Formation of virions is strictly required for turnip yellows virus long-distance movement in plants

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Viral genomic RNA of the Turnip yellows virus (TuYV; genus Polerovirus; family Luteoviridae) is protected in virions formed by the major capsid protein (CP) and the minor component, the readthrough (RT*) protein. Long-distance transport, used commonly by viruses to systemically infect host plants, occurs in phloem sieve elements and two viral forms of transport have been described: virions and ribonucleoprotein (RNP) complexes. With regard to poleroviruses, virions have always been presumed to be the long-distance transport form, but the potential role of RNP complexes has not been investigated. Here, we examined the requirement of virions for polerovirus systemic movement by analysing CP-targeted mutants that were unable to form viral particles. We confirmed that TuYV mutants that cannot encapsidate into virions are not able to reach systemic leaves. To completely discard the possibility that the introduced mutations in CP simply blocked the formation of RNP complexes, we tested in trans complementation of TuYV CP mutants by providing WT CP expressed in transgenic plants. WT CP was able to facilitate systemic movement of TuYV CP mutants and this observation was always correlated with the formation of virions. This demonstrated clearly that virus particles are essential for polerovirus systemic movement.

INTRODUCTION

Members of the genus Polerovirus belonging to the family Luteoviridae are phloem-restricted viruses transmitted from plant to plant by aphids. Virus replication occurs in the nucleated companion and phloem parenchyma cells, where virions have been observed (Esau & Hoefert, 1972; Mutterer et al., 1999; Shepardson et al., 1980), and cell-to-cell movement should take place between these two types of cell. To initiate new infection sites, the virus is transported to distant tissues through the sieve elements. Long-distance transport, also called systemic movement, is a common step shared by all plant viruses, which use the phloem flow to invade the whole plant. Two viral forms of long-distance transport have been described: virions protecting the viral genome in a shell formed by the assembly of capsid protein (CP) subunits, and ribonucleoprotein (RNP) complexes in which the viral genome is associated with viral and/or cellular proteins (for a review, see Hipper et al., 2013). Whilst most viruses use only one transport form, some others adopt multiple forms to be transported to non-inoculated tissues. The three genomic RNAs (gRNAs) of the Potato mop-top virus (genus Pomoavirus; family Virgaviridae) are transported individually in the same host using different viral structures: RNA1 and RNA2 can spread as RNP complexes in the absence of the CP, but formation of virions is strictly required for the long-distance movement of RNA3 (McGeachy & Barker, 2000; Savenkov et al., 2003; Torrance et al., 2009, 2011; Wright et al., 2010). Other viruses seem to use preferentially one of the two viral structures depending on the host. As an example, Cymbidium ringspot virus (CymRSV; genus Tombusvirus; family Tombusviridae) moves in Nicotiana clevelandii in a virion form, but it can also be transported in a CP-independent manner in Nicotiana benthamiana (Dalmay et al., 1992).

Turnip yellows virus (TuYV; genus Polerovirus; family Luteoviridae) has icosahedral virions of ~25 nm of diameter that encapsidate the viral RNA genome. TuYV virions are composed of the major CP (23 kDa) and the minor component, the readthrough protein (RT* protein, ~54 kDa), that arises from a C-terminal cleavage of the native product of 74 kDa (RT protein). The RT protein is a C-terminal extension of CP that is produced by a bypass of the CP stop codon. CP, RT* and RT proteins are involved in polerovirus long-distance transport (Brault et al., 2000,
2003; Bruyère et al., 1997; Peter et al., 2008). Poleroviruses also encode a protein of 17 kDa (P4 protein) that shares biochemical and cellular characteristics with conventional cell-to-cell movement proteins, such as its ability to bind ssRNA, target plasmodesmata, increase the size exclusion limit of plasmodesmata, form homodimers and become phosphorylated (Hofius et al., 2001; Schmitz et al., 1997; Sokolova et al., 1997; Tacke et al., 1993). Although P4-defective Potato leafroll virus (PLRV; genus Polerovirus; family Luteoviridae) mutants were, in some hosts, impaired in their ability to reach systemic leaves (Lee et al., 2002), the exact role of P4 protein in the polerovirus cycle remains to be determined.

Polerovirus particles were detected in plasmodesmata connecting nucleated phloem cells and sieve elements, suggesting that virions are the long-distance mobile form of poleroviruses (Esau & Hoefert, 1972; Mutterer et al., 1999; Shepardson et al., 1980). Moreover, polerovirus virions were also observed in sap collected from infected plants (V. Brault, unpublished results), which represents the virus reservoir sampled by aphids that allows virus transmission. Mutations in the CP gene of TuYV that disrupt virion formation also inhibit systemic transport (Brault et al., 2003), reinforcing the hypothesis that virus particles are required in polerovirus long-distance movement. Nevertheless, it is conceivable that RNP complexes may participate, together with virions, in this process. Indeed, accumulation of TuYV CP mutants in systemic leaves has not been evaluated by reverse transcriptase (RT)-PCR, leaving the possibility that RNP complexes could migrate to non-inoculated leaves, but less efficiently than virions.

Therefore, we reinvestigated the requirement of virions for polerovirus systemic movement by generating new CP-targeted mutants unable to form viral particles. Infection of non-inoculated leaves was followed by RT-PCR. We confirmed that TuYV CP mutants unable to form virus particles were also blocked in their systemic movement. To discard the possibility that the mutations introduced into the capsid protein gene inhibited the formation of RNP complexes, we tested in trans complementation of TuYV CP mutants defective in movement by providing WT CP expressed in transgenic plants. The ectopically expressed CP was able to promote systemic movement of the tested TuYV CP mutants. Moreover, this long-distance transport was always correlated with the formation of virions, which confirms the requirement of virus particle formation for polerovirus systemic movement.

RESULTS

Mutations in CP

All the observations and experiments performed so far have been in favour of a role of virions in the long-distance transport of poleroviruses (Brault et al., 2003; Esau & Hoefert, 1972; Kaplan et al., 2007; Lee et al., 2005; Mutterer et al., 1999; Shepardson et al., 1980; Ziegler-Graff et al., 1996). To determine whether RNP complexes can also participate in the systemic transport of poleroviruses, we introduced point mutations in the CP sequence of TuYV to obtain virus mutants unable to form virus particles, but which could retain their potential to form RNP complexes. Two domains of the TuYV CP sequence corresponding to surface epitopes on PLRV CP (previously referred to as epitopes 5 and 10; Torrance, 1992; Terradot et al., 2001) were targeted by site-directed mutagenesis. These epitopes are situated in the shell domain (S) forming the capsid structure and located downstream of the N-terminal arginine-rich domain (R). This latter domain is thought to be oriented towards the inner capsid where it can associate with viral RNA (Chavez et al., 2012; Terradot et al., 2001; Torrance, 1992). The high sequence homology between PLRV and TuYV CP (62 % amino acid identity) allowed the positioning of these epitopes between aa 77–92 and 165–175 on the TuYV CP sequence (Brault et al., 2003; Mayo & D’Arcy, 1999; Terradot et al., 2001). Seven TuYV CP mutants bearing one or two amino acid changes in the potential TuYV epitope 5 were obtained (Fig. 1). The mutations affect either the overall CP charge (S79R/G80R and D90N/C91R), or modify the aromatic phenylalanine residue (F84Q/G85W, T83S/F84L and F84V) or proline (T83P/P86T) positions. A conservative mutation that does not change the overall charge of the CP was also introduced in one of the mutants (S87G). Additionally, the previously described mutant W166R affecting the putative TuYV epitope 10 (Brault et al., 2003) was also studied. In the mutant NoCPRT, the expression of CPs was completely abolished by deleting 37 nt in the CP sequence spanning the CP start codon. None of the aforesaid mutations in the ORF3 alter the amino acid sequence of the P4 protein encoded by the overlapping ORF4 (Fig. 1).

Effect of CP mutations on virus accumulation and virion formation

To evaluate the ability of TuYV CP mutants to infect plants locally, Montia perfoliata leaves were infiltrated with a suspension of Agrobacterium tumefaciens transformed by the corresponding binary plasmids containing the full-length viral mutated sequences. Six days post-infiltration (p.i.), virus replication and accumulation were assessed by ELISA, Northern blotting and RT-PCR in the infiltrated leaves. Among all mutants, only mutant S87G was able to infect the infiltrated leaves to a virus titre similar to that of WT TuYV, as measured by ELISA (Table 1). None of the other mutants were detectable by ELISA (Table 1). However, the presence of the viral gRNA and subgenomic RNA (sgRNA) of all TuYV CP mutants in the infiltrated leaves was confirmed by Northern blotting (Fig. 2a), indicating that all CP mutants, also detectable by RT-PCR in the infiltrated leaves (Fig. 2b, upper panel), were able to replicate. The viral RNAs synthesized by TuYV CP mutants accumulated at a much lower level compared with WT

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viruses, except in the case of the S87G mutant for which gRNA and sgRNA accumulation was comparable to that of WT virus (Fig. 2a). In addition, replication of the mutants S79R/G80R, D90N/C91R, T83P/P86T, F84Q/G85W and W166R was confirmed by the detection of both CP and RT proteins in transcript-transfected Chenopodium quinoa protoplasts (Fig. S1a, available in JGV Online). Accumulation of CP and RT proteins in the leaves agro-infiltrated by all CP mutants confirmed their replication ability, but the detection level varied between experiments, e.g. as shown in the Western blot presented in Fig. S1(b).

An unexpected observation was that although the mutated CP and RT proteins synthesized by the different CP mutants were detected by Western blotting, they were not detectable by ELISA. As ELISA and Western blot analyses were performed using different antibodies (commercial polyclonal antiserum raised against whole-virus particles and polyclonal antisera directed against TuYV CP or RT polypeptides, respectively), we hypothesized that the different antisera could recognize distinct protein conformations. To address this question, TuYV WT virions were disrupted by an SDS treatment before being assayed by ELISA. As shown in Fig. S2(a), using the serum raised against virions and similar amounts of protein, disassembled CP subunits gave a lower absorbance than whole virions. Disruption of virions by SDS treatment was confirmed by immunosorbent electron microscopy (ISEM) observations.

**Fig. 1.** Position of amino acid changes (in black letters) in the TuYV CP sequence shown below the genetic organization of TuYV gRNA and sgRNA. The CP protein (202 aa) is represented by a white rectangle, and the position of the putative epitopes 5 (ep.5) and 10 (ep.10) on the TuYV CP sequence is indicated. In TuYV mutant XnY, the ‘X’ amino acid in the original sequence is substituted for a ‘Y’ at the position ‘n’. The deletion introduced in the NoCPRT mutant, spanning the CP start codon, is represented by a discontinuous line.

**Table 1.** Detection of TuYV CP mutants by ELISA in infiltrated and systemic leaves of *M. perfoliata*

<table>
<thead>
<tr>
<th>Inocula</th>
<th>Infiltrated leaves (6 days p.i.)</th>
<th>Systemic leaves (15 days p.i.)</th>
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<tbody>
<tr>
<td></td>
<td>Plants infected/total inoculated*</td>
<td>OD&lt;sub&gt;405&lt;/sub&gt;†</td>
</tr>
<tr>
<td>WT</td>
<td>31/31</td>
<td>1.53±0.50</td>
</tr>
<tr>
<td>NoCPRT</td>
<td>0/21</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td>W166R</td>
<td>0/31</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>S79R/G80R</td>
<td>0/21</td>
<td>0.12±0.00</td>
</tr>
<tr>
<td>D90N/C91R</td>
<td>0/21</td>
<td>0.12±0.02</td>
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<tr>
<td>T83R/P86T</td>
<td>0/21</td>
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<td>F84Q/G85W</td>
<td>0/21</td>
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<tr>
<td>F84V</td>
<td>0/21</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>S87G</td>
<td>21/21</td>
<td>1.55±0.36‡</td>
</tr>
<tr>
<td>Mock§</td>
<td>0/5</td>
<td>0.17±0.02</td>
</tr>
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*Number of *M. perfoliata* plants positive by ELISA/total number of plants agroinoculated with the viral construct.
†Mean OD<sub>405</sub>±SD of the infected plants after 30 min of substrate incubation.
‡P>0.05 (Student’s t-test; Minitab software) comparing S87G accumulation with that of WT TuYV.
§Non-inoculated plants.
(Fig. S2b), and the quantity of CP in each sample (untreated and treated virions) was evaluated by SDS-PAGE (Fig. S2c). Therefore, the absence of detection of CP mutants in inoculated leaves by ELISA suggested strongly an inability of the mutated virus to form virions. This hypothesis was further evaluated by performing ISEM on infiltrated leaf homogenates to observe virions. Typical icosahedral virus-like particles of ~25 nm in diameter were observed in leaves infiltrated with the WT virus or the S87G mutant, but no virus-like particles were seen in leaves infiltrated with any of the other TuYV CP mutants (Fig. 3 and data not shown).

Taken together, these results show that all TuYV CP mutants were replication-competent in infiltrated leaves, and synthesized detectable amounts of mutated CP and RT proteins. However, except for mutant S87G, for which the modified CP could still assemble into virions, none of the CP mutants formed virus particles.

**Effect of CP mutations on systemic infection**

The capacity of TuYV CP mutants to reach systemic leaves of *M. perfoliata* was further analysed by ELISA and RT-PCR at 15 days p.i. As in inoculated leaves, the S87G mutant was the only mutant that could be detected by ELISA in systemic leaves (Table 1). The accumulation level was similar to the WT virus level as indicated by Student’s *t*-test. RT-PCR further confirmed the ability of the S87G mutant to move to the upper leaves (Fig. 2b, lower panel). Conversely, none of the other TuYV CP mutants were detected in the systemic leaves by either of the two methods (Table 1, Fig. 2b, lower panel). The stability of the nucleotide substitution introduced into the CP sequence of the S87G mutant was investigated in the systemically infected leaves by RT-PCR followed by sequencing of the DNA fragment encompassing the mutation. In the two plants analysed, the viral population was homogeneous and carried the original mutation (data not shown), confirming that the long-distance movement of this mutant was not affected by the modification of the CP sequence. Viral particles of the S87G mutant purified from systemic infected leaves were also fully aphid transmissible (data not shown), suggesting that the modified amino acid in the CP sequence did not affect virus acquisition or inoculation by the aphids.

Taken together, these results showed a strong correlation between viral particle formation and virus long-distance movement, as the only mutant virus able to reach systemic leaves was the sole mutant capable of forming virions.

**In trans complementation of movement-defective TuYV CP mutants**

In previous experiments, we found evidence supporting the notion that virus particles are the transport form of TuYV.
However, the possibility that CP mutants were unable to produce RNP complexes could not be discarded completely. To address this issue, we expressed WT TuYV CP in transgenic *Arabidopsis thaliana* Col-0 plants using a binary plasmid containing the TuYV CP sequence under the control of the cauliflower mosaic virus 35S promoter. These plants were referred to as P35S:CP. TuYV CP expressed in the transgenic plants was detected by Western blotting in the F3 homozygous P35S:CP line (Fig. 4), whilst no signal was obtained by ELISA (Table S1, mock P35S:CP). The absence of detection by ELISA of TuYV CP expressed in the transgenic plants may result from a low accumulation of the ectopically expressed CP or, as discussed previously, from the absence of virions in these CP-expressing plants. We confirmed by ISEM that no virus-like particles were observed in non-inoculated transgenic plant extracts that underwent a virus purification procedure (Fig. 7a, P35S:CP mock).

The P35S:CP plants were agro-infiltrated with two CP mutants, W166R and NoCPRT, or with the WT TuYV. The level of virus accumulation was assayed by ELISA at 6 days p.i. in the infiltrated leaves, or at 12 days p.i. in the systemic leaves. WT TuYV accumulated at similar levels in P35S:CP and Col-0 plants, whereas no mutants were detected (Table S1). The viral genome of both CP mutants was, however, detected by RT-PCR (Fig. 5a) and Northern blotting (Fig. 6) in the infiltrated leaves. To address whether these mutants were able to move to systemic leaves in the CP-expressing plants, the presence of viral RNA in non-inoculated leaves was evaluated by RT-PCR. W166R viral RNA was detected in 16 out of 51 inoculated plants; similarly, the NoCPRT genome was detected in four out of 26 inoculated plants (Fig. 5b). Conversely, the viral RNA of the two mutants was not detected by RT-PCR in any of the 26 Col-0-inoculated plants (Fig. 5b). The gRNA and sgRNA of both mutants could also be observed by Northern blotting in the systemic leaves of the P35S:CP plants identified as positive by RT-PCR, but the level of RNA accumulation was considerably lower compared with that of the WT virus and the gRNA of both mutants was only visible clearly on a longer exposure blot (Fig. 6 and data not shown). At 18 days p.i., the viral RNA of both CP mutants was still detected by RT-PCR in systemic leaves of P35S:CP, showing the ability of the mutant to persist in transgenic plants (data not shown).

To discard the possibility that virus movement arose from a reversion of the mutation introduced in the CP sequence, either from secondary mutations appearing on the CP sequence or from a recombination event between the CP sequence on the viral genome and the transgene mRNA, the viral progenies present in the P35S:CP systemically infected plants were analysed by sequencing a RT-PCR-amplified DNA fragment encompassing the CP sequence. At 12 days p.i., the introduced W166R mutation was present in the 16 P35S:CP plants systemically infected with the W166R mutant. Only one of them showed an additional change (S108T) upstream of the introduced mutation (data not shown). Similarly, the deletion introduced in the sequence of the NoCPRT mutant was still present in the virus progeny systemically infecting the four P35S:CP plants and no other modification was observed on the analysed sequence (data not shown).

![Fig. 4. Analysis of the expression of the ectopically expressed TuYV CP in transgenic *Arabidopsis thaliana* (P35S:CP) by Western blotting. The blot was incubated with antibodies raised against TuYV CP. Protein extracts were prepared from non-infiltrated Col-0 plants (Mock Col-0), from Col-0 inoculated with WT TuYV (TuYV Col-0) or from non-infiltrated P35S:CP plants (Mock P35S:CP). Coomassie blue-stained gel served as a loading control. RT, RT protein.](image-url)
To finally address whether the long-distance transport of the W166R and NoCPRT mutants in P35S:CP plants was due to formation of virions or to systemic propagation of RNP complexes containing WT CP expressed in the transgenic plants, viral particles were purified from systemic leaves of W166R- or NoCPRT-inoculated plants. Indeed, because of the phloem restriction of poleroviruses, detecting virus particles of low-accumulating viruses, as is the case of both CP mutants, from ground leaf extracts is very inefficient and requires a purification step to concentrate the virions. Typical virus particles were observed in the purified extracts prepared from W166R- or NoCPRT-infected P35S:CP plants, whereas no such virus particles were present in similar extracts prepared from Col-0-inoculated plants (Fig. 7), suggesting that the long-distance movement of the virus mutants was a consequence of virion formation in the transgenic plants. The RNA content of the virions formed in the transgenic plants infected with the W166R and NoCPRT mutants was further analysed by RT-PCR, and showed that the mutation or the deletion introduced into the respective virus mutants was indeed present.

Although P35S:CP plants can be infected with the WT and TuYV CP mutants, showing an absence of pathogen-derived resistance, we cannot discard completely the hypothesis that endogenous CP mRNA is targeted by the RNA-silencing mechanism. Such a mechanism would interfere with the CP complementation test. To address whether TuYV infection could induce degradation of CP mRNA, transgene expression was assayed by Northern blotting on both infiltrated and systemic leaves. Whilst CP mRNA was detected in the non-inoculated transgenic plants, and in plants inoculated with the W166R and NoCPRT mutants, it was no longer observed in plants infected with the WT virus (Fig. 6). This result shows that the virus-triggered RNA-silencing mechanism is active on endogenous CP mRNA expressed in P35S:CP plants only when the plants were infected by WT virus and not by CP mutants. Whether the absence of CP mRNA targeting was related to the lower accumulation of the two CP mutants in the transgenic plants or to another process needs to be investigated further.

**Fig. 6.** Detection of both viral RNA and CP mRNA in Arabidopsis thaliana P35S:CP plants after infection with WT TuYV or CP mutants. Total RNA was extracted from non-inoculated Col-0 and P35S:CP plants (NI), or from P35S:CP plants infected with WT TuYV, or W166R or NoCPRT mutants. Both infiltrated (I) and systemic (S) leaves were analysed. Viral gRNA and sgRNA, and transgene CP mRNA were detected by Northern blotting using a digoxigenin-labelled probe complementary to 312 nt of the TuYV ORF3 sequence. Ethidium bromide staining of the agarose gel before the transfer onto the membrane was used as a loading control.

**Fig. 7.** Visualization by ISEM of virus particles in P35S:CP transgenic plants inoculated with TuYV CP mutants. A virus purification procedure was performed on non-inoculated (mock) P35S:CP (a) or Col-0 (e) plants, or on systemic leaves of P35S:CP (b–d) and Col-0 plants (f–h) agro-infiltrated with WT TuYV or CP mutants (W166R or NoCPRT). Typical virus particles are indicated by arrows. Bar, 30 nm.
DISCUSSION

From previous studies on TuYV and PLRV CP mutants, it was proposed that poleroviruses are transported to non-inoculated leaves in the form of virions (Brault et al., 2003; Kaplan et al., 2007; Lee et al., 2005; Mutterer et al., 1999; Ziegler-Graff et al., 1996). However, because the presence of the CP mutants in systemic leaves was never evaluated by RT-PCR, the possibility that RNP complexes could migrate to non-inoculated leaves at a level not detectable by ELISA or Northern blotting still remained plausible. Furthermore, if RNP complex formation requires a functional CP, these viral structures might not be formed when a mutated CP is synthesized. For these reasons, we addressed the requirement of CP for long-distance movement of TuYV by studying the transport of newly engineered CP punctual mutants unable to form virions and adopted more sensitive techniques to detect the virus mutants in systemic tissues. One epitope, that by homology with PLRV CP, is located potentially on the surface of the TuYV particle, was targeted specifically in the seven engineered mutants. One additional mutant (W166R) displaying an encapsidation defect, described previously by Brault et al. (2003), was included in the experiment. Among the eight TuYV point mutants studied, only one mutant (S87G) was able to produce virus particles and was fully competent for systemic transport. On the contrary, none of the seven other CP mutants were detected by RT-PCR in the systemic leaves of M. perfoliata plants, even though they replicated in inoculated leaves, and produced the mutated CP and RT proteins. Moreover, their inability to form particles in inoculated leaves reinforced the hypothesis that virions are necessary for the long-distance transport of TuYV. Interestingly, detection of RT protein in leaves agroinoculated with the encapsidation-deficient TuYV CP mutants differs from the observations made with the assembly-deficient PLRV CP mutants, which did not produce or accumulate RT protein at a detectable level in agroinoculated leaves of N. benthamiana (Lee et al., 2005). The authors proposed that the non-incorporated RT protein could be degraded and, in this respect, TuYV would differ from PLRV by having a more stable non-incorporated RT protein.

To assess whether the mutations in the TuYV CP could be detrimental to the formation of both virions and RNP complexes, transgenic plants expressing WT CP were generated in order to perform complementation assays. Strikingly, although CP was expressed constitutively and detected easily as shown by Western blotting, no virus-like particles were observed. This suggests either that TuYV RNA is required to initiate virus particle formation or that the amount of CP subunits is not sufficient to form empty particles. In a previous report, Lamb et al. (1996) showed that virus-like particles could be formed in insect cells expressing the CP of PLRV, which seems contrary to our results. However, the amount of CP synthesized and/or the addition of 17 non-viral amino acids at the N terminus of PLRV CP may explain the formation of particles under these specific heterologous conditions.

When two of the encapsidation-defective mutants (W166R and NoCPRT) were inoculated into these TuYV CP-expressing Arabidopsis thaliana, the TuYV CP synthesized in the transgenic plants was able to complement the defective movement of the two CP mutants as confirmed by the detection by RT-PCR of the viral RNAs in the systemic leaves of inoculated plants. This complementation was, however, poorly efficient, as only 31 and 16% of transgenic plants became infected systemically with the W166R and NoCPRT mutants, respectively. This gain-of-movement function of the two mutants was correlated with the formation of particles that contained only the mutated viral genome, as confirmed by the sequence of the viral progeny present in the purified particles. First, these data support strongly the idea that TuYV viral RNA is required for virion formation by providing potentially an anchor for CP assembly. Secondly, the necessity of TuYV to form virus particles to move systemically is heavily reinforced by the complementation experiments. With regard to the W166R mutant, whether the particles formed in the transgenic plants are only composed of WT CP expressed by the plant or are hybrid particles formed by a hetero-association of mutated and WT CPs is not known. Although virion formation restored the long-distance movement of the two CP mutants, the amount of virions accumulating in infiltrated and systemic leaves was too low to be detected by ELISA; however, virions could be observed after a virus purification step performed from systemically infected leaves. This might be due to the low amount of CP expressed in the transgenic plants, which was below the level of the protein expressed from the replicating virus (Fig. 4). The absence of virus detection by ELISA may also be due to the lower replication rate of the two CP mutants, as shown by the lower detection of gRNA and sgRNA in infected leaves when compared with the WT virus (Fig. 6). We observed that the WT TuYV induced disappearance of the CP transcript in inoculated transgenic plants. In transgenic N. benthamiana expressing the TuYV RT protein, we reported previously a disappearance of the RT protein mRNA by RNA silencing after challenging the plants with the WT virus or with other viruses sharing sequence homology with the TuYV RT protein sequence (Brault et al., 2002). This silencing mechanism had only a moderate effect on the virus accumulation in the plant, probably because of the expression of the RNA-silencing suppressor protein, the P0 protein (Pfeffer et al., 2002). Owing to the high sequence homology between the CP of the two viral mutants, W166R and NoCPRT, and the transgene sequence, such a silencing mechanism was also expected to occur in the inoculated and systemic leaves of the transgenic plants. However, contrary to the observation in the plants infected with WT TuYV, disappearance of the CP transgene was not observed in the mutant-infected plants. The low accumulation of both viral mutants could induce the production of small interfering RNA of too low abundance to efficiently promote the silencing of the CP transgene, resulting in the maintenance of the CP mRNA in the tissues infected with the two CP mutants.
In conclusion, by investigating the long-distance movement of TuYV CP mutants, we acquired additional evidence that virions constitute the sole systemic transport form of TuYV. However, this observation does not dismiss the possibility that additional viral and cellular proteins contribute to the long-distance transport of TuYV. In this respect, TuYV is similar to a broad range of plant viruses that require virion formation for long-distance movement (for a review, see Hipper et al., 2013), as exemplified by Tobacco mosaic virus (TMV; genus Tobamovirus) in Nicotiana tabacum (Holt & Beachy, 1991; Osbourn et al., 1990; Saito et al., 1990). In addition, as we showed that TuYV virions were required for systemic infection of both M. perfoliata and Arabidopsis thaliana, virions seem to be a general transportation form of TuYV whatever the host. This is in contrast with CymRSV, which only requires CP for long-distance transport in N. clevelandii, but not in N. benthamiana (Dalmay et al., 1992).

Although we showed clearly in this study that virions are required for TuYV long-distance transport in sieve tubes, the question of the role of RNP complexes in cell-to-cell movement remains to be addressed.

**METHODS**

**Construction of TuYV mutants.** The transcription vector BW0 (Veidt et al., 1992) and the agro-infection vector pBinBW0 (Braut et al., 1995; Leiser et al., 1992) containing full-length TuYV (formerly called Beet western yellows virus) DNA sequence were used as templates for cloning. Construction of the W166R mutant was described previously (Braut et al., 2003). The deletion of 37 nt in the NoCPRT mutant sequence encompassing the ORF3 start codon was obtained by directed mutagenesis on BW0 using the primer 5′-TCTTCATGATACTAGTGTGTTTGG-3′ (Reutenauer, 1994). The seven mutants T83P/P86T, S79R/G80R, D90N/C91R, F84Q/G85W, T83S/F84L, F84V and S87G were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Primers were designed according to the manufacturer’s instructions. The mutated full-length sequences of S79R/G80R, D90N/C91R, T83S/P86T and F84Q/G85W were first introduced into BW0 to perform *in vitro* transcription and protoplast inoculation. Vectors for agro-infection corresponding to the mutants T83P/P86T, S79R/G80R, D90N/C91R and F84Q/G85W were made by replacing the SpeI/Sall fragment of pBinBW0 with the SpeI/Sall fragment of the mutated sequence. Mutants T83S/F84L, S87G and F84V were created directly from pBinBW0 using the QuikChange Lightning Site-Directed Mutagenesis Kit. The different constructs were sequenced to confirm the presence of the mutations. pBin-derived constructs were obtained by directed mutagenesis on BW0 using the primer 5′-TCTTCCATTGATACGTACGTGTTTGG-3′ (Reutenauer, 1994). The templates for cloning. Construction of the W166R mutant was obtained by chemiluminescence (Lumi-LightPLUS kit; Roche) after addition of a goat anti-rabbit IgG-HRP conjugate (Invitrogen). Double-antibody sandwich ELISA was performed on infiltrated and systemic leaves as described previously (Bryüere et al., 1997), TuYV-specific antiserum (Loewe), recognizing TuYV virions, was used at a dilution of 1/400 (v/v).

**Detection of viral RNA.** Detection of both gRNA and sgRNA was performed using RT-PCR on total RNA extracted using a commercial RNA purification kit (RNeasy Plant Mini Kit; Qiagen). Two primers bordering the ORF3 sequence were used, the forward primer complementary to the intergenic region (3331-GTAAGAGACCTAAGCACCACC-3531) and the reverse primer corresponding to the ORF3 sequence (4116-TGGAGAGGAGAAGGCGCTTG-4136). The amplified cDNA sequences were then visualized on a 1 % agarose gel in 0.5 \( \times \) Tris/acetate/EDTA buffer after ethidium bromide staining. Viral gRNA and sgRNA were detected by Northern blotting using a digoxigenin-UTP-labelled RNA probe (Roche) complementary to the 3′ terminal 196 nt of TuYV gRNA (Reutenauer et al., 1993) or to the 312 nt complementary to the ORF3 sequence (nt 3632–3944).

**Virus purification.** Virus particles were purified from 300 g systemically infected *M. perfoliata* as described previously (van den Heuvel et al., 1991) or from 3 g systemically infected *Arabidopsis thaliana* using the same protocol that we adapted for small volumes.

**ISEM.** Virus particles were visualized by ISEM using a modified protocol described previously (Reutenauer et al., 1993). TuYV-specific antiserum (Loewe) at a dilution of 1/200 (v/v) in PBS was deposited onto the grids for 30 min at 25 °C. The grids were washed again in PBS and stained with uranyl acetate 3 % before visualization using an electron microscope (EM 208; Philips) operating at 80 kV.

**Transformation of Arabidopsis thaliana plants by floral dips.** Arabidopsis thaliana (Col-0) was transformed by floral dip as described by Beckhold & Pelletier (1998) using recombinant Agrobacterium tumefaciens C58C1. The CP sequence of TuYV was introduced between the left and right borders of the T-DNA into the binary plasmid vector pBTIC (Erhardt et al., 1999). The CP sequence was also flanked by a 355 promoter and a TMV omega translation enhancer sequence on the 5′ side, and by the nopaline synthase (NOS) termination signal on the 3′ side (Braut et al., 2002). The pBTIC vector possesses a neomycin resistance gene (Kan) under the control of the NOS promoter and terminator to select both transformed bacteria and plants. A homozygous line was obtained by successive self-crossings and selection of the transgenic plants on selective Murashige–Skoog medium containing kanamycin.

**SDS treatment of virions.** A sample of 1 µg WT TuYV purified virions was treated with 1 or 5 % SDS in a total volume of 1 ml. After
1 min of vortexing and incubation for 10 min at 65 °C, the samples were loaded onto a centrifugal filter device (Centricon; Millipore) with a cut-off of 10 kDa to eliminate the SDS. The centrifugation was performed at 5000 g and the final volume (300 μl) was dispatched in a 96-well plate for the ELISA test. Citrate buffer (1 x) was used as a negative control. In parallel, a similar sample was centrifuged until the volume was 10 μl for ISEM observation of virions in comparison with untreated viroins.

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