

Interaction between the New World begomovirus *Euphorbia yellow mosaic virus* and its associated alphasatellite: effects on infection and transmission by the whitefly *Bemisia tabaci*

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Abstract

The majority of Old World monopartite begomoviruses (family *Geminiviridae*) are associated with satellite DNAs. Alphasatellites are capable of autonomous replication, but depend on the helper virus for movement, encapsidation and transmission by the insect vector. Recently, *Euphorbia yellow mosaic alphasatellite* (EuYMA) was found in association with *Euphorbia yellow mosaic virus* (EuYMV) infecting *Euphorbia heterophylla* plants in Brazil. The geographical range of EuYMA was assessed in a representative sampling of *E. heterophylla* plants collected in several states of Brazil from 2009 to 2014. Infectious clones were generated and used to assess the phenotype of viral infection in the presence or absence of the alphasatellite in tomato, *E. heterophylla*, *Nicotiana benthamiana*, *Arabidopsis thaliana* and *Crotalaria juncea*. Phenotypic differences of EuYMV infection in the presence or absence of EuYMA were observed in *A. thaliana*, *N. benthamiana* and *E. heterophylla*. Symptoms were more severe when EuYMV was inoculated in combination with EuYMA in *N. benthamiana* and *E. heterophylla*, and the presence of the alphasatellite was determinant for symptom development in *A. thaliana*. Quantification of EuYMV and EuYMA indicated that EuYMA affects the accumulation of EuYMV during infection on a host-dependent basis. Transmission assays indicated that EuYMA negatively affects the transmission of EuYMV by *Bemisia tabaci* MEAM1. Together, these results indicate that EuYMA is capable of modulating symptoms, viral accumulation and whitefly transmission of EuYMV, potentially interfering with virus dissemination in the field.

INTRODUCTION

The family *Geminiviridae* consists of plant viruses with genomes comprised of one or two molecules of circular, single-stranded (ss) DNA encapsidated by a single structural protein into twinned, quasi-icosahedral particles. The family includes nine genera based on the type of insect vector, host range, genome organization and phylogenetic relationships [1–3]. Viruses in the genus *Begomovirus* are transmitted by whiteflies of the *Bemisia tabaci* cryptic species complex (Hemiptera: Aleyrodidae) and infect dicot plants. Most begomoviruses fit into two major groups, Old World (OW; Europe, Africa, Asia and Australasia) and New World (NW; the Americas) based on genome features, phylogeny and geographical distribution [4, 5]. The vast majority of NW

begomoviruses possess a bipartite genome comprised of two ssDNA molecules named DNA-A and DNA-B [4, 6]. OW begomoviruses are mostly monopartite but can also be bipartite.

Monopartite OW begomoviruses are frequently associated with circular ssDNA satellites or satellite-like DNA molecules of half the size of begomovirus genome components, named betasatellites and alphasatellites [7]. Recently, a third class of DNA satellites, the deltasatellites, has been described in association with begomoviruses [8]. DNA satellites require a helper begomovirus for replication (except in the case of alphasatellites), systemic spread in plants and insect transmission.

Betasatellites (previously known as DNA-β) have a highly conserved genome organization, including a sequence

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Abbreviations: d.p.i., days post-inoculation; EuYMA, *Euphorbia yellow mosaic alphasatellite*; EuYMV, *Euphorbia yellow mosaic virus*; MEAM1, Middle East-Asia Minor 1; NW, New World; OW, Old World.

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Two supplementary tables and six supplementary figures are available with the online Supplementary Material.

conserved amongst all betasatellites, named satellite conserved region (SCR), an A-rich region and a single gene in the complementary-sense encoding the β C1 protein [9]. β C1 is a pathogenicity determinant [10, 11], and acts as a transcriptional and post-transcriptional suppressor of gene silencing [12, 13]. The presence of betasatellites is usually a determinant of symptoms by the helper begomovirus [7, 14]. Betasatellites have not been reported in association with NW begomoviruses.

Alphasatellites (previously known as DNA-1) are frequently associated with OW monopartite begomovirus/betasatellite complexes [15]. The genome of alphasatellites contains a stem-loop structure predicted to be the origin of virion-sense DNA replication, and a single virion-sense gene encoding a replication-associated protein named alpha-Rep. Alpha-Rep is similar to the master-Rep protein encoded by the DNA-R component of nanoviruses (family *Nanoviridae*). Therefore, alphasatellites can replicate autonomously in plant cells. The stem-loop structure contains the nonanucleotide sequence TAGTATTAC, identical to that of nanoviruses [7].

Although alphasatellites were discovered more than 15 years ago, little is known about their effects on begomovirus infection. They are generally described as having no obvious effect on symptoms induced by begomoviruses or by begomovirus/betasatellite complexes, and may affect betasatellite replication, but not replication of the helper begomovirus [7, 16]. However, there is evidence that alphasatellites may be involved in pathogenicity. The alpha-Rep of two alphasatellite isolates can suppress post-transcriptional gene silencing [17]. Also, an alphasatellite isolate from Oman was shown to attenuate symptoms by reducing betasatellite accumulation [18]. This alphasatellite from Oman is closely related to an unusual (DNA-2-type) alphasatellite reported in Singapore, and both are phylogenetically divergent from typical (DNA-1-type) alphasatellites [18, 19].

Recently, alphasatellites have been found in association with NW begomoviruses infecting non-cultivated plants in Brazil [20] and Cuba [21], and watermelon in Venezuela [22]. Alphasatellites were also detected by vector-enabled metagenomics (VEM) in insect samples from Guatemala and Puerto Rico [23]. The NW alphasatellites are more closely related to DNA-2-type alphasatellites and were classified as a third phylogenetically distinct group (DNA-3-type) by Rosario *et al.* [23].

In Brazil, DNA-3-type alphasatellites were found in *Euphorbia heterophylla* (Euphorbiaceae) and *Cleome affinis* (Cleomaceae) plants infected by bipartite begomoviruses [20]. Interestingly, and in contrast to the DNA-2-type alphasatellite from Oman, the presence of which attenuated symptoms induced by the helper begomovirus [18], the DNA-3-type alphasatellites described in Brazil were reported to increase symptom severity [20]. The DNA-3-type alphasatellites had 51–55% amino acid identity with DNA-2-type alphasatellites from Singapore and Oman [23].

Understanding the dynamics of the interaction between begomoviruses and DNA satellites in non-cultivated plants is important as they can be transferred to cultivated plants. Although sap transmission of a begomovirus/alphasatellite complex was demonstrated for the DNA-3-type alphasatellite described in Venezuela [24], transmission studies by *B. tabaci* are lacking in the literature. In free-choice tests, adult preference and oviposition indicated that *E. heterophylla* was the most suitable host for *B. tabaci* among seven non-cultivated plants tested [25]. Given the polyphagous habit and preference of *B. tabaci* for *E. heterophylla*, the acquisition of DNA satellites associated with bipartite begomoviruses could be facilitated and may result in the emergence and spread of new disease complexes [7, 25, 26].

The objectives of this study were to assess the occurrence and prevalence of satellites in *E. heterophylla* plants infected by begomoviruses, and to investigate their influence in the infection phenotype, viral accumulation and transmission. The geographical range of Euphorbia yellow mosaic alphasatellite (EuYMA) was assessed in Brazil, and its phylogenetic relationship with typical (DNA-1-type) and unusual (DNA-2- and DNA-3-type) alphasatellites was described. Dimeric constructs of *Euphorbia yellow mosaic virus* (EuYMV) genomic components (DNA-A and DNA-B) and EuYMA were generated to perform the biological characterization. Phenotypic differences of EuYMV infection in the presence of EuYMA were found in *Arabidopsis thaliana*, *Nicotiana benthamiana* and *E. heterophylla*. Whitefly transmission assays indicated that EuYMA negatively affects the transmission of EuYMV, potentially affecting the spread of the virus in the field.

RESULTS

Geographical range and phylogeny of EuYMV and EuYMA

A total of 165 *E. heterophylla* samples, collected over a period of six years in locations throughout the Brazilian states of Amazonas, Goiás, Mato Grosso do Sul, Minas Gerais, Paraná, Pernambuco, Rio Grande do Sul and Santa Catarina, were tested for the presence of begomoviruses and associated DNA satellites. While EuYMV DNA-A and DNA-B were cloned from 129 and 41 samples, respectively [27], EuYMA was detected in only six samples collected in the states of Rio Grande do Sul and Paraná [Fig. 1a and Table S1 (available in the online Supplementary Material)]. Pairwise sequence comparisons indicated $\geq 93\%$ identity amongst EuYMA sequences (Fig. S1 and Table S1).

A Bayesian phylogenetic tree based on complete nucleotide sequences separated EuYMA isolates according to sampling location (Fig. 1a, b). A tree based on nucleotide sequences of the *alpha-Rep* gene showed two major clades, confirming the phylogenetic distinction between typical (DNA-1-type) and unusual (DNA-2-type and DNA-3-type) alphasatellites (Fig. 1c). A tree based on deduced amino acid sequences had equivalent topology (Fig. S2). The *alpha-Rep* genes of DNA-2-type and DNA-3-type alphasatellites share $\geq 58\%$

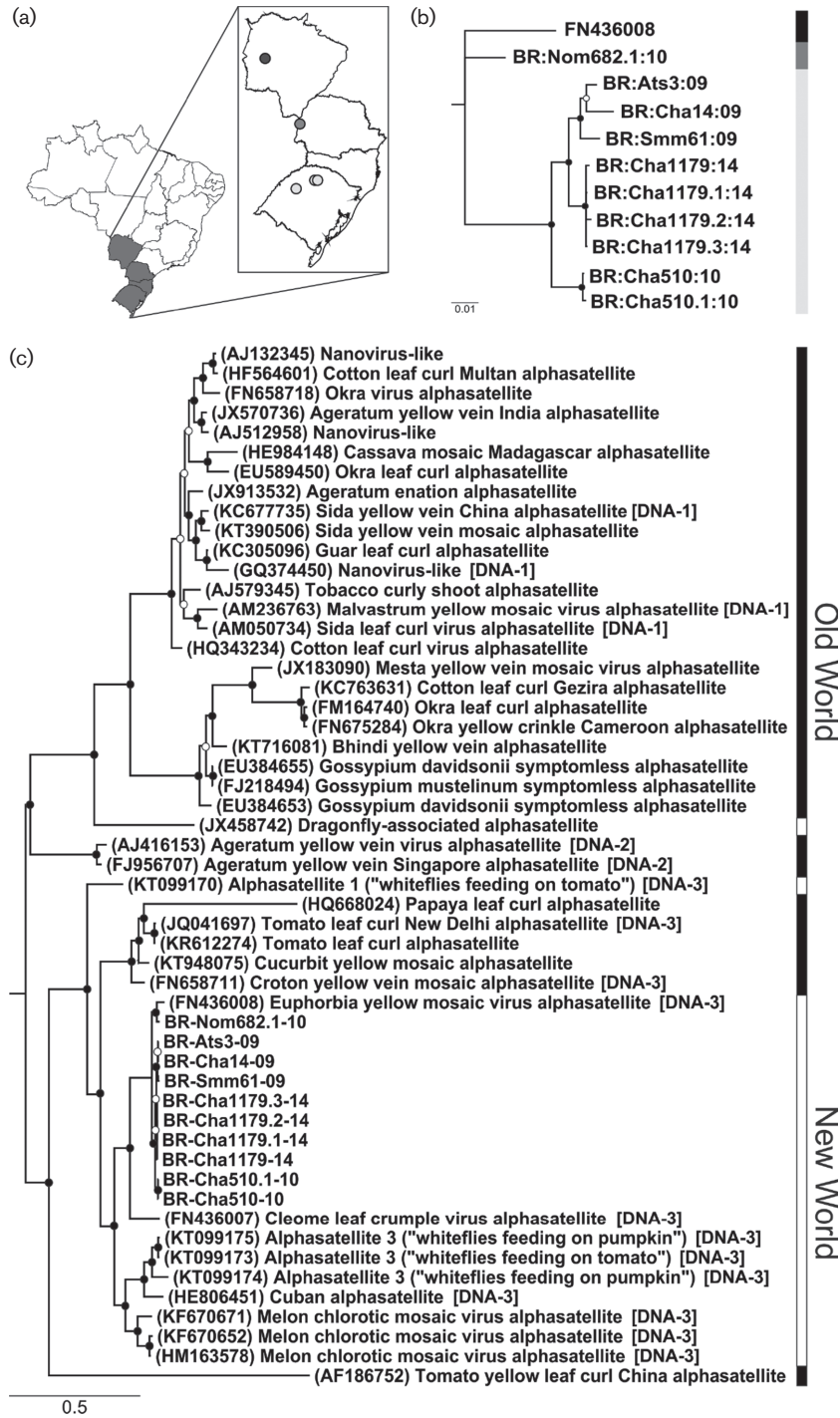


Fig. 1. (a) Map of Brazil indicating the locations where Euphorbia yellow mosaic alphasatellite has been detected in the states of Rio Grande do Sul, Paraná (light and medium grey, respectively; this study) and Mato Grosso do Sul (dark grey; Paprotka et al. [20]). (b, c) Midpointed-rooted Bayesian phylogenetic trees based on full-length nucleotide sequences of EuYMA (b) and *alpha-Rep* gene nucleotide sequences (c). Nodes to the right of branches with posterior probabilities ≥ 0.8 are indicated by filled circles and those with values < 0.8 and > 0.5 by empty circles. The scale bars indicate substitutions per site. In (b), the vertical bar at the right indicates the geographical regions represented on the map. In (c), the vertical bar indicates Old World and New World begomoviruses, and the classification proposed by Rosario et al. [23] is shown in square brackets.

approx. 90 % of the plants infected with EuYMV (Table 1, Fig. 2b). In general, the severity of symptoms increased in the presence of EuYMA (Figs 3 and S4). Moreover, in the absence of EuYMA, some plants recovered from the symptoms (Fig. S3).

The same number of infected *E. heterophylla* plants was detected by PCR at either 14 or 28 d.p.i. When EuYMV was inoculated alone, infection was detected in 40 % of the plants (18/45), while inoculation of the two agents lead to infection in approx. 30 % of the plants (13/45) (Fig. 2a, Tables 1 and S2). Similar to *N. benthamiana*, from a total of 45 *E. heterophylla* inoculated with both virus and alphasatellite, EuYMV alone was detected in six plants (data not shown). The first symptoms were observed at 4 d.p.i. and by 28 d.p.i. all infected plants showed symptoms consisting of mosaic and vein chlorosis (Fig. 2b and Table 1). A significant difference in symptoms was observed in the presence of EuYMA. In addition to mosaic and vein chlorosis, leaf curling was also observed in *E. heterophylla* infected with both EuYMV and EuYMA (Figs 3 and S3).

The presence of EuYMA influences EuYMV accumulation in three hosts

Considering the influence of EuYMA in the EuYMV infection phenotype in *A. thaliana*, *E. heterophylla* and *N. benthamiana*, the accumulation of each viral genomic component (DNA-A and DNA-B) was quantified at 14 and 28 d.p.i. to

compare viral accumulation in the presence or absence of EuYMA.

The accumulation of EuYMV DNA-A increased in *E. heterophylla* and *N. benthamiana* in the presence of EuYMA, compared with plants infected with the virus alone at both 14 and 28 d.p.i. (Figs 4 and S5). Viral accumulation varied widely among plants of *E. heterophylla*. Also, the number of *E. heterophylla*-infected plants was lower compared with *A. thaliana* and *N. benthamiana*, impairing the statistical analysis (Fig. S5). Interestingly, and unlike what was observed in *E. heterophylla* and in *N. benthamiana*, the presence of EuYMA did not cause an increase in the accumulation of EuYMV DNA-A in *A. thaliana* (Fig. 4). In fact, the results point to a reduction, albeit with weak statistical support (Figs 4 and S5).

The results did not indicate significant differences in the accumulation of EuYMV DNA-B in the presence or absence of EuYMA in *N. benthamiana* and *A. thaliana* (Figs 4 and S5). However, comparisons in *E. heterophylla* pointed to an increase of EuYMV DNA-B accumulation in the presence of EuYMA at both 14 and 28 dpi (Fig. 4).

EuYMA accumulated at high levels in the systemically-infected leaves of *A. thaliana*, with a significantly higher accumulation compared to both EuYMV components at 28 d.p.i. (Fig. S6). In *N. benthamiana* and *E. heterophylla*, EuYMA accumulated at higher levels than EuYMV DNA-B (Fig. S6).

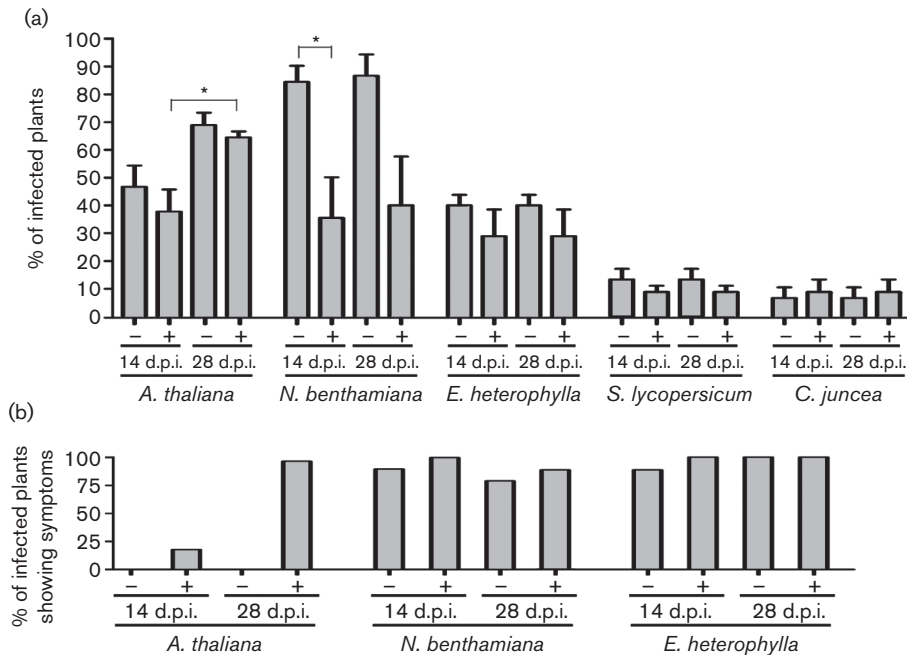


Fig. 2. Experimental host range of *Euphorbia yellow mosaic virus* (EuYMV) and its associated alphasatellite, Euphorbia yellow mosaic alphasatellite (EuYMA). (a) The percentage of infected plants (detected by PCR) with standard errors is indicated for each host at 14 and 28 days post-inoculation (d.p.i.) in the presence (+) or absence (-) of EuYMA (three independent replications, 15 plants per replication). Statistically significant differences (Student's *t*-test) are indicated with horizontal bars (*, $P < 0.05$). (b) Percentage of PCR-positive plants showing symptoms at 14 and 28 d.p.i.

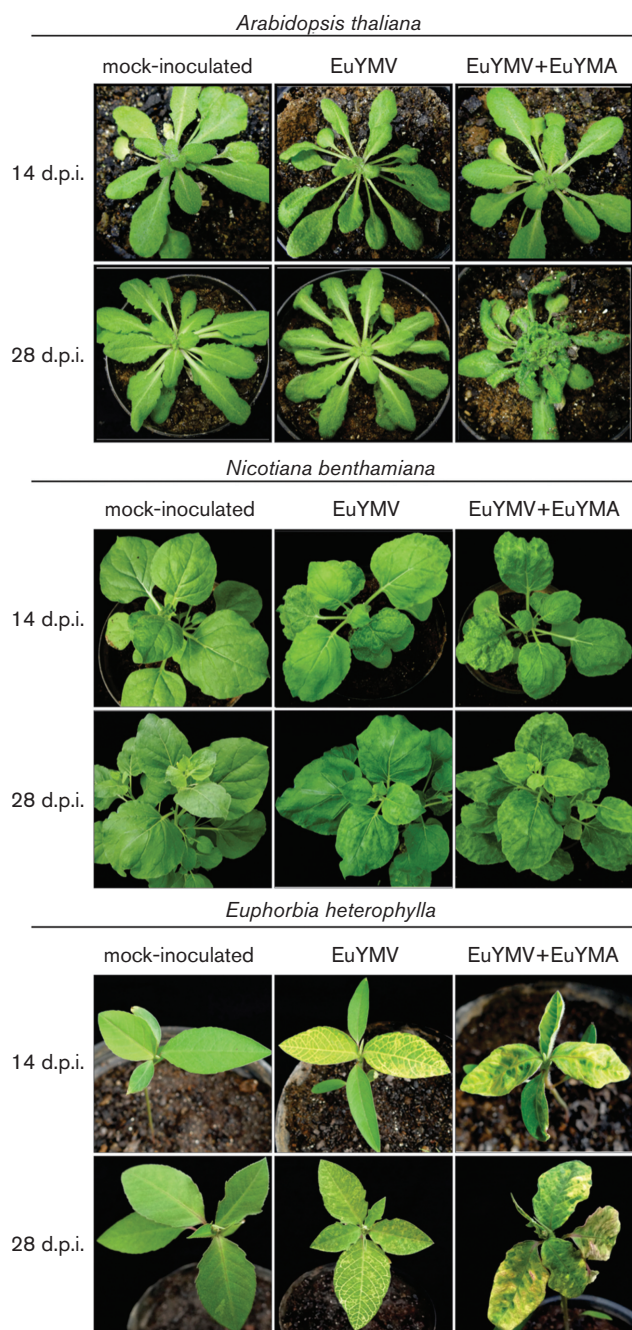


Fig. 3. Symptoms induced by *Euphorbia yellow mosaic virus* (EuYMV) alone or in the presence of *Euphorbia yellow mosaic alphasatellite* (EuYMA) at 14 and 28 days post-inoculation (d.p.i.) by biolistics.

EuYMA negatively affects the transmission of EuYMV by the insect vector

The transmission of EuYMV by *B. tabaci* Middle East-Asia Minor 1 (MEAM1) was compared in the presence or absence of EuYMA. The plants were PCR-tested for the presence of the EuYMV genomic components and for EuYMA. In both replications of the experiment (for a total

of 40 inoculated plants in each treatment), EuYMV was transmitted more efficiently when the source plants were infected with EuYMV alone (Table 2). This result indicates that EuYMA could negatively affect the vector transmission of EuYMV.

Similarly to what was observed in the host range assay (when the plants were inoculated by biolistics), *E. heterophylla* plants infected with EuYMV alone showed symptoms of interveinal chlorosis and yellow mosaic (Fig. 5a). When the virus was inoculated in combination with EuYMA the symptoms were more severe, characterized by a more intense yellow mosaic, leaf crumpling and stunting (Fig. 5b, c). Interestingly, after 28 d.p.i., some plants recovered from the symptoms (EuYMV alone and EuYMV + EuYMA; nine and five plants, respectively) (Fig. 5d). The presence of all molecules was confirmed by PCR in all symptomatic plants (including those that recovered), with oligonucleotides for the CP and MP genes of EuYMV and for the *alpha-Rep* of EuYMA (Fig. 5f).

Interestingly, in the transmission assay, only the EuYMV DNA-A was detected in some *E. heterophylla* plants, at both time points (in one plant the DNA-A was detected at 14 d.p.i. but no genomic components were detected at 28 d.p.i.). This was observed in three plants in each treatment, in the two experiments (3 out of 40 plants inoculated with EuYMV alone and 3 out of 40 plants in which EuYMV was inoculated together with EuYMA). Mild mosaic symptoms were observed in these plants (Fig. 5e). For the purpose of statistical analyses, these plants were considered negative for the presence of the virus.

DISCUSSION

In a representative sampling encompassing six years and locations throughout eight Brazilian states, EuYMA was detected in only six samples collected in the states of Rio Grande do Sul and Paraná. It seems clear that the association of EuYMV with EuYMA is not common. Nevertheless, EuYMA was detected in four additional locations besides that previously reported (in the state of Mato Grosso do Sul; [20]), in agreement with the large geographical range of alphasatellites in South America. A recent study corroborates these conclusions. Ferro *et al.* [29] detected EuYMA in one out of 43 samples of *Sida* spp. collected in an area overlapping that covered in our study. Although the associated begomovirus was not identified at the species level, EuYMV was detected in two other *Sida* spp. samples, indicating that EuYMV can infect *Sida* spp. Together, these results suggest that the association between the EuYMV and EuYMA could be specific.

Pairwise sequence comparisons indicated that alphasatellite sequences were more variable than the helper begomovirus components [27]. The EuYMA *alpha-Rep* gene is most closely related to those from alphasatellites found in association with bipartite begomoviruses in the Americas (DNA-3-type), corroborating the analysis of Rosario *et al.* [23].

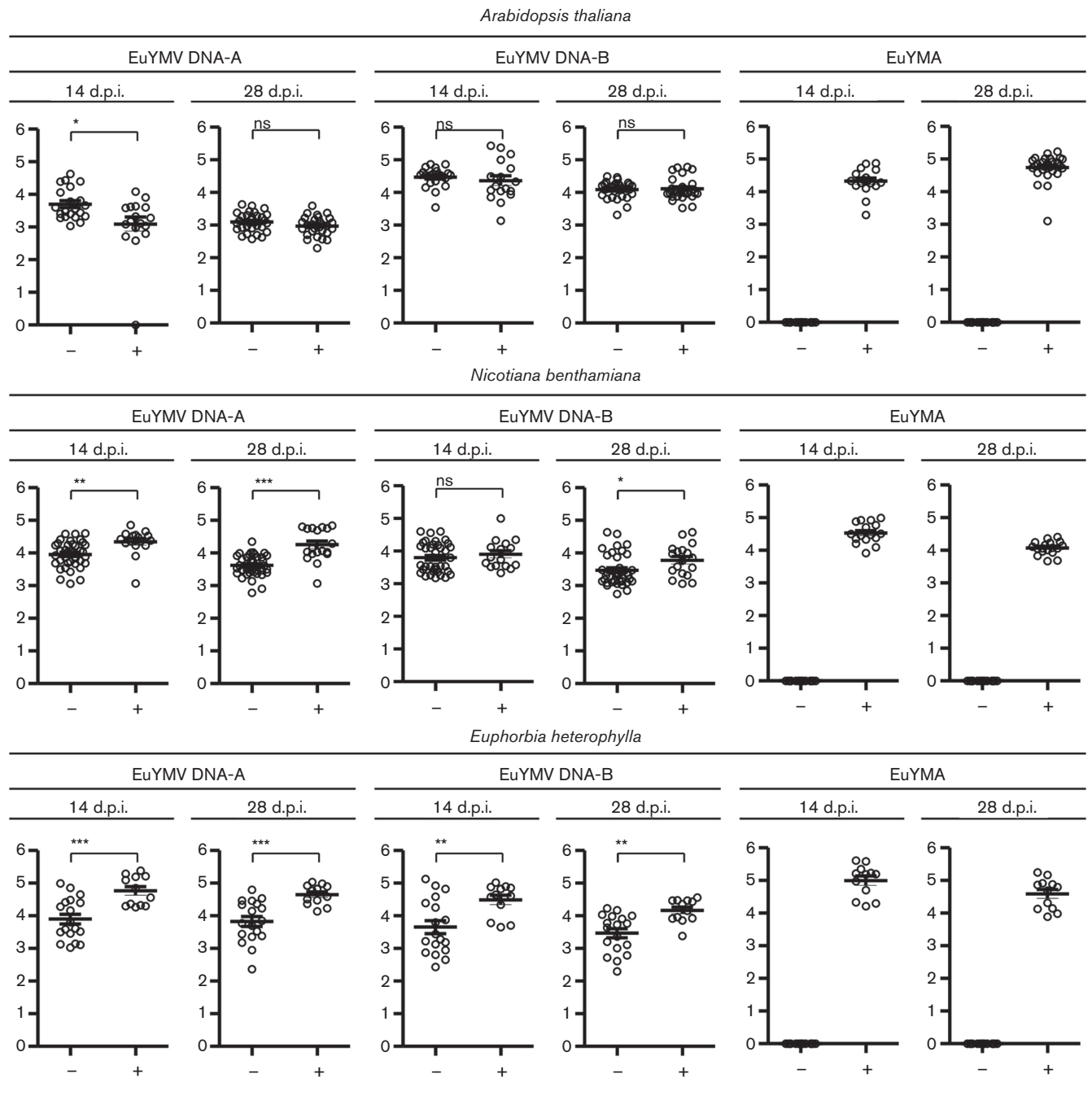


Fig. 4. Absolute quantification by quantitative PCR of *Euphorbia yellow mosaic virus* (EuYMV) DNA-A and DNA-B and of *Euphorbia yellow mosaic alphasatellite* (EuYMA) in different hosts. Viral accumulation is presented as the log of the number of molecules, and values below the detection threshold were considered as background. Each circle represents one infected plant. (–) represents infection by EuYMV alone; (+) represents infection by EuYMV together with EuYMA. Data refers to all plants of three independent replicates, evaluated at 14 and 28 days post-inoculation (d.p.i.) and the mean with standard error is indicated. Significance of comparisons between the log of the number of molecules is indicated by horizontal bars (ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

The presence of EuYMA correlated with an increase of symptom severity in *N. benthamiana* and *E. heterophylla*, and was determinant for the development of severe leaf curling in *A. thaliana*. Also, the EuYMV/EuYMA complex can

systemically infect *C. juncea* and tomato. Besides the characteristic mosaic symptoms and interveinal chlorosis induced by EuYMV in *E. heterophylla*, leaf crumpling, downward leaf rolling and stunting symptoms were observed when EuYMA

Table 2. Transmission of *Euphorbia yellow mosaic virus* (EuYMV) to *Euphorbia heterophylla* plants, alone or in the presence of *Euphorbia yellow mosaic alphasatellite* (EuYMA), by *Bemisia tabaci* Middle East-Asia Minor 1 (MEAM1)

Experiment	No. infected plants/no. inoculated plants		
	EuYMV*	EuYMV and EuYMA†	Negative control‡
1	17/20	15/20§	0/3
2	18/20	14/20§	0/3

*PCR detection of EuYMV DNA-A and DNA-B in plants inoculated with EuYMV alone.

†PCR detection of EuYMV DNA-A and DNA-B and EuYMA in plants inoculated with EuYMV and EuYMA.

‡Transmission with aviruliferous whiteflies.

§Statistical significance ($P \leq 0.05$) according to Barnard's unconditional test.

was present. This is the opposite of what was observed for the DNA-2-type *Ageratum yellow vein* Singapore alphasatellite from Oman, which is associated with begomovirus/betasatellite complexes. In that case, the presence of the alphasatellite led to attenuated symptoms in *N. benthamiana* and substantially reduced betasatellite accumulation [18]. Symptom attenuation is likely due to reduced betasatellite titre, leading to lower accumulation of the $\beta C1$ protein, a suppressor of gene silencing [12, 18].

One possible explanation for the increased severity of symptoms of EuYMV in combination with EuYMA is that the alpha-Rep protein of EuYMA may possess silencing suppressor activity, as shown by Nawaz-ul-Rehman *et al.* [17] for alpha-Rep proteins encoded by two alphasatellites from Pakistan. However, preliminary experiments did not support a role as post-transcriptional gene silencing suppressor for the alpha-Rep protein of EuYMA in *N. benthamiana* leaves (data not shown). It is possible that it may suppress transcriptional silencing, as recently reported for the alpha-Rep of Cotton leaf curl Multan alphasatellite [30].

The presence of EuYMA affects the accumulation of EuYMV in *E. heterophylla* and *N. benthamiana*. Considering all classes of DNA satellites that can be found in association with begomoviruses, an increase in helper begomovirus accumulation is known to occur only in the presence of betasatellites [31]. The recently described deltasatellites have been shown to decrease accumulation of viral genome components in agro-inoculated *N. benthamiana* plants [32, 33], and some of the unusual alphasatellites can affect only betasatellite replication without affecting the helper virus [17, 18]. Therefore, at this time, the pathosystem *E. heterophylla*/EuYMV/EuYMA is the only one in which the presence of an alphasatellite increases symptom severity and DNA accumulation of the helper begomovirus. The mechanism(s) by which alphasatellites act during begomovirus infection remains unknown [7]. For EuYMV/EuYMA, both symptom modulation and changes in DNA accumulation

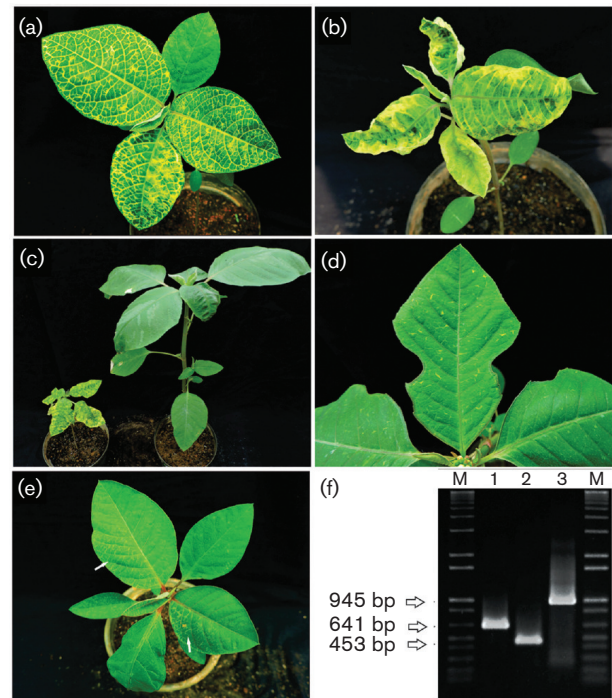


Fig. 5. Whitefly transmission of *Euphorbia yellow mosaic virus* (EuYMV) to *E. heterophylla* plants, alone or in the presence of *Euphorbia yellow mosaic alphasatellite* (EuYMA). (a) Yellow mosaic and vein chlorosis induced by EuYMV alone at 14 days post-inoculation (d.p.i.). (b, c) Severe mosaic, leaf shrivelling and stunting induced by EuYMV in the presence of EuYMA at 14 d.p.i. (d) Recovering symptoms at 28 d.p.i. in plants infected with EuYMV alone, with similar observations also in plants infected with EuYMV and EuYMA. (e) Symptoms caused by infection with EuYMV DNA-A only. The arrow points to mild mosaic in plants infected with the DNA-A alone. (f) PCR-based amplification of fragments of the CP (1), MP (2) and alpha-Rep (3) genes for detection of EuYMV DNA-A and DNA-B and of EuYMA, respectively. M, size marker (1 kb Plus DNA Ladder).

were observed in the three hosts, emphasizing the need for further studies to determine the role of alphasatellites in begomovirus infections.

We attempted to clarify the role of alphasatellites in the transmission of begomoviruses by their insect vector, and found that the presence of EuYMA negatively affects the transmission of EuYMV by *B. tabaci* MEAM1 (the prevalent species in Brazil; [34]). Alphasatellites are capable of autonomous replication in plant cells as they encode their own, nanovirus-like, Rep. However, they do not encode a coat protein gene and thus depend on their helper begomovirus for insect transmission [7]. It is possible that alphasatellites compete with begomoviruses for encapsidation of the genome. Thus, depending on the relative amount of alphasatellite and begomovirus DNA, many particles may contain alphasatellite molecules rather than viral genomic components, reducing the chances of virus particles being acquired and transmitted by the vector. Moreover, all things being equal, a small molecule will replicate faster than a large molecule, possibly leading to a

preponderance of alphasatellites in infected tissues. In agreement with these hypotheses, a high level of EuYMA accumulation was observed in the upper leaves of *A. thaliana*, *N. benthamiana* and *E. heterophylla*. Transmission was favoured in this experiment by using 30 whiteflies per plant, since the number of infected plants increases as whitefly numbers increase [35]. A statistically significant difference between the number of infected plants in the presence and absence of EuYMA was observed. Studies using gradually lower numbers of whiteflies and also a higher number of plants could lead to even greater differences, providing more conclusive results.

EuYMA was efficiently transmitted by *B. tabaci* MEAM1, being detected in all plants in which EuYMV (DNA-A +DNA-B) was also detected. Thus, the reason why it is not widespread in the field is probably not due to low efficiency in the transmission by the insect vector. The encapsidation of EuYMA probably did not alter the structural properties of the CP (the only essential protein for vector transmission), and thus did not affect the CP interaction with GroEL chaperones produced by endosymbionts, which is important for particle stability during their passage through the gut of whiteflies [36–39].

A small number of *E. heterophylla* plants were infected by EuYMV DNA-A alone, displaying mild mosaic symptoms. Systemic infection by begomovirus DNA-A alone is not without precedent. Previous reports indicate that, in exceptional cases, the DNA-A is capable of infecting and moving in plants in the absence of the DNA-B [40–42]. Weigel *et al.* [43] demonstrated that the DNA-A can reach the cell nucleus of upper, systemically infected leaves and replicate therein, independently of the DNA-B. However, such infections are usually asymptomatic. The occurrence of mild symptoms in *E. heterophylla* plants infected by EuYMV DNA-A alone suggests that the virus is very well adapted to this host, while at the same time reinforcing the role of the DNA-B in symptom induction by bipartite begomoviruses.

Our results indicate that the association of the alphasatellite EuYMA with the bipartite begomovirus EuYMV is not common, although their geographical range is large. Evidence is also provided that alphasatellites can increase the symptom severity of bipartite begomovirus infections and promote changes in DNA accumulation, although the mechanisms remain to be elucidated. The presence of EuYMA negatively affected transmission by the vector, which can negatively affect the spread of the virus. EuYMV seems to be well adapted to *E. heterophylla*, and can also infect a number of other non-cultivated hosts. This is an interesting system to study the interaction between begomovirus and DNA satellites in non-cultivated hosts, and to assess the possibility and consequences of these being transferred to cultivated plants.

METHODS

Cloning and sequencing of DNA satellite genomes

The same samples of *E. heterophylla* described for the genetic variability study of EuYMV [27] were used for the

detection and cloning of DNA satellites. Circular DNA molecules were amplified using rolling-circle amplification (RCA) with phi29 DNA polymerase according to Inoue-Nagata *et al.* [44]. Restriction fragments of approximately 1300 nucleotides (nt), corresponding to one full-length copy of satellite DNAs, were ligated in the pBLUESCRIPT-KS+ (Stratagene) plasmid vector. Inserts of selected clones were completely sequenced at Macrogen (Seoul, South Korea). Pairwise sequence comparisons were performed with Sequence Demarcation Tool (SDT) v. 1.2 [45].

Phylogenetic analysis

Multiple sequence alignments were performed using the MUSCLE algorithm [46] implemented in the MEGA 6 software package [47]. Phylogenetic reconstruction using Bayesian inference was performed with MrBayes 3.2 [48] available at the CIPRES Science Gateway [49], with the models selected by MrModeltest2.2 [50] in the Akaike Information Criterion. Two independent analyses were conducted, each running at least 10 000 000 generations. Phylogenetic trees were visualized using FigTree 1.3 (tree.bio.ed.ac.uk/software/figtree).

Construction of infectious clones and host range assay

Tandem dimeric constructs of each genomic component of EuYMV-[BR-Cha510-10] [27] and of the associated EuYMA (Table S1) were obtained by partial restriction digestion [51] and were used in host range assays with biolistics [52]. Fifteen plants each of tomato (cv. Santa Clara), *E. heterophylla*, *N. benthamiana*, *A. thaliana* and *C. juncea* were inoculated with EuYMV alone (DNA-A+DNA-B), and 15 were inoculated with EuYMV and EuYMA. Three independent replications of the experiment were conducted, for a total of 45 plants of each species/treatment. The youngest upper leaves were collected at 14 and 28 days post-inoculation (d.p.i.) for total DNA extraction [53]. Plants were also visually evaluated for symptoms and PCR-tested for the presence of virus and satellite at both time points.

Virus and alphasatellite titres were determined by quantitative PCR. Total DNA was quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). A standard curve was obtained by dilution of known quantities of plasmids containing one copy of the EuYMV DNA-A and DNA-B and of the EuYMA genome. Primers were designed with Primer Express Software 3.0.1 (Applied-Biosystems), based on the sequences of the EuYMV CP gene (to estimate the DNA-A titre; 5'-AAGGCCTCTTCATGGG TGAA-3' and 5'-TCCGGTACATCTGGGCCTCTA-3'), the EuYMV MP gene (to estimate the DNA-B titre; 5'-TTGGCGCTCTGAAAGGGATA-3' and 5'-GACACA TTTCGCGAAGTTCAA-3') and the EuYMA *alpha-Rep* gene (to estimate the alphasatellite titre; 5'-ACAAGAG-GAAGACGATGGAACGT-3' and 5'-GCAGCGACGATA-CAGCTTAGG-3'). Quantitative PCR was carried out using Fast SYBR Green Master Mix in a StepOnePlus Real-Time PCR System (Applied Biosystems). Each sample was

analysed in triplicate, in a reaction containing 0.1 ng total DNA. Data analysis was performed with GraphPad Prism Software 5 [54] and the significance between means was calculated with Student's *t*-test.

Whitefly transmission assay

Seedlings of *E. heterophylla* (2–3 days post-germination) were biolistically inoculated with EuYMV (DNA-A+DNA-B) alone or with EuYMV and EuYMA. Total DNA was extracted at 14 d.p.i. [53]. The presence of the two agents was assayed by conventional PCR with oligonucleotides for the *CP* and *MP* genes of EuYMV and the *alpha-Rep* gene of EuYMA. One set of plants infected by the virus alone and one set infected by the virus and the alphasatellite were used as sources for acquisition by *B. tabaci* Middle East-Asia Minor 1 (MEAM1), as described by Polston and Capobianco [55]. Non-viruliferous whitefly adults (maintained on cabbage plants) were released in insect-proof cages containing the source plants. After an acquisition access period of 48 h, the whiteflies were transferred to healthy *E. heterophylla* plants with the first pair of true leaves fully expanded. Each plant was maintained in an insect-proof cage with 30 whiteflies per cage for an inoculation access period of 48 h. Twenty plants were used in each treatment (EuYMV transmission in the presence or absence of EuYMA), with three healthy plants as negative controls. Two independent replications of the experiment were performed.

Total DNA was extracted at 14 and 28 days after the inoculation access period, and the presence of the two agents was assessed by PCR as described above. Transmission rates were compared using a 2×2 contingency table constructed based on infection determined by PCR diagnostics. To determine whether the variation in transmission efficiency was dependent on the presence of EuYMA, the independence between rows and columns was tested using an unconditional test, a more powerful alternative than a conditional test. As recommended by Mato and Andrés [56], independence test statistics were performed with the *exact* test from the R package, through Barnard's unconditional test [35, 57].

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The research reported in this paper did not involve animals or humans.

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