Pita, J.S., Fondong, V.N., Sangaré, A., Otim-Nape, G.W., Ogwal, S. and Fauquet, C.M. 2001b. Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda. *J. Gen. Virol.* 82: 655–665.
- Ratcliff, F.G., MacFarlane, S.A. and Baulcombe, D.C. 1999. Gene silencing without DNA: RNA-mediated cross-protection between viruses. *Plant Cell* 11: 1207–1215.
- Ratcliff, F., Martin-Hernandez, A.M. and Baulcombe, D.C. 2001. Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant J.* 25: 237–245.
- Ruiz, M.T., Voinnet, O. and Baulcombe, D.C. 1998. Initiation and maintenance of virus-induced gene silencing. *Plant Cell* 10: 937–946.
- Salcedo, A., Mancilla, L., Cortés, D., Chavariaga, P. and Tohme, J. 2004. Cloning carotene synthesis genes from cassava roots. In: International Scientific Meeting of the Cassava Biotechnology Network. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia, p. 122.
- Siritunga, D. and Sayre, R.T. 2003. Generation of cyanogen-free transgenic cassava. *Planta* 217: 367–373.
- Storey, H.H. and Nichols, R.F.W. 1938. Studies of the mosaic disease of cassava. *Ann. Appl. Biol.* 25: 790–806.
- Thomas, C.L., Jones, L., Baulcombe, D.C. and Maule, A.J. 2001. Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a potato virus X vector. *Plant J.* 25: 417–425.
- Turnage, M.A., Muangsan, N., Peele, C.G. and Robertson, D. 2002. Geminivirus-based vectors for gene silencing in *Arabidopsis*. *Plant J. Cell Mol. Biol.* 30: 107–114.
- Vanitharani, R., Chellappan, P., Pita, J.S. and Fauquet, C.M. 2004. Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and posttranscriptional gene silencing suppression. *J. Virol.* In press.