The Potyvirus Particle Recruits the Plant Translation Initiation Factor eIF4E by Means of the VPg covalently Linked to the Viral RNA

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The viral protein genome-linked (VPg) of potyviruses is a protein covalently linked to the 5' end of viral RNA. It interacts with eIF4E, a component of the cellular translation initiation complex. It has been suggested that the 5' RNA-linked VPg could mimic the cellular mRNA cap, promoting synthesis of viral proteins. Here, we report evidence for recruitment of the plant eIF4E by *Lettuce mosaic virus* (LMV, potyvirus) particles via the 5' RNA-linked VPg. Analysis of the viral population was performed by enzyme-linked immunosorbent assay-based tests, either with crude extracts of LMV-infected tissues or purified viral particles. In both cases, LMV-VPg and LMV-eIF4E subpopulations could be detected. After reaching a maximum within the first 2 weeks postinoculation, these populations decreased and very few labeled particles were found later than 3 weeks postinoculation. The central domain of VPg (CD-VPg) was found to be exposed at the surface of the particles. Using a purified recombinant lettuce eIF4E and CD-VPg-specific antibodies, we demonstrate that the plant factor binds to the VPg via its central domain. Moreover, the plant eIF4E factor could be imaged at one end of the particles purified from LMV plant extracts, by immunoredox atomic force microscopy coupled to scanning electrochemical microscopy. We discuss the biological significance of these results.

The genus *Potyvirus* represents one of the largest and most economically destructive genera of plant viruses. The potyviral genome encodes a small set of 11 different proteins responsible for hijacking host resources for its replication. Many efforts have been made to better understand the molecular aspects of the cellular virus cycle and to analyze the virus-host interactome (Elena and Rodrigo 2012; Ivanov et al. 2014; Jiang and Laliberté 2011; Mäkinen and Hafrén 2014; Martínez et al. 2016; Wang 2015). Although the literature does not report many studies on potyvirus particle contribution to the infectious cycle, the mature viral particle is a key player in processes such as protein translation and genome replication control (Besong-Ndika et al. 2015), cell-to-cell and systemic movement within the plant (Dolja et al. 1994), or interaction with an aphid vector

(Ruiz-Ferrer et al. 2005). It is likely that a more detailed characterization of nonstructural protein factors copurifying with virus particles could help to decipher some molecular mechanisms of the viral cycle. However, although promising (Brizard et al. 2006), this approach has not been much explored so far. In the present work, we looked for the presence of eIF4E, a subunit of the host plant translation initiation complex, on native potyvirus particles. Potyviruses possess a single-stranded, polyadenylated, positive-sense genomic RNA that is covalently linked at its 5' end to a viral protein, the viral protein genome-linked (VPg) (Murphy et al. 1991). The potyviral VPg is a virulence factor that has been shown to interact with several viral and host factors and is assumed to be a multifunctional protein involved in all essential steps of the virus infection cycle, translation, replication, and movement (Bosque et al. 2014; Ivanov et al. 2014; Jiang and Laliberté 2011). Among cellular host factors necessary to the virus, VPg recruits the eukaryotic translation initiation factor 4E (eIF4E) or its isoform, eIF(iso)4E, in an interaction that is crucial for infection (Charron et al. 2008; Léonard et al. 2000). Eukaryotic mRNAs are capped at their 5' end with 7-methylated guanine (m7G). The recognition of this m7G functional group by eIF4E leads to the recruitment of the 40S ribosomal subunit onto the mRNA (Browning and Bailey-Serres 2015). The general structural similarity between the potyvirus genomic RNA and eukaryotic mRNAs suggests that VPg may serve as a cap-like structure, allowing the virus to recruit the cell translation machinery. A VPg-mediated enhancement of viral genome expression was observed concomitant with a cellular mRNA expression shut-down. It was suggested that VPg could inhibit endogenous mRNA translation by complexing available eIF4E (Eskelin et al. 2011). It was previously reported that VPg is exposed at one end of the virus particle (Nault et al. 2015; Torrance et al. 2006). In this study, we investigated the possible eIF4E binding to the LMV particle. An analysis of the particle population was conducted on LMV-infected plants. By looking for VPg and eIF4E proteins associated to the viral particle and locating the eIF4E binding site, we demonstrated that plant eIF4E interacts with the VPg linked to the RNA of the LMV potyvirus particle. The possible functional implications of this interaction are discussed.

RESULTS

The LMV VPg and cellular eIF4E interact with viral forms associated with coat proteins (CPs) in planta.

The presence of VPg and eIF4E within complexes, including CPs, was first assessed in crude extracts from infected plants.

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A two-sites triple antibody sandwich–enzyme-linked immunosorbent assay (2S-TAS-ELISA) (Roudet-Tavert et al. 2002) was performed on extracts of *Nicotiana benthamiana* systemically infected with the LMV-AF199 isolate (Krause-Sakate et al. 2002), using anti-eIF4E (α eIF4E) and anti-VPg (α VPg) antibodies (described below). These antibodies were used as baits in plastic wells (primary antibody), and anti-LMV (α CP) antibodies directed against the CP were used for CP detection (secondary antibody: conjugate). A significant signal was observed in both cases, revealing that populations of CPs present in infected plant extracts were physically associated with VPg, eIF4E, or both proteins (Fig. 1). Values obtained from CP complexes trapped in plastic wells through α CP were comparatively much higher than values obtained when eIF4E or VPg antibodies were used, indicating signal amplification due to an excess of CP with respect to eIF4E and VPg. Indeed, as it uses α CP for detection, the ELISA test probes for the total amount of CP in whatever form it participates. No significant signal was observed when performing the test with infected plant extracts using anti-green fluorescent protein (α GFP) antibodies in place of eIF4E/VPg antibodies or with healthy plant extracts (negative control).

In planta, the amount of VPg- and eIF4E-labeled LMV particle subpopulations varies in the course of systemic infection.

Within the infected plant, the virus is present as various nucleoprotein complexes, including transient CP-RNA structures and fully assembled particles. VPg proteins of *Potato virus A* (PVA) and *Potato virus Y* (PVY) (Torrance et al. 2006) and, more recently, LMV (Nault et al. 2015) were previously found, associated with a subpopulation of particles purified from infected plants. Consequently, it is reasonable to assume that a part of the α VPg trapped structures containing CPs revealed in the infected plant extracts are composed of virus particles. In preliminary experiments, the analysis of eIF4E and VPg labeled subpopulations originating from heterogeneous leaf batches, in particular, samples originating from various harvesting times postinoculation or a leaf at various growth stages within a given plant, returned poorly reproducible results. Consequently, we assessed if the size of the VPg/eIF4E-LMV subpopulations varied during virus systemic infection. 2S-TAS-ELISA was performed on plant extracts from leaves located at different levels above the inoculated leaf, at 13, 21, and 28 days postinoculation (dpi). As illustrated in Figure 2A, the VPg- and eIF4E-labeled subpopulations reached a maximum during the first 13 dpi in leaves 2 and 3. At 21 and 28 dpi, the proportion of these populations within the total virus load was reduced. This kinetic feature was observed in several independent experiments, showing that the subpopulations of VPg- and eIF4E-labeled particles reached a maximum between 10 and 13 dpi (Supplementary Table S1). Such a synchronous occurrence of VPg- and eIF4E-labeled viral forms suggests a structural link between VPg and eIF4E on the virus. We then looked at the presence of these labeled forms in purified particle preparations. As high-quality particles were required, we used a soft purification protocol (described below). The viral RNA associated to these particles can be translated in vitro with high efficiency (Gabrenaitė-Verkhovskaya et al. 2008). Following this procedure, potyviral particles of high physical integrity could be recovered, as was confirmed by transmission electron microscopy and atomic force microscopy (AFM) imaging. A time-dependency in the proportion of VPg- and eIF4E-labeled subpopulations was observed in purified LMV particles, with a maximum of labeled forms between 10 and 13 dpi (Fig. 2B). A possibility exists that the extraction procedure put in closer contact virus particles, VPg, and eIF4E, thereby favoring interactions. However, the time-dependency in the

proportion of VPg- and eIF4E-labeled subpopulations in purified LMV particles substantiates a biological trait. Hence, we are inclined to consider that neither leaf tissue grinding nor the mild conditions used for particle preparation had a significant impact on the original VPg and eIF4E interaction with the particle.

Mapping of VPg regions accessible on the viral particle surface.

We investigated the trapping of the particles by α 21DC, a monoclonal antibody previously described as recognizing an epitope of 17 amino acids within the VPg central region. This structural feature was demonstrated as being involved in the interaction between VPg and eIF4E (Roudet-Tavert et al. 2007). LMV particles, either originating from plant extracts (not shown) or from purified preparations, were efficiently trapped with both polyclonal α VPg and α 21DC (Fig. 3). Consequently, a part of the particle population exposes the VPg central domain, which is available for α 21DC recognition. The subpopulation trapped by α VPg could represent the totality of VPg-labeled LMV particles, including both particles with free VPg and particles with VPg bound to cellular eIF4E or other proteins, such as HCPro (the potyvirus helper component). The latter is known to compete with eIF4E for binding to the VPg central domain (Roudet-Tavert et al. 2007). As a matter of fact, the presence of HCPro was previously imaged together with VPg in a tip structure at one end of PVA and PVY particles by AFM and immuno-electron microscopy (Torrance et al. 2006). We previously showed that α 21DC is a competitive inhibitor of eIF4E association with VPg (Roudet-Tavert et al. 2007). A part of the particles could be trapped by α 21DC, which indicated that an eIF4E-free VPg was exposed at their surface. We investigated the ability of this type of particle to bind to an exogenous eIF4E.

Fig. 1. In planta immunodetection of eIF4E and VPg associated with *Lettuce mosaic virus* (LMV) coat proteins. Immunodetection was performed using two-sites triple antibody sandwich–enzyme-linked immunosorbent assays. Wells were coated with antibodies as indicated. Crude extracts from LMV-inoculated plants (IP) (gray bars) or healthy plants (HP) (dotted bars). *N. benthamiana* plant extracts were added to the coated wells. The amount of LMV particles trapped in the wells was measured by colorimetry at 405 nm, using LMV conjugate (α CP). A_{405} = absorbance at 405 nm, mean values $\pm$ standard deviation of three replicates obtained in a typical experiment. Negative control, anti-green fluorescent protein (α GFP) (coating). Student test: α CP/HP, P value < 0.00001 ; α VPg/HP, P value < 0.0001 ; α eIF4E/HP, P value < 0.005 ; α GFP/HP, P value > 0.1 .

A recombinant lettuce eIF4E binds to the LMV particle.

The interaction of a recombinant form of lettuce eIF4E with LMV particles was tested in a 2S-TAS-ELISA experimental set-up. Wells were precoated with α eIF4E to retain the recombinant eIF4E, and plant extracts or purified virus particles were

added. A significant and reproducible signal was obtained, showing that the extract contained an eIF4E-free subpopulation of LMV particles (Fig. 4A). In the absence of recombinant eIF4E, the subpopulation of eIF4E-labeled particles was also revealed. The eIF4E-free subpopulation was analyzed. When purified particles were

Fig. 2. Variation of populations of VPg and eIF4E-associated *Lettuce mosaic virus* (LMV) particles during virus migration in planta. **A**, Leaves from immediately above the inoculated leaves, numbered L1 through L7, were harvested at 13, 21, and 28 days postinoculation (dpi), and their virus load was quantified by two-sites triple antibody sandwich–enzyme-linked immunosorbent assay (2S-TAS-ELISA); 13 dpi: L1, 2, 3 (white bars); 21 dpi: L1, 2, 3, 4, 5 (gray bars); 28 dpi: L1, 2, 3, 4, 5, 6+7 (black bars); healthy plant (dotted bars). Inset: to facilitate comparisons, absorbance at 405 nm (*A405*) values corresponding to α VPg- or α eIF4E-trapped LMV populations were divided by *A405* values corresponding to the total α CP-trapped LMV population, corrected from healthy-plant unspecific signal (dotted bars). The highest ratios (13 dpi) are plotted above bars. **B**, LMV particles were purified from a pool of (L2 + L3) leaves of infected plants harvested at 10 to 13 dpi (pLMV-1, white bars) or at 21 to 25 dpi (pLMV-2, black bars) and the virus load was quantified by 2TAS-ELISA. Unspecific signal: PB = phosphate buffer (dotted bars); *A405* = mean values $\pm$ standard deviation of three and four replicates (A and B, respectively).

incubated with recombinant eIF4E before deposition into wells, a significant increase (P value < 0.05) of the amount of trapped particles was observed (Fig. 4B). It is likely that this exogenous eIF4E supplementation permitted the recruitment of eIF4E-free particle subpopulations. It was deduced that at least two types of particles, eIF4E-free and pre-existing eIF4E-labeled particles, were present in the preparation.

Our results indicate that a subpopulation of virus particles could physically interact with an exogenous eIF4E. An eIF4E-interacting domain was previously mapped in the VPg central region (Roudet-Tavert et al. 2007). We show above (Fig. 3) that this region is exposed at the surface of the LMV particle. It is likely that the eIF4E-binding site displayed on the particle, is the VPg central domain. *In planta*, this domain could trap the endogenous eIF4E that we found associated to the particle. In order to validate our hypothesis, we performed ELISA competition experiments.

Lettuce eIF4E competes with a monoclonal anti-VPg central domain antibody for binding to the LMV particle.

We investigated whether LMV particle incubation with purified eIF4E could affect their trapping in wells coated with $\alpha 21DC$, the monoclonal antibody directed toward the VPg central domain. A significant decrease of the signal was observed both from plant extracts (Fig. 5A) and purified particles (Fig. 5B). This suggests that eIF4E and $\alpha 21DC$ compete for the binding to the LMV particle at a site located in the central region of the VPg. In a complementary assay, a preincubation of the plant extract with $\alpha 21DC$ prevented the trapping, in eIF4E-coated wells, of a significant amount of particles (Fig. 5A). From these results, we concluded that eIF4E binds to LMV particles through an interaction with the VPg central domain. Similarly, when purified particles were preincubated with a noninfected plant extract, a signal decrease was also observed (Fig. 5B). We hypothesize the involvement of cell proteins such as eIF4E or other factors competing with $\alpha 21DC$ for the binding to VPg central domain.

The binding of eIF4E on LMV particle is imaged by AFM-scanning electrochemical microscopy (SECM).

In order to visualize the location of eIF4E on the LMV particle surface, we made use of immunoredox molecule touching (Mt)/AFM-SECM, a high-resolution correlative in-situ imaging technique

we developed previously (Anne et al. 2011) and recently used to locate VPg at the extremity of LMV particles (Nault et al. 2015). This technique requires that the virus is first labeled by primary antibodies, specific to the sought viral protein. These primary antibodies are then made electrochemically “visible” using secondary antibodies carrying viewable tags consisting of redox ferrocene polyethylene glycol (Fc-PEG) chains. Hence, in the present work, LMV particles were adsorbed onto an ultraflat gold surface. The surface was subsequently saturated with bovine serum albumin (BSA), was then exposed to α eIF4E primary antibodies, and finally, was exposed to redox-labeled secondary antibodies. Mt/AFM-SECM imaging of the surface then allowed topography and current images to be simultaneously acquired in situ. As exemplified in Figure 6A, individual intact LMV particles could easily be identified from topography images. Interestingly, for a subpopulation of the particles examined, the correlated current image concomitantly showed a single bright current spot, which was specifically located at only one of the extremities of the virus particle (Fig. 6B and C; Supplementary Fig. S1A and B). The ELISA test used in this study cannot allow an accurate quantitation of the eIF4E-labeled particle subpopulation; however, AFM-SECM by simple counting gives access to a reliable value of 30% of the total particles within the sample (purification from 10- to 15-dpi extracts). For the remaining population of particles, no such current spot was visible anywhere in the current image. Therefore, the observed current spot corresponds to the selective electrochemical detection by the Mt/AFM-SECM probe of the immune complex formed between the redox-labeled antibody, α eIF4E antibodies, and eIF4E. Localization of this immune complex at one of the extremities of the LMV virus particles, where VPg was previously found (Nault et al. 2015), strongly supports, on a structural basis, the hypothesis of binding of eIF4E to VPg.

DISCUSSION

The potyvirus particle is made up of about 2,000 identical CP subunits, but, despite its filamentous monotonous structure, it harbors, at its very end, a set of viral factors (VPg, HcPro, cylindrical inclusion [CI]) whose role is poorly understood (Gabrenaite-Verkhovskaya et al. 2008; Torrance et al. 2006). Among these proteins, the VPg was experimentally demonstrated

Fig. 3. VPg labeled *Lettuce mosaic virus* (LMV) subpopulation analysis. Monoclonal $\alpha 21DC$ and polyclonal αVPg antibodies raised against VPg were coated into the wells as indicated. The trapping of VPg labeled particles from LMV purified solution (pLMV) was quantified in a two-sites triple antibody sandwich–enzyme-linked immunosorbent assay. 21CD: monoclonal antibody directed against the VPg central domain. Unspecific signal: PB = phosphate buffer (dotted bars); A405 = absorbance at 405 nm, mean values $\pm$ standard deviation of three replicates.

as being an intrinsically disordered protein (IDP) (Chroboczek et al. 2012; Hébrard et al. 2009; Rantalainen et al. 2008). IDPs are devoid of stable tertiary structures under physiological conditions and gain a stable three-dimensional structure only when they interact with their target molecules (Boehr and Wright 2008; Habchi et al. 2014). The VPg can be considered as a hub protein, interacting with both various host and viral factors as well as with the viral RNA (Charon et al. 2016; Jiang and Laliberté 2011; Martínez

et al. 2016). Of special interest is the VPg-disordered central domain, shown in this study to be exposed to the particle surface. This domain interacts with various partners, such as HcPro and eIF4E (Roudet-Tavert et al. 2007). In this study, we have shown that eIF4E is found associated to the viral particle through a specific interaction with the VPg. This observation was made both from infected plant extracts and from purified viral particles. It is legitimate to ask what could be the biological meaning of this

Fig. 4. In vitro interaction between a recombinant form of lettuce eIF4E and *Lettuce mosaic virus* (LMV) particles. **A**, Purified eIF4E was incubated, as indicated, into α eIF4E precoated wells and LMV-infected plant extract (IP) or purified particles (pLMV) were added. HP = healthy plant. **B**, Purified LMV particles (pLMV), either alone or preincubated with purified eIF4E, were incubated into α eIF4E precoated wells. The LMV-eIF4E particles retained in the wells were revealed by performing two-sites triple antibody sandwich–enzyme-linked immunosorbent–based assays as describe above. A405 = absorbance at 405 nm, mean values $\pm$ standard deviation of between three and five replicates. PB = phosphate buffer, *P* value < 0.05. Dotted bars show unspecific signal.

feature. Virus genome replication requires the synthesis of the viral replicase (RNA-dependent RNA polymerase [RdRP]). Consequently, RNA decapsidation and its subsequent translation into polyprotein must be two of the earliest events of the virus cycle. The potyvirus infectious cycle relies on a successful interaction between the VPg and eIF4E (Charron et al. 2008; Léonard et al. 2000). Because cellular mRNAs translation processes through an initiation step involving an interaction between the mRNA 5' cap and eIF4E, it is tempting to hypothesize that the viral VPg could mimic this cap, thus hijacking the translation machinery. But it was clearly observed that, for several potyviruses, translation initiation of the viral RNA mainly processes through the positioning of the ribosome small subunit on an internal ribosome entry site (IRES) within the 5' untranslated region (UTR) and this independently of eIF4E (Zhang et al. 2015). However, mutations that, in the VPg from *Tobacco etch virus*, disturb eIF4E

binding, abolish stimulation of IRES-mediated translation in wheat germ extract (Khan et al. 2008). In fact, the VPg was found to play a dual role in favoring viral 5' UTR-mediated translation while inhibiting cellular mRNA cap-dependent translation by decreasing the affinity of the eIF4E for the cap structure (Khan et al. 2008; Miyoshi et al. 2008). Perhaps most interestingly, an overexpression in trans of the PVA VPg in planta is associated with an enhancement of the viral RNA translation mediated by the IRES and this effect requires eIF4E (Eskelin et al. 2011). Alternatively, VPg covalently linked to the RNA could be only necessary for the first round of translation after disassembly of viral particles (Revers and García 2015). Regardless of the precise role of the VPg-eIF4E complex, taken together, these data provide strong evidence that this interaction is a key player in one or both genome translation or replication. Translation initiation factors are tightly regulated. It is likely that not

Fig. 5. Competition between a lettuce eIF4E and an anti VPg monoclonal antibody for binding to the *Lettuce mosaic virus* (LMV) particle. Purified lettuce eIF4E or α 21DC were coated into the wells and an overlay of **A**, LMV-infected plant crude extract (IP) or **B**, LMV purified particles (pLMV) was added. When indicated, the extract was supplemented with purified eIF4E (α 21DC coating), or α 21DC (purified eIF4E coating), or healthy plant extract (α 21DC coating), before trapping. A405 (absorbance at 405 nm), mean values $\pm$ standard deviation of five (A) or three replicates (B). HP = healthy plant. Unspecific signal: PB = phosphate buffer (dotted bars).

much free eIF4E is available for viral genome translation initiation. Indeed, it was observed that up to 68% of eIF4E forms (Iborra et al. 2001) are sequestered in the nucleus and are involved in various functions such as premRNA splicing or non-sense mediated decay, among others (Strudwick and Borden 2002). In the cytoplasm, eIF4E is the mRNA cap target within the eIF4F translation initiation complex. Given the strong affinity (K_D in the nanomolar range) of the plant eIF4F subunits to each other, it seems unlikely that these complexes will dissociate once formed (Mayberry et al. 2007). In potyviruses, the natural mode of plant infection is virus particle transmission by aphid vectors. It was demonstrated that very few infectious particles (one to three) are introduced in the plant by the vector (Moury et al. 2007). In addition, the average number of potyvirus particles infecting a cell was estimated to be one (Tomas et al. 2014). This implies the presence of very few genome-associated VPg within an infected cell. Owing to the probable low concentration of available free eIF4E, the hypothesis that viral particles could bring into the cell a preassembled VPg-eIF4E binary complex linked to the viral RNA is worth considering. It can be inferred from our AFM-SECM data that about one-third of the particles are eIF4E-labeled at 13 dpi, which gives a high probability for the aphid to transfer this type of particle for acquisitions early after inoculation (discussed below). Immediately after cell penetration, the preassembled eIF4E-VPg complex could highjack cytoplasmic free eIF4G, allowing a translation initiation similar to that of mRNAs. This path could be subsequently replaced by the more competitive IRES-mediated translation.

The temporal distribution of CP, RdRP, and VPg at various time periods after inoculation was precisely investigated (Baunoch et al. 1991). It reveals that all proteins are detected simultaneously in systemic leaves from the fifth day after inoculation. All proteins increase in concentration until the seventh day and, then, show no further increase, with the exception of the CP. Although small concentrations of VPg and RdRP are detected in the cytoplasm, most of these proteins are relocated just after synthesis in the nucleus, in which they are sequestered as nuclear inclusions NIa (VPg-Pro) and NIb (RdRP). At 14 days after infection, both proteins are found only as inclusions in the nucleus. The CPs are found at all stages of infection only in the cytoplasm. Our

observations are in accordance with these data, as the subpopulation of VPg and eIF4E-labeled particles increase up to 14 dpi, after which their amount decreases progressively with respect to the population of nonlabeled particles. Several features of virus biology suggest that a low translation efficiency could be required in the early step of infection. First, maintaining a low CP concentration within the cell is necessary. The single or so viral genome present within the cell at the start needs to be amplified. This amplification requires the biosynthesis of the virus replicase (RdRP) and other viral factors, such as CI and VPg, shown to be involved in replication. However, owing to the strict stoichiometry of the viral RNA polycistronic organization, each time a translation event occurs, the arising polypeptide contains one copy of each viral factor, including the CP. The presence on the RNA strand of very few CP monomers will prevent either the 5', 3' progression of the ribosomes in the translation process or the 3', 5' RdRP progression for replication. It is likely that a weak translation efficiency mediated by the VPg-eIF4E interaction will favor a low concentration of CP. The particle assembly precludes the genome translation-replication steps, and it seems that the amount of CP is a regulator of the balance between genome amplification and particle formation through its phosphorylation state (Ivanov et al. 2003, 2001) and sequestration by the plant HSP70 and its cochaperone (Besong-Ndika et al. 2015; Hafren et al. 2010). Second, maintaining a low concentration of RdRP should favor successful infection. Indeed, RdRP, owing to its high mutational rate (10^{-5}), could easily jeopardize infection efficiency, as, for instance, 40% of potyviral genomes bearing a single nucleotide substitution are lethal (Carrasco et al. 2007). As the mutation rate is an intrinsic feature of the replicase, there is a tradeoff between the necessity of virus amplification and a low number of RdRP available in the cell, tunable through a low translation efficiency (Thébaud et al. 2010). Third, it was previously reported that high viral loads within primary infection sites prevent systemic movement (Lafforgue et al. 2012; Tomas et al. 2014). In the early stage, a part of the VPg produced could be coupled through phosphoester bound to the 5' end of newly synthesized genomic RNAs (Murphy et al. 1991). Following the systemic spread of virus into the plant, more active translation would take over, favoring CP production and particle assembly

Fig. 6. In situ immunoredox molecule touching atomic force microscopy coupled to scanning electrochemical microscopy (Mt/AFM-SECM) imaging of an eIF4E-labeled *Lettuce mosaic virus* (LMV) particle immobilized on a gold surface. **A**, Topography image; **B**, simultaneously acquired current image; and **C**, overlay of the topography and current images showing the precise location of the current spot at one of the extremities of the virus particle. Bottom, Cross-sections of the topography (red trace) and current (blue trace) images taken along the dotted line shown. The position where both topological and current signals correlate is shown by green arrows.

around new-born genomes, while excess VPg and RdRP would be sequestered into the nucleus.

In conclusion, we provide, in this study, the first example of an interaction of potyviral particles with a cellular protein. Binding occurs via the central region of VPg, the viral protein covalently linked to the 5' end of the viral genomic RNA. This result supports the hypothesis of cap mimicry by the VPg. It suggests a sequestration of eIF4E by the particle, readily available for viral cap-dependent translation during the early steps of the viral life in planta.

MATERIALS AND METHODS

Biological material.

Nicotiana benthamiana four-leaves-old seedlings were mechanically inoculated (excluding the first oldest one) with *Lettuce mosaic virus* (LMV) strain AF199 (Krause-Sakate et al. 2002). Leaves were collected at 7 to 35 dpi and were used as crude extracts for ELISA-based assays and particle purification. LMV purified particles were obtained from infected leaves as previously described (Nault et al. 2015). Symptomatic leaves (300 g) were homogenized in cold 0.1 M phosphate buffer, pH 8, containing 0.15% 2-mercaptoethanol and 0.01 M EDTA (1 g of leaves in 2 ml of buffer). Plant debris was spun down at $7,000 \times g$ for 20 min. After filtration through two layers of cheese cloth, Triton X-100 was added to the supernatant (final concentration 3%, vol/vol) and was gently stirred at 4°C for 3 h. The remaining insoluble material was spun down at $12,000 \times g$ for 10 min. Virus particles were precipitated overnight by the addition of PEG6000 (40 g per liter of supernatant) and 0.2 M NaCl, under gentle stirring at 4°C in the dark. Virus particles were recovered by centrifugation at $12,000 \times g$ for 20 min. The virus pellet was suspended in one-tenth of the starting supernatant in 0.1 M cold phosphate buffer, pH 8, containing 1% Triton X-100. This extract was centrifuged at $16,000 \times g$ for 1 h at 4°C. The pellet was gently suspended overnight at 4°C in 0.1 M phosphate, pH 8 (5 ml for 100 g of starting leaves). Ultracentrifuge tubes (Beckman rotor SW41 TI, 13-ml volume) were filled with 5 ml of 30% sucrose in 0.1 M phosphate buffer, pH 8. The virus suspension (2 ml per tube) was gently layered on top of this sucrose cushion and was centrifuged at $150,000 \times g$ for 3 h at 4°C. The pellet containing the virus particles was gently suspended overnight at 4°C in 2 ml of 0.1 M phosphate buffer, pH 8. The virus particles obtained were purified through a 5 to 40% discontinuous sucrose gradient as follows. Centrifugation tubes (Beckman rotor SW 41 TI, 13-ml volume) were filled with sucrose solutions in phosphate buffer: from bottom to top, 1.5 ml of 40%, 3 ml of 30%, 3 ml of 15%, and 1.5 ml of 5%. A volume of 0.5 to 1 ml of virus suspension was carefully layered on top of the gradient. The virus preparation was centrifuged at $80,000 \times g$ at 4°C for 1 h. The pellet was suspended in the minimum amount of phosphate buffer (0.1 M), pH 8. The virus suspension was quantified by absorption spectrometry ($\epsilon_{0.1\% \text{ } 260 \text{ nm}} = 2.6$). Aliquots of 200 μ l of virus (5 to 6 mg/ml) were stored at -80°C.

Lettuce eIF4E was expressed in *Escherichia coli* fused with a N-ter hexahistidine tag, purified according to (German-Retana et al. 2008), and was used in ELISA interaction and competition assays (Roudet-Tavert et al. 2007).

ELISA-derived binding assays.

Monoclonal antibody directed against the VPg from LMV (α 21DC), polyclonal α VPg, and polyclonal rabbit α eIF4E or against LMV coat protein (α -LMV) (Roudet-Tavert et al. 2007) were used as trapping antibodies in a 2S-TAS-ELISA assay (Roudet-Tavert et al. 2002). LMV-infected crude plant extract or purified LMV particles were added (50 μ g/ml in phosphate buffered saline [PBS] Tween 0.2%). Polyclonal rabbit antibodies

against LMV virus particles coupled to alkaline phosphatase (LMV conjugate) were used for particle detection. In some experiments, a specific GFP antibody (α GFP) was used as a negative control.

ELISA test conditions for eIF4E interaction and competition assays.

For interaction tests, purified recombinant eIF4E was incubated, either alone (2 μ g/ml) or after a 2-h premix at 37°C (1 μ g/ml) with LMV particles in α eIF4E pre-coated wells. For ELISA competition assays, purified eIF4E (4 μ g/ml) or α 21DC (5 μ g/ml) were coated into the wells, followed by a saturation step (2% BSA in PBS). LMV plant extract or purified LMV particles (10 μ g/ml in PBS Tween 0.2%), preincubated (2 h 37°C), respectively, with α 21DC (2 μ g/ml) or purified eIF4E (1 μ g/ml) added. After a washing step, specifically retained LMV particles were revealed by the addition of LMV conjugate (dilution 1:500).

Immunoredox Mt/AFM-SECM imaging.

Polyclonal antirabbit antibody tagged with Fc-terminated PEG chains was used as a redox secondary antibody to reveal the location of the primary α eIF4E rabbit antibody. The Fc-PEGylated secondary antibody conjugate was prepared by reacting home-synthesized NHS-PEG₃₄₀₀-Fc chains with an α -rabbit antibody and purifying, following a procedure detailed elsewhere (Nault et al. 2015). For all operations including imaging, the medium was 10 mM phosphate buffer (PB), pH 7.4. Purified LMV particles (from 12-dpi infected plants, content in eIF4E-labeled LMV, 20% ELISA quantitation) (60 μ g/ml) were first adsorbed onto a freshly peeled ultraflat gold surface for 10 min. A 1-h BSA (1% in PB) saturation step was then carried out. Subsequently, the surface was exposed to the primary α eIF4E antibody (5 μ g/ml) for 1 h. Finally, the surface was exposed overnight to the redox Fc-PEGylated secondary antibody (20 μ g/ml). The Mt/AFM-SECM instrumental set-up, imaging protocol and conditions were as previously described (Nault et al. 2015).

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