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An attempt to identify recombinants between two sobemoviruses in doubly infected oat plants

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Recombination in RNA viruses is considered to play a major role as a driving force in virus variability to counterbalance loss in fitness that can be due to the accumulation of detrimental mutations. Studies on mixed infections are pertinent for understanding the role of recombination in virus evolution. They also provide important baseline information for studying the biosafety of plants expressing viral sequences. To investigate the possibility of RNA recombination occurrence between two sobemoviruses under little or no selection pressure, we co-infected test plants with *Cocksfoot mottle virus* (CfMV) and *Ryegrass mottle virus* (RGMoV). CfMV and RGMoV were selected because of their overlapping host range and geographical distribution. First, symptom development of both viruses in barley (*Hordeum vulgare*) and oat (*Avena sativa*) was examined. Both viruses generated quite strong infection symptoms in oat, but synergism was not detected. RGMoV was lethal for barley, whereas CfMV infection in barley was nearly symptomless. RT-PCR analysis revealed 100% infection with both viruses in oat but not in barley. Therefore, an RNA recombination study of CfMV and RGMoV was performed in oat. 105 plants were co-inoculated with both viruses and putative recombinational hot spot regions were screened for recombination events by RT-PCR analysis at a sensitivity level down to 0.1–100 pg of viral genomic RNA. No recombination events between the two sobemoviruses were detected.

Keywords: *Cocksfoot mottle virus* / *Ryegrass mottle virus* / RNA recombination / RT-PCR

INTRODUCTION

Recombination between homologous viral genomes is considered to be common in plants during natural co-infection of viruses (Garcia-Arenal et al., 2001). Genome sequencing data, as well as studies on virus gene functions, have suggested frequent recombination of geminiviruses, caulimoviruses, bromoviruses, tobamoviruses, potyviruses, closteroviruses, luteoviruses and their related genera (tombusviruses, poleroviruses and sobemoviruses) during evolution (Hull, 2002).

The unassigned genus *Sobemovirus* consists of plant viruses with a single (+)-stranded RNA genome whose 5' end is covalently bound to VPg, and packed into icosahedral particles (Hull and Fargette, 2005). The 5' terminus of the sobemovirus genome encodes the non-conserved P1 protein from ORF1 (Tamm and Truve, 2000). The middle part of the sobemovirus genome (encoding Pro-VPg-RdRp) is similar to that of the genus *Polerovirus* belonging to the family *Luteoviridae*, whereas the 3' part of the genome – encoding the coat protein (CP) from a subgenomic RNA – is related to the genus *Necrovirus* belonging to the family *Tombusviridae* (Hull and Fargette, 2005). The viruses of the genera *Polerovirus* and *Enamovirus* are classified in the family *Luteoviridae* according to their homology with genus *Luteovirus* at the 3' parts of their genomes, whereas their 5' parts are clearly distant from luteoviruses. At the same time, the products of ORF1 and ORF2 of viruses from genus *Luteovirus* are most similar to those of the viruses of the genus *Dianthovirus* from the family *Tombusviridae* (D'Arcy and Domier, 2005). Taking into account all of these homologies, it has been suggested that the key mechanism in the evolution of "the supergroup" composed of luteo-, sobemo- and tombusviruses is RNA recombination (Martin et al., 1990). A recent report of the sequence of *Poinsettia cryptic virus* described a virus showing a close relationship to poleroviruses within the

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first three quarters of its genome, but rather to sobemoviruses in the last quarter. The authors suggested to change the name of the virus to *Poinsettia latent mottle virus,* and to put it in the new genus *Polemovirus* (aus dem Siepen et al., 2005).

Sobemoviruses have narrow host ranges. Among the sequenced sobemoviruses, there are three monocot-infecting species: *Cocksfoot mottle virus* (CfMV), *Ryegrass mottle virus* (RGMoV) and *Rice yellow mottle virus* (RYMV). The natural host plants for CfMV are cocksfoot (*Dactylis glomerata*) and wheat (*Triticum aestivum*) (Serjeant, 1964). In experimental conditions, it is also propagated in barley (*Hordeum vulgare*) and oat (*Avena sativa*). RGMoV has been reported from cocksfoot and annual ryegrass (*Lolium multiflorum*) (Toriyama et al., 1983). In experimental conditions, it can also infect wheat, oat, barley, perennial ryegrass (*Lolium perenne*), red fescue (*Festuca rubra*), foxtail bristlegrass (*Setaria italica*), and rye (*Secale cereale*). The host range of *Rice yellow mottle virus* (RYMV) does not overlap with the previous ones at all. Furthermore, RYMV is occurs in tropical Africa, whereas the other two species are dispersed in areas with moderate climate, like the majority of known sobemoviruses. The geographical distribution of RGMoV and CfMV overlaps at least in Japan.

According to the suggestion that recombinational shuffling of genes and gene blocks has played an important role in the formation of contemporary species of "the supergroup" of luteo-sobemo-tombusviruses, the biosafety of using these viruses for biotech applications, such as viral vectors expressing foreign genes or pathogen-derived virus resistance, must be considered.

To identify recombination potential in the sobemovirus group, 105 oat plants were co-inoculated with CfMV and RGMoV. The experiments were carried out under little or no selection pressure (*i.e.* non-transgenic plants were infected with two wild-type viruses), to avoid positive selection for escape-recombinations that may reflect the results of selective pressure rather than the mechanism of recombination itself. Also, the real frequency of recombinational events may be underestimated if the recombinants with higher fitness are selected and those with negatively selected are lost (reviewed by Aaziz and Tepfer, 1999). Regions of the –1 ribosomal frameshifting signal and a putative subgenomic RNA promoter were selected to screen for recombination events, because they probably possess (Tamm, 2000) secondary structure elements that have been suggested to be potential hot spots for a RNA recombination. This study is the first survey of recombination between sobemoviruses.

RESULTS

Symptom development

In order to evaluate their appropriateness for virus recombination studies, two experimental hosts, oat and barley, were examined for virus propagation and symptom development in the case of single or double infections with wild-type CfMV and RGMoV. In barley, CfMV infection was nearly symptomless, causing slight mottling of leaves, whereas RGMoV induced a rapid yellowing of systemic leaves (Fig. 1A). Infection with RGMoV was usually lethal two weeks after inoculation (Fig. 1B). In oat, the infections of CfMV and RGMoV caused quite strong chlorotic mottling of leaves (Fig. 1A), but with no fatal consequences (Fig. 1B). Oat plants infected with RGMoV became slightly stunted (Fig. 1B), as described previously (Yao et al., 2002). In both hosts, the symptoms of RGMoV prevailed if co-inoculated with CfMV (Fig. 1A). The RT-PCR analysis of systemic leaves of co-inoculated plants indicated that both viruses were present in oat, whereas CfMV was absent roughly in half of the doubly inoculated barley plants (Fig. 1C). Therefore, oat plants were chosen for further experiments. Synergistic effects in symptom development were observed neither in barley nor in oat in the case of double infections.

Specificity and sensitivity of RT-PCR

When the entire genome sequences of CfMV and RGMoV were aligned, the identity was of 48%, with score 481 and *E* value e^{-132}. This alignment was used to design virus-specific primer pairs for amplifying the regions of the –1 ribosomal frameshifting signal and the putative subgenomic RNA promoter. In the frameshift region, between CfMV (1343–1942 nt) and RGMoV (1527–2136 nt), the identity was of 53.9%, with score 1681. In the putative subgenomic RNA promoter region, between CfMV (2818–3380 nt) and RGMoV (3009–3593 nt), the identity was of 50.2%, with score 1421.5. The largest blocks of sequence identity were 10 bp and 8 bp, respectively.

The primers were named as follows: the first letter (C or R), refers to CfMV or RGMoV, sg or fs designate the subgenomic promoter or frameshift region, and 5' or 3' corresponds to the position of the primer relative to the putative recombination hot spot. Specific detection of viruses was achieved both with CfMV- and RGMoV-specific primer pairs when amplifying the regions of the –1 ribosomal frameshifting signal or the putative subgenomic RNA promoter in the RT-PCR analysis of

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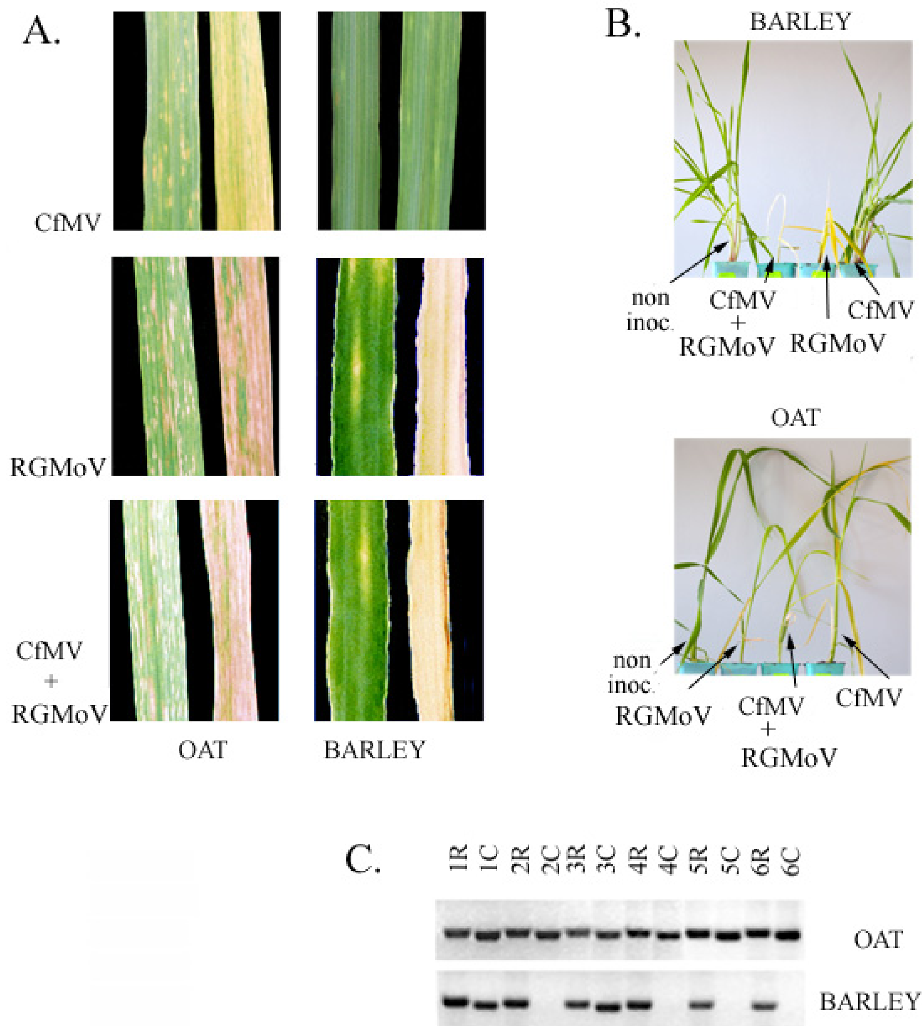

Figure 1. A. Macroscopic symptoms in oat and barley leaves inoculated with CfMV, RGMoV or both at 14 dpi. **B.** Barley and oat plants infected with CfMV, RGMoV or both at 21 dpi. **C.** RT-PCR analysis of systemic leaves of 6 plants of oat and barley co-inoculated with CfMV and RGMoV at 21 dpi. Primers Rsg5'+Rsg3' were used to detect RGMoV (R), and primers Cfs5'+Cfs3' were used to detect CfMV (C).

singly infected oat plants. No unspecific amplification was observed with these primer pairs when the RNA extracted from the CfMV-infected plant was artificially mixed with that extracted from the RGMoV-infected plant before using it as a template for RT-PCR (Fig. 2).

Recombinant primer pairs Csg5'+Rsg3', Rsg5'+ Csg3' and Cfs5'+Rfs3' did not amplify any fragment from the RNA isolated from singly CfMV- or RGMoV-infected oats or from the artificial mixture of these two (Fig. 2). Thus, these primer pairs were appropriate for the recombinant virus screen. The primer combination of Rfs5'+Cfs3' amplified several non-specific fragments from the RNA extracted from plant material with a single infection of RGMoV as well as from its mixture with the RNA from CfMV-infected oat. Therefore, the primer pair Rfs5'+Cfs3' was not suitable for a recombination study.

The whole genome alignment of CfMV and RGMoV was also used design and then clone artificial hybrid virus templates (Fig. 3A) that were used to test the sensitivity of recombinant primer pairs. The primer pairs Cfs5'+Rfs3' and Rsg5'+Csg3' had good sensitivity – they detected down to 0.1 pg of the *in vitro* synthesized recombinant template RNA from the mixture of 0.5 µg total RNA of non-inoculated oat (Fig. 3B). In contrast, the

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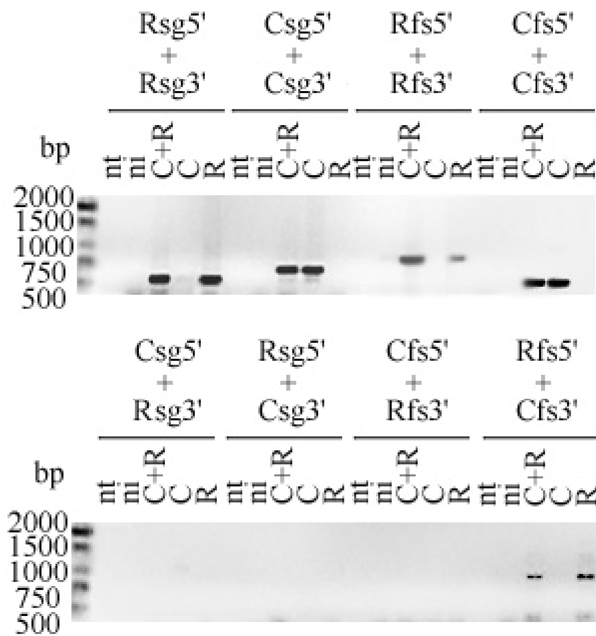

Figure 2. Specificity of primer pairs in combinations specific to parental and recombinant viruses. Templates used in RT-PCR: nt, no template; ni, RNA extracted from non-inoculated oat; C+R, RNA extracted from CfMV-infected oat mixed with RNA extracted from RGMoV-infected oat; C, RNA extracted from CfMV-infected oat; R, RNA extracted from RGMoV-infected oat.

primer pair Csg5'+Rsg3' detected the presence of template RNA in the mixture at a sensitivity level down to only 100 pg (Fig. 3B).

For the recombination study, 105 oat plants were doubly inoculated with CfMV and RGMoV in five groups within three days: 25 plants were co-inoculated simultaneously on day 0, whereas 20 singly CfMV- or RGMoV-inoculated plants were inoculated with the other virus on day 1, and 20 of singly CfMV- or RGMoV-inoculated plants were inoculated with the other virus on day 2. According to the RT-PCR analysis, 99 of the 105 plants contained both viruses at 7 days post-inoculation (dpi) (Tab. 1). No differences were observed in the development of co-infection when the five inoculation groups were compared. Plant material was collected from the inoculated leaves to obtain an original pool of possible recombinant molecules and to avoid loss of those with lower fitness. However, no RT-PCR products were detected using primer pairs specific to the recombinant viruses at a sensitivity level down to 0.1–100 pg of the recombinant template RNA from any of doubly infected oats (Tab. 1).

DISCUSSION

Previous *in vivo* recombination studies on bromo-, carmo- and tombusviruses have established that recombination does not occur randomly within viral RNA genomes, but there are recombination hot spots. These include short AU-rich sequences, inter- and intramolecular secondary structures (stem-loop structures and heteroduplexes formed between complementary stretches present in separate RNAs) and cis-acting RNA elements with high affinity toward the viral replicase (5' end replicase pausing sites, replication enhancers, genomic and subgenomic promoters). Also, the presence of non-templated nucleotides at recombination junction sites is essential (reviewed by White and Nagy, 2004).

In this study, sobemovirus regions of the –1 ribosomal frameshifting signal and the putative subgenomic RNA promoter were selected to screen for potential recombinational hot spots. The –1 ribosomal frameshift signal characteristic of CfMV consists of a slippery sequence (UUUAAAC) and a stem-loop structure several nucleotides downstream from it. It can be found in all sequenced sobemovirus genomes (Tamm and Truve, 2000). The slippery sequence positioned in CfMV at nt 1634–1640 aligned well with the corresponding sequence of RGMoV at nt 1842–1848. In contrast, the exact position of the sobemoviral subgenomic promoter is not known. By analogy with 5' end of the genomic sequence, a transcription start point for subgenomic RNA was suggested to be ACAAA for SBMV, RYMV and LTSV, located a few nucleotides upstream the CP gene start codon (Tamm and Truve, 2000). This sequence motif is not present in CfMV (Tamm and Truve, 2000). In this study, the subgenomic RNA promoter was expected to lie in the range of –150 and +150 nt from the CP translation

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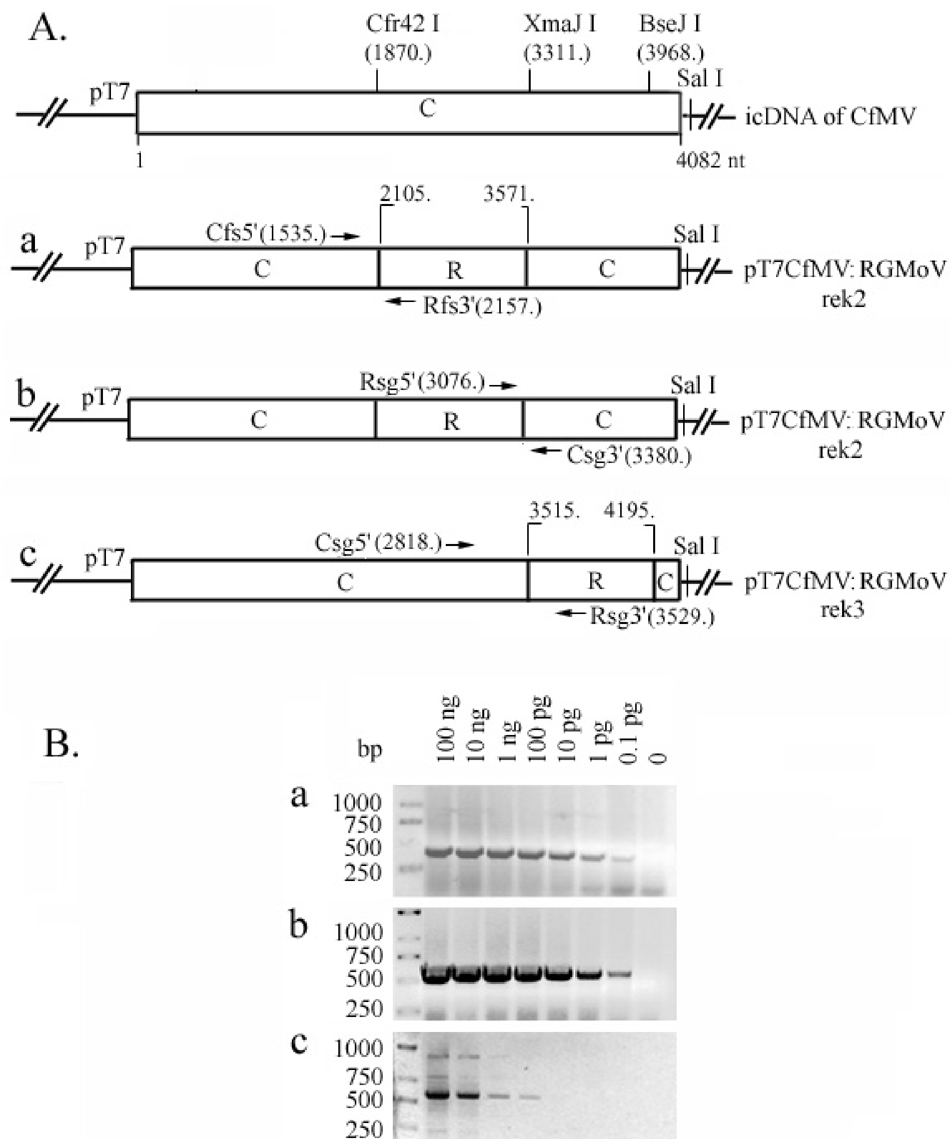

Figure 3. A. Schematic presentation of recombinant CfMV:RGMoV clones constructed for *in vitro* RNA synthesis. Arrows show primer pairs specific to recombinant viral RNAs. C, CfMV, R, RGMoV. **B.** Sensitivity of primer pairs (marked with a, b and c according to panel A) specific to recombinant viral RNA. The RT-PCR analysis was performed using primer pairs and *in vitro* synthesized RNA templates diluted in 10-fold series (from 100 ng to 0.1 pg). 0.5 µg of RNA extracted from non-inoculated oat was added into all RT-PCR reactions to mimic the natural proportions of cellular and viral RNAs.

Table 1. RT-PCR analysis of inoculated oat plants at 7 dpi.

No. of plants infected			No. of recombinants detected			
CfMV[1]	RGMoV[2]	CfMV + RGMoV[1,2]	Rfs5' + Cfs3'	Cfs5' + Rfs3'	Rsg5' + Csg3'	Csg5' + Rsg3'
100/105	102/105	99/105	NS[3]	0/99	0/99	0/99

[1]Cfs5'+ Cfs3'.
[2]Rsg5' + Rsg3'.
[3]NS- non-specific RT-PCR products appeared.

start point, as characteristic of other plant viral subgenomic promoters (reviewed by Miller and Koev, 2000).

All together, the areas between 1535 nt and 1967 nt, as well as between 2818 nt and 3380 nt in the CfMV genome, and between 1440 nt and 2157 nt, as well as between 3076 nt and 3529 nt in the RGMoV genome, were screened by RT-PCR for possible recombination

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products, using combinations of appropriate primer pairs. However, when the 99 CfMV and RGMoV co-infected oat plants were screened, no RT-PCR products were detected using primer pairs specific to recombinant viruses at a sensitivity level down to 0.1–100 pg of the recombinant template RNA. It remains unknown whether recombination could occur elsewhere between the two genomes.

No intra-specific or inter-specific recombinant sobemoviruses have been described so far. Based on the phylogenetic analysis of 14 RYMV full-length isolates and 58 capsid protein genes sequences, it has been concluded that RYMV evolved in the absence of recombination events (Chare and Holmes, 2006; Fargette et al., 2004). Similarly, on the basis of the phylogenetic comparison of six sobemovirus genomes, including CfMV, RYMV, Lucerne transient streak virus (LTSV), Sesbania mosaic virus (SeMV), Southern cowpea mosaic virus (SCPMV), and Southern bean mosaic virus (SBMV), it was suggested that recombination is neither frequent nor significant in the sobemovirus group (Lokesh et al., 2001).

On the other hand, the presence of viral defective interfering molecules (DI) is considered to be proof of a replicase-driven template switching mechanism (reviewed by White and Morris, 1999). Five DI RNAs of CfMV have been found, corresponding to 35–40 nucleotides of the 5'-proximal end of genomic RNA linked with 850–950 nucleotides of the 3' terminus (Mäkinen et al., 2000).

There can be various reasons for the poor detection of recombinant virus molecules. Generally, five steps must be passed to generate a viable recombinant between different viruses or virus strains: co-infection of the host, co-infection of the cell, replication, template switching, and selection (reviewed by Worobey and Holmes, 1999). Since the length of a replication cycle of CfMV and RGMoV is not known, co-inoculations were performed in five groups within three days to provide the possibility of simultaneous replication and potential recombination between these two viruses. No differences were observed in the development of co-infection in these five inoculation groups. The results presented here are based on the RT-PCR analysis, which confirm the co-infection of the same leaf and replication of both viruses. However, co-infection of the same cell may only be assumed. If there exist any restrictions (*e.g.* cross-protection between related viruses, reviewed by Roossinck, 2005) for the co-infection of the cell, then it would automatically diminish a chance for recombination events and further selection. Therefore, it would be necessary to study the co-localization of CfMV and RGMoV at the cellular level using specific antibodies or any other application to distinguish them in the co-infected plants.

Discrete distribution of related virus strains or species has been described at least for *Tobacco mosaic virus* (TMV), *Alfalfa mosaic virus* (AMV), *Potato virus X* (PVX), *Plum pox virus* (PPV), and *Tobacco vein mottling virus* (TVMV) (Dietrich and Maiss, 2003; Divéki et al., 2002; Hull and Plaskitt, 1970; McKinney, 1929). Spatial separation patterns with very few cells expressing both viruses have been documented for different potyvirus species – PPV, TVMV and *Clover yellow vein virus* (ClYVV) (Dietrich and Maiss, 2003). However, there is substantial evidence for intra- and interspecific recombination between potyviruses derived from sequencing and phylogenetic analysis of natural virus populations (Bousalem et al., 2000; Cervera et al., 1993; Chare and Holmes, 2006; Desbiez and Lecoq, 2004; Fanigliulo et al., 2005; Ohshima et al., 2002; Tan et al., 2004; Zhong et al., 2005).

Studies on plant virus recombination demonstrate that the properties of the viral replicase and several host factors also play a role in RNA recombination. When complementary RNA synthesis and template switching of carmo- (*Turnip crinkle virus*, TCV) and tombusvirus (*Cucumber necrosis virus*, CNV) replicases were tested *in vitro*, TCV replicase was stimulated by a CNV replication enhancer element, whereas CNV replicase discriminated against the TCV replication enhancer element (Cheng et al., 2005). *Tomato bushy stunt virus* (TBSV) replicase can conduct RNA recombination at an AU-rich signal that constitutes a recombination hot spot in *Brome mosaic virus* (BMV) and retroviruses (Shapka and Nagy, 2004). Mutations within the RNA-binding domains of a replicase affect the frequency of recombination – for CNV replicase it was shown that mutations could both delay or accelerate the formation of recombinants (Panaviene and Nagy, 2003). These studies imply that recombination between two viruses and post-recombinational amplification depends on the template switching ability of a specific replicase.

The recombination potential of TBSV, *Turnip mosaic virus* (TuMV), *Yam mosaic virus* (YMV) and BMV has been shown to depend on the host (Bousalem et al., 2000; Desvoyes and Scholthof, 2002; Dzianott and Bujarski, 2004; Ohshima et al., 2002). Screening the recombination efficiency of TBSV using a yeast single-knockout library revealed that host genes involved in RNA degradation were suppressing the generation of new viral RNA recombinants. In contrast, genes contributing to the intracellular transport of proteins were identified as viral RNA recombination accelerators (Serviene et al., 2005; Serviene et al., 2006).

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In this study, approximately one quarter of the CfMV and RGMoV genomes were monitored with highly sensitive and specific RT-PCR for the generation of recombinant molecules in co-inoculated oat plants. No recombination was detected between the two sobemoviruses in the regions of the –1 ribosomal frameshifting signal and a putative subgenomic RNA promoter. These regions were selected because of their content of secondary structures and homologous blocks (8–10 bp). The priming between the donor and acceptor strands as well as re-initiation of the dissociated replicase/nascent RNA complex have specific requirements. For example, CNV replicase favors base-paired regions of 4–5 bp in length, being less effective with shorter or longer regions (Cheng et al., 2002). In contrast, recombination between two cucumoviruses (*Bromoviridae*), *Cucumber mosaic virus* (CMV) and *Tomato aspermy virus* (TAV), occurred more frequently in longer blocks, and particularly in ones of at least 15–20 identical nucleotides (De Wispelaere et al., 2005; Suzuki et al., 2003). As no recombinants were detected between the two sobemoviruses, it is not clear whether the reason is the extent and/or the content of identical blocks or not. It was surprising that no recombination events in the putative subgenomic promoter area were identified, as the evolution of Pro-VPg-RdRp/CP region in the "supergroup" of luteo-sobemo-tombusviruses is modular (aus dem Siepen et al., 2005; Gibbs and Cooper, 1995; Martin et al., 1990; Mayo and Jolly, 1991; Mayo and Ziegler-Graff, 1996; Miller and Rasochova, 1997; Moonan and Mirkov, 2002; Moonan et al., 2000). Thus, although the evolutionary analysis supports the idea of frequent recombinations within this supergroup, this study was unable to confirm that RNA recombinations take place during the replication of sobemoviruses.

MATERIALS AND METHODS

Plants, viruses and virus inoculation

Oat cv. Jaak and barley cv. Kymppi were grown in soil mix (vermiculite:peat:soil, 1:1:2) in a climate chamber (60% relative humidity, 16 h light at 23 °C, 8 h dark at 16 °C).

CfMV Norwegian isolate (Mäkinen et al., 1995) and RGMoV Japanese isolate (obtained from MAFF GeneBank, 307043) were used throughout the study. The stocks of infected plant material were frozen in liquid nitrogen and stored at –70 °C.

The inoculums were prepared by grinding symptom-expressing leaves from the stock of frozen material in a mortar with 1 ml 100 mM phosphate buffer (pH = 7.0), supplemented with 0.5% celite per 1 g of leaf material. 50 µl of freshly prepared sap containing CfMV or RGMoV or a 1:1 mixture of both inoculums was rubbed onto the leaves of 2-leaf stage plants 10 days after sowing. Six plants of barley and oat, respectively, were used with each inoculum in parallel for monitoring symptom development. In the recombination study, 105 oat plants were mechanically inoculated with CfMV and RGMoV in five groups. On day 0, 25 plants were co-inoculated with both viruses, 40 plants were singly inoculated with RGMoV and 40 with CfMV. Half of the singly inoculated plants were inoculated with the other virus on day 1 and another half on day 2.

RNA extraction

0.2 g of inoculated leaf material was harvested at 7 dpi, followed by total RNA extraction according to Logemann et al. (1987). Systemically infected upper leaves of doubly inoculated oat and barley were collected at 21 dpi. The integrity of extracted RNA was checked by electrophoresis in a 6% formaldehyde, 0.8% agarose gel buffered with 1 × MOPS pH 7.0.

Sequence alignment

The nucleotide sequences of CfMV (GeneBank accession no. Z48630) and RGMoV (GeneBank accession no. AB040446) were aligned pairwisely using NCBI-BLAST2 version BLASTN 2.2.4 (Tatusova and Madden, 1999). To make the alignment, the reward for match was raised (from 1 to 2) and the penalty for mismatch was lowered (from –2 to –1). Based on this alignment, CfMV- and RGMoV-specific primer pairs were designed to amplify the areas of the –1 ribosomal frameshifting signal and the putative subgenomic RNA promoter.

The same alignment was used to design primers for the amplification of RGMoV cDNAs, in order to exchange them to the corresponding CfMV sequences in the CfMV infectious cDNA (icDNA) clone.

Plasmids

The cDNA fragments of RGMoV were generated from total RNA isolated from RGMoV infected plants by RT-PCR, using primers 5'REK2 (5'-AGA*GCCGGC*AG-GCAGATTCCGC-3') and 3'REK2 (5'-GA*CCTAGG*A-GAGCACCGTGCCG-3') to amplify nt 2105 to 3571 (cDNA2), 5'REK3 (5'-CAT*CCTAGG*TTAGTACGC-GTCACAT-3') and 3'REK3 (5'-GA*GATTGGTATC*-CCCCTACGCTAG-3') to amplify nt 3515 to 4195

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(cDNA3). The restriction endonuclease sites (*Pdi*I, *Xma*JI, *Bse*JI) flanking the RGMoV sequence are shown in italics. All the cDNAs generated were inserted into the pTZ57R/T cloning vector (Fermentas) and sequenced.

To construct recombinant CfMV:RGMoV molecules (Fig. 3A), the CfMV icDNA (Meier et al., 2006) was linearized with *Cfr*42I, and the cohesive ends were filled by T4 DNA polymerase (Fermentas) treatment. RGMoV cDNA2 digested with *Bsp*68I and *Xma*JI was ligated into CfMV icDNA, cut by *Cfr*42I and *Xma*JI to create pT7CfMV:RGMoVrek2. pT7CfMV:RGMoVrek3 was obtained by inserting *Xma*JI and *Bse*JI digested RGMoV cDNA3 into similarly cut CfMV icDNA.

RT-PCR

SuperScript One-Step RT-PCR kit with Platinum Taq Polymerase (Invitrogen) was used throughout the study. The reactions were carried out as suggested by the manufacturer: first-strand cDNA synthesis was accomplished in 30 min at 55 °C, PCR was carried out in 30 cycles (15 sec at 94 °C, 30 sec at 55 °C, 30 sec at 72 °C) after the 2 min denaturation at 94 °C. A 10 min incubation at 72 °C was performed at the end of the program.

pT7CfMV:RGMoVrek2 and pT7CfMV:RGMoVrek3 were linearized with *Sal*I and used as templates for RNA synthesis carried out with T7 RNA polymerase (Fermentas). Thereafter, template DNA was degraded with DNase I (Ambion). RNA was purified using the RNAEasy kit (Qiagen) and quantified. The 10-fold serial dilutions of *in vitro* transcribed RNA were mixed with 0.5 µg of total plant RNA extracted from non-infected oat leaves to measure the sensitivity of RT-PCR for the detection of recombinant virus molecules.

pT7CfMV:RGMoVrek2 was used to test the sensitivity of the primer pair of Cfs5' (5'-AGCTGAGGCGTTGCGTGTCG-3' corresponding to CfMV nt 1535 to 1554) and Rfs3' (5'-CTCGGCACGCGCTGTCG-3' complementary to RGMoV nt 2157 to 2141) as well as the sensitivity of the primer pair of Rsg5' (5'-CGGACATACGTGAGCGGGAG-3' corresponding to RGMoV nt 3076 to 3095) and Csg3' (5'-CAATGCAGCGGGTGACACAA-3' complementary to CfMV nt 3380 to 3361), pT7CfMV:RGMoVrek3 was used to test the sensitivity of the primer pair of Csg5' (5'-GGTCGATGATGCTCCCAGGA-3' corresponding to CfMV nt 2818 to 2837) and Rsg3' (5'-TGTGACGCGTACTAAAGAGCCA-3' complementary to RGMoV nt 3529 to 3508).

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