P1-independent replication and local movement of *Rice yellow mottle virus* in host and non-host plant species

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**A R T I C L E I N F O**

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Barley
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**A B S T R A C T**

Sobemovirus P1 protein, characterized previously as a suppressor of posttranscriptional gene silencing, is required for systemic virus spread and infection in plants. Mutations in the ORF1 initiation codon do not affect viral replication indicating P1 is not necessary for this process. Wild type, recombinant and P1 deletion mutants of Cocksfoot mottle virus and *Rice yellow mottle virus* were used to infect oat, rice, wheat, barley, *Arabidopsis thaliana* and *Nicotiana benthamiana* plants. Wild type RYMV, RYMV without P1 and RYMV with CfMV P1 were detected in inoculated leaves of all tested plant species. We found that RYMV does not need P1 for replication and for local movement neither in host nor non-host species tested in this study. However, it is crucial for successful systemic spread of the virus in its host plant rice. Moreover, adding CfMV P1 into RYMV genome did not help it to overcome restriction to the inoculated leaf.

1. Introduction

*Cocksfoot mottle virus* (CfMV) and *Rice yellow mottle virus* (RYMV) belong to the genus Sobemovirus. The genome of sobemoviruses consists of a 4–4.5 kb polycistronic positive single-stranded RNA, in which open reading frame 1 (ORF1) encodes P1 protein that has been characterized as suppressor of posttranscriptional gene silencing (Fusaro et al., 2012; Sarmiento et al., 2007; Siré et al., 2008). Furthermore, even though P1 does not seem to be needed for replication of sobemoviruses, it is necessary for systemic infection (Bonneau et al., 1998; Meier et al., 2006; Sivakumaran et al., 1998) and has therefore been suggested to serve as viral movement protein (MP).

P1 of Sesbania mosaic sobemovirus (SeMV) interacts with the genome-bound VPg and P10 in vitro indicating there is active transport of viral RNA complex facilitated by P1 acting as a MP and supported by hydrolysis of ATP by P10 (Chowdhury and Savithri, 2011). However, the amino acid sequence of P1 is very variable within the genus – the only conserved feature seems to be the zinc-finger motifs (Sõmera et al., 2015) related to suppression of posttranscriptional silencing activity of P1 (Gillet et al., 2013; Siré et al., 2008). It remains unclear whether P1 RNA silencing suppressor activity can be uncoupled from its possible movement function.

Sobemoviruses infect both monocotyledonous and dicotyledonous species, but the host range for each sobemovirus species has generally been considered quite narrow and specific for each virus species. CfMV and RYMV are known to only infect plants from the family *Gramineae*. The main natural host of CfMV is cocksfoot (*Dactylis glomerata*) (Mohamed, 1980; Rabenstein and Schmidt, 1979; Serjeant, 1967). Latest reports state that CfMV infection naturally transmits from cocksfoot grasslands to native New Zealand grass-species like *Poa aniceps*, *P. cita*, *Festuca novae-zelandiae*, and *Chionochloa rubra* (Delmiglio et al., 2010). In laboratory experiments CfMV infects, via infectious sap, wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rye (*Secale cereale*) (Serjeant, 1967; Toriyama, 1982) and also several natural grass species (Benigno and A’Brook, 1972; Mohamed and Mossop, 1981; Rabenstein and Stanarius, 1984). The host range of CfMV seems to be restricted to the tribes *Avenae*, *Bromeae*, *Danthonieae*, *Festuceae*, *Hordeeae* and *Phalarideae* (Benigno and A’Brook, 1972; Delmiglio et al., 2010).

RYMV is known as the most important pathogen of rice (*Oryza sativa*) grown in Africa. It has been shown that RYMV infects several species of the genera *Oryza* and *Leersia* from an *Oryzeae* tribe and also a few species from the tribes *Andropogoneae*, *Bromeae*, *Chlorideae*, *Eragrostidaceae*, *Paniceae* and *Phalarideae* (Allarangaye et al., 2007). According to these studies, RYMV and CfMV lack a common host species.

Sobemovirus proteins have not been studied as potential host-range determinants, as has been the case for certain other plant virus groups.
where non-conserved viral proteins have been found to act as host range determinants. For example, *Citrus tristeza virus* proteins p13, p18 and p33 play a role in permitting the infection of different host species (Tatineni et al., 2011). The role of *Potyvirus* protein P1 (which is not related to sobemovirus P1) in host-range determination has been investigated via P1 exchange between *Plum pox virus* (PPV) and *Tobacco vein mottling virus* (TVMV). While P1 from TVMV does not affect PPV infectivity in the common hosts of the two viruses, it does obstruct pathogenicity of PPV in peach that is not a host for TVMV (Salvador et al., 2008). Additionally, amino acid variances within PPV P1 population display particular host-dependent pathogenicity features (Maliogka et al., 2012).

The purpose of this study was to experimentally test whether P1 is the host-range determinant for sobemoviruses and to evaluate a role of P1 in virus movement. Wild type, recombinant and P1 deletion mutants of CfMV and RYMV were used to infect oat, wheat, barley, rice, *Nicotiana benthamiana* and *Arabidopsis thaliana* plants. The experiments unravelled new data on the need of P1 for replication and movement in host and non-host plant species.

2. Results

To assess viral determinants of sobemovirus host range, we used infectious cDNA clones of CfMV and RYMV, their recombinants and P1 deletion mutants to infect host and non-host species. Using RT-PCR, CfMV infection was detected in inoculated leaves of oat, wheat and barley but not in rice, as expected. However, infection of RYMV was detected in inoculated rice leaves but surprisingly also in oat, wheat and barley leaves (Fig. 1). CfMV mutants, where P1 gene was deleted or replaced with RYMV P1 were not able to infect any of the host plants. However, RYMV without P1 or carrying the P1 gene of CfMV, similarly to the wild-type RYMV, established local infection in all four tested monocotyledonous hosts (Fig. 1).

As RYMV and mutated versions of RYMV unexpectedly replicated both in host and non-host species, we analysed also the potential infectivity of RYMV, CP1-RYMV and ΔP1-RYMV in dicotyledonous plants *N. benthamiana* and *A. thaliana*. Again, all three RYMV-based constructs are able to establish infection in the inoculated leaves, whereas CfMV-based ones are not adept at doing so (Fig. 1).

In order to verify that the detected viral sequences were truly replicating viruses and not the *in vitro* transcribed RNA bombarded to leaves, we tested the inoculated leaves for the presence of the minus strand of corresponding constructs. Indeed, the minus strand RNA of RYMV, CP1-RYMV and ΔP1-RYMV was detectable in all six experimental plant species, indicating that these viruses were replicating in tested monocotyledonous as well dicotyledonous plants. CfMV replication in oat and wheat served as a positive control for detecting viral minus strand (Fig. 2).

Moreover, when we analysed the presence of viral RNA in inoculated leaves outside of the bombarded area, we still succeeded in detecting RYMV, CP1-RYMV and ΔP1-RYMV in rice, as well as in oat, wheat and barley leaves. This indicates that RYMV with or without the P1 gene is capable of moving from cell-to-cell in broad range of monocotyledonous hosts. Again, CfMV was able to move from cell to cell only in oat, barley and wheat, as expected (Fig. 3).

Finally, we analysed whether the generated infectious clones or mutated viruses could establish systemic infection. CfMV achieved systemic infection in oat and wheat whereas RYMV managed to systemically infect rice plants, as expected. RYMV and its mutants could not establish systemic infection in any other plant species tested (Supplementary Fig. 1). We also analysed whether RYMV and its mutants replicating in the inoculated leaves were sap transmissible or not. RYMV was transmissible only from rice whereas the RYMV-based mutants were not able to do so in any of the tested plant species (Supplementary Fig. 2).

Throughout the study, sequenced RT-PCR products confirmed that...
detected viruses did not contain any de novo mutations or rearrangements.

3. Discussion

Sobemovirus P1 is needed for systemic infection, but not for replication, as previously described in several studies. For example, *Southern bean mosaic virus* (SBMV) P1 initiation codon mutants can replicate in host protoplasts (Sivakumaran et al., 1998) and RYMV P1 initiation codon mutant replicates in rice protoplasts, but not in rice plants whereas P1 deletion mutant fails to replicate at all (Bonneau et al., 1998). Transgenic rice plants expressing exogenous P1 complement the systemic movement of the RYMV P1 initiation codon mutant (Bonneau et al., 1998). Controversially, in this study we demonstrate that mutant RYMV without P1, can still replicate in rice plants. This discrepancy most likely stems from the fact that (Bonneau et al., 1998) used Northern blot methodology instead of sensitive strand-specific RT-PCR. On the other hand, CMV without P1 cannot be detected at all, although it has been shown that CMV P1 initiation codon mutant can replicate in inoculated leaves of oat plants, although it cannot move to the upper leaves (Meier et al., 2006). Deletion of the whole P1 gene area may lead to disruption of the CMV genomic RNA secondary structure and therefore, RNA replication. Finally a recently discovered ORFX of sobemoviruses (Ling et al., 2013), partially overlapping with ORF1, may be needed for the replication of CMV. In the current study, both ΔP1-CMV and RP1-CMV included a deletion of the putative initiation codon of ORFX. Interestingly, ORFX in RYMV is not overlapping with ORF1 (Ling et al., 2013).

The P1 protein of RYMV is thought to have a role in viral accumulation and either suppressing or enhancing RNA silencing to maintain the balance between host and virus (Lacombe et al., 2010). In addition to the finding that RYMV can replicate in rice and non-host species – oat, wheat, barley, *A. thaliana* and *N. benthamiana*, we show that it can do so without P1 and, interestingly, also with CMV P1 gene. Furthermore, all three RYMV constructs were detected outside the inoculated area of all tested monocots, which means RYMV can move from cell to cell without P1. Hence, RYMV P1 is not a local movement protein. However, absence of P1 does not permit systemic spread of RYMV in host plant. This feature is most likely related to silencing suppression activity, as it has previously been recorded that RYMV P1 acts as a silencing suppressor and its effect is even stronger at systemic level (Fusaro et al., 2012; Sarmiento et al., 2007). Similarly to RYMV P1, CMV P1 also has a role in a silencing suppression (Sarmiento et al., 2007). Interestingly, CMV P1 gene in RYMV did not complement for the lack of systemic movement ability of RYMV in oat, wheat or barley, the hosts of CMV. Most probably, CMV P1 is not solely related to suppression of the host antiviral defence system but it also has to interact with other CMV proteins to achieve suppression of the host antiviral defence and a successful infection.

Our analysis reveals that experimental host range supporting RYMV replication in inoculated leaf is quite different from another sobemovirus, CMV (Table 1). It has never been reported before that RYMV can replicate in oat, wheat, barley, *N. benthamiana* and *A. thaliana*. However, these species do not act as the subliminal hosts, enabling only the replication and very restricted movement of virus to few neighbouring cells, but all these species facilitated both the replication and the movement of RYMV on a level of local infection. Also, in non-host species, RYMV is not sap-transmissible from one plant to another. However, since previous reports indicate that RYMV isolates may have different host ranges (Allarangaye et al., 2007), it cannot be ruled out that our findings do not apply to all RYMV isolates.

Additionally, our finding that the most widely studied model organism among plants, *A. thaliana*, is susceptible to RYMV, opens new possibilities in sobemovirus research, especially considering virus replication or RNAi suppression.
4. Experimental procedures

4.1. Virus isolates and viral constructs

The RYMV infectious clone (RYMV) and constructs containing RYMV sequence used in this study originate from the Ivory Coast isolate (Brugidou et al., 1995). The RYMV construct without P1 has been previously described as PL5ΔΔ88-547 (Bonneau et al., 1998). Here, we named it ΔP1-RYMV. Both RYMV and ΔP1-RYMV were kindly provided by Dr. Christophe Brugidou (Institut de Recherche pour le Developpement, Montpellier, France). CfMV infectious clone (CfMV) and constructs containing CfMV sequences originate from Norwegian isolate (Mäkinen et al., 1995; Meier et al., 2006). All primer sequences are described in the Supplementary Table 1. Enzymes used throughout the study are from Thermo Scientific if not stated otherwise.

CfMV construct without P1 (ΔP1-CfMV) was obtained by amplifying CfMVic sequence, which was put in pUC19 vector, with DNA enzyme DNA polymerase so that the primers CfMV SphI S and CfMV SphI AS would be complementary to the bordering sequences of P1. The PCR fragment was cut with Bsp1407I and Bsp119I, the ends of the fragment were filled via T4 DNA polymerase and the ends were ligated with T4 DNA ligase.

To obtain the chimeric constructs of both viruses the P1 gene sequences from CfMV and RYMV were amplified using Taq DNA polymerase (Thermo Scientific) and CfMV SphI S and CfMV SphI AS and 2RYMV S and 3RYMV AS primers, respectively. The P1 sequence of CfMV was ligated into ΔP1-RYMV and named CP1-RYMV. P1 from RYMV was ligated into ΔP1-CfMV and named RP1-CfMV.

All DNA constructs used in this work were verified by sequencing.

4.2. In vitro RNA transcription

RYMV, ΔP1-RYMV, and CP1-RYMV plasmids were linearized with HindIII and CfMV, ΔP1-CfMV, and RP1-CfMV plasmids with SalI. In vitro RNA transcription was carried out with T7 RNA polymerase with m7G(5')ppp(5')G RNA cap structure analog (New England Biolabs). Template DNA was degraded with TURBO™ DNase (Ambion), RNA was extracted with phenol:chloroform and precipitated with ethanol.

4.3. Plant inoculation and virus detection

In vitro transcribed RNA was bound to 1.0 μm gold particles (chemPUR®) and plants were inoculated biolistically (Helios, BioRad). Plant species and cultivars used in this study were oat ‘Jaak’, wheat ‘Zebra’, barley ‘Viire’, rice ‘IR4’, N. benthamiana and A. thaliana (Col-0).

Inoculation was performed with 11–13 d old cereals, 18–20 d old A. thaliana plants and 25–27 day old N. benthamiana plants, respectively. Inoculated leaves were collected 7 days post inoculation (dpi) to analyze the local infection and upper leaves were collected 21 dpi to detect a systemic infection. Non-inoculated plants were used as negative control. Total RNA was extracted by homogenizing plant leaves with Tissue Lyzer (Qiagen) and isolating RNA according to Oñate-Sánchez and Vicente-Carbajosa (2008). The analysis of viral RNA was carried out by RT-PCR. For detection of positive and negative strand of the RNA, virus-specific primers were used (Supplementary Table 1). Revert Aid Reverse Transcriptase for the complementary strand DNA synthesis and 5x FIREPol® Master Mix Ready to Load (Solis Biodyne) for the PCR reaction were used.

For the analysis of virus movement, the plants were biolistically inoculated and samples were collected outside the bombarded area, whereas the sampled part of the leaves was protected during the bombardment with folium shield. After 7 days, leaf samples, situated apically and basally 1–2 cm outside of the inoculated area, were collected and virus was detected as described above.

For the analysis of sap transmission, the inoculated area of leaves was collected from each plant species at 7 dpi. For additional positive control, upper leaves of previously infected rice plants that were showing symptoms, were used. All samples were ground in liquid nitrogen and suspended in 10 volumes (w/v) of 20 mM sodium phosphate and potassium phosphate buffer (pH 7.2) containing 0.7% Celite. This suspension was used to mechanically inoculate plants and the virus was detected as described above.

All the experiments were carried out at least in two biological replicates and the viruses were verified by sequencing.

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Table 1

Overall results. Results obtained with all plant species and virus constructs used in this work, showing whether virus plus or minus RNA strand was detected from inoculated leaves or from upper leaves (I/U). Virus movement is also shown via identification of viral plus strand RNA outside the inoculated area.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Strands</th>
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<th>Viruses</th>
<th>RYMV</th>
<th>ΔP1-RYMV</th>
<th>CP1R</th>
<th>CFMV</th>
<th>ΔP1-CF MV</th>
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nt – not tested.
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Appendix A. Supplementary material
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2016.12.007.

References