Recombination in natural populations of watermelon mosaic virus: new agronomic threat or damp squib?

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Since their introduction in south-eastern France around 1999, new, ‘emerging’ (EM) strains of watermelon mosaic virus (WMV) coexist with the ‘classic’ (CL) strains present for more than 40 years. This situation constitutes a unique opportunity to estimate the frequency of recombinants appearing in the few years following introduction of new strains of a plant RNA virus. Molecular analyses performed on more than 1000 isolates from epidemiological surveys (2004–2008) and from experimental plots (2009–2010), and targeting only recombinants that became predominant in at least one plant, revealed at least seven independent CL/EM or EM/EM recombination events. The frequency of recombinants involving at least one EM parent in the natural populations tested was on the order of 1 %. No new recombinant was detected for more than 1 year, and none but one in more than one location. In tests comparing host range and aphid transmissibility, the new recombinants did not display a better fitness than their ‘parental’ isolates. No recombinant was detected from artificial mixed infections of CL and EM isolates of various hosts after testing more than 1500 subcultures obtained after single-aphid transmission. These results constitute one of the first estimations of the frequency of recombinants in natural conditions for a plant RNA virus. This suggests that although viable recombinants of WMV are not rare, and although recombination may potentially lead to new highly damaging strains, the new recombinants observed so far had a lower fitness than the parental strains and did not emerge durably in the populations.

INTRODUCTION

Mutation and recombination are the major evolutionary forces in plant viruses generating genetic variability, afterwards shaped by selection and genetic drift. Although recombination induces more drastic genomic changes than point mutations, its frequency in natural plant virus populations is not well estimated. Recent studies have shown that recombination is very common in plant DNA viruses (Froissart et al., 2005; Lefeuvre et al., 2009). In begomoviruses, interspecific recombination has led in the last years to the emergence of new virus strains or species particularly damaging to crops (García-Andrés et al., 2007a; Padidam et al., 1999; Zhou et al., 1997). In plant RNA viruses, recombination has long been considered as rare. The recent increase in viral sequences available and the improvement of methods of analysis have shown that recombination events can be traced in many viral families, particularly in the family Potyviridae containing the genus Potyvirus, the most numerous and economically devastating genus of plant viruses (Revers et al., 1996; Chare & Holmes, 2006). At least three members of the family Potyviridae have acquired plant genes by heterologous recombination (Mbanzibwa et al., 2009; Susaimuthu et al., 2008). Interspecific recombination, particularly in the 5’ end of the genome, has led in several cases to the appearance of novel strains (Larsen et al., 2005) or even to novel virus species, as for watermelon mosaic virus (WMV) that is an interspecific recombinant between two legume-infecting viruses belonging to the ‘bean common mosaic virus’ cluster (Desbiez & Lecoq, 2004; Valli et al., 2007). In laboratory conditions of high selective pressure (defective parents), recombinants appear within a few days or weeks (Gal-On et al., 1998). In natural conditions, according to sequence analyses, recombination in potyviruses is quite common (Chare & Holmes, 2006; Revers et al., 1996; Tan et al., 2004), with or without recombination hotspots along the viral genome (Desbiez & Lecoq, 2008; Moury et al., 2006; Ohshima et al., 2007).

Some recombinants tend to invade populations (Glais et al., 2002; Glasa et al., 2004; Moreno et al., 2004), but since the timing of contact between the parental strains is usually unknown, the real recombination frequency and kinetics of recombinant emergence in natural conditions remains an open question. Studying the emergence of
recombinants in a defined environment shortly after the introduction of novel viral strains and characterizing their fitness can help to estimate these parameters, and infer the agronomic risks associated with recombination. Such was the situation of WMV in south-eastern France. Three molecular groups have been defined for this virus at the world level based on coat protein (CP) sequences with intergroup nucleotide divergence around 10%. Only two of them, group 1 or ‘classic’ (CL) and to a much lower extent group 2 (G2), were found in France until 1999 (Desbiez et al., 2009). Most ‘CL’ isolates – not only in France, but also all along Europe and the Mediterranean basin (Desbiez & Lecoq, 2008; Moreno et al., 2004) – are actually G2/CL recombinants with the recombination point located in the 5' half of the genome, but the timing of appearance of such recombinants is unknown. Strains from group 3 (‘emerging’ or EM) have been introduced in the last 10 years in south-eastern France (Desbiez et al., 2009), probably from Asia. Four subgroups of EM isolates, EM1–EM4, with molecular divergence of 5–8%, have been defined (Desbiez et al., 2009). Within a few years, the relative proportion of EM isolates has increased tremendously, so that they have now nearly replaced the local ‘classic’ (CL or G2/CL) strains in some locations of south-eastern France (Desbiez et al., 2009). Data on the worldwide structure of WMV populations suggest that indigenous (CL) and new (EM) strains were not in contact before (Desbiez et al., 2007, 2009). Thus, the situation of WMV in south-eastern France constitutes a unique opportunity to estimate the frequency of recombinants in the few years following introduction of new strains. In this study, we revealed the presence of at least seven independent CL/EM or EM/EM recombination events in natural conditions. We also studied the maintenance and spread of the recombinants in the field, as well as their appearance and their fitness in experimental conditions, in order to estimate the agronomic risks associated with recombination.

RESULTS

Detection and molecular characterization of natural recombinants

Of the 600 isolates from the 2004 to 2008 epidemiological surveys for which partial CI sequences were obtained, three isolates C04-106, C07-014 and C07-284 (Table 1) had an obvious phylogenetic discrepancy between the partial CI and CP sequences, and were shown by specific RT-PCR in the P1-coding region to be devoid of mixed infections. This was also the case for isolate Cg09-640 collected from an experimental plot in 2009. Several other isolates that displayed an apparent CI/CP discrepancy were discarded as artefacts related to mixed infections (data not shown). Isolates C05-463, C07-349 and C07-350 had already been found to have a CL/EM recombination breakpoint in the CP-coding region (Desbiez et al., 2009). The full-length sequences of isolates C05-463, C07-349, C07-014, C07-284, Cg09-640 and C04-106 were obtained (Table 1). Full-length sequences were also obtained for the putative ‘parents’ of C05-463 (isolates C05-464 and C05-465 found in the same field at the same sampling date) and of C07-349 (isolates C06-257 and C06-526 found in the same location but on the previous year) (Table 1).

Recombination analyses confirmed the presence of a single CL/EM recombination breakpoint in the N-terminal part of the CP-coding region for isolates C05-463 ($P=10^{-15}$ to $P=10^{-55}$ depending on the method used in RDP3) and C07-349 ($P=10^{-14}$ to $P=10^{-55}$) (Fig. 1). Regarding the ‘CL’ part of the sequence, C05-463 had the G2/CL recombination breakpoint in the CI-coding region typical for all ‘CL-B’ isolates (Desbiez et al., 2009), and C07-349 had a G2/CL breakpoint in the 6K1 region found in several ‘CL-A’ isolates (Desbiez et al., 2007, 2009) (Fig. 1). C05-464 and C05-465, collected in the same field as C05-463, share 99.5%/90.5% and 95.8%/100% identity with this isolate before and after the recombination breakpoint, respectively. Their very high similarity with C05-463 suggests that they are indeed representative of the ‘parents’ of this recombinant. Isolates C06-526 and C06-257, collected in the same area as C07-349 but in a previous year, shared 98.2%/89.1% and 95.3%/99.8% identity with C07-349 before and after the breakpoint, respectively. They did not display a higher similarity with C07-349 than other isolates from their respective molecular subgroups (data not shown). C06-526 did not have exactly the same G2/CL breakpoint as C07-349 in the 6K1/CI region (Fig. 1), indicating that it is not representative of the ‘true parent’ of this recombinant isolate.

Among the other putative recombinants analysed, C04-106 had a unique CL/EM1 breakpoint in the N-terminal extremity of the VPg-coding region ($P=10^{-41}$ to $P=10^{-121}$), and the same G2/CL breakpoint as C07-349 (Fig. 1). C07-014 and C07-284 were found to be EM1/EM2 recombinants, with a unique breakpoint in the NIB and CI region, respectively ($P=4.10^{-20}$ to $P=5.10^{-56}$ and $P=8.10^{-20}$ to $P=7.10^{-60}$ for C07-014 and C07-284, respectively) (Fig. 1). Isolate Cg09-640 found in 2009 in an experimental plot in Montfavet, Vaucluse, was shown to be an EM1/EM3 recombinant with a unique breakpoint in the CI-coding region ($P=4.10^{-10}$ to $P=5.10^{-101}$) (Fig. 1).

Prevalence of recombinants in and around places where they were first detected

Table 2 shows the number of similar CL/EM or EM/EM recombinant isolates observed in the fields where such recombinants were first detected, and the number of samples tested for the same recombinants in and around the same locations on different years.

Except C05-463, C07-349 and C07-350, none of the 2000 WMV samples from 2004 to 2008, including the 125 samples
specifically collected in or around St Chaptes in 2008, was found to have a recombination breakpoint in the 273 bases of the Nib–CP-coding region sequenced for all isolates. The six isolates collected in Aubignan in 2007, including C07-014, were all EM1/EM2 recombinants with the same recombination breakpoint as C07-014 (data not shown). In

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Geographical origin</th>
<th>Year of collection</th>
<th>Host of origin</th>
<th>GenBank accession no.</th>
<th>Molecular group</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi87-620</td>
<td>Chile</td>
<td>1987</td>
<td>Zucchini</td>
<td>EU660580</td>
<td>G2</td>
<td>Desbiez et al. (2007)</td>
</tr>
<tr>
<td>FMF00-LL1</td>
<td>Montfavet (Vaucluse)</td>
<td>2000</td>
<td>Zucchini</td>
<td>EU660581</td>
<td>EM1</td>
<td>Desbiez et al. (2007)</td>
</tr>
<tr>
<td>FMF03-141</td>
<td>Montfavet (Vaucluse)</td>
<td>2003</td>
<td>Zucchini</td>
<td>EU660583</td>
<td>EM2</td>
<td>Desbiez et al. (2007)</td>
</tr>
<tr>
<td>FBR04-37</td>
<td>Tarascon (Bouches du Rhône)</td>
<td>2004</td>
<td>Zucchini</td>
<td>EU660586</td>
<td>EM3</td>
<td>Desbiez et al. (2007)</td>
</tr>
<tr>
<td>C05-270</td>
<td>Bourdic (Gard)</td>
<td>2005</td>
<td>Melon</td>
<td>EU660585</td>
<td>EM4</td>
<td>Desbiez et al. (2007)</td>
</tr>
<tr>
<td>Ita00-G</td>
<td>Italy</td>
<td>2000</td>
<td>Zucchini</td>
<td>EU660590</td>
<td>G2/CL (CL-A)*</td>
<td>Desbiez et al. (2007)</td>
</tr>
<tr>
<td>FMF00-LL2</td>
<td>Montfavet (Vaucluse)</td>
<td>2000</td>
<td>Zucchini</td>
<td>EU660578</td>
<td>G2/CL (CL-A)</td>
<td>Desbiez et al. (2007)</td>
</tr>
<tr>
<td>C05-337</td>
<td>Marmande (Lot et Garonne)</td>
<td>2005</td>
<td>Zucchini</td>
<td>EU660589</td>
<td>G2/CL (CL-B)</td>
<td>Desbiez et al. (2007)</td>
</tr>
<tr>
<td>C05-463</td>
<td>Garons (Gard)</td>
<td>2005</td>
<td>Zucchini</td>
<td>JF273458</td>
<td>CL-B/EM4</td>
<td>Desbiez et al. (2009)</td>
</tr>
<tr>
<td>C05-464</td>
<td>Garons (Gard)</td>
<td>2005</td>
<td>Zucchini</td>
<td>JF273459</td>
<td>CL-B</td>
<td>This work</td>
</tr>
<tr>
<td>C05-465</td>
<td>Garons (Gard)</td>
<td>2005</td>
<td>Zucchini</td>
<td>JF273460</td>
<td>EM4</td>
<td>This work</td>
</tr>
<tr>
<td>C07-349</td>
<td>St Chaptes (Gard)</td>
<td>2007</td>
<td>Melon</td>
<td>JF273461</td>
<td>CL-A/EM3</td>
<td>Desbiez et al. (2009)</td>
</tr>
<tr>
<td>C06-526</td>
<td>Bourdic (Gard)</td>
<td>2006</td>
<td>Melon</td>
<td>JF273462</td>
<td>CL-A</td>
<td>This work</td>
</tr>
<tr>
<td>C06-257</td>
<td>St Chaptes (Gard)</td>
<td>2006</td>
<td>Melon</td>
<td>JF273463</td>
<td>EM5</td>
<td>This work</td>
</tr>
<tr>
<td>C07-014</td>
<td>Aubignan (Vaucluse)</td>
<td>2007</td>
<td>Melon</td>
<td>JF273464</td>
<td>EM1/EM2</td>
<td>This work</td>
</tr>
<tr>
<td>A08-160, A08-170</td>
<td>Aubignan (Vaucluse)</td>
<td>2008</td>
<td>Zucchini</td>
<td>JF273465 JF273466</td>
<td>EM1/EM2</td>
<td>This work</td>
</tr>
<tr>
<td>Cg09-640</td>
<td>Montfavet (Vaucluse)</td>
<td>2009</td>
<td>Zucchini</td>
<td>JF273467</td>
<td>EM1/EM3</td>
<td>This work</td>
</tr>
<tr>
<td>C07-284</td>
<td>La Tour d’Aigues (Vaucluse)</td>
<td>2007</td>
<td>Zucchini</td>
<td>JF273468</td>
<td>EM1/EM2</td>
<td>This work</td>
</tr>
<tr>
<td>C04-106</td>
<td>Maillane (Bouches du Rhône)</td>
<td>2004</td>
<td>Melon</td>
<td>JF273469</td>
<td>CL-A/EM1</td>
<td>This work</td>
</tr>
</tbody>
</table>

*’CL-A’ isolates, although they cluster together in the CP-coding region, can have different G2/CL recombination breakpoints, e.g. Ita00-G and FMF00-LL2.

Fig. 1. Location of recombination points in WMV isolates, as estimated with GARD and RDP.
Table 2. Prevalence of the recombinants involving at least one ‘EM’ parent, in and around the places where they were first detected

<table>
<thead>
<tr>
<th>Recombinant</th>
<th>Nature of the recombinant</th>
<th>Place of origin</th>
<th>Year</th>
<th>No. recombinants/no. isolates tested in the same location, same year</th>
<th>No. recombinants/no. isolates tested in the same location, other years</th>
<th>No. recombinants/no. isolates tested in a 10 km radius around the initial location (all years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C04-106</td>
<td>CL-A/EM1</td>
<td>Maillane</td>
<td>2004</td>
<td>1/23</td>
<td>0/0</td>
<td>0/57</td>
</tr>
<tr>
<td>C05-463</td>
<td>CL-B/EM4</td>
<td>Garons</td>
<td>2005</td>
<td>1/22</td>
<td>0/36</td>
<td>0/136</td>
</tr>
<tr>
<td>C07-349</td>
<td>CL-A/EM3</td>
<td>St Chaptes</td>
<td>2007</td>
<td>2/3</td>
<td>0/71</td>
<td>0/85</td>
</tr>
<tr>
<td>C07-014</td>
<td>EM1/EM2</td>
<td>Aubignan</td>
<td>2007</td>
<td>6/6</td>
<td>0/168</td>
<td>0/198</td>
</tr>
<tr>
<td>A08-160</td>
<td>EM1/EM2</td>
<td>Aubignan</td>
<td>2008</td>
<td>3/157</td>
<td>0/17</td>
<td>0/198</td>
</tr>
<tr>
<td>C07-284</td>
<td>EM1/EM2</td>
<td>La Tour d’Aigues</td>
<td>2007</td>
<td>2/11</td>
<td>0/74</td>
<td>0/70</td>
</tr>
</tbody>
</table>

*The same recombinant was observed in 2009 in another experimental plot later in season, 4 km apart from the first one.

Aubignan in 2008, three samples of 157 belonged to subgroup EM2 in the CP-coding region, whereas specific RT-PCR indicated that the P1 region belonged to subgroup EM1. Restriction fragment analysis and partial sequencing showed that these isolates did not have the same recombination breakpoint as C07-014 found in 2007 on the same farm. The complete sequences of two isolates, A08-160 and A08-170, were obtained (Table 1). These two sequences shared 99.9% nt sequence identity. Sequence analyses revealed that A08-160 and A08-170 were indeed EM1/EM2 recombinants, with a unique recombination breakpoint located in the CI-coding region (P=6.10^{-18} to P=8.10^{-58} depending on the method) (Fig. 1). Partial sequencing indicated that the third isolate A08-175 had the same recombination breakpoint as A08-160 and A08-170 (data not shown). Among the 198 additional isolates collected in a 10 km radius around Aubignan in 2004–2006 and 2008, there was no evidence for the presence of EM1/EM2 recombinant.

In La Tour d’Aigues, isolate C07-286, collected in the same field and at the same time as C07-284, was the only sample that showed a discrepancy in the P1/CP clustering. Partial sequencing showed that it had the same recombination breakpoint as C07-284 (data not shown). The other EM1 and EM2 samples collected in and around La Tour d’Aigues did not appear to be EM1/EM2 recombinants.

Also, none of the samples collected in and around Maillane showed any evidence of being a CL/EM recombinant like C04-106.

No isolate belonging to the EM3 subgroup regarding its CP sequence was detected between 2004 and 2008 or in 2010, in and around Montfavet where the EM1/EM3 recombinant Cg09-640 was detected in 2009. However, this recombinant was found in 2009 in two experimental plots in Montfavet separated by 4 km, first in the summer 2009 (isolate Cg09-640, one isolate of 27 tested in the plot) and the other in the autumn 2009 (5 isolates of 78). This recombinant was not detected in other nearby plots or sampling sites in 2009 (0 isolate of 276).

Biological properties of recombinants

Host range. As observed previously (Lecoq et al., 2011), little variability could be detected in the host range of WMV isolates. No differential host was detected between EM isolates or with the EM1/EM2 recombinants C07-014 and A08-160 (data not shown). Also, no difference in the host range was observed between CL isolates and G2/CL recombinants CL-A and CL-B. In an extensive host range comparison, 96% of 48 different plant species or cultivars tested reacted similarly after inoculation by CL-A, CL-B or EM isolates (Lecoq et al., 2011). Only two hosts were differential: Chenopodium quinoa was systemically infected by CL-A and CL-B but not by the EM isolates, and Ranunculus sardous was systemically infected by EM but not by the CL-A or CL-B isolates (Lecoq et al., 2011).

The two CL/EM recombinants, C05-463 and C07-349, behaved like their CL parent on R. sardous. Isolate C07-349 infected systemically C. quinoa like CL-A or CL-B isolates. C05-463 did not infect C. quinoa, although it has almost the same CL/EM recombination breakpoint as C07-349 (Fig. 1).

The symptoms induced by the CL/EM or EM/EM recombinants in cucurbit hosts were similar to those induced by the other isolates (data not shown).

Aphid transmission. The results of aphid-transmission experiments with recombinant isolates C05-463, C07-349, C07-014, A08-160 and Cg09-640, as well as with their putative ‘parental’ strains belonging to the different molecular groups, are presented on Fig. 2. The two G2/CL/EM recombinants C05-463 and C07-349 presented a significantly lower transmissibility compared with both their ‘parents’ (P<0.001 in both cases). As observed previously (Lecoq et al., 2011), the two ‘parental’ isolates of C05-463 and C07-349, i.e. C05-464 + C05-465 and C06-526 + C06-257, did not have significantly different transmission rates (P=0.967 and P=0.964, respectively).

The transmission rate of EM1/EM2 recombinant C07-014 was significantly lower than the one of the EM1 and EM2 isolates used as reference (P<0.001). For A08-160 and A08-175, the
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Search for recombinants in laboratory experiments

Single-aphid transmissions from plants co-infected with C05-464 + C05-465 and C06-526 + C06-257 yielded 794- and 765-infected melon plantlets, respectively. Triple antibody sandwich (TAS)-ELISA and RT-PCR tests performed on the leaves used for aphid transmission confirmed that both strains were present in the source plants (data not shown). Also, transmission of both strains was obtained from 86 % of the source plants (data not shown). Twenty-nine and 21 % of the test plants, respectively, had mixed infections after single-aphid transmission. After TAS-ELISA tests, 575 and 603 plants that presented no evidence of co-infections were tested by RT-PCR with P1-specific primers. No evidence for differential P1/CP clustering was detected in the 1178 samples tested. In the second set of experiments, 508 plants showed symptoms after aphid transmission; 345 plants that did not appear to be co-infected were tested by specific RT-PCR. No recombinant was observed among the 1523 singly infected plants tested in the two sets of experiments.

DISCUSSION

At least seven recent independent recombination events were detected in natural WMV populations in south-eastern France, either between local and recently introduced strains (three cases) or between two subgroups of introduced strains (four cases). The number of recombination events is too limited to determine if a recombination hotspot exists, as observed for G2/CL recombinants (Desbiez & Lecoq, 2008), but four of seven breakpoints were located within or very close to the CI-coding region. The other breakpoints were located in the 3’ half of the genome, whereas the G2/CL recombination hotspot is in the 5’ part of the genome (Desbiez & Lecoq, 2008). EM1/EM2 and CL-A or CL-B/EM recombinants were the most common ones, probably in relation to the fact that these isolates are frequently found in the same locations. Mixed infections are common in the field, particularly between CL and EM isolates, in relation to an inefficient cross-protection between both types of strains (Fabre et al., 2010; Lecoq et al., 2011) and to the possibility of their simultaneous transmission by aphids from plants with double infections.

When the information was available, the CL/EM or EM/EM recombinant isolates represented between 2 (3/157, Aubignan 2008) and 100 % (6/6, Aubignan 2007) of the isolates collected from the same site on the same date. The recombinants were in most cases found in a few adjacent plants from the same field, but they were not detected in several locations or several years. In four of seven cases, plants infected by each of the ‘parental’ isolates, as well as plants presenting mixed infections with both ‘parents’, were detected in the same field as their respective recombinants. In two of the other cases, the number of non-recombinant isolates collected from the same field was very limited (0 and 1 for C07-014 and C07-349, respectively). The most intriguing case was that of the EM1/EM3 recombinant Cg09-640, because EM3 isolates, which represent only 5 % of all EM isolates from the 2004 to 2008 epidemiological surveys, are only exceptionally present in the same sites as the EM1 isolates (Desbiez et al., 2009; Joannon et al., 2010). This recombinant was also the only one that was detected in several fields, suggesting an efficient aphid transmission; however, it was not detected in 2010 among 231 samples from the same and from two other experimental plots in Montfavet, indicating that it apparently failed to be maintained durably.

Since the same recombinants were frequently found in several neighbouring samples, the percentage of CL/EM or EM/EM recombinants observed in the epidemiological surveys (21 recombinant isolates, corresponding to the seven recombination events) is in the order of magnitude of 1 % of the total isolates collected. This is probably a gross underestimation of the total number of recombination events, since (i) not all samples from the survey were actually tested, (ii) not all recombinants can be detected with the protocol used (double or multiple recombinants with limited parts of the genome exchanged outside the P1, CI and CP would pass unnoticed), and (iii) only recombinants accumulating at high levels in at least one or a few neighbouring plants – i.e. the ones that have a chance to
emerge in the populations – are detected this way. This indicates that intraspecific recombination in the field is not a rare event, and new recombinants involving at least one EM parental isolate appear every year in WMV populations without becoming durably established. The recent introductions of EM isolates in France – and by now, in several European countries and in the USA (C. Desbiez & H. Lecoq, unpublished data; Vincelli & Seebold, 2009) – will probably continue to favour recombination, by putting into contact highly divergent strains displaying inefficient cross-protection.

Although recombinants involving at least one EM parent appear quite frequently in natural WMV populations (seven independent events), none was observed in laboratory conditions after single-aphid transmissions from mixed-infected plants; the difference in frequency was significant (Fisher’s exact test, \( P<0.015 \)). The experimental approach in this case, like in the screening for natural recombinants in the field, targeted only recombinants fit enough to accumulate in their parental plants and be aphid-transmitted, i.e. the ones that may be agronomically important. Other approaches targeting all recombinants without any selection also yielded negative results from mixed infections of two potyviruses (Dietrich et al., 2007b), contrary to mixed infections of cucumoviruses where recombinants were indeed detected (Aaziz & Tepfer, 1999; de Wispelaere et al., 2005) although they seemed to have a poor fitness (Pierrugues et al., 2007). Nevertheless, cucumovirus recombinants were also found in natural populations (Bonnet et al., 2005). In the case of potyviruses, it has been shown that in plants mixed infected by two viruses or two almost identical strains of the same virus, the number of doubly infected cells is very limited (Dietrich & Maiss, 2003). Since recombination requires the replication of the two parental genomes in the same cell (Morilla et al., 2004), it could account for the much higher frequency of recombinants in plant DNA versus RNA viruses (García-Andrés et al., 2007b; Morilla et al., 2004; van der Walt et al., 2009). It is also possible that recombination requires some conditions (plant genotype or age, length of infection, biotic or abiotic stresses...) different from the ones used in our laboratory experiments. It has been shown recently that plant and environmental factors can in certain cases affect the recombination frequency in plant RNA viruses (Jaag & Nagy, 2010). However, our results confirm that recombination in potyviruses is less common than in plant DNA viruses where it can be observed in experimental as well as in natural conditions (Froissart et al., 2005; García-Andrés et al., 2007b).

The fitness of the recombinants is at least as important as their frequency of appearance to determine their potential agronomic impact. In our case, no recombinant presented a higher fitness than the parental isolates under the experimental conditions tested. CL/EM recombinants were less efficiently aphid transmitted than most CL and EM isolates. This may be related to the fact that in both cases, recombination took place in the N-terminal extremity of the CP involved in aphid transmissibility, even if the DAG triplet required for aphid transmission of potyviruses (Atreya et al., 1991) was not affected. Since the CP and the helper component of aphid transmission (HC-Pro) belong to different molecular groups in the recombinants, their interaction for transmission might be less efficient than in non-recombinant isolates. In the EM/EM recombinants, CP and HC-Pro also belong to different subgroups, but are less divergent molecularly and the interaction may be unaffected, resulting in transmission efficiencies similar to those of the parental isolates. The lower transmission of recombinants could also be related
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to lower titre in plants, even if no quantitative data are available. This would be in itself more evidence for fitness reduction. There was also no obvious gain in host range related to the recombinant nature of the isolates, contrary to what was observed for some begomoviruses (García-Andrés et al., 2006). However, the diversity in host range of WMV appears very limited, and the determinants for the infectivity on the only two differential hosts detected, C. quinoa and R. sardous, remain unknown. The genome of recombinants C05-463 and C07-349, behaving like CL-A or CL-B isolates on R. sardous, belongs to the ‘CL’ group (or G2/CL) except in its CP-coding region and 3’ non-coding extremity. This indicates that the determinants for infecting R. sardous are probably not located in the 3’ end of the genome. Although C05-463 and C07-349 have almost the same recombination point (Fig. 1), only C07-349, like CL isolates, infects systemically C. quinoa. The main difference between C05-463 and C07-349 lies in the fact that C07-349 has a ‘KEA’ motif in the N-terminal extremity of the CP like most CL isolates, whereas EM isolates usually have a ‘KEKET’ (Desbiez et al., 2007). The recombination breakpoint of C05-463 is located on this motif, resulting in a ‘KET’ sequence for this isolate. These data suggest that the ‘KEA’ motif may be involved in the systemic infection of C. quinoa. In another potyvirus, pea seedborne mosaic virus, a single amino acid in the N-terminal part of the CP, although more internal than the ‘KEA’ of WMV, was found to modulate the ability to infect C. quinoa systemically (Andersen & Johansen, 1998).

In potato virus A, artificial intraspecific recombinants displayed a fitness – measured as virus accumulation – similar or lower to that of parental strains (Dietrich et al., 2007a; Paalme et al., 2004), even though they could occasionally display new symptoms (Paalme et al., 2004). In the case of WMV, the symptoms induced by the natural recombinants in the field where they were collected were usually indistinguishable from those of their parental strains. Changes in WMV symptoms had been observed around 2000 in relation to the introduction and spread of EM isolates in south-eastern France leading to rapid population shifts, suggesting that in this case migration – i.e. introduction(s) from other origins – had a much stronger direct effect on WMV agronomic impact than recombination (Desbiez et al., 2009).

This work constitutes one of the first quantitative estimates of the frequency and kinetics of appearance of recombinants in natural plant RNA virus populations. We showed that recombinants fit enough to accumulate and spread in a few neighbouring plants appeared every year in the populations but have not become established so far. More often than not, recombination appeared to decrease viral fitness compared with parental strains. Even if one cannot exclude that particularly damaging strains may emerge by recombination, recombination in WMV populations does not seem so far to generate new and particularly important agronomic threats compared with mutations and migration, the other means of generating genetic changes in populations.

METHODS

Biological material. Two thousand one hundred and thirteen WMV isolates were collected during an epidemiological survey performed in France between 2004 and 2008, and were characterized molecularly by partial sequencing of their CP-coding region (Desbiez et al., 2009; Joannon et al., 2010). All isolates except some from 2004 were kept as dried plant material over calcium chloride. Two recombinants in the CP-coding region, C05-463 and C07-349, had been detected in these analyses (Desbiez et al., 2009), and were used here for further characterization (Table 1).

Seven hundred and ten samples from experimental plots at Montfavit, Vaulcuse, collected between 2004 and 2010, were also included in this study. Specific surveys were performed in 2008 in Saint Chaptes (Gard) and in Aubignan (Vaulcuse) where recombinants had been detected in the previous years (Table 2). All samples were tested by double antibody sandwich-ELISA for the presence of WMV. Partial CP sequences of the WMV-positive samples were obtained. The collection points of the different isolates from south-eastern France used in Table 1 are presented in Fig. 3.

Search for recombinants. The Nb–CP fragment sequenced for all WMV-infected samples overlaps the recombination breakpoints of C07-349 and C05-463, thus allowing a direct search for isolates with the same breakpoints. To look for other recombinants, since the P3–CI region was shown previously for WMV to be a recombination hotspot (Desbiez & Lecoq, 2008), partial sequences of this region were obtained for 592 of the isolates from the surveys that did not show evidence for mixed infections in the CP fragment. Among these, 491 were isolated from south-eastern France, the only area where EM isolates were detected repeatedly at a high frequency. The RNAs previously extracted (Desbiez et al., 2009) were submitted to one-step RT-PCR with primers W-VVEES-5′:5′-GTTGTAGAAAGAAAAGCAGCGC-3′ and W-AENV-3′:5′-AACTGTGTTGCTAATTCCTT- GC-3′ before sequencing with primer W-AENV-3′. Sequences were aligned with CLUSTAL W included in DAMBE (Xia, 2000), and distance analyses (Desbiez et al., 2009) were submitted to one-step RT-PCR with primers W-VVEES-5′:5′-GTTGTAGAAAGAAAAGCAGCGC-3′ and W-AENV-3′:5′-AACTGTGTTGCTAATTCCTT-GC-3′ before sequencing with primer W-AENV-3′. Sequences were aligned with CLUSTAL W included in DAMBE (Xia, 2000), and distance analyses (Desbiez et al., 2009), using reference sequences from isolates belonging to the different molecular groups or subgroups and G2/CL recombinants: CL (WMV-FR, GenBank accession no. AY437609), G2 (Chi87-620, GenBank accession no. EU660580), CL-A (FMF00-1L2, GenBank accession no. EU660578), CL-B (C05-337, GenBank accession no. EU660589), EM1 (FMF00-1L1, GenBank accession no. EU660581), EM2 (FMF03-141, GenBank accession no. EU660583), EM3 (FB04-37, GenBank accession no. EU660586) and EM4 (C05-270, GenBank accession no. EU660585).

For isolates showing discrepancies between CI and CP phylogeny, further partial amplifications, digestions with restriction enzymes and partial sequencings were performed in order to localize the putative recombination points. Recombination detection analyses were first performed visually with SimPlot (Ray, 1998), then confirmed with GARD (Kosakovsky Pond et al., 2006) and RDP3 (Martin et al., 2005), using the same reference sequences as above. When recombination points were detected, the full-length sequence of the isolate, and if possible of putative parental isolates originating from the same field or area, were obtained as described previously (Desbiez & Lecoq, 2008).

To look for EM2/EM1 recombinants, the 157 WMV-positive samples from Aubignan that did not show any mixed infections were tested with specific EM1 and EM2 primers (Supplementary Table S1,
available in IGV Online) targeting the P1-coding region. Among 198 partially sequenced isolates collected between 2004 and 2008 within a 10 km radius around Aubignan that did not show evidence for mixed infections (178 EM isolates and 20 CL isolates), all EM isolates were tested with the EM1 and EM2 primers in order to estimate the spatial spread of recombinants. Seventy-four samples from La Tour d’Aigues (Vaucluse) and 70 samples collected in a 10 km radius around La Tour d’Aigues, as well as 57 samples collected within a 10 km radius around Maillane (Bouches-du-Rhône), were also tested (Table 2).

Biological characterization of recombinants

Mechanical inoculations. All isolates stored as dried leaf material on calcium chloride were mechanically inoculated to zucchini squash cv. Diamant according to the current protocol used in the laboratory and maintained in this host in insect-proof individual cages. Isolate C04-106 had not been kept as dried material on calcium chloride, and could not be tested biologically.


Single-aphid transmission. Green peach aphids (Myzus persicae) reared on healthy pepper plants were starved for 1 h before being deposited on the lower face of zucchini leaves infected with ‘parental’ strains C06-526, C06-257, C05-464, C05-465, MFT03-91 (EM1), C06-097 (EM2), FBR04-37 (Lecoq et al., 2011), and with the recombinants C05-463, C07-349, C07-014, A08-160, A08-175 and Cg09-640. After a 1 min acquisition period, the aphids were deposited individually on 30 healthy melon plantlets at the cotyledonary stage, using one aphid per plant. After 2 h of transmission, the plants were sprayed with an insecticide and kept in an insect-proof greenhouse until symptoms developed. The experiments were repeated 10 times. Transmission rates were estimated by counting plants with symptoms 4 weeks after inoculation.

Four groups of isolates containing the recombinants and their putative parents were defined (Fig. 1). For each group, a generalized linear model testing the effect of the fixed effect factor ‘Isolate’ on the binomial response variable ‘No. of plant infected’ was fitted to the data. The factor ‘Isolate’ was significant for each group. A Tukey means separation test was then performed to identify which pairs of isolates significantly differ for their transmission rate. Analyses were done with the R software environment (http://cran.r-project.org/) using the package ‘multcomp’.

Laboratory experiments under minimal selection pressure. Two pairs of isolates, (A) C05-464 + C05-465 and (B) C06-526 + C06-257, were inoculated mechanically with crude extracts at a 50/50 ratio to eight plants of zucchini squash cv. ‘Black Beauty’, melon cv. ‘Védranais’, bean cv. BTS2, N. benthamiana, N. clevelandii and C. bursa-pastoris, representing a range of the cultivated and wild hosts of WMV, as described in Lecoq et al. (2011). Four weeks after inoculations, single-aphid transmissions were performed from two leaves of each source plant to 15 melon plantlets. All symptomatic plants after aphid transmission were tested in TAS-ELISA with mAbs 2C6 and EB11, specific for EM and CL strains, respectively (Desbiez et al., 2007; Lecoq et al., 2011), and as a control with mAb EG2 detecting both strains, using the standard protocol from the laboratory (Desbiez et al., 2007).

Total RNA was extracted from all ELISA-positive plants after single-aphid transmission that did not appear to be co-infected by CL and EM isolates. Two pairs of specific primers were defined, using the sequences of the parental strains, in the 5’ end of the genome (Supplementary Table S1). One-step RT-PCRs were performed according to standard protocols (Desbiez et al., 2009), with an annealing temperature set at 55 °C in all cases. The specificity of the primers and expected fragment sizes are presented in Supplementary Table S1.

A second set of experiments was performed 3 months later with the same protocol, using two different sets of source plants: (i) 30 of the mechanically co-infected plants that had been used previously for the first series of experiments, and (ii) 30 melon plants that presented evidence of co-infections after the first aphid-transmission experiments.

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