MARCOS VINÍCIUS VIEIRA MATTOS

A REVIEW OF WHITEFLIES AS VECTORS OF PLANT VIRUSES AND A MOLECULAR ANALYSIS OF VECTOR TRANSMISSION OF COWPEA MILD MOTTLE VIRUS

Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Simon Luke Elliot

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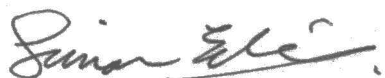

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ABSTRACT

MATTOS, Marcos Vinícius Vieira, D.Sc., Universidade Federal de Viçosa, August, 2022**. A review of whiteflies as vectors of plant viruses and a molecular analysis of vector transmission of cowpea mild mottle virus**. Advisor: Simon Luke Elliot.

The whitefly is one of the main vectors of plant viruses. With its status as a pest, this insect causesdamage to crops such as soybeans, beans, cotton and tomatoes. Research efforts have focussed on control measures, vector biology and the interactions of these insects with the viruses they carry. Regarding the relationships established during viral transmission, many studies have shown how these interactions occur at the molecular level. At the same time, much information still needs to be elucidated. In chapter 1 of this study we review the virus families known so far to be transmitted by the whitefly, the mechanisms involved in the interaction, and we raise hypotheses that may explain some points in the transmission process. In Chapter 2, we experimentally assessed whether a cysteine-rich protein (CRP) encoded by *Cowpea mild mottle virus* (CPMMV) can act as an helper component of capsid protein (CP) in the process of viral transmission by the whitefly, *Bemisia tabaci*. We found evidence that this does not occur and we conclude that CRP probably does not participate in the transmission process and CP probably acts alone. With this study we hope to contribute with more information to the knowledge of the interactions between viruses and their vectors.

Keywords: Whitefly. Aphid. Disease vectors - Control. Plant virus.

RESUMO

MATTOS, Marcos Vinícius Vieira, D.Sc., Universidade Federal de Viçosa, agosto de 2022. **Revisão sobre moscas-brancas como vetores de vírus de plantas e análise molecular da transmissão vetorial do cowpea mild mottle virus**. Orientador: Simon Luke Elliot.

A mosca branca é um dos principais vetores de vírus que causam doenças em plantas. Com o status de pragas, esse inseto tem provocado danos a culturas como soja, feijão, algodão e tomate. Pesquisadores têm se esforçado em medidas de controle, no estudo sobre a biologia e na compreensão das relações que os insetos mantêm com os vírus que carregam. Acerca das relações estabelecidas durante a transmissão viral, muitos trabalhos têm mostrado como essas interações ocorrem a nível molecular. Ao mesmo tempo, muitas informações ainda carecem de elucidações. No capítulo 1 deste trabalho revisamos as famílias de vírus conhecidas até o momento por serem transmitidas pela mosca branca, os mecanismos envolvidos na interação, e levantamos hipóteses que podem explicar alguns pontos do processo de transmissão. No capítulo 2, fizemos um trabalho empírico para avaliar se uma proteína rica em cisteína (CRP) do *Cowpea mild mottle virus* (CPMMV), pode atuar com um componente auxiliar para a proteína capsidial (CP) no processo de transmissão viral pela mosca branca, *Bemisia tabaci*. Encontramos evidências de que isso não ocorre e concluímos que provavelmente a CRP não participa do processo de transmissão e a CP provavelmente atua sozinha. Com este estudo esperamos contribuir com mais informações para o conhecimento das interações entre os vírus e seus vetores.

Palavras-chave: Moscas-branca. Afídeo. Vetores de doença - Controle. Vírus de planta.

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Chapter 1: Whiteflies as vectors of plant viruses: a review of transmission mechanisms

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Abstract

Among insects, aphids and whiteflies are the best known for transmitting a large number of viruses that cause diseases in a diversity of crops. Studies have addressed the range of viruses, vectors and hosts involved in transmission, as well as the mechanisms employed during this process, however, some points still need attention. In this study, we review the virus families known to date to be transmitted by the whitefly and the transmission mechanisms, including viral localization in the vector body and the proteins involved in the virus-vector interaction. We also compare this interaction with that of viruses and aphids, to raise hypotheses that may help to clarify some points of the transmission process. Our observations mainly point out that (1) for the begomoviruses transmitted in a persistent manner, proteins that participate in the viral transmission process and the viral path in the insect body are already clarified; (2) for viruses transmitted semipersistently, only the protein related to the transmission of criniviruseses is known, viral location has been elucidated for criniviruseses and torradoviruseses while for ipomoviruseses neither of these is known; (3) for nonpersistent carlaviruses, neither viral localization nor proteins participating in transmission are known. We also raise the following hypotheses (1) the interaction between whitefly and nonpersistent viruses occurs in a region homologous to the aphid acrostyle (2) mutation events and mixed infection have led to transmission of viruses by whiteflies. In addition, we emphasize the emergence of new viruses being transmitted by whiteflies and viruses that were once transmitted by aphids that are now transmitted by whiteflies.

Key-words: Hemiptera, whitefly, aphid, virus transmission, transmission mechanisms

1. Introduction

Insects play key roles in ecosystems as pollinators, pest control, or food for reptiles, birds and mammals. On the other hand, their often high reproduction rate, easy locomotion and voracity affords many insects the status of pests through direct damage to crop plants or via the transmission of diseases between animals or plants as vectors. Insects can act as vectors of parasitic viruses, bacteria, fungi, protozoa and nematodes. In the case of plant diseases, they are of particular importance as vectors of viruses: it is estimated that insects are responsible for the transmission of 80% of plant viruses (Sarwar, 2020), the remainder being transmitted by mites, fungi and nematodes. Within the class Insecta, the most important viral vectors of phytosanitary importance are to be found within the order Hemiptera (Ng and Falk, 2006), although orders such as Coleoptera and Thysanoptera are also recognized as vectors of plant viruses (Fereres and Moreno, 2009; Sarwar, 2020). To feed, herbivorous hemipterans introduce their piercing-sucking stylet directly into the leaf tissue or phloem where they can acquire or spread viruses. Of the Hemiptera, homopterans (aphids, whiteflies, leafhoppers, and planthoppers) are responsible for transmitting more than 380 plant viruses (Sarwar, 2020), in large part due to their short life cycle, high rate of dispersion, frequent polyphagy, all added to their feeding mode. Within this group, aphids (Aphididae) and whiteflies (Aleyrodidae) are the ones that transmit most viruses, about 150 and 114 species respectively (Sarwar, 2020).

There are good reviews of whiteflies and aphids as viral vectors, especially regarding the range of vector, viral and host species, transmission mechanisms and proteins involved in this process, although knowledge for aphids is broader than for whiteflies (Ng and Falk, 2006; Brown and Czosnek, 2009; Navas-Castillo *et al.*, 2011; Fiallo-Olivé *et al.*, 2019). For the first time in this review we highlight commonalities between these two insects, explore transmission mechanisms for whiteflies known so far, and apply what we know about aphids to raise hypotheses that might help explain some key features of the transmission of viruses by whitefly.

We focus particularly on three observations that are plain from the literature: (1) there are very few species of whitefly that transmit viruses; (2) there are cases in which viruses transmitted by aphids are now transmitted by whiteflies (but not *vice versa*, to the best of our knowledge); (3) there is a wide diversity of geminiviruses transmitted by a single species of whitefly. To take steps towards explaining these observations, we first provide a context from an overview of the mechanisms involved in viral transmission and present biological similarities between aphids and whiteflies. From this, we focus on virus-whitefly relationships already reported in the literature as well as on the emergence of new viruses being transmitted by this insect.

2. Mechanisms involved in viral transmission

To categorize viral transmission according to the acquisition time of viral particles from host plants by the vector and the period of retention of these particles in the vector before being transmitted to new hosts, Watson and Roberts (1939) coined the terms “nonpersistent”, for viruses that have a short acquisition and retention time and “persistent”, for viruses that have longer acquisition and retention times. Later, Sylvester (1985) designated relationships with intermediate times as “semipersistent”. Nonpersistent viruses comprise those that the vector acquires from the host in seconds or minutes, including in probe feeding, and where the vector retains viral particles in its mouthparts for minutes to hours. These viruses do not have a latent period, i.e. time required by the vector after acquiring viral particles until it is able to transmit these particles to new hosts. Furthermore, this type of virus does not reach the vector´s haemolymph and does not undergo replication or vertical transmission in its vector (Watson and Roberts, 1939; Nault, 1997; Ng and Falk, 2006; Casteel and Falk, 2016). In semipersistent transmission, the vector takes time to acquire new viral particles, this ranging from minutes to hours; after acquisition, the retention of these particles can last from hours to days. As with

nonpersistent transmission, there is no latent period, the viruses do not reach the haemolymph, do not replicate in the vector, and are not vertically transmitted (Sylvester, 1985; Nault, 1997; Ng and Falk, 2006; Casteel and Falk, 2016). In persistent transmission, optimal acquisition of the virus by the vector takes hours; similarly, retention time is long and may vary from days to the lifetime of the vector. In contrast to nonpersistent and semipersistent transmission, in persistent transmission, virus particles cross the barriers of the foregut, reaching the midgut, hindgut and haemolymph, being carried to the salivary glands to be inoculated into their host. Persistent viruses can be circulative, with no replication in the vector, or propagative, with replication in the vector. Furthermore, persistent propagative viruses can reach the vector gonads, so that they are transmitted vertically, but this is not a rule (Watson and Roberts, 1939; Nault, 1997; Ng and Falk, 2006; Casteel and Falk, 2016).

Another classification refers to the places that viruses are retained inside the insect body during the transmission process. Kennedy *et al.* (1962) listed nonpersistent viruses as stylet-borne, Nault and Ammar (1989) listed semipersistent viruses as foregut-borne and Kennedy *et al.* (1962) described persistent viruses as circulative, that is, those that pass from the intestine to the haemolymph and reach the vector salivary glands. Despite this relationship, we now know that nonpersistent and semipersistent viruses can be stylet-borne or foregut-borne. For example, *Cauliflower mosaic virus* (CaMV) is transmitted semipersistently and localizes to the tip of the aphid stylet (Uzest *et al.*, 2010).

Proteins that interact to anchor and stabilize viral particles during transmission mediate the virus-vector relationship. The viral capsid protein (CP) may be responsible for attaching the particles directly to the vector structures (capsid-strategy) or non-structural proteins may be a helper component in this function (helper-strategy). Likewise, vector proteins have been shown to interact directly with CP or the helper component. Uzest *et al.* (2007) showed for the first time that for CaMV, the viral helper protein (P2) directly interacts with a protein receptor

located in the chitin matrix of the stylet; further, P2 binds to another protein P3 which in turn binds to viral CP, anchoring this viral complex to the aphid stylet protein. Since then, other vector proteins have been discovered as part of this interaction (Linz *et al*., 2015; Webster *et al.,* 2018). Although it was not explicitly discussed in the first of these studies, the fact that the vector bears proteins that the virus uses for anchorage opens the possibility that the vector is selected to carry the virus, essentially in an offensive mutualism. This is now known to be the case in several systems and has been reviewed extensively (Mauck *et al*., 2012).

Although some mechanisms have been well studied at the molecular level in virus vectors, especially in aphids, many of these mechanisms remain unknown, principally in whiteflies. Thus, in this review, we will focus on the whitefly, the viruses transmitted by this vector, the known information about the mechanisms involved during transmission and the lacunae that need further study.

3. General characteristics of whitefly biology and similarities to aphids

Whiteflies, as well as other hemipteran insects, have piercing-sucking mouthparts, feed on plant sap and excrete a substance known as honeydew (excess sugar and carbohydrates derived from plant sap). Adults are winged and lay their eggs on the abaxial surface of the leaf. The first nymphal instar moves until it settles in a place on the plant where it begins to feed. The next three nymphal stages are completely sessile (unlike aphids that are partially sessile) and increase in size until they reach adulthood (Perring *et al*., 2018). Whiteflies currently have a worldwide distribution, although they were first described in the tropics (CABI, 2017a and b; Perring *et al*., 2018). These insects may be polyphagous and are known to feed on more than 900 species of host plants (McKenzie *et al*., 2014).

Byrne and Bellows (1991) described the whitefly as "the aphid of the tropics", due to their commonalities with aphids and prevalence in a tropical climate. In fact, there are many similarities between these insects, especially with regard to aspects of their feeding. Rosell *et al*. (1995) concluded, based on transmission electron microscopy analyses, that the mouthparts of whiteflies are morphologically similar to those of other homopterans, especially those of aphids. The mouthparts of these insects are formed by two sets of external mandibular stylets, which have the function of piercing the plant tissue, and two sets of internal maxillary stylets, which form the alimentary canal, through which the sap is ingested, and the salivary channel, through which saliva is injected into the plant. The distal part of these stylets is interconnected and allows for the mixing of salivary and food content (Rosell *et al*., 1995). Furthermore, behaviour during feeding also presents similarities: both insects feed on phloem sap and can causally ingest xylem sap (Pompon et. al, 2010), continuously for hours, through intracellular stylet punctures. That said, puncture frequencies have been shown to be lower in whiteflies than in aphids (Johnson and Walker, 1999; Ng and Walker, 2016; Garzo *et al*., 2020).

Beyond morphology, phylogenetic studies from the analysis of 185 rDNA nucleotide sequences indicate that the order Sternorrhyncha is a monophyletic group, i.e. with a single common ancestor, and that whiteflies form a sister group to the aphids (Campbell *et al*., 1994; von Dohlen and Moran 1995; Thao *et al*., 2004). This can explain the similarities such as the morphology of the mouthparts, feeding behaviour and even regions in the genome that are conserved and possess the same function in the two groups, such as regions responsible for the production of chemosensory proteins and siRNA machinery (Morin *et al*., 1999; Upadhyay *et al.,* 2013; Li *et al.,* 2021).

4. Virus-carrying whiteflies

Whiteflies are among the most important pests for many crops. Due to the polyphagy of these insects, many plant species are affected directly, through the feeding on sap that reduces general vigor, and indirectly through the excretion of honeydew. The latter substance covers the leaf surface, reducing photosynthesis especially as saprophytic fungi grow upon it. Most plant damage caused by whiteflies, however, is related to their ability to transmit viruses that cause diseases such as necrosis, mosaic, chlorosis and dwarfism.

There are more than 1,550 known whitefly species (Liu *et al*., 2015) yet only three are known to transmit viruses: *Trialeurodes vaporariorum*, *Trialeurodes abutilonia* and the species complex *Bemisia tabaci*. While *T. vaporariorum* and *T. abutilonia* transmit three virus species (see below), *B. tabaci* transmits at least 111 (Tiwari *et al*., 2013). Thus, this last species has received greater attention for its high potential to transmit viruses.

Bemisia tabaci is currently recognized as a complex of morphologically indistinguishable but genetically distinct species. Divergences in the sequence of the mitochondrial cytochrome oxidase one (mtCO1) gene has been the best approach to separate the species that are part of the complex. Dinsdale *et al*. (2010) suggest that there are 24 species of *B. tabaci* and that 3.5% divergence in mtCO1 gene is sufficient for this distinction. The most important *B. tabaci* species reported to transmit virus are the widespread Mediterranean (MED), Middle-East Asia Minor 1 (MEAM1), New World 1 (NW1), New World 2 (NW2), in addition to species endemic in the Americas and rates of viral transmission vary across the *B. tabaci* species complex (Polston *et al*., 2014; De Marchi *et al*., 2017; Shi *et al*., 2018).

The transmission of viruses by whiteflies is species-specific, so that each species of the vector is responsible for transmitting certain viral species. Currently, five viral genera

transmitted by whiteflies are recognized: *Begomovirus*, *Carlavirus*, *Crinivirus*, *Ipomovirus* and *Torradovirus*.

5. Nonpersistent carlaviruses

The genus *Carlavirus* belongs to the family *Betaflexiviridae*, which currently contains 13 genera, 2 subfamilies and 108 species, according to the International Committee on Virus Taxonomy (ICTV). The representatives of this family have filamentous, flexuous and encapsidated particles, with a length of 600 to 1000nm, and a single-stranded positive-sense RNA. Of all known carlaviruses, aphids transmit the vast majority of species in a nonpersistent mode and only two are known to have whitefly as their vector: *Cowpea mild mottle virus* (CPMMV) and *Melon yellowing-associated virus* (MYaV); for the second of these, the mode of transmission has not been described so we will focus on CPMMV.

CPMMV is known to cause the economically important disease soybean stem necrosis, despite infecting other plants such as beans and peanuts, and causing other symptoms such as mosaic, chlorosis and dwarfism (Almeida *et al*., 2003; Zanardo *et al*., 2014). This virus is the only one recognized as being transmitted nonpersistently by whiteflies (*B. tabaci* MEAM1) today. Viral acquisition by the vector takes just 10 minutes and transmission of this virus to new hosts can take 5 minutes, without the presence of a latent period (Iwaki *et al*., 1982; Marubayashi *et al*., 2010; Mansour *et al*., 1998). The CPMMV RNA encodes a protein responsible for replication, three proteins responsible for the intercellular movement of the virus in the host plant, a structural protein, CP, and another protein rich in cysteine, with no known function (Menzel *et al*., 2010; Zanardo *et al*., 2014).

Although acquisition and inoculation times define CPMMV as a nonpersistent virus (table 1), information about possible interactions at the molecular level between the virus and

its vector during transmission remains unstudied. We do not know whether the virus actually lodges in the whitefly stylet (figure 1), whether there is a specific region in the stylet capable of interacting with viral particles, nor whether the viral capsid itself is capable of interacting with the vector mouthparts or whether it needs a helper component during transmission, i.e. we do not know if the virus has a helper or capsid strategy (this is what we try to clarify in chapter 2).

The nonpersistent transmission of viruses has been more widely studied in aphids than in whiteflies and we can imagine that similarities can also occur during the viral transmission process and raise hypotheses to help explain lacunae in our understanding of the virus-vector interaction. It is reasonable to suppose, for example, that during nonpersistent transmission, viral particles interact with regions of the whitefly stylet homologous to the acrostyle known from aphids (figure 2). This is an organ located in the common duct of the alimentary and salivary channel of the aphid stylet, which has cuticular proteins capable of binding to the helper component of the CaMV (P2) (Uzest *et al.,* 2007; Uzest *et al.,* 2010; Webster *et al.,* 2018). Furthermore, it is possible to question the type of strategy employed by CPMMV, the only known virus that is transmitted nonpersistently by the whitefly: Is the strategy of this transmission helper or capsid? In aphids, there are nonpersistent viruses transmitted in both forms, like potyviruses, which have HCPro as a helper (Blanc *et al*., 1998), or bromoviruses, which use the CP itself to anchor in the insect stylet (Ng *et al*., 2000). For whiteflies, the same may occur with as yet undiscovered viruses.

Since the vast majority of caralaviruses are transmitted by aphids, one has to considerwhy only two species within this genus have come to be transmitted by whiteflies. Zanardo and Carvalho (2017) hypothesized that mixed infections generated recombination in viral CP between these two carlaviruses and another virus already transmitted by whiteflies,

allowing them to start being transmitted by whiteflies. However, evolutionary studies need to confirm this from phylogenetic analyses to find CP homologies of these viruses.

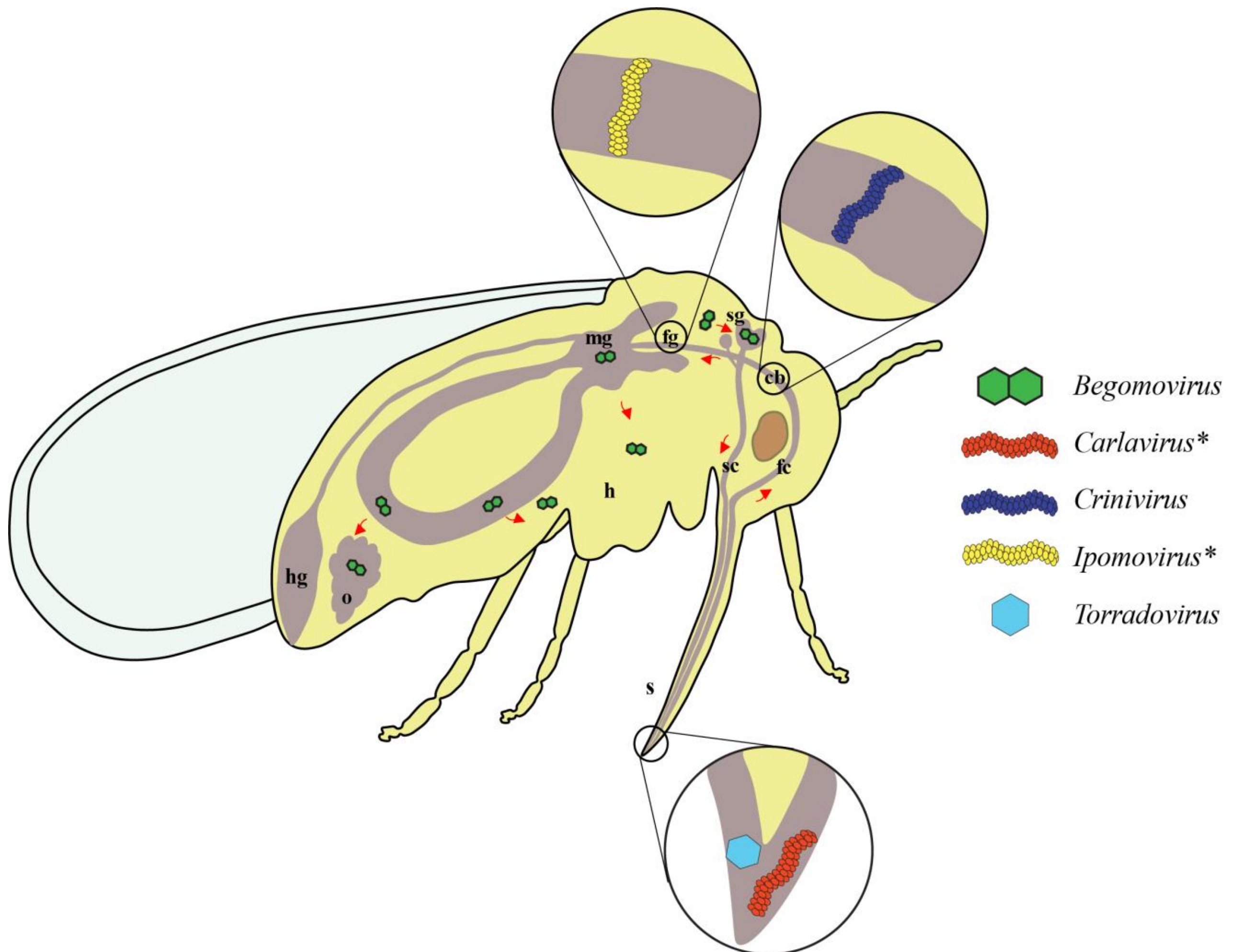

Figure 1. Schematic representation of the location where whitefly-borne viruses are retained. *The location for these viruses is only estimated based on the mode of transmission; further studies need to elucidate the true location. The red arrows indicate the paths that viruses can travel through the insect's body. s, stylet; fc, food channel; sc, salivary channel; cb, cibarium; fg, foregut; sg, salivary gland; mg, midgut; hg, hindgut; o, ovary; h, haemolymph.

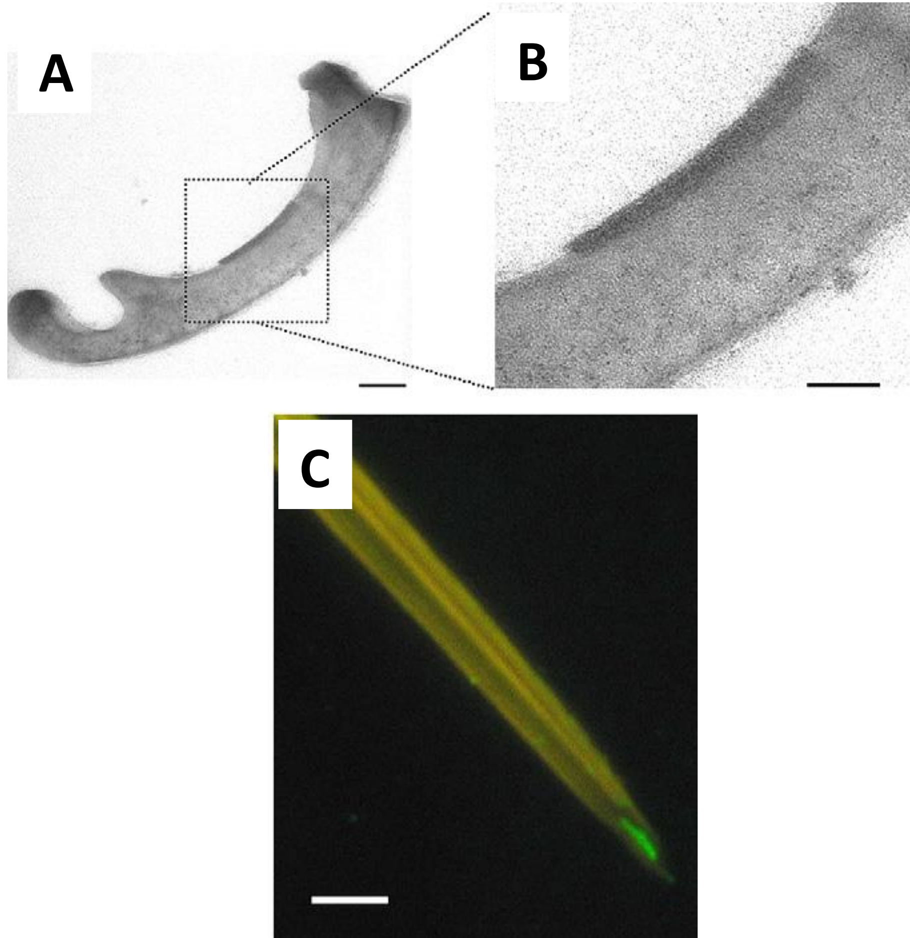

Figure 2. The acrostyle evidenced by transmission electron microscopy of the maxillary stylet, which presents an area with a dense chitin surface, where viral particles anchor (A and B) and by epifluorescence microscopy that detected the *Cauliflower mosaic virus* P2 bound to the stylet (C). Modified from Uzest *et al*. (2010).

Table 1. Characteristics of viral transmission by whiteflies

Virus Family	**Virus Genus**	**Vector whitefly species**	**Retentio n site in vector**	**Transmission mode**	**Proteins involved in transmissio n**	**Helper or capsid strategy**
Betaflexiviridae	*Carlavirus*	*B. tabaci*	Unknown	Nonpersistent	Unknown	Unknown
Closteoviridae	*Crinivirus*	*B. tabaci* *T. vaporariorum* *T. abutiloneus*	Cibarium	Semipersistent	mCP	Capsid
Potyviridae	*Ipomovirus*	*B. tabaci*	Unknow	Semipersistent	Unknown	Unknown
Secoviridae	*Torradovirus*	*B. tabaci* *T. vaporariorum*	Stylet	Semipersistent	Unknown	Unknown
Geminiviridae	*Begomovirus*	*B. tabaci*	Midgut	Persistent	CP	Capsid

6. Semipersistent criniviruses, ipomoviruses and torradoviruses

6.1.Crinivirus

The genus *Crinivirus* is one of the four genera within the family *Closteroviridae*. This familycomprises a total of 59 species (ICTV) whose particles are flexuous, helical and encapsidated, with a length of 650nm to over 2,200nm. The genome is single-stranded positive-sense RNA, and may be monopartite or fragmented . Only the genus *Crinivirus* is semipersistently transmitted by whiteflies, while the other three genera are transmitted by aphids, mealybugs and soft scales. Although there are other species transmitted by whitefly, here we will focus on the *Lettuce infectious yellows virus* (LIYV), as this is the most studied representative of its mode of transmission at the molecular level.

LIYV was initially described by Duffus *et al*. (1986) causing the yellowing of lettuce, sugarbeet and carrots, among other crops, causing great economic impact in the southwestern United States, being transmitted semipersistently by *B. tabaci* NW (table 1). The vector was able to acquire new viruses after 10 minutes of feeding on the host, however this acquisition was more efficient after 48 hours. The same occurred for inoculation (Duffus *et al*., 1986; Wisler *et al*., 1998).

The genome of LIYV is bipartite, such that RNA1 encodes proteins related to RNA replication and RNA2 encodes six proteins related to viral encapsidation, movement and transmission (Klaassen *et al*., 1995). Four proteins present in the coat, HPS70, p59, major cap protein (CP) and minor coat protein (mCP) have been studied as possible components involved in viral transmission. An interesting study conducted by Tian *et al*. (1999) showed that when LIYV particles were purified to eliminate possible non-structural proteins external to the capsid that could act as a helper component, virus transmission by the whitefly continued to be efficient, suggesting that LIYV has a capsidal strategy. Furthermore, the work went deeper and

in an infectivity neutralization analysis, using specific antisera for HSP70, p59, CP and mCP, showed that only the antiserum for mCP was able to neutralize the transmission of LIYV by *B. tabaci*. These results indicate that LIYV mCP is essential for virus transmission. In a more recent study, Chen *et al.* (2011), using an immunofluorescent localization technique, demonstrated that viral retention occurs in the cibarium, a region close to the *B. tabaci* foregut (figure 1). Furthermore, the authors performed a mutation in mCP that caused the loss of retention, reinforcing the role of this protein in the viral transmission process.

6.2.Ipomovirus

The genus *Ipomovirus* is included in the family *Potyviridae*, which contains another 11 genera and 235 described species (ICTV). The viral particles of the family are filamentous, flexuous and encapsidated, of 800 to 940 nm in length, with a single-stranded positive-sense RNA molecule. The vast majority of potyviruses are transmitted by aphids, while ipomoviruses are an exception, being transmitted by whiteflies (*A. dispesus*, *B. tabaci* and *T. vaporariorum*) (table 1). Within this genus are the species *Cassava brown streak virus* (CBSV), *Coccinia mottle virus* (CocMoV), *Cucumber vein yellowing virus* (CVYV), *Squash vein yellow virus* (SqVYV), *Sweet potato mild mottle virus*, *Tomato mild mottle virus* and *Ungandan cassava brown streak virus* (UCBSV). Among the species transmitted by *B. tabaci*, only SqVYV has been shown to be transmitted by MEAM1, while for other species the identity of the vector is not yet clear (Polston *et al.,* 2014). Likewise, there are still no studies that elucidate the molecular components that participate in the transmission process, although genomic studies point out differences and similarities between ipomoviruses and other potyviruses that may be related to vector transmission. For CVYV, a part of the sequence encoding the helper component (HC-Pro) that is conserved among aphid-transmitted potyvurses and important for their transmission was not found (Janssen *et al*., 2005). On the other hand, it was found that

CBSV and CocMoV have a DAG (Asp-Ala-Gly) motif in their coat protein that is highly conserved among aphid-borne potyviruses, suggesting that these viruses may also establish relationships with this vector (Ateka *et al*., 2017).

6.3.Torradovirus

The genus *Torradovirus* belongs to the family *Secoviridae*, which comprises 1 subfamily, 8 genera, 3 subgenera and 86 species (ICTV). Members of this family are icosahedral, 25 to 30nm long, with a single-stranded positive-sense RNA, and may have a mono- or bipartite genome. In case of bipartite genomes, they are ecapsidated in two different particles (Thompson *et al*., 2017). Of the six torradoviruses species, three are nonpersistently transmitted by whiteflies: *Squash chlorotic leaf spot virus* (SCLSV), *Tomato marchitez virus* (ToMarV) and *Tomato roast virus* (ToTV) (table 1), while *Carrot torradovirus* and *Lettuce necrotic leaf curl virus* are transmitted by aphids (Rozado-Aguirre *et al*., 2016; Verbeek *et al*., 2017). SCLSV and ToTV can be transmitted by *B. tabaci* MED and *T. vaporariorum* (Pospieszny *et al*., 2007; Amari *et al*., 2008; Lecoq *et al*., 2016), while ToMarV is transmitted by *B. tabaci* MEAM1, *T. vaporariorum* and *T. abutiloneus* (Verbeek et al., 2014) (table 1). In a study of ToMarV, Verbeek *et al*. (2014) noted that this virus needs an acquisition time of 16 hours and an inoculation time of 8 hours for optimal transmission, retaining particles for at least 8 hours. Furthermore, through RT-PCR analysis in insects that were dissected in stylet, head, thorax and abdomen, they demonstrated that only the stylet contained the virus, confirming stylet-borne transmission (figure 1).

7. Persistent begomoviruses

The genus *Begomovirus* is included within the family *Geminiviridae*, which has another 13 genera and 520 described species. The members of the family are geminate, in the form of 2 incomplete icosahedrons, 22 to 38 nm in size, which contain a circular single-stranded DNA (ICTV). In begomoviruses, the genome can be mono- or bipartite, and in the latter case, they have an DNA-A (homologous to the monopartite genome), which encodes proteins related to replication, control of gene expression, assembly of viral particles and vector transmission, and a DNA-B, which encodes proteins related to viral movement in the host (Hanley-Bowdoin *et al*., 1999).

Diseases caused by these viruses have damaged crops of great economic value such as beans, tomatoes, peppers and soybeans, and among the common viral symptoms are mosaic, yellowing and curling of the leaves (Faria *et al*., 2000). begomoviruses are persistently transmitted by different species of whiteflies species (*B. tabaci* MEAM1, MED and an indigenous Asia II 1) (table 1) with some specificity (Guo *et al*., 2018; Pan *et al*., 2018).

Virus-vector relationships have been well studied in the case of begomoviruses, so there is information available on their location in the vector body, as well as molecular components that participate in the process. Hunter *et al*. (1998), using indirect immunofluorescence, showed that the begomoviruses *Tomato mottle virus* and *Cabbage leaf curl virus* were located in the anterior portion of the midgut, in the filter chamber and in the salivary glands (figure 1). With this result, it was possible to create a hypothetical model for the viral pathway in the insect body, so that the first two regions are the gateway to the haemolymph, from which the virus can infect other organs and the salivary gland, being then transmitted to new hosts. Höhnle *et al*. (2001), by exchanging three CP amino acids from an *Abutilon mosaic virus* (AbMV) isolate, not naturally transmissible by whitefly, obtained a mutant that was transmitted by the insect, demonstrating the importance of CP in transmission. Caciagli *et al*. (2009) observed that the

Tomato yellow leaf curl Sardinia virus with a mutation in its CP, accumulated in the midgut, haemolymph and salivary glands of the whitefly, however the virus was not transmitted to new plants, leading the authors to argue that the mutation meant that the virus was no longer able to bypass the salivary gland towards the host. In turn, Wei *et al*. (2014), exchanged a CP fragment of *Tomato yellow leaf curl China virus* (TYLCCNV), not transmitted by *B. tabaci* MED, by CP fragment of *Tomato yellow leaf curl virus* (TYLCV), transmitted by this vector, and TYLCCNV became transmissible. Furthermore, using confocal microscopy, the authors showed the accumulation of this virus in cells in the central region of the insect salivary gland. Other research has shown the interaction between CP of begomoviruses and proteins present in the midgut and salivary glands of the whitefly, showing the cellular components of the vector responsible for viral transmission (Rana *et al*., 2016, 2019; Kanakala *et al*., 2016).

Although some points in the virus-vector relationship for begomoviruses are well established, other questions, such as whether the virus is circulative and whether it can be transmitted vertically, remain under discussion. Pakkianathan *et al*. (2015) and Wang *et al*. (2016) showed, through quantitative PCR, an increase in the viral genome of TYLCV in whiteflies after feeding on infected plants. On the other hand, Becker *et al*. (2015), using real-time PCR, found no evidence of TYLCV accumulation after viral acquisition. Likewise, Sánchez-Campos *et al*. (2016) did not identify an increase in viral DNA 96 hours after viral acquisition in a quantitative PCR analysis. Ghanim *et al*. (1998) through PCR and Southern blot hybridization detected the presence of TYLCV in whitefly progeny, raising evidence for vertical transmission of begomoviruses. However, further research has indicated that despite the presence of viral DNA in eggs and nymphs, in adults this presence is much lower, and infectivity does not seem to be inherited by the progeny (Bosco *et al*., 2004.; Wang *et al*., 2010; Gadhave *et al*., 2020). Despite these clues, the expression of viral genes related to replication when they are in the vector, still need to be investigated.

In addition to the virus-vector relationship, proteins from whitefly symbionts are known to facilitate the process of viral transmission. The protein GroEL, found in whitefly haemolymph, is produced by symbiotic bacteria, as occurs with aphids, and may facilitate viral transmission (Morin *et al.*, 1999). In interaction studies between proteins, GroEL interacted with the CP of TYLCV (Morin *et al.*, 2000) and this suggests that the symbiont protein can carry viral particles from the gut to the host haemolymph, protecting these particles from degradation of proteases and nucleases present in the haemolymph (Gottlieb *et al.*, 2010; Czosnek *et al.*, 2017).

We still do not know how begomoviruses came to be transmitted strictly by whiteflies and evolutionary studies are needed to clarify this. However Krupovic *et al.* (2009), through phylogenetic analysis demonstrated that the gene encoding the CP of current geminiviruses have similarities with genes encoding the phytoplasma genome, including in the region known to be responsible for the transmission of geminiviruses by whiteflies. As geminiviruses and phytoplasmas occupy the same ecological niche, it is reasonable to assume that recombination events in viral CP may have allowed transmission by whitefly.

8. Emergence of new whitefly-transmitted viruses

New research has brought to light the emergence of new viruses being transmitted by whiteflies. These viruses belong to families that were not previously known to havewhitefly as a vector and shows how many new viruses are yet to be discovered.

The genus *Poleovirus*, belonging to the family *Luteoviridae*, comprises icosahedral, encapsidated viruses, with 25 to 30nm in diameter, monopartite genome and a single-stranded positive-sense RNA molecule (ICTV). Viruses of this genus were considered to be transmitted strictly by aphids in a persistent non-propagative manner, but recent work has reported two

species of poleoviruses being transmitted exclusively by whitefly: *Pepper whitefly-borne vein yellows virus* (PeWBVYV) (Ghosh *et al*. 2019) and *Cucurbit whitefly-borne yellows virus* (CWBYV) (Costa *et al*. 2019). Transmission of both viruses has been characterized as circulatory, similarly to begomoviruses (Gosh and Ghanim, 2021). Genome analyses of emerging viruses show that despite the high similarity with aphid-borne poleoviruses, the identity of transmission-related proteins (CP and mCP) are very divergent among viruses transmitted by these insects (Bello *et al*. 2021). This evidence strongly suggests that evolutionary events, such as mutations or recombinations caused by mixed infections with other viruses, may have led to differences in these proteins that allowed the transmission of these viruses by whiteflies.

9. Speculations and conclusions

Studies of viral transmission mechanisms in whiteflies still need advances, especially in relation to carlaviruses, ipomoviruses and torradoviruses. For the carlavirus MYaV there are not even data on the mode of transmission by the vector, while for CPMMV there is a lack of studies on the molecular components involved in the interaction. Likewise, for ipomoviruses and torradoviruses, despite clues about the components that participate in transmission, deeper investigations remain to be done. Studies on the transmission mechanisms of criniviruses and begomoviruses are more advanced, but in the first case studies are still needed on the vector proteins involved in transmission and in the second case, issues such as replication and vertical transmission need to be elucidated due to contradictory studies.

In general, studies of virus-vector interactions are necessary for the biological understanding of this relationship that can help in new control strategies through the use of techniques that disrupt this interaction and viral transmission, such as the use of molecules that

block viral receptors of vector insects or the use of iRNA that silence genes of the insects responsible for this transmission (Whitfield and Rotenberg, 2015).

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Chapter 2: Analysis of cysteine-rich protein from *Cowpea mild mottle virus* as a helper in viral transmission by the vector, *Bemisia tabaci*

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Abstract

The relationship between viruses and insect vectors is mediated by molecular interactions that guarantee the successful transmission of viral particles and host infection. For some viruses, the structural capsid protein (CP) interacts directly with effectors present in the insect cuticle, while for others, a non-structural protein, defined as helper-component, binds the viral CP to these insect effectors. For potyviruses, the helper-component is HCPro, a protein rich in cysteine and with the ability to bind to nucleic acids. Mutations in cysteine residues of this protein abolished viral transmission by the vector. The carlavirus, *Cowpea mild mottle virus* (CPMMV) also has a cysteine-rich protein (CRP) capable of binding to nucleic acids. We therefore hypothesized that CRP acts as a helper in CPMMV transmission by its vector, the whitefly, *Bemisia tabaci*. To test this hypothesis, we constructed a mutant defective for CRP encoding, to test whether viral transmission by the vector would be affected but, contrary to our expectation, it was unaffected. We also analysed the interaction between CP and CRP using yeast two-hybrid, bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation and found no evidence for interaction. Our findings indicate that CRP does not act as a helper in transmission process; moreover, although CRP can act as a transcription factor, its absence in the mutant does not affect host infection. We therefore suggest that the CP binds directly to the vector during the transmission process. These results may point the way to new studies on the real functions of this protein in the virus-vector-host interaction.

Key-words: CPMMV, helper-component transmission, *Bemisia tabaci*

1. Introduction

The vector-virus interaction is crucial to the cycle of many viral diseases. In the case of plant hosts, vectors are essential in viral dissemination asplants are sessile(Casteel and Falk, 2016; Dietzgen *et al.,* 2016). Hemipteran insects that feed on sap, including aphids, whiteflies, leafhoppers and planthoppers, are the most common vectors of plant viruses, these causing a large number of diseases in a wide range of crops, vegetable and ornamental plants (Nault, 1997; Hogenhout *et al.,* 2008; Whitfield *et al.,* 2015; Zanardo and Carvalho, 2017).

Viral transmission by the insect goes beyond passive adhesion of virions to the vector body and molecular interactions are required for successful binding (Dietzgen *et al.* 2016). While for some viruses, the structural capsid protein (CP) appears to interact directly with insect cuticle proteins ("capsid strategy"), for other viruses, non-structural proteins, defined as helper components (Kassanis and Govier 1971), can act as "bridges" between vector cuticle and capsid proteins ("helper strategy").

These strategies are already known from members of the genera *Cucumovirus, Potyvirus*, *Caulimovirus* and others. The CP of *Cucumber mosaic virus* interacts directly with its vector stylet (Pirone and Megahed 1996; Chen and Francki 1990; Liu *et al.* 2002); on the other hand, the HCPro of potyviruses and the P2 of caulimoviruses interact with their respective CPs and bind them to the vector stylets (Wang *et al.* 1996; Blanc *et al.* 1998; Leh *et al.* 1999; Bak *et al.* 2013). While for some viruses the molecular interaction with the vector is well understood, for others there is still information missing. This is the case for the relationship between *Cowpea Mild Mottle Virus* (CPMMV) and its vector, the whitefly *Bemisia tabaci*.

CPMMV belongs to genus *Carlavirus*, family *Betaflexiviridae*. In soybeans, CPMMV can cause mild symptoms such as mosaic, chlorosis and mottle, or severe symptoms such as dwarfism and stem necrosis (Zanardo *et al.* 2014). This virus is non-enveloped, has a flexuous particle and a size of approximately 610-700 nm in length and 12-15 nm in diameter. Its genome

is a positive-sense single-stranded RNA, with 6.48-8.6 kb, a cap structure at the 5' end and a polyadenylated tail at the 3' end, (Menzel *et al.* 2010; King *et al.*, 2012; Zanardo *et al.* 2014). Like the other viruses belonging to the genus *Carlavirus*, CPMMV has six open reading frames (ORFs), which are regions within the genome that can be translated and are bounded by a start codon and a stop codon (Sieber *et al.* 2018). ORF 1 encodes a polypeptide of 211.6 kDa that constitutes the viral replicase (Menzel *et al.* 2010; Zanardo *et al.* 2014). ORFs 2, 3 and 4 (25 kDa, 12 kDa and 7 kDa, respectively) compose the triple gene block (TGB), responsible for viral intercellular movement. ORF 5 encodes the coat protein (CP) with 32 kDa that has a structural function. In turn, ORF 6 encodes a cysteine-rich protein (CRP) of 15.2 kDa, with nucleic acid-binding activity (Menzel *et al.* 2010; Zanardo *et al.* 2014).

The whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) is a cryptic species with more than 24 species that are morphologically indistinguishable but are reproductively isolated and genetically distinct (Dinsdale *et al.* 2010). This insect is polyphagous and sap-sucking on a variety of plant families. It can transmit more than 300 species of plant viruses, and as such is a pest of great economic importance (Byrne and Bellows 1991).

Among the carlaviruses that have already been described, most are transmitted by aphids while only CPMMV and *Melon yellowing-associated virus* are transmitted by whitefly (Nagata *et al.* 2003; King *et al.* 2011). This may represent specificity in the virus-vector relationship. It is known that CPMMV is transmitted in a non-persistent manner by whiteflies, and studies have shown that the period of access to vector acquisition can be only 10 minutes while the period of access to inoculation can be 5 minutes, with no latent period of the virus (Iwaki *et al.* 1982; Marubayashi *et al.* 2010; Mansour *et al.* 1998). However, the molecular components involved in this interaction remain undocumented. Foster and Mills (1991) postulated that the CRP of carlaviruses could have a function homologous to the potyvirus helper due to cysteine residues

present in both proteins, while mutations in these cysteine residues abolished transmission of potyvirus by aphid vectors (Atreya, 1992).

Based on previous information, we hypothesized that CRP will act as a helper in CPMMV transmission by the whitefly. To determine if the protein encoded by ORF 6 (CRP) can interact with the protein encoded by ORF 5 (CP) we set up a yeast two-hybrid assay. Additionally, we created a defective mutant for CRP translation to determine if whitefly transmission will be affected.

2. Materials and methods

2.1.Insects and plants

Bemisia tabaci were reared on cabbage plants (*Brassica oleracea* var. capitata) in gauze cages under greenhouse conditions. The whiteflies were previously identified as *B. tabaci* MEAM1 by partial sequencing of the mitochondrial cytochrome oxidase 1 gene (mtCOI).

The soybean cultivar CD206 was used in this study. Seeds were planted in 50ml plastic cups containing substrate. After germination, they were transferred to 430ml pots. Plants were kept in a greenhouse (26±4 °C) and watered daily. After 10 days, once the two first unifoliate leaves had developed, the plants were used in the transmission experiment.

Nicotiana benthamiana seeds were planted in seed trays. Aftertwo weeks, seedlings were transferred to 430ml pots and used in experiments after 4 weeks. These plants were kept in a greenhouse at a temperature of 26 °C (±4 °C) and watered daily.

2.2.Plasmids

The regions corresponding to CP (ORF 5) and CRP (ORF 6), previously cloned into the entry vector (pENTR11) and the destination vectors used for the yeast two-hybrid, BiFC and co-immunoprecipitation assay were obtained from the collection belonging to the *Laboratório de Ecologia e Evolução de Vírus* and *Laboratório de Vírus*, Universidade Federal de Viçosa.

The constructs obtained from the entry vector were used for cloning in the destination vector by the LR clonase reaction (Gateway LR Clonase II Enzime Mix - Invitrogen). One microliter of each reaction was transformed into electrocompetent *Escherichia coli* DH5α cells and clones were confirmed by restriction pattern analysis.

2.3.Transmission assay of a defective mutant for CRP encoding

2.3.1. Construction of a defective mutant for CRP encoding

To disrupt CRP translation, we made a substitution of four nitrogen-containing bases to create two stop codons (TGA) immediately after the start codon (ATG) of ORF 6. The full-length infectious cDNA clone, pUB19:SP6.CPMMV, from CPMMV genomic RNA designed by Carvalho *et al.* (2017), was used as a template for performing this site-direct mutagenesis. The region corresponding to ORF 6 was amplified in two overlapping fragments by separate PCR reactions. An external primer that annealed at the end of the target sequence and an internal primer that annealed to the mutation site and exchanged bases were used to amplify each fragment (Table 1). The fragments were gel-purified with Wizard SV Gel and PCR Clean-Up System (Promega) and used as templates in the overlap extension PCR reaction, using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). The resulting PCR product was ligated into the pCR-Blunt vector (Thermo Fisher Scientific) transformed using the electrocompetent *E. coli* DH5α cells and, after plasmid DNA extraction and purification, Sanger

sequencing (Macrogen Inc., Seoul, South Korea) confirmed the mutation. As a final step, the infectious cDNA clone and plasmid DNA with the mutation were both digested first with AdeI (Thermo Fisher Scientific) and then with SexAI (NEB) becoming complementary. Finally, the region with functional ORF 6 of the infectious clone was replaced by the region with defective ORF 6 through a ligation reaction performed with T4 DNA Ligase (NEB). After ligation, electrocompetent *E. coli* DH10B cells were transformed and Sanger sequencing (Macrogen Inc., Seoul, South Korea) confirmed insertion of the mutation and maintenance.

Table 1. Internal and external primers used to build a mutant CPMMV that does not encode the cysteine-rich protein (CRP). Green letters represent the region corresponding to the start codon of OFR 6 and red letters represent the substituted amino acids to create two stop codons.

Name	Sequence 5'-3'
orf6_int_FW	TAAGAAGTAAAG**ATGTGATGA**TGTAAGTTAATCGCC
orf6_int_RW	GGCGATTAACTTACA**TCATCACAT**CTTTACTTCTTA
orf6_ext _FW	CATCGGCACTCAGTGGAGTTTCTG
orf6_ext _RW	GTAGTGACCGGTTCTCATCTCAAC

2.3.2. *In vitro* transcription of the infectious cDNA clone and mutant

The mutant and the infectious cDNA clone were linearized with NotI (Promega), gel-purified with Wizard SV Gel and PCR Clean-Up System (Promega) and used as templates for the *in vitro* transcription reaction with RiboMAX large-scale RNA production system-SP6 (Promega).

2.3.3. Inoculation of plants used as a source of inoculum in the transmission assay

Ten-day-old soybean plants were mechanically inoculated in their first unifoliate leaves with the infectious cDNA clone and mutant transcripts mixed in 0.1M phosphate buffer with sodium sulphite (pH 7.2) through carborundum friction damage. Plants inoculated with CPMMV-infected plant extract were used as a positive control while plants inoculated with buffer and water (instead of RNA) were used as a negative control. Five plants were used for each treatment (positive control, negative control, infectious cDNA clone and mutant). The plants remained in the greenhouse for 15 days, to allow viral replication, before being used in the transmission assay.

2.3.4. Transmission assay

150 whiteflies were placed in each of the five inoculum source plants for each treatment. These insects were collected by suction, based on the methodology of Polston and Capobianco (2013), and were then placed in clipcages to feed on the first trifoliate leaf for 30 minutes. After that period, 50 whiteflies from each plant were collected for subsequent total RNA extraction to confirm that they were able to acquire the virus or confirm that they were virus-free after feeding on the negative control. Second trifoliate leaves of each inoculum source plant were also collected for RNA extraction to confirm their infection.

The remaining 100 whiteflies collected from each plant were transferred to new virus-free plants and allowed to feed for 30 minutes before being removed. These plants were then left in a greenhouse for 15 days until their leaves were collected for RNA extraction to confirm that whiteflies were able to transmit the virus from inoculum source plants to the new plants. This experiment was repeated twice

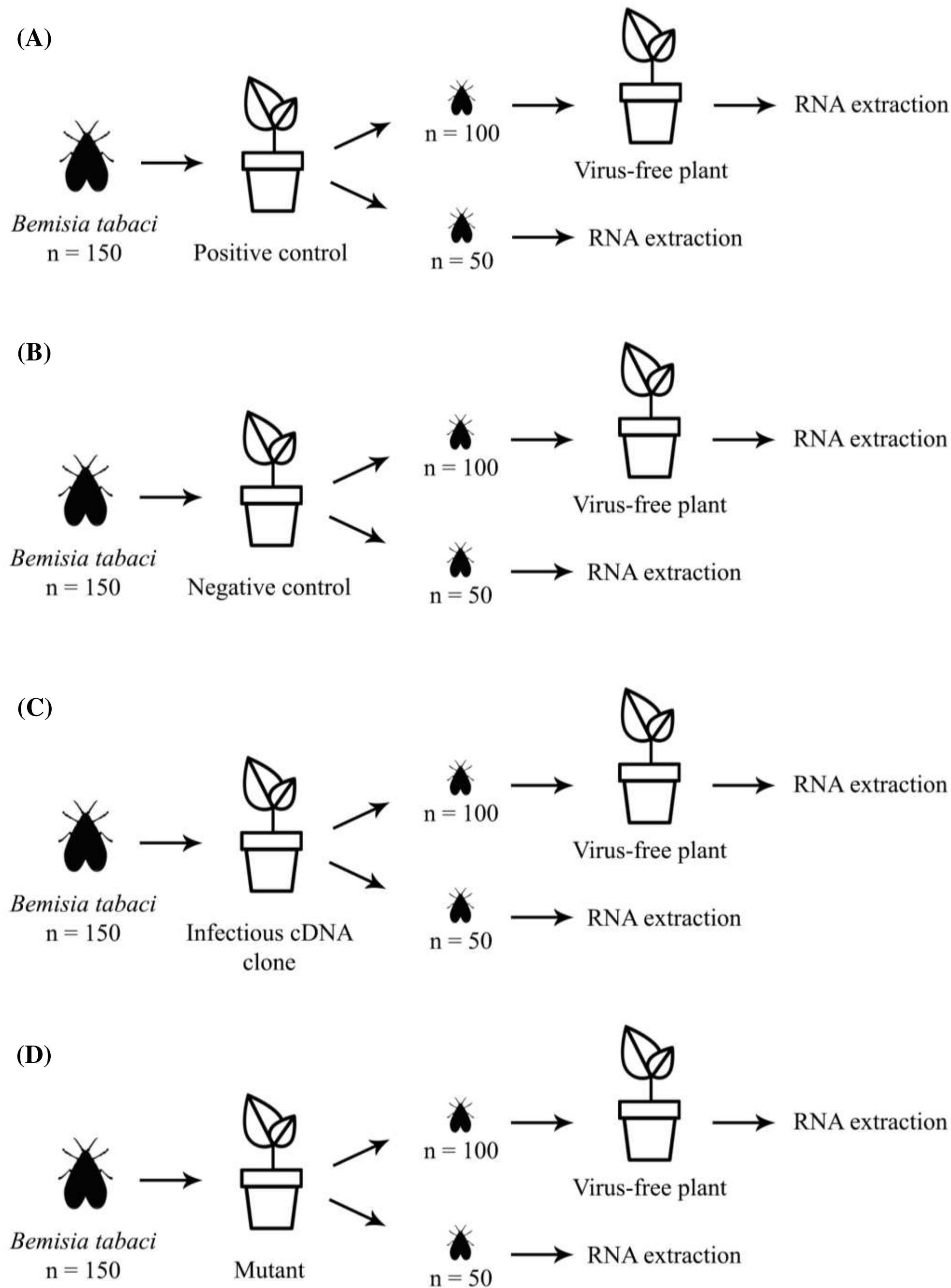


Figure 1. Schematic representation of experimental set-up. 150 whiteflies were placed on soybean plants (CD206) previously inoculated with the positive control (CPMMV-infected plant extract) (A), negative control (inoculation buffer) (B), infectious cDNA clone (C) and mutant (D). After 30 minutes, 100 whiteflies from each treatment were transferred to new virus-free plants, while the remaining 50 insects were used for RNA extraction to detect the presence of virus. Whiteflies that were transferred to new plants were allowed to feed for 30 minutes before being completely removed with insecticide. The plants were then kept in a greenhouse for 15 days, after this RNA was extracted to detect the presence of CPMMV.

2.3.5. RNA extraction, cDNA synthesis and RT-PCR analysis of collected samples

Viral presence was confirmed by RNA extraction of all collected samples with TRI Reagent (Sigma-Aldrich) followed by synthesis of cDNA with Superscript III Reverse Transcriptase (Invitrogen), using the reverse primer ORF6R (Zanardo *et al.* 2014), and PCR amplification with Platinum Taq DNA Polymerase (Invitrogen) and primers forward and reverse for ORF 6. Electrophoretic analysis of RT-PCR products was performed on agarose gel (1%).

2.4. Confirmation of the CP-CRP interaction

2.4.1. Yeast two-hybrid assay

The yeast two-hybrid system is based on complementarity between a DNA-binding domain (BD) and a transcriptional activation domain (AD), two functional domains of the yeast GAL4 transcription factor. When there is an interaction between a protein of interest (bait) fused to a vector containing BD, and a protein to be tested (prey) fused to a vector containing AD, the transcription factor is restored and transcription of a reporter gene responsive to GAL4 occurs. The reporter gene is usually an auxotrophic marker that allows yeast to grow in a selective medium (Rodríguez-Negrete et al. 2014).

In this study we used the yeast strain AH109 which is auxotrophic for the amino acids leucine (*leu2*), tryptophan (*trp1*) and histidine (*his3*). Auxotrophic markers *leu2* and *trp1* allow the selection of yeast cells transformed with the vectors BD and AD, respectively, while auxotrophic marker *his3* is used as a reporter gene, since its transcription is activated by the interaction between proteins fused to the BD and AD domains that complement each other.

Here we use the pDEST22 vector, which has the GAL4 DNA activation domain, and the pDEST32 vector, which has the GAL4 DNA binding domain. Both regions (ORF 5 and

ORF 6) encoding the proteins studied (CP and CRP) were cloned into pDEST22 and pDEST32 vectors. The following constructions were obtained: pDEST22+CP, pDEST22+CRP, pDEST32+CP, pDEST32+CRP.

First, the bait regions, cloned into pDEST32 (BD(CP) and BD(CRP)) and empty pDEST32 (BD (Ø)) were transformed in yeast AH109 by heat shock, plated in selective medium (SD without leucine) and incubated at 30 °C for 4 days. After this, when desired transformants appeared, colonies were used for cotransformation with prey regions, cloned into pDEST22 (AD(CP) and AD(CRP)) and empty pDEST22 (AD(Ø)). Cotransformants were selected in SD medium without leucine and tryptophan, after growing at 30 °C for 4 days. The selected colonies were transferred to YPD (yeast peptone dextrose) liquid medium and incubated overnight at 30°C. Finally, a serial dilution of the cell suspension was performed and dripped on to solid TD medium (suplemented SD medium, 2% glycosis, deficient in leucine, tryptophan and histidine). The interaction was analysed by the growth of colonies in TD medium. Three different concentrations of 3-amino-1,2,4-triazole (3-AT in 25nM, 50nM and 100nM) were used to prevent growth of false positives. The mouse p53 antitumor protein (P53) and lamin C (Lam) were used as positive and negative controls, respectively. This experiment was repeated twice.

2.4.2. Bimolecular fluorescence complementation (BiFC) assay

BiFC is the technique in which candidate proteins for interaction are fused into separate fragments of a fluorescent reporter protein. If the candidate proteins interact, the reporter protein fragments approach, recovering their structure and emitting a fluorescent signal that can be captured by confocal microscopy in plant cells. Candidate proteins fused to reporter protein

fragments are delivered to plant cells through infiltration of *Agrobacterium tumefaciens* pre-transformed with these constructs.

We used the pSITE-cEYFP-C1, pSITE-cEYFP-N1, pSITE-nEYFP-C1 and pSITE-nEYFP-N1 vectors, which have fragments with the n and c-terminal regions of the yellow fluorescent protein (YFP) and the n and c-terminal regions for the fusion of candidate proteins for interaction. The ORF5 and ORF6 regions encoding the CP and CRP proteins were fused to BiFC vectors and the following constructs were obtained: pSITE-cEYFP-C1 (CP), pSITE-cEYFP-C1 (CRP), pSITE-cEYFP-N1 (CP), pSITE-cEYFP-N1 (CRP), pSITE-nEYFP-C1 (CP), pSITE-nEYFP-C1 (CRP), pSITE-cEYFP-N1 (CP) and pSITE-cEYFP-N1 (CRP).

The obtained constructs and empty vectors were transformed into *Agrobacterium tumefaciens* strain AGL1 by electroporation, plated in LB medium with specific antibiotics and grown at 28°C for 2 days. After this period, the colonies that grew were resuspended in liquid LB medium with appropriate antibiotics and kept in a shaker for 2 days at 28°C. Finally, the pellet from each culture was resuspended in an infiltration buffer (10 mM $MgCl_2$; 10 mM MES; Acetosyringone 150 μM) for a final OD_{600} of 1 (Yang *et al*. 2000). After 1 hour incubation in the dark, 6-week-old *N. benthamiana* leaves were infiltrated into the abaxial surface with a 1ml syringe. The plants were kept in greenhouse conditions before being analyzed 24 and 48 hours after agroinfiltration.

The analyses were carried out under a confocal laser-scanning microscope (Zeiss LSM510 META) on slides with previously infiltrated leaf fragments. Cell images were captured with a 40X objective. GFP excitation occurred at 488nm and YFP excitation occurred at 514nm. Experiments and analyses involving confocal microscopy were performed in the *Núcleo de Microscopia e Microanálises* at the *Universidade Federal de Viçosa, Viçosa*, MG, Brazil (http://www.nmm.ufv.br/).

2.4.3. Co-immunoprecipitation assay

In co-immunoprecipitation, a bait protein is bound to antibody-labeled agarose beads that precipitate during the reaction. If any trapped protein interacts with the bait protein, it is also precipitated and detected during analysis.

We used CRP fused to pGWB405 and pGWB406 vectors as bait (which have a GFP tag) and CP fused to vector pK7WGR2 as prey (which has an RFP tag). The constructs were transformed into *A. tumefaciens* strain AGL1 by electroporation, plated in LB medium with appropriate antibiotics and kept for 2 days at 28°C. Then the colonies were transferred to liquid LB medium and kept in a shaker for 2 days at 28°C. Pellets from each construct were resuspended in infiltration buffer (10 mM $MgCl_2$; 10 mM MES; Acetosyringone 150 μM) (final OD_{600} set to 1) and infiltrated on the abaxial surface of 6-week-old *N. benthamiana* leaves. After 3 days leaves were harvested and total proteins were extracted with TRI Reagent (Sigma-Aldrich). Finally, the extracted proteins were submitted to co-immunoprecipitation using the GFP-Trap® kit (Chromotek) and the samples obtained were analyzed by Western Blot with anti-GFP (Sigma-Aldrich) and anti-RFP (Sigma-Aldrich) antibodies in the 1:1000 dilutions. Membranes have been revealed using the ECL Prime kit (GE Healthcare) and detected by the LAS-3000 Luminescent Image Analyzer (Fujifilm). This experiment was repeated twice.

3. Results

3.1.Transmission assay of a defective mutant for CRP encoding

Electrophoretic analysis of RT-PCR products on agarose gel confirmed that the plants used as a source of inoculum were successfully infected by the positive control (CPMMV-infected plant extract), by the infectious cDNA clone (pUB19:SP6.CPMMV) and by the mutant (CPMMV-defective mutant for CRP encoding) (Figure 2A). There was no CPMMV infection in the negative control (plants blank-inoculated with water) (Figure 2A).

In turn, whiteflies, collected after feeding on the inoculum-source plants, were able to acquire the positive control, the infectious cDNA clone and continued to acquire the mutant (Figure 2B). There was no infection for the negative control, showing that the whiteflies used in the transmission assay did not previously carry CPMMV (Figure 2B).

Finally, RT-PCR analysis of the plants used in the transmission assay also showed infection for the positive control, for the infectious cDNA clone and for the mutant, but not for the negative control (Figure 2C). Together, these results shows that whiteflies were able to transmit viruses from all positively infected plants.

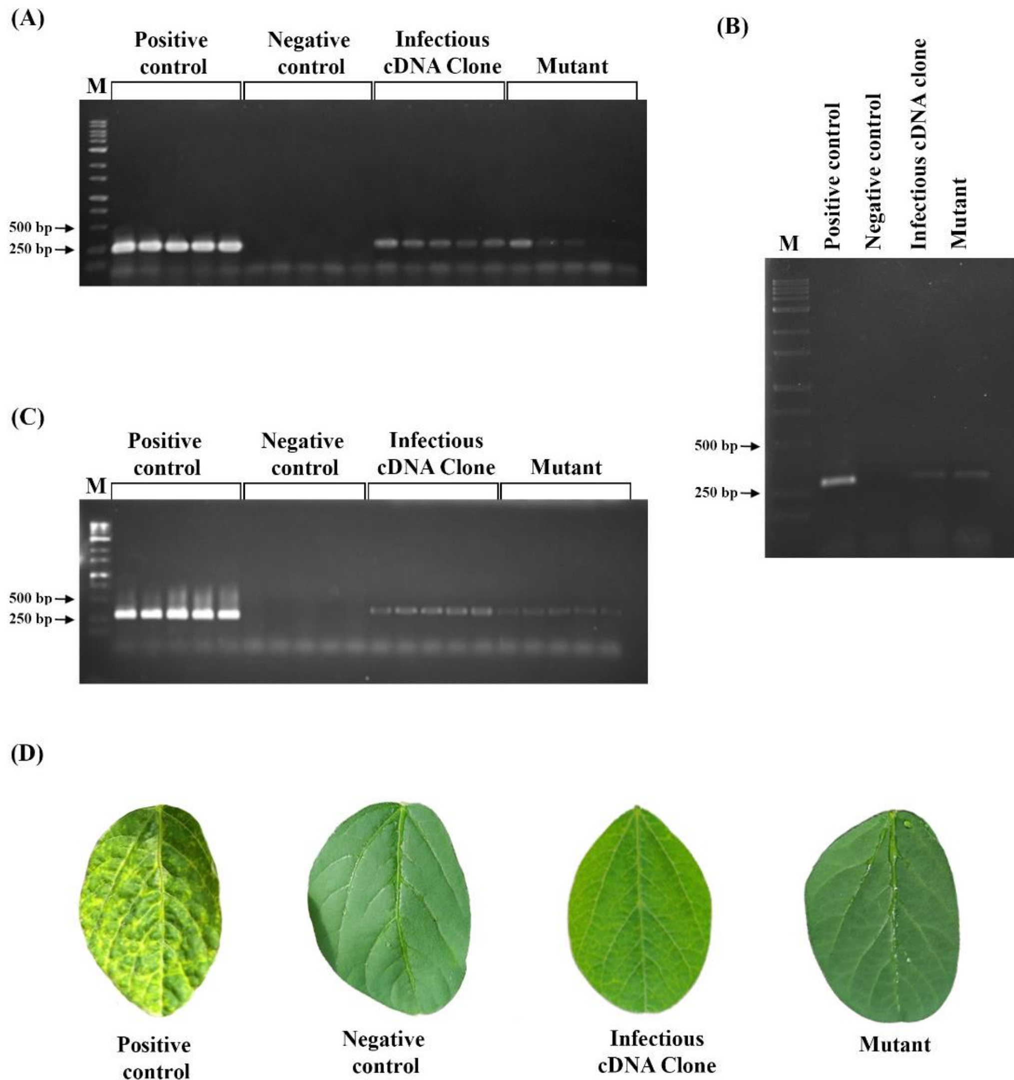


Figure 2. Agarose gel electrophoresis of RT-PCR to detect *Cowpea Mild Mottle Virus*, CPMMV (Betaflexviridae: *Carlavirus*) in soybean CD 206, *Glycine max* (Glicyne: Fabales) (A and B) and in whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae) (C). 10-day-old plants, used as a source of inoculum for the transmission assay, were inoculated with CPMMV-infected plant extract (positive control), water (negative control), CPMMV-infectious cDNA clone or CPMMV-defective mutant for CRP encoding, five plants were used for each treatment. 15 days after inoculation, about 150 virus-free whiteflies were placed on each plant and fed for 30 minutes. After this period, 50 insects and leaves of each plant were collected for subsequent RNA extraction. The other whiteflies were transferred to virus-free plants, where they fed for 30 minutes before being removed. Then, the plants were left in a greenhouse for 15 days until their leaves were collected for RNA extraction. Viral infection was confirmed by synthesis of cDNA from the previously collected RNA followed by the amplification PCR. Electrophoretic analysis of RT-PCR on agarose gel (1%) showed the presence of virus for soybean used as a transmission inoculum (A), whiteflies (B) and soybean used in the transmission assay (C). Plants of each treatment (D). M: 1KB Marker; bp: base pairs.

3.2.Yeast two-hybrid system to identify protein-protein interactions

While the positive control showed an interaction and grew at different dilutions and concentrations of 3-AT, the negative control was unable to grow under any conditions (Figure 2). These results were expected and demonstrate that we had adopted appropriate conditions for the assay. The cotransformed empty vectors (BD and AD) were also unable to grow, since there were no fused proteins capable of mediating an interaction between them (Figure 2).

When proteins were fused only to AD there was also no growth, and this shows that they are not able to self-activate in this domain (Figure 2). However, when the regions were fused only to BD, there was self-activation for CRP, while CP did not show this ability. Thus, interactions in which CRP was fused to BD (i.e. *BD (CRP) + AD (CRP) and *BD (CRP) + AD (CP)), were considered false positives (Figure 2).

CP showed some self-interaction when fused to BD and AD. Finally, there was a noticeable growth of yeast when CP fused to BD was combined with CRP fused to AD (Figure 2). This result may reflect an interaction between these proteins or a false positive that is common in this type of analysis. Other analyses, such as BiFC and co-immunoprecipitation, are therefore needed to corroborate this result.

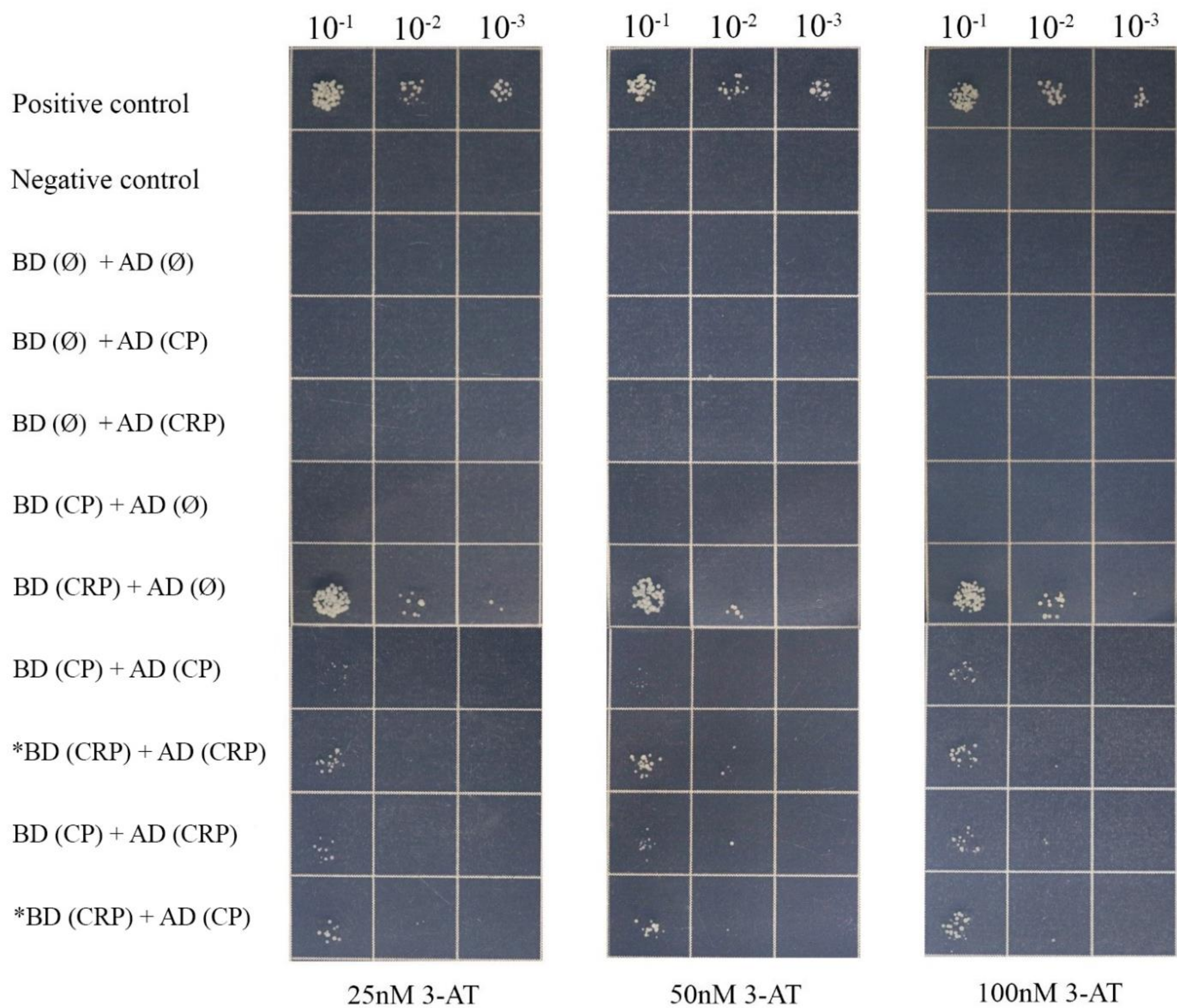
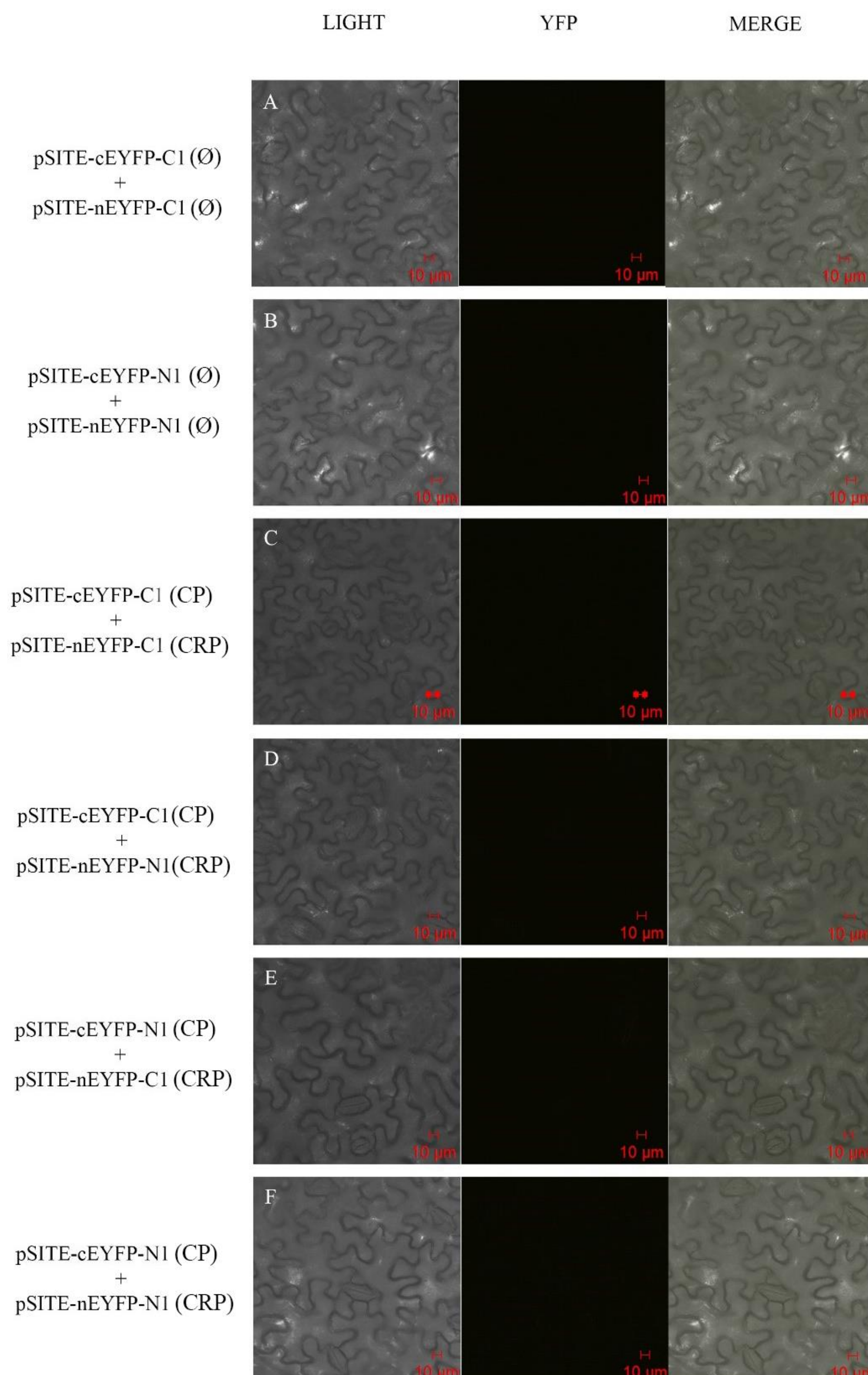


Figure 2. Yeast two-hybrid assay to evaluate interaction between CP and CRP of *Cowpea mild mottle virus*, CPMMV (Betaflexviridae: *Carlavirus*). Yeast strain AH109 was co-transformed with plasmids containing vectors BD and AD fused or not with CP and CRP. Serial dilutions of each transformation were dripped on to a selective medium (SD/ -Trp/ -Leu/ -His), containing different concentrations of 3-amino-1,2,4-triazole (3-AT) to prevent growth of false positives. After 4 days at 30º C, selected colonies were registered. The mouse p53 antitumor protein (P53) and lamin C (Lam) were used as positive and negative controls, respectively. *: false positive interactions.

3.3.Analysis of protein-protein interaction by Bimolecular fluorescence complementation (BiFC) assay

We did not observe the reestablishment of YFP fluorescence either for the combinations of empty vectors, used as negative controls (Figure 3A and B), or for all possible combinations of BiFC vectors fused to CP and CRP (Figure 3C- J). These results strongly indicate that there is no interaction between CP and CRP.



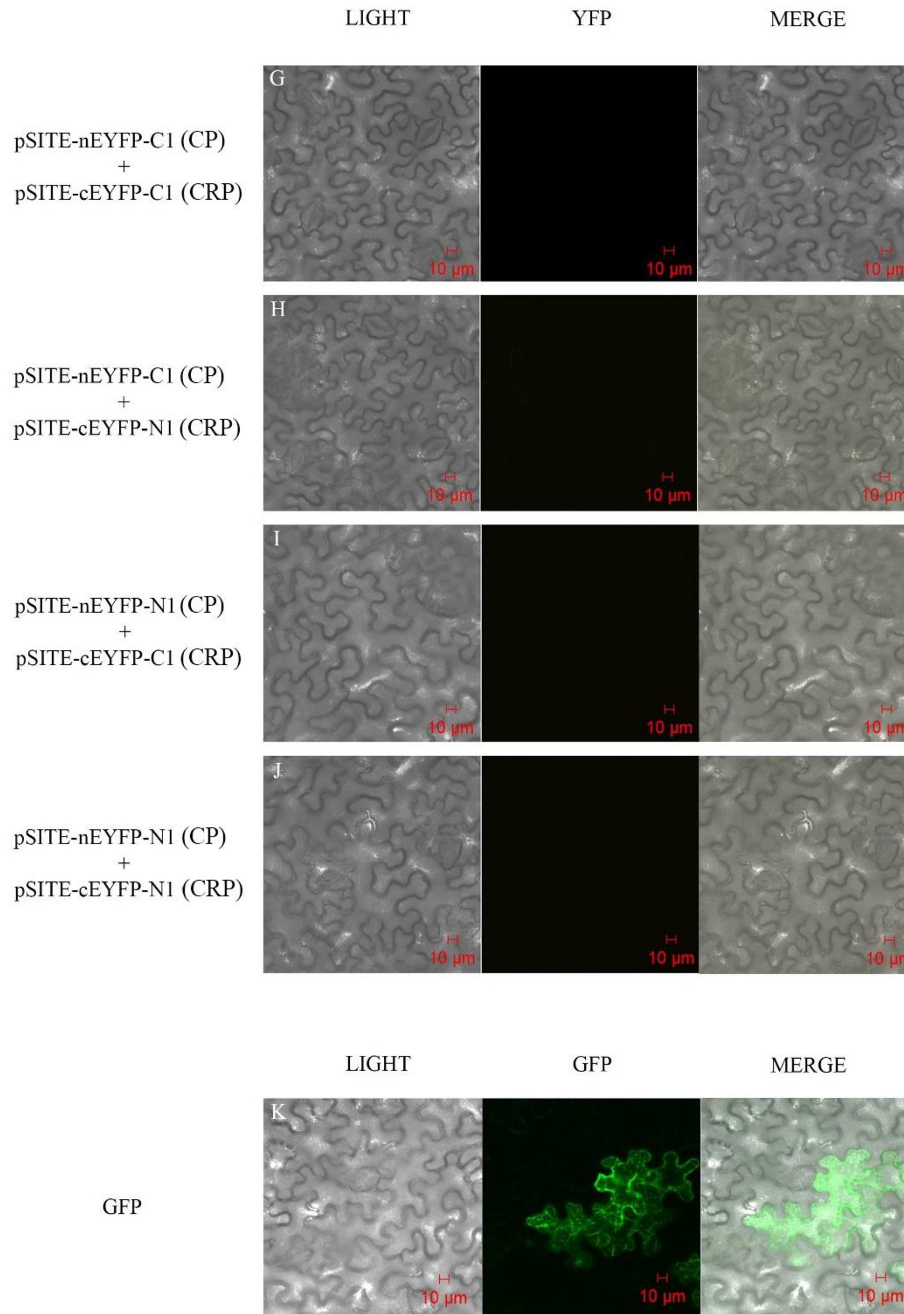


Figure 3. Bimolecular fluorescent complementation (BiFC) assay to evaluate interaction between ORFs 5 and 6 of *Cowpea mild mottle virus*, CPMMV (Betaflexviridae: *Carlavirus*). *Agrobacterium tumefaciens* AGL1 were transformed with BiFC plasmids fused or not with CP and CRP. The constructions were infiltrated in leaves of *Nicotiana benthamiana* in the presence of the p19 suppressor. The images were acquired under a confocal microscope 48h after agroinfiltration. (A and B) Negative control. (C-J) All possible combinations between CP and CRP fused to BiFC vectors. (K) Control of the infiltration process with *A. tumefaciens* transformed with GFP.

3.4. Analysis of protein-protein interaction by Co-Immunoprecipitation (Co-IP) assay

The protein encoded by ORF 6 (CRP) fused to GFP and used as bait was confirmed in the immunoprecipitated complex when in the presence of the anti-GFP antibody (Figure 5A), which was already expected since the bait protein binds to the GFP-trap which is responsible for precipitation. However, we did not identify the presence of the bait protein encoded by ORF 5 (CP) fused to RFP in the co-immunoprecipitated complex and subjected to Western Blot (Figure 5B) in the presence of the anti-RFP antibody. This indicates that there was no interaction between the proteins and therefore the CP was not identified in the Western Blot analysis.

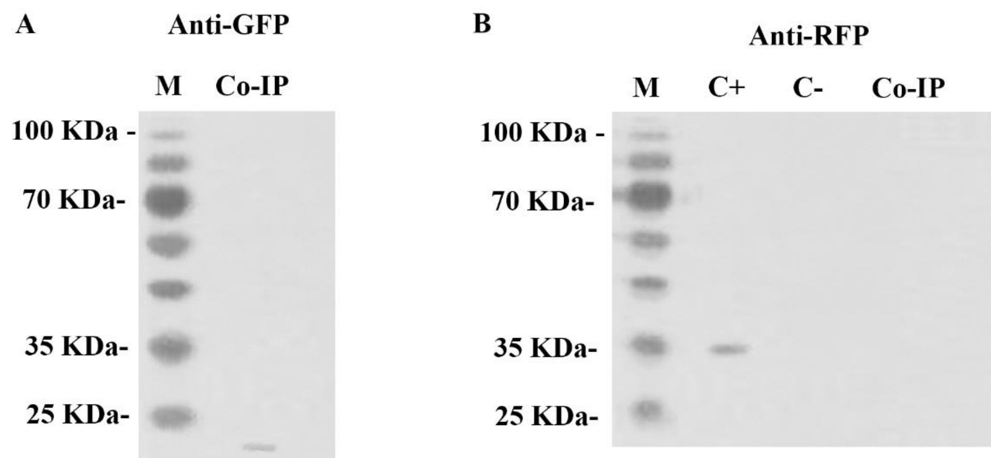


Figure 5. Co-immuneprecipitation (Co-IP) assay followed by Western blot analysis to evaluate interaction between CP and CRP of *Cowpea mild mottle virus*, CPMMV (Betaflexviridae: *Carlavirus*). Western blot analysis of the co-immunoprecipitated protein complex in the presence of anti-GFP (A) and anti-RFP (B) antibodies. M: molecular mass marker; Co-IP: Co-immunoprecipitated complex; C+: total protein extract; C-: immunoprecipitation reaction control.

4. Discussion

4.1.Transmission assay of a defective mutant for encoding CRP

Our results show that whitefly remained capable of both acquiring and inoculating CPMMV that had a mutation in ORF 6 and did not encode the protein CRP, postulated to be involved in the process of viral transmission. Thus, CRP does not seem to have a role as a helper in the process of CPMMV transmission by the whitefly, which would make us reject our initial hypothesis.

CPMMV CRP has been reported as a transcription factor, regulating its host genes, due to its nuclear localization signal and zinc finger motif capable of binding to nucleic acids (Martelli *et al.*, 2007; Zanardo *et al.*, 2014). Our study seems to be in agreement with this evidence, since during the experiments it was noticeable that the mutant virus caused much less apparent symptoms than the wild type virus and the infectious clone (Figure 2D). The absence of CRP in the mutant virus may have affected the transcription process and viral accumulation in the host, so resulting in milder symptoms. Further studies are needed to investigate the biological role of this protein and how it can modulate the process of viral infection.

CP may be directly related to the transmission process of CPMMV by the whitefly and further studies need to be conducted considering this perspective. Mutations can be designed to change possible domains conserved within the protein of carlaviruses transmitted by whitefly to test whether the vector fails to acquire and inoculate the virus. There are already studies for other viral genera following this methodology. Liu *et al.* (2002), for example, mutated conserved domains of CP of the cucumovirus, *Cucumber mosaic virus*, which affected the transmission of this virus by aphids. Therefore, after evaluating our results to date, we suggest that CPMMV uses a capsid strategy in viral transmission and we can test that.

4.2.Confirmation of the CP-CRP interaction

In the yeast two-hybrid assay, self-activation of CRP fused to BD limits the interaction analysis, since this system is based on the reconstitution of reporter gene transcription factors. So transcription-related proteins, when used as baits, can activate these genes, generating false positives (Foster *et al*., 2008). The possible role of CRP in the transcription process can explain this self-activation. As mentioned before, other studies provide evidence that CPMMV CRP can act as a transcription factor, being able to regulate the genes of its host (Martelli *et al.*, 2007; Zanardo *et al.*, 2014a, Carvalho, 2016, Paiva, 2016, Lima 2018). Lukhovitskaya et al. (2013) showed that for carlavirus *Chrysanthemum virus B* the CRP acts as a transcription factor modulating the gene of its host responsible for leaf cell proliferation. CRP of CPPMV also localizes to the nucleus of the host (Carvalho, 2016). Additionally, a mono-hybrid assay showed its interaction with the promoter region of the soybean SNC1 gene, related to host immunity (Paiva, 2016), and a chromatin immunoprecipitation assay confirmed this interaction (Lima, 2018). Thus, our results seems to point in the same direction as these studies.

BiFC results showed no interaction between CP and CRP. This conflicts with the yeast two-hybrid results and supports our expectations that the interactions of this assay are false positives. The main advantage of BiFC in this case is that the candidate proteins for interaction are not able to self-activate, since they are in different fragments of the reporter protein that will only fluoresce if these fragments are in the same complex, i.e. if the candidate proteins interact physically (Zilian and Maiss, 2011). Another advantage of this technique over other *in vitro* and *in vivo* protein interaction systems is the visualization of protein interactions in real time, through intrinsic fluorescence that does not require the use of exogenous reagents (Hu *et al*., 2002). However, BiFC requires that the results be well interpreted through prior knowledge. For example, in this study, we detected samples that showed stomatal autofluorescence and background fluorescence (Figure S1) and could be confused with true interactions, however

these results are already described in the literature (Eisele *et al*., 2016; Kodama and Den Hu, 2018) and therefore, they were not included in the study.

The results of the co-immunoprecipitation assay also support the possibility that there is no interaction between CP and CRP of CPMMV. One of the disadvantages of co-immunoprecipitation is the occurrence of false-negative in the case of low-affinity interactions (Wang *et al*., 2019). However, BiFC analyses would be sufficient to demonstrate interactions of this type, since stabilization in the protein complex in this type of approach allows a more sensitive analysis of interactions (Bischof *et al*., 2018).

5. Conclusion

This study shows that a CPMMV mutant that does not produce CRP continued to be acquired and transmitted by its vector and to infect its plant host. In our analysis of protein interactions, we detected a putative interaction between CRP and CP by the yeast two-hybrid system, however, when performing the BiFC and co-immunoprecipitation assays, we did not detect this same interaction, so it is reasonable to assume that this it is a case of false positive. Therefore, our results lead us to reject our initial hypothesis that CRP could act as a helper component, interacting with CP during the viral transmission process.

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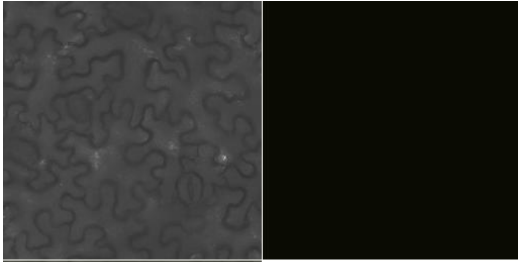
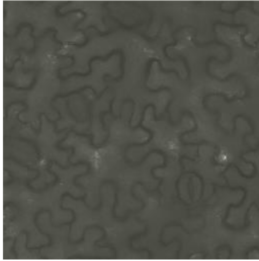
Supplementary material

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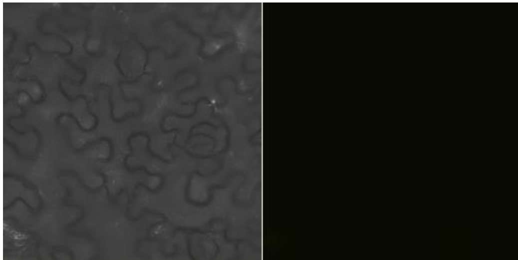
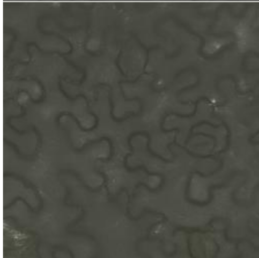

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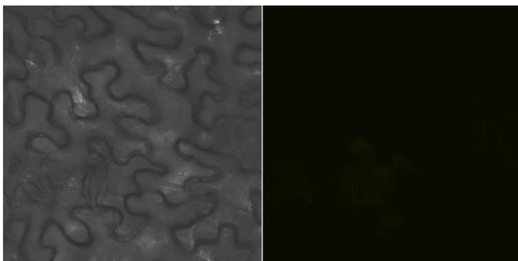
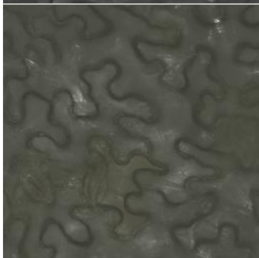

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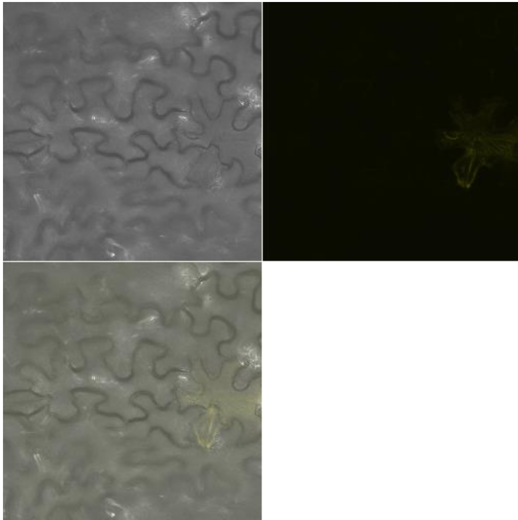

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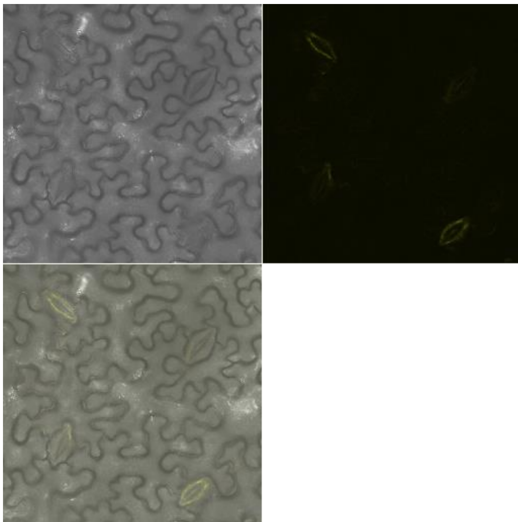

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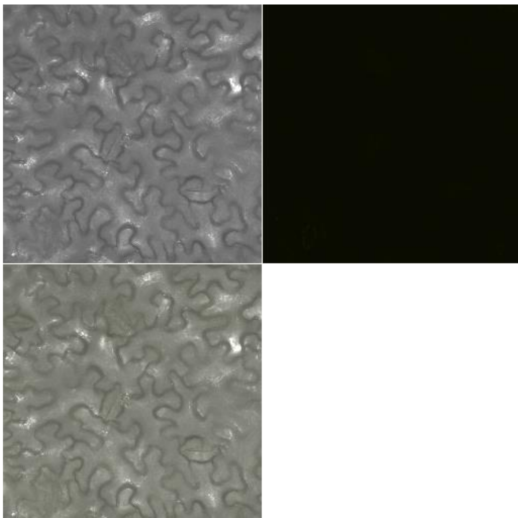

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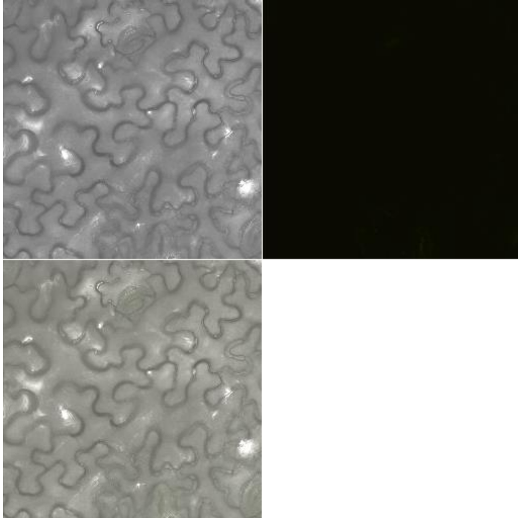

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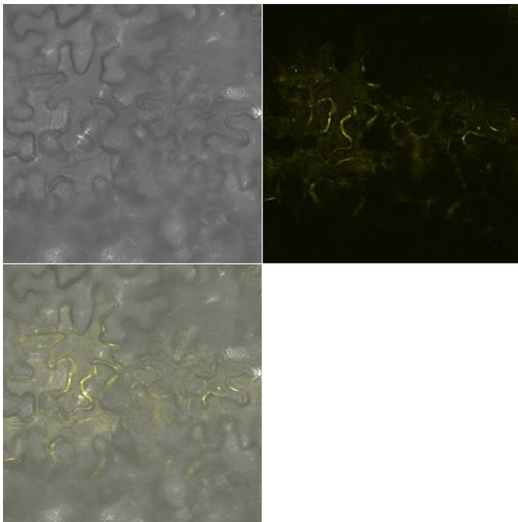

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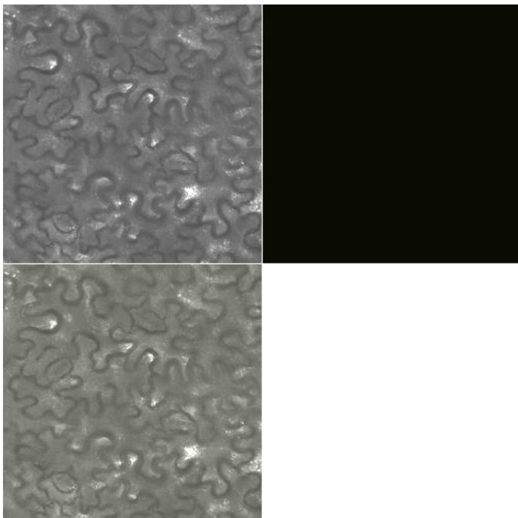

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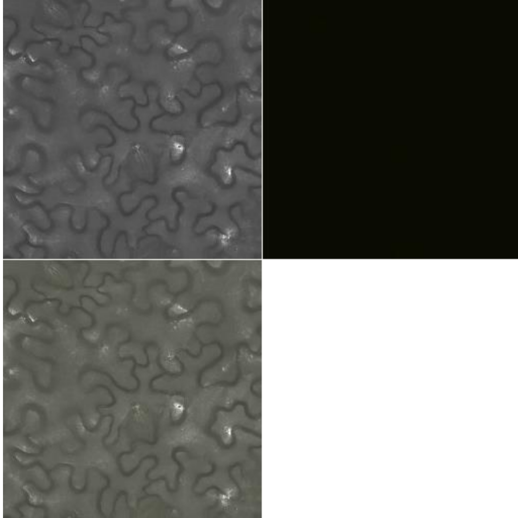

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Figure S1. Bimolecular fluorescent complementation (BiFC) assay to evaluate interaction between ORFs 5 and 6 of *Cowpea mild mottle virus*, CPMMV (Betaflexviridae: *Carlavirus*). *Agrobacterium tumefaciens* AGL1 were transformed with BiFC plasmids fused or not with CP and CRP. The constructions were infiltrated in leaves of *Nicotiana benthamiana* in the presence of the p19 suppressor. The images were acquired under a confocal microscope 12h after agroinfiltration.