

Gene silencing and applications for functional gene validation: The case of Geminiviruses

Silenciamiento génico y aplicaciones para la validación funcional de genes: el caso de los Geminivirus

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ABSTRACT

Plants are able to recognize and degrade double-strand RNA molecules employing the mechanism of post-transcriptional gene silencing (PTGS). PTGS is both a vegetal defense strategy against viral infections and a conserved eukaryotic mechanism for regulate endogenous gene expression. The VIGS methodology (Virus Induced Gene Silencing) uses this mechanism to selectively silence genes employing viral vectors, which contain the target gene, becoming a tool for functional gene validation. *Geminiviridae* family, with the *Begomovirus*, *Curtovirus*, *Mastrevirus* and *Topocuvirus* genera, encompass viruses of circular, single-strand DNA packed in icosahedric geminated particles, and some of them had been evolved to infect particular plant species. Classification of these genera is based in the genome organization, the type of host plants and the insect vector that transmits the virus. Geminiviruses are able to induce gene silencing (GS) and therefore they have been used to develop VIGS-based methodologies for functional gene silencing. This review describes the molecular mechanism of gene silencing, with emphasis in gene silencing induced by Geminiviruses and the applications for a staple crop as cassava.

Key words: gene silencing, functional genomics, Geminivirus, *Manihot esculenta*.

RESUMEN

Las plantas reconocen y degradan moléculas de ARN de doble cadena a través del mecanismo de silenciamiento génico post-transcripcional (PTGS). Se trata de una estrategia de defensa vegetal contra infecciones virales y, simultáneamente, de un mecanismo conservado en los eucariotas para regular la expresión endógena de genes. La metodología VIGS (*Virus Induced Gene Silencing*) aprovecha el PTGS para silenciar genes selectivamente empleando vectores virales que contienen el gen blanco, constituyéndose en una herramienta para la validación funcional de genes. La familia *Geminiviridae*, con los géneros *Begomovirus*, *Curtovirus*, *Mastrevirus* y *Topocuvirus* comprende virus con ADN de cadena circular sencilla empaquetado en partículas icosaédricas geminadas, algunos de los cuales han evolucionado para infectar especies particulares de plantas. La clasificación de estos géneros se basa en la organización del genoma, el rango de plantas hospederas y el insecto vector que los transmite. Los Geminivirus inducen silenciamiento génico (SG) y han sido empleados como herramientas de VIGS para la validación funcional de genes. Esta revisión describe la base molecular del mecanismo de silenciamiento génico con énfasis en el silenciamiento inducido por Geminivirus y las aplicaciones a un cultivo de seguridad alimentaria como la yuca.

Palabras clave: silenciamiento génico, genómica funcional, Geminivirus, *Manihot esculenta*.

Introduction

Viral diseases represent great limitations in crop production of agronomic interest. In response to viral infection, plants have developed different kinds of immunities, one of which involves gene silencing (GS), which produces the degradation of the double-strand RNA molecules of viral origin produced in the process of virus replication. This phenomenon has been used to implement strategies for the functional validation of genes, inhibiting gene expression and evaluating the resulting phenotype. This is how the biotechnological exploitation of plant-virus interactions

has permitted elucidating the function of multiple genes. This review conducts a description of the GS mechanism with emphasis to that produced by Geminiviruses and its application for the functional validation of genes in plants.

Types of silencing

Gene silencing controls viral replication within the host cells and avoids the invasion of characteristic DNA fragments like transposons and regulates gene endogenous expression (Chapman and Carrington, 2007). GS is highly conserved evolutionarily and involves the degradation of

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RNA molecules (Chen and Rajewsky, 2007). It was initially reported in plants, where it was denominated as Post Transcriptional Gene Silencing (PTGS) (Napoli *et al.*, 1990), but it was subsequently described in fungus (quelling) (Cogoni and Macino, 1997) and recently in animals, where it is known as RNA interference (RNAi) (Fire *et al.*, 1998).

GS consists of the junction between a short RNA sequence of approximately 20-30 nucleotides (nts) with an RNA target sequence for its degradation (Fire *et al.*, 1998). There are several paths activating the degradation mechanism of RNA molecules, which is related to the evolutionary histories of each of the different organisms and the functions GS must fulfill (Voinnet, 2009). GS starts with the presence of a double-strand RNA (dsRNA) recognized by the Dicer enzyme, which has RNAase III activity and, specifically cuts dsRNAs producing duplex RNAs of 21 to 24 nts called siRNA (*small interfering RNAs*). Dicer is evolutionarily conserved in diverse organisms (Bernstein *et al.*, 2001). The siRNAs generated by Dicer are recruited by the multi-enzyme RISC complex (*RNA-Induced Silencing Complex*), which contains Argonaut (AGO) (Hammond *et al.*, 2001). Within RISC, one of the siRNA strands remains joined to the complex and the complementary strand is hydrolyzed, possibly by AGO (Hutvagner and Simard, 2008). When RISC has been charged with siRNA, it begins to recognize messenger RNA sequences (mRNA) complementary to siRNA, cutting them off or repressing their translation (Baulcombe, 2005) (Fig. 1). Silencing may extend due to a *RNA-dependent RNA polymerase* (RDRP), which amplifies the signal producing additional siRNAs migrating from the infection site to remote cells (Voinnet, 2008).

The origin of the dsRNAs defines the different GS mechanisms in the organisms. RNA silencing may occur at the transcriptional level (*Transcriptional Gene Silencing*, TGS), as well as at the post-transcriptional (PTGS) level (Brodersen and Voinnet, 2006). In *Drosophila* germ cells, a type of small (between 24-30 nts), noncoding RNA has been described, denominated piwiRNA, which originates from a single-strand RNA precursor without needing the Dicer. The Piwi complex would join the transcripts emerging from the transposable elements (TE), recruiting chromatin remodeling or assembly factors and activating the transcription (Klattenhoff and Theurkauf, 2008).

TGS involves the methylation of promoter regions reducing or inactivating the genetic expression (Bao *et al.*, 2004). This silencing is related to controlling the TE invasion in the genome (Obbard *et al.*, 2009). Methylation-based GS is begun by small RNAs (sRNAs) of 24 nts from transpo-

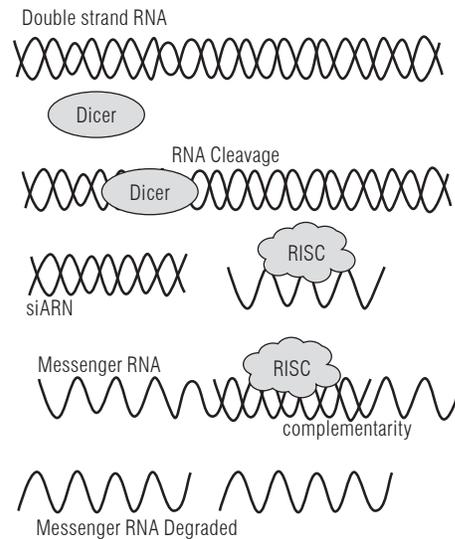


FIGURE 1. Scheme of gene silencing process mediated by siRNAs. Dicer recognizes the dsRNA, cutting it in siRNAs, which are loaded in the RISC complex that degrades the RNA strand complementary to siRNA, and subsequently recognizes the messenger RNA to be silenced through complementarity of bases with loaded siRNA. Once the double-strand fragment is generated between the siRNA and the complementary region of mRNA, the cut and degradation of the mRNA is produced by RISC.

sons (Mosher *et al.*, 2008). The sRNAs induce epigenetic silencing by generating RNA-directed DNA methylation (RdDM) (Chan *et al.*, 2005).

PTGS is mediated by microRNAs (miRNAs). The miRNAs are a fundamental part for the regulation of the expression of endogenous genes in plants and animals and participate in multiple processes and responses to biotic and abiotic stresses (Brodersen and Voinnet, 2009). miRNAs are transcribed by RNA polymerase II, forming the primary miRNA (Lee *et al.*, 2002). These primary miRNAs are processed in the nucleus by Drosha-type proteins making up miRNAs precursors, which are exported to cytoplasm and processed by Dicer, forming mature miRNAs (Chapman and Carrington, 2007). As with siRNA from the virus (see below), miRNAs are recruited by RISC where one of the RNA strands (denominated passenger RNA*) is lost. The miRNA:RISC complex interacts with the complementary mRNA molecule producing its cut and degradation (Voinnet, 2009).

The first PTGS case was discovered in plants as a defense mechanism against viral infections (Hamilton and Baulcombe, 1999). Many virus infecting plants are made up of single-strand RNA, which in order to replicate go through an intermediary stage where double-strand molecules are produced, which are recognized by Dicer and cut into siRNAs (Voinnet, 2009). These siRNAs are incorporated to RISC, where a strand of the siRNA is cut and degraded.

The single-strand siRNA remaining in the RISC, being complementary to the viral RNA sequence, will make up a duplex where the viral RNA is digested, generating resistance to the infection (Baulcombe, 2005). In various eukaryotes, there is RDRP, which permit producing more siRNAs, amplifying the signal and making it systemic (Ding and Voinnet, 2007).

Suppressors

The interaction between plants and virus can be seen as an “arms race” (Waterhouse and Fusaro, 2006). In this sense, viruses have generated mechanisms to block the GS and in consequence the resistance of the host (Deleris *et al.*, 2006) through silencing suppressors, initially characterized as viral pathogenicity factors (Voinnet *et al.*, 1999). GS suppressor proteins are not similar at the sequence level, but act at different levels within the same GS route, suggesting they have originated via convergent evolution processes (Soosaar *et al.*, 2005). The first suppressor identified was HC-Pro in Potyvirus (Pruss *et al.*, 1997). HC-Pro acts as a suppressor joining Dicer, interfering with its activity and trapping the siRNAs (Voinnet, 2005). Other suppressors acting similarly are P19 from the Tombusvirus (*Tombusviridae*) and P21 from BYV (*Beet yellow virus*) (Lakatos *et al.*, 2006), P38 from TCV (Turnip crinkle virus), and 2b from CMV (Cucumber mosaic virus) (Deleris *et al.*, 2006). Recently, P0 was characterized from BWYV (Beet western yellow virus), which presents an F-box motive implicated in protein degradation and interacts with AGO degrading it and blocking GS (Bortolamiol *et al.*, 2007).

Gene silencing and functional genomics

The most widely used mechanism to determine gene function is the generation of mutants of genes of interest, causing the loss of function or expression through mutations in the promoters (Page and Grossniklaus, 2002). The collection of mutants generated is evaluated to identify the resulting phenotype. The way of applying this methodology involves the insertion of DNA sequences (transposons or T-DNA elements in plants) (Page and Grossniklaus, 2002), homologous recombination (bacteria and animals) (Kile and Hilton, 2005; Shuman and Silhavy, 2003), treatment with mutagenic agents like sodium azide (Rines, 1985), ethyl methanesulfonate (EMS) (Katavic *et al.*, 1995), or gamma radiation exposure (Vizir *et al.*, 1994) to just name the most important. Identifying the GS mechanism in plants and animals generated new methodological strategies that have permitted guided silencing of target genes for their functional validation (Boutros and Ahringer, 2008).

In *Drosophila melanogaster* and *Caenorhabditis elegans*, silencing has been implemented by RNAi (Dorsett and Tuschl, 2004). Initially long dsRNA were employed, which were cleaved in intra-cellular manner by Dicer in functional siRNAs (Fire *et al.*, 1998; Hammond *et al.*, 2000). Nevertheless, these long dsRNAs are not very efficient in activating the silencing in many mammal cells (Grimm, 2004). Alternatively, cells are transfected with synthetic siRNAs or cloned in plasmids (Jackson *et al.*, 2006; Berns *et al.*, 2004). Thus using for example, siRNA libraries, functional genomics experiments have been conducted to determine the phenotypic effect of inactivating the genes annotated through complete genome sequencing strategies (Berns *et al.*, 2004; Boutros *et al.*, 2004; Paddison *et al.*, 2004). There are siRNA libraries available in public and private research centers (Boutros and Ahringer, 2008).

For *C. elegans*, the ingestion of bacteria containing plasmids with siRNAs permits introducing these into the nematode cell cytoplasm, inducing silencing of the endogenous target gene (Jorgensen and Mango, 2002), while in *Drosophila* or cell cultures it is necessary to transfect to incorporate siRNAs into the cytoplasm (Boutros and Ahringer, 2008).

In plants, the Virus-Induced Gene Silencing (VIGS) strategy was developed in which a gene or part of such is introduced into a viral vector in antisense direction (Eamens *et al.*, 2008), is inoculated in plants and a couple of weeks later the silencing of the endogenous gene occurs, by producing siRNAs generated by Dicer from dsARN produced during viral replication, and which includes the production of dsRNAs complementary to the target gene to be silenced. The VIGS system has been widely used mainly in plants from the *Solanaceae* family while in others it has been difficult to implement. It can be explained considering the difficulty finding a balance between the replication capacity of the viral construct and the plant's relative resistance against infection. To avoid a phenotype product of the virus instead of the target gene to be silenced is troublesome (Purkayastha and Dasgupta, 2009). Alternatively, the strategy known as RNAi has been developed, which consists of introducing, via genetic transformation, a sense-antisense construction of the target gene to be silenced. This way, the transcription of the “transgen” in the plant cell will produce dsRNAs, which will be recognized by Dicer and siRNAs are generated complementary to the endogenous gene to silence (Wesley *et al.*, 2001). This strategy takes up more time given that transgenic lines must be generated with the silencing construct; this is a rather inefficient process in plants recalcitrant to genetic transformation (Purkayastha and Dasgupta, 2009).

Geminiviruses are part of the game

Although most viruses infecting plants have RNA genomes, there are exceptions like viruses from the *Caulimoviridae*, *Geminiviridae*, and *Circoviridae* families; curiously, in prokaryotes, invertebrates, and vertebrates, most viral infections are caused by DNA viruses (Rojas *et al.*, 2005). Caulimoviruses are formed by dsDNA, which is replicated via an RNA intermediary by reverse transcription (Schoelz, 2008). Geminiviruses and Nanoviruses have single-strand DNA or ssDNA, replicating via an intermediary dsDNA molecule through the rolling circle mechanism (Hanley-Bowdoin *et al.*, 2000).

Since the beginning of 20th century, the first plant infections caused by Geminiviruses were recognized, although they were much later associated to vector insects. Isolation and characterization of Geminiviruses was undertaken in the 1970s (Mumford, 1974). Geminiviral diseases affect production of crops like corn, bean, cassava and tomato (Fargette *et al.*, 2004; Mansoor *et al.*, 2006), to name just some cases.

Geminiviruses have a genome composed of one (monopartite) or two (bipartite) circular ssDNA molecules whose sizes range between 2.5 and 3.0 kb. These viruses use a mechanism for bidirectional transcription and genetic overlap that maximizes the use of their genome (Rojas *et al.*, 2005). Structurally, they present twin icosahedral virions, which gives them their name (from the Latin *geminis*: twin) (Matthews and Hull, 2002). Geminiviruses present the REP protein (replication-associated protein), essential for replication, and whose genomic position, sequence and function is conserved in all the Geminiviruses (Matthews and Hull, 2002). Geminiviruses are grouped in the *Geminiviridae* family, made up of four genus according to their genomic arrangement, host range, and vector specificity: Geminiviruses with monopartite genomes and transmitted by insects from the *Cicadellidae* family to monocotyledon plants belong to the *Mastrevirus* genus. Geminivirus with the characteristics of *Mastrevirus* but transmitted to

dicotyledon plants are *Curtovirus*. The *Topocuvirus* genus is monopartite and infects dicotyledon plants through insects from the *Membracidae* family. The *Begomovirus* genus is one of the most diverse Geminiviruses; it is transmitted exclusively by the *Bemisia tabaci* whitefly complex to dicotyledon plants and contains mono and bipartite members (Vanitharani *et al.*, 2005).

One of the evolutionary advantages permitting Geminiviruses (like DNA virus) to infect plants may have been their bipartite genomic structure (Rojas *et al.*, 2005). As with other virus families, there is a close co-evolution of Geminiviruses with their vector insects to ensure expansion and transmission, which is evidenced in the generation of symptoms that produce yellowing of the leaves of plants infected by Geminiviruses, attracting plant sucking insects and allowing the propagation of these viruses (Rojas *et al.*, 2005).

Silencing produced by Geminiviruses: we are part of the solution

Although Geminiviruses are made up of DNA and do not present RNA intermediaries during their replication, they can induce PTGS, as initially observed when using Tomato golden mosaic virus (TGMV) as vector to silence reporter genes (Kjemtrup *et al.*, 1998). Then came reports of virus-induced gene silencing (VIGS) in tomato (Lucioli *et al.*, 2003), as in Geminiviruses infecting cassava like the African cassava mosaic virus Cameroon (ACMV-CM), the East African cassava mosaic Cameroon/Uganda (EACMC/UG/V), the Sri-Lanka cassava mosaic virus (SLCMV), and the Indian cassava mosaic virus (ICMV) (Patil and Fauquet, 2009). Currently, the VIGS strategy is being used in several plants (Tab. 1).

Several hypotheses suggest how Geminiviruses could induce GS. The most accepted of these states that bidirectional transcription of transcripts produced by regions with opposing polarity would produce overlaps that would be recognized by Dicer as dsRNAs (Vanitharani *et al.*, 2005).

TABLE 1. Geminiviruses used as genetic silencing vectors.

Virus	Silencing host	Natural transmission mode	Pathogenesis host	Infection mechanism for VIGS	Reference
TGMV	<i>N. benthamiana</i> Tomato	White fly	Tomato	Microparticle bombardment	Lucioli <i>et al.</i> , 2003
CaLCuV	<i>Arabidopsis</i> Broccoli	White fly	Cabbage, broccoli, cauliflower	Microparticle bombardment	Hill <i>et al.</i> , 1998
PHYVV	Pepper	Mechanical	Peppers	Agroinfiltration	Stewart <i>et al.</i> , 2005
CLCrV	Cotton	Mechanical	Cotton	Microparticle bombardment	Tuttle <i>et al.</i> , 2008
ACMV	Cassava	White fly	Cassava	Microparticle bombardment	Fofana <i>et al.</i> , 2004

Geminiviruses could induce or suppress GS. The Potato virus X (PVX) TrAP protein (Voinnet *et al.*, 1999) was one of the first suppressors identified. Then C2, from certain Begomoviruses and AC4 from ACMV-CM, and SLCMV were characterized as PTGS suppressors (Vanitharani *et al.*, 2004), given that transgenic plants expressing AC2 or C2 were more susceptible to the virus (Rojas *et al.*, 2005).

Geminiviruses are used as vectors to carry out VIGS. Some examples are Tobacco curly shoot virus (TbCSV), Tomato yellow leaf curl China virus (TYLCCNV), Tomato golden mosaic virus (TGMV), and Cabbage leaf curl virus (CaLCuV) (Peele *et al.*, 2001; Turnage *et al.*, 2002; Tuttle *et al.*, 2008; Golenberg *et al.*, 2009). These vectors offer some advantages over RNA-based viruses: high sequence conservation among Geminiviruses permits adapting a virus to be used in VIGS through information from another related virus, greater genetic stability with respect to vectors based on RNA virus and can infect meristematic tissue (Padmanabhan and Dinesh-Kumar, 2008). They also have a broader range of hosts, including plants of agronomic interest (Golenberg *et al.*, 2009). There are disadvantages for Geminiviruses as VIGS inducers: symptoms can be present in the inoculated plants, it is necessary to co-inoculate plasmids with the A and B genomes (Padmanabhan and Dinesh-Kumar, 2008) and the size of the inserts the geminiviral vectors can host is small. Nevertheless, to trigger silencing big fragments are not necessary (100 base pairs may be enough) (Vanitharani *et al.*, 2005).

Geminiviruses and cassava

Cassava (*Manihot esculenta*) originated in the Amazon River basin and constitutes the nutritional base for almost one billion people (FAOSTAT, 2009). Cassava is attacked by *Cassava mosaic geminiviruses* (CMGs) causing the Cassava mosaic disease (CMD) (Patil and Fauquet, 2009). CMD is transmitted by the *Bemisia tabaci* whitefly complex (Legg and Fauquet, 2004), with isolates of such in Africa and India (Maruthi *et al.*, 2002). The genome of the virus responsible for this disease, African cassava mosaic virus (ACMV) was sequenced in 1983 (Stanley and Gay, 1983). CMD has been reported in at least nine African countries, causing losses near US\$2-billion (Legg and Fauquet, 2004). Recently, various African and Indian strains have been identified and classified (Patil and Fauquet, 2009).

CMD is found in Africa and India but not in America, although the vector, the white fly (*Bemisia tabaci*) is present in the Old and the New Worlds. It is possible the fly's specific biotype (B) cannot colonize cassava in America

(Carabali *et al.*, 2005). Cassava arrived to the African continent in the 16th century (Cock, 1982). It is possible that the Geminivirus, already present in this continent and infecting other types of hosts, took advantage of the extension of the cassava cultivation in Africa for its colonization. CMD expansion in Africa has been facilitated by the use of susceptible varieties, the exchange of material among geographical regions and the exchange among geminiviruses (Patil and Fauquet, 2009). One of the influential factors in the expansion of CMGs is the high diversity they present in the A genome due to the frequent recombination processes between strains and species (Fondong *et al.*, 2000; Patil and Fauquet, 2009), among which there is also high synergy, increasing the severity of the CMD symptoms (Fondong *et al.*, 2000).

Genetic composition

CMGs belong to the *Begomovirus* genus, *Geminiviridae* family; they have two molecules of single-strand DNA, A and B forms (Patil and Dasgupta, 2006). The A-DNA presents six open reading frames, each one for a specific protein. *AC1* is the protein associated with replication (Rep), *AC2* is the transcriptional activator protein (TrAP). Gene *AC3* codifies for a protein that enhances replication (REn). Finally, *AC4* is a silencing suppressor (Patil and Dasgupta, 2006). In the complementary sense, there are *AV1* and *AV2* codifying for capsid proteins. In B-DNA there is *BV1* codifying nuclear-shuttle protein (NSP) and *BC1* codifying movement protein (MP) implied in intra- and-intercellular movement (Pita *et al.*, 2001).

The A and B components of Begomoviruses have a common region of approximately 200 nucleotides with 80% identity (Harrison and Robinson, 1999), which contain several regulatory elements and "iteron" copies, junction sites for the protein associated to replication (Rep) (Hanley-Bowdoin *et al.*, 2000).

CMGs have used strategies that permit them to generate a high diversity for their adaptation, among which there is a high recombination rate between A and B components (Lefevre *et al.*, 2009), which may be favored by the mixed infection present in cassava cultivations where different CMG strains or species can cohabit in the same plant (Patil and Fauquet, 2009).

Silencing in cassava by Geminiviruses

Geminiviruses can induce GS or PTGS. Among those inducing PTGS, there are CMGs. In infected tobacco and cassava

plants siRNAs associated to PTGS are produced (Chellappan *et al.*, 2004). For some viruses, like ACMV and SLCMV, there is the *recovery* phenomenon (Rodríguez-Negrete *et al.*, 2009), which has been correlated with PTGS and with siRNA production (Padmanabhan and Dinesh-Kumar, 2008). However, in other cases, although siRNAs have been identified, the plants do not show *recovery*, suggesting that there must be other viral or suppressor genetic factors that could counter silencing (Patil and Fauquet, 2009).

Most siRNAs identified correspond to the C-terminal of *ACI* that overlaps with the N-terminal region of the *AC2* gene from the A-DNA component. In some cases, siRNAs from the *BCI* gene of the B-DNA component have been detected (Chellappan *et al.*, 2004).

VIGS in cassava: silencing the genome!

The VIGS system has been utilized with CMGs for functional validation of genes in cassava. Fofana *et al.* (2004) introduced a multi-cloning region (MCS) within the ORF codifying for the capsid protein of the genome A component of ACMV-CM; subsequently, it was subcloned in pBluescript to generate a silencing construct. To validate the correct functioning of silencing in cassava, phytoene desaturase (PDS) and sulfur (*su*) genes were used (Fofana *et al.*, 2004) whose silencing phenotype is easily visible, plants showing bleaching and/or yellowing, respectively (Kumagai *et al.*, 1995). From *Nicotiana benthamiana* DNA constructions were made of these genes within the ACMV vector (ACMV+SU and ACMV+PDS, respectively) and cassava 60444 variety and tobacco plants were infected. In tobacco photo-bleaching was observed in plants silenced with PDS; unlike cassava did not show photo-bleaching (Fofana *et al.*, 2004). By using the *su* gene, yellowing was observed in cassava and tobacco leaves, revealing that the VIGS system using ACMV works adequately (Fofana *et al.*, 2004). To demonstrate that the system works for gene validation, Fofana *et al.* tested the silencing of *CYP79D2*, a gene involved in linamarin biosynthesis, and through RT-PCR they verified the *CYP79D2* and *CYP79D1* silencing, which shares 89% identity with *CYP79D*. Additionally, linamarin content was reduced by close to 70% in cassava leaves silenced plants (Fofana *et al.*, 2004).

Recently, we used the VIGS strategy in cassava to silence *RXam1*, a candidate gene for resistance to vascular cassava bacterial blight. The ACMV-RXAM1 construct was generated and gene silencing was sought in varieties 60444 and SG107-35. Although yellowing was clearly observed in plants from the 60444 variety, only one of seven plants from the SG107-35 variety revealed a clear phenotype, sug-

gesting that VIGS can be dependent on the variety of the plant used (Cortés and López, 2010).

Conclusions

Geminiviruses are among the main limitations in the production of several agricultural importance crops, including cassava. Plants activate GS in response to Geminiviruses, which has been used for functional validation of genes. This strategy has been implemented in cassava and has proven to be a useful tool in this plant, as well as being a great aid in the new era of functional genomics in cassava, with the recent release of its complete genome sequence (<http://www.phytozome.net/cassava>). ACMV has not been reported in America and for bio-security, these kinds of strategies can only be carried out in countries where CMD is present or in regions of the world where cassava is not cultivated and under strict control measures. It would be interesting to try to establish if from the viruses infecting cassava in the American continent, which present a very low incidence, viral vectors could be developed for VIGS in cassava.

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Literature cited

- Bao, N., K.W. Lye, and M.K. Barton. 2004. MicroRNA binding sites in *Arabidopsis* class III HD-ZIP mRNAs are required for methylation of the template chromosome. *Dev. Cell* 7, 653-662.
- Baulcombe, D. 2005. RNA silencing. *Trends Biochem. Sci.* 30, 290-293.
- Berns, K., E.M. Hijmans, J. Mullenders, T.R. Brummelkamp, A. Velds, M. Heimerikx, R.M. Kerkhoven, M. Madiredjo, W. Nijkamp, B. Weigelt, R. Agami, W. Ge, G. Cavet, P.S. Linsley, R.L. Beijersbergen, and R. Bernards. 2004. A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature* 428, 431-437.
- Bernstein, E., A.A. Caudy, S.M. Hammond, and G.J. Hannon. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363-366.
- Bortolamiol, D., M. Pazhouhandeh, K. Marrocco, P. Genschik, and V. Ziegler-Graff. 2007. The Polerovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing. *Curr. Biol.* 17, 1615-1621.

- Boutros, M., A.A. Kiger, S. Armknecht, K. Kerr, M. Hild, B. Koch, S.A. Haas, R. Paro, and N. Perrimon. 2004. Genome-wide RNAi analysis of growth and viability in *Drosophila* cells. *Science* 303, 832-835.
- Boutros, M. and J. Ahringer. 2008. The art and design of genetic screens: RNA interference. *Nat. Rev. Genet.* 9, 554-566.
- Bridson, R., J. Brown, E. Moriones, J. Stanley, M. Zerbini, X. Zhou, and C.M. Fauquet. 2008. Recommendations for the classification and nomenclature of the DNA-b satellites of begomoviruses. *Arch. Virol.* 153, 763-781.
- Brodersen, P. and O. Voinnet. 2006. The diversity of RNA silencing pathways in plants. *Trends Genet.* 22, 268-280.
- Brodersen, P. and O. Voinnet. 2009. Revisiting the principles of microRNA target recognition and mode of action. *Nat. Rev. Mol. Cell Biol.* 10, 141-148.
- Brown, J.K. 2008. Plant Resistance to viruses: Geminiviruses. pp. 52-58. In: Mahy, B.W.J, and M.H.V Van Regenmortel (eds.). *Desk encyclopedia of plant and fungal virology*. Academic Press, San Diego, CA.
- Carabali, A., A. Bellotti, J. Montoya-Lerma, and M. Cuellar. 2005. Adaptation of *Bemisia tabaci* biotype B (Gennadius) to cassava, *Manihot esculenta* (Crantz). *Crop Prot.* 24, 643-649.
- Chan, S.W., I.R. Henderson, and S.E Jacobsen. 2005. Gardening the genome: DNA methylation in *Arabidopsis thaliana*. *Nat. Rev. Genet.* 6, 351-360.
- Chapman, E.J. and J.C. Carrington. 2007. Specialization and evolution of endogenous small RNA pathways. *Nat. Rev. Genet.* 8, 884-896.
- Chellappan, P., R. Vanitharani, J. Pita, and C.M. Fauquet. 2004. Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. *J. Virol.* 78, 7465-7477.
- Chen, K., and N. Rajewsky. 2007. The evolution of gene regulation by transcription factors and microRNAs. *Nat. Rev. Genet.* 8, 93-103.
- Cock, J.H. 1982. Cassava: a basic energy source in the tropics. *Science* 218, 755-762.
- Cogoni, C., and G. Macino. 1997. Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*. *Proc. Natl. Acad. Sci. USA* 94, 10233.
- Cortés, S. and C. López. 2010. Estrategia de silenciamiento génico en yuca para la validación de genes de resistencia. *Acta Biol. Colomb.* 15(2), 1-23.
- Deleris, A., J. Gallego-Bartolome, J. Bao, K.D. Kasschau, and J.C. Carrington. 2006. Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* 313, 68-71.
- Ding, S.W. and O. Voinnet. 2007. Antiviral immunity directed by small RNAs. *Cell* 130, 413-426.
- Dorsett, Y. and T. Tuschl. 2004. siRNAs: applications in functional genomics and potential as therapeutics. *Nat. Rev. Drug. Discov.* 3, 318-329.
- Eamens, A., M.B. Wang, N.A. Smith, and P.M. Waterhouse. 2008. RNA silencing in plants: yesterday, today, and tomorrow. *Plant Physiol.* 147, 456-468.
- FAOSTAT. 2009. FAO database. Food and Agriculture Organization of the United Nations, <http://faostat.fao.org/site/567/Desktop-Default.aspx?PageID=567#ancor>; consulted: December, 2010.
- Fargette, D., G. Konaté, C. Fauquet, E. Muller, M. Peterschmitt, and J.M. Thresh. 2006. Molecular ecology and emergence of tropical plant viruses. *Annu. Rev. Phytopathol.* 44, 235-60.
- Fire, A., S. Xu, M.K. Montgomery, S.A. Kostas, and S.E. Driver. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806-811.
- Fofana, I.B., A. Sangare, R. Collier, C. Taylor, and C.M. Fauquet. 2004. A Geminivirus-induced gene silencing system for gene function validation in cassava. *Plant Mol. Biol.* 56, 613-624.
- Fondong, V.N., J.S. Pita, M.E.C. Rey, A. de Kochko, R.N. Beachy, and C.M. Fauquet. 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.
- Golenberg, E., D. Sather, L. Hancock, K. Buckley, and N. Villafranco. 2009. Development of a gene silencing DNA vector derived from a broad host range Geminivirus. *Plant Methods* 5, 9.
- Grimm, S. 2004. The art and design of genetic screens: mammalian culture cells. *Nat. Rev. Genet.* 5, 179-189.
- Hamilton, A.J. and D.C. Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950-952.
- Hammond, S.M., E. Bernstein, D. Beach, and G.J. Hannon. 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 404, 293-296.
- Hammond, S.M., S. Boettcher, A.A. Caudy, R. Kobayashi, and G.J. Hannon. 2001. Argonaute2, a link between genetic
- Peele, Ch., Ch.V. Jordan, N. Muangsan, M. Turnage, E. Egelkrout, P. Eagle, L. Hanley-Bowdoin, and D. Robertson. 2001. Silencing of a meristematic gene using geminivirus-derived vectors. *Plant J.* 27, 357-366.
- Pita, J.S., V.N. Fondong, A. Sangare, G.W. Otim-Nape, S. Ogwal, C. M. Fauquet. 2001. 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.
- Pruss, G., X. Ge, X.M. Shi, J.C. Carrington, and V. Bowman. 1997. Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* 9, 859-868.
- Purkayastha, A., and I. Dasgupta. 2009. Virus-induced gene silencing: a versatile tool for discovery of gene functions in plants. *Plant Physiol. Biochem.* 47, 967-976.
- Rines, H.W. 1985. Sodium azide mutagenesis in diploid and hexaploid oats and comparison with ethyl methanesulfonate treatments. *Environ. Exp Bot.* 25, 7-16.
- Rodríguez-Negrete, E.A., J. Carrillo-Tripp, and R.F. Rivera-Bustamante. 2009. RNA Silencing against geminivirus: complementary action of posttranscriptional gene silencing and transcriptional gene silencing in host recovery. *J. Virol.* 83, 1332-1340.
- Robertson, D. 2004. VIGS vectors for gene silencing: many targets, many tools. *Annu. Rev. Plant Biol.* 55, 495-519.

- Rojas, M.R., C. Hagen, W.J. Lucas, and R.L. Gilbertson. 2005. Exploiting chinks in the plant's armor: evolution and emergence of Geminiviruses. *Annu. Rev. Phytopathol.* 43, 361-394.
- Shuman, H.A., and T.J Silhavy. 2003. The art and design of genetic screens: *Escherichia coli*. *Nat. Rev. Genet.* 4, 419-431.
- Schoelz, J.E. 2008. Caulimoviruses: general features. pp. 147-155. En: Mahy, B.W.J., and M.H.V. Van Regenmortel (eds.). *Desk encyclopedia of plant and fungal virology*. Academic Press, San Diego, CA.
- Soosaar, J.L., T.M. Burch-Smith, and S.P. Dinesh-Kumar. 2005. Mechanisms of plant resistance to viruses. *Nat. Rev. Microbiol.* 3, 789-798.
- Stanley, J., and M. Gay. 1983. Nucleotide sequence of cassava latent virus DNA. *Nature* 301, 260-262.
- Stewart, C.S., B.C. Kang, K. Liu, M. Mazourek, S.L. Moore, I. Paran, M. Jahn. 2005. The Pun1 gene for pungency in pepper encodes a putative acyltransferase. *Plant J.* 42, 675-688.
- Turnage, M.A., N. Muangsang, Ch. Peele, and D. Robertson. 2002. Geminivirus-based vectors for gene silencing in *Arabidopsis*. *Plant J.* 30, 107-114.
- Tuttle, J.R., A.M. Idris, J.K. Brown, C.H. Haigler, and D. Robertson. 2008. Geminivirus-mediated gene silencing from cotton leaf crumple virus is enhanced by low temperature in cotton. *Plant Physiol.* 148, 41-50.
- Umbach, J.L., and B.R. Cullen. 2009. The role of RNAi and microRNAs in animal virus replication and antiviral immunity. *Gene Dev.* 23, 1151-1164.
- Vanitharani, R., P. Chellappan, J.S. Pita, and C.M. Fauquet. 2004. Differential roles of AC2 and AC4 of cassava Geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. *J. Virol.* 78, 9487-9498.
- Vanitharani, R., P. Chellappan, and C.M. Fauquet. 2005. Geminiviruses and RNA silencing. *Trends Plant Sci.* 10, 144-151.
- Vizir, I.Y., M.L. Anderson, Z.A. Wilson, and B.J. Mulligan. 1994. Isolation of deficiencies in the *Arabidopsis* genome by gamma-irradiation of pollen. *Genetics* 137, 1111-1119.
- Voinnet, O. 2005. Induction and suppression of RNA silencing: insights from viral infections. *Nat. Rev. Genet.* 6, 206-220.
- Voinnet, O. 2008. Use, tolerance and avoidance of amplified RNA silencing by plants. *Trends Plant Sci.* 13, 317-328.
- Voinnet, O. 2009. Origin, biogenesis, and activity of plant microRNAs. *Cell* 136, 669-687.
- Voinnet, O., Y.M. Pinto, and D.C. Baulcombe. 1999. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc. Natl. Acad. Sci. USA.* 96, 14147-14152.
- Waterhouse, P.M., and A.F. Fusaro. 2006. Plant science. Viruses face a double defense by plant small RNAs. *Science* 313, 54-55.
- Wesley, S.V., C.A. Helliwell, N.A. Smith, M.B. Wang, D.T. Rouse, Q. Liu, P.S. Gooding, S.P. Singh, D. Abbott, P.A. Stoutjesdijk, S.P. Robinson, A.P. Gleave, A.G. Green, P.M. Waterhouse. 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J.* 27, 581-590.