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Analysis of external factors that affect formation and accumulation of D-RNAs in Bromovirus infections

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Abstract

Defective RNA molecules (D-RNAs) are being studied in several plant RNA virus groups. In the genus *Bromovirus*, D-RNAs have been described for *Broad bean mottle virus* (BBMV), they are formed by a single internal deletion in the RNA2 and have been generated *de novo* by serial passages. In contrast, in *Brome mosaic virus* (BMV) D-RNAs are generated by a single or double internal deletion in the RNA3 without serial passages in their hosts. In this work the external effects of the host and the growth temperature in the generation and accumulation of D-RNAs in BBMV, BMV and *Cowpea chlorotic mottle virus* (CCMV) are studied. The BBMV and BMV D-RNAs were generated and accumulated with or without serial passages of the viruses in different hosts and cultivars. Plants grown at 12, 16, 20 and 24°C in a growth chamber and in a greenhouse (22 ± 5°C) generated D-RNAs after being inoculated with virus-free D-RNAs, with and without passages. The D-RNAs observed in BBMV and BMV presented some common characteristics: both were formed *de novo* after serial passages or without passages, their deletion borders had short repeated and palindrome sequences that favour recombination. In addition, growing inoculated plants at lower temperatures greatly facilitated the generation and accumulation of D-RNAs. The CCMV did not generate defective molecules in cowpea (*Vigna unguiculata)* and *Nicotiana benthamiana* plants after several serial passages or without passages. This is the first time that D-RNAs have been generated in BBMV without passages and BMV with serial passages.

Additional key words: BBMV, BMV, CCMV, host effects, temperature conditions.

Resumen

Análisis de los factores externos que afectan a la formación y acumulación de los D-RNAs en infecciones de Bromovirus

Los RNAs defectivos (D-RNAs) están siendo estudiados en diversos virus RNA de plantas. En el género *Bromovirus* se han descrito D-RNAs en el virus del moteado del haba (BBMV), formados mediante una delección interna en el RNA2, que se generan *de novo* mediante pases seriados del virus sobre un nuevo huésped. En contraste, en el virus del mosaico del bromo (BMV), los D-RNAs se forman por una o dos delecciones en el RNA3 sin necesidad de pases en sus huéspedes. En este trabajo estudiamos algunos factores externos, como los huéspedes, las inoculaciones y las temperaturas de crecimiento en la generación y acumulación de D-RNAs en BBMV, BMV y en el virus del moteado clorótico de la carilla (CCMV). Los D-RNAs de BBMV y BMV fueron generados y acumulados mediante pases seriados y sin pases de los virus en diferentes huéspedes y cultivares. Plantas inoculadas con BBMV y BMV sin D-RNAs, mantenidas a 12, 16, 20 y 24°C en cámaras de crecimiento a temperatura constante o en un invernadero (22 ± 5°C), generaron D-RNAs con o sin pases sobre nuevos huéspedes. Los D-RNAs observados en ambos virus presentan algunas características en común: son formados *de novo* mediante pases seriados o purificados de hojas infectadas algunas semanas después de la inoculación; los bordes de la delección tienen secuencias repetidas, y secuencias palindrómicas que favorecen la recombinación; además, el crecimiento de las plantas inoculadas a bajas temperaturas favorece la formación y acumulación de D-RNAs. CCMV, después de diversos pases del virus o sin pases, no generó moléculas defectivas en plantas de *Vigna unguiculata* o *Nicotiana benthamiana*. Es la primera vez que se han generado D-RNAs en BBMV sin necesidad de pases y en BMV mediante pases seriados.

Palabras clave adicionales: BBMV, BMV, CCMV, condiciones de temperatura, efectos del huésped.

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Introduction[1]

Defective molecules and defective interfering RNAs (D-RNAs) have been studied recently, mainly to understand the mechanism of their formation, and the role they play in the control of virus replication. It is important to analyse the relationships that exist among these molecules, the helper virus, the host and the effect of environmental conditions on their generation. Many D-RNAs have been well characterized for most animal RNA virus groups (Holland, 1990) and several plant RNA virus groups including tombusvirus (Hillman *et al*., 1987), carmovirus (Li *et al.*, 1989), potexvirus (White *et al.*, 1991), closterovirus (Mawassi *et al.*, 1995; Rubio *et al.*, 2000), tospovirus (Inoue-Nagata *et al.*, 1998), tobamovirus (Lewandoski and Dawson, 1998), cucumovirus (Graves and Roosinck, 1995) and bromovirus (Romero *et al.*, 1993; Pogany *et al.*, 1995). All of them are derived from the helper virus genome by simple internal deletions or by complex rearrangements of genomic sequences (Simon and Bujarski, 1994). The D-RNA retains the cis-acting components necessary for efficient replication but depends on helper virus replication and encapsidation functions (Graves *et al.*, 1996). The presence of D-RNAs has important effects on host symptoms, ranging from amelioration to enhancement, to no effect at all (White and Morris, 1999).

Viruses belonging to the genus *Bromovirus* are widely distributed affecting different crops such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), corn (*Zea mays* L.) or legumes [*Phaseolus vulgaris* L.*, Vicia faba* L.*, Pisum sativum* L., and *Glycine max* (L.) Merr] (Lane, 1981; Ahlquist *et al*., 1984; Allison *et al*., 1989; Dzianott and Bujarski, 1991; Romero *et al*., 1992). The type member is *Brome mosaic bromovirus* (BMV). Their genome consists of three species of messenger-sense single-stranded RNA1, 2 and 3 (Ahlquist, 1992). The RNA1 and RNA2 encode the 1a and 2a proteins required for virus replication (Kibertis *et al.*, 1981), RNA3 encodes the 3a protein required for virus movement (Schmitz and Rao, 1996) and the coat protein which is synthesized from the subgenomic RNA4 by virus replicase from a promoter present in the (–) strand of RNA3 (Miller *et al*., 1985). Other members of this genus are *Cowpea chlorotic mottle virus* (CCMV), and *Broad bean mottle virus* (BBMV) (Lane, 1981). Defective molecules have been described and characterized for some of these viruses. In BMV D-RNAs are generated by a single or a double internal deletion in the RNA3 (Damayanti *et al*., 1999). They decrease accumulation of 3a protein but have no apparent effect on virus symptoms and were found in systematically infected barley leaves 8 weeks post inoculation (p.i.) or later but not before, when barley plants were inoculated with virions containing D-RNA. In BBMV several RNA2 – derived D-RNAs have been characterized (Romero *et al*., 1993), they are generated *de novo* after serial passages and their presence reduced the accumulation of RNA2 of the helper virus. They also exacerbated the severity of symptoms and have a negative effect on production, reducing yield by 50% (Romero *et al*., 1993; Pogany *et al*., 1995, 1997; Llamas *et al*., 2004; Sandoval *et al.,* 2007). Considerable progress has been made in the identification factors which may contribute to, or influence formation of D-RNAs (Graves *et al.*, 1996; White, 1996) but there is still much to learn about events involved in the generation of naturally occurring D-RNAs. In this work the generation and accumulation of D-RNAs was studied in BBMV, BMV and CCMV. They were characterized and some external factors which affects its accumulation such as plant host, environment and temperature analysed.

Material and Methods

Plant material, virus strains, plant inoculations and growing conditions

Broad beans (*Vicia faba*) cv. Agua Dulce, peas (*Pisum sativum*) cvs. Alderman, Froy and Voluntario, barley (*Hordeum vulgare*) cv. Scarlet, cowpea (*Vigna unguiculata*) and *Nicotiana benthamiana* plants were used in the experiments. The BBMV-Mo was a gift from K. Makkouk (ICARDA, Syria), BMV-M1 and CCMV-T were provided by J.J. Bujarski (NIU, Dekalb, USA). The BBMV was maintained by mechanical inoculation in broad beans, BMV in barley and the CCMV in cowpea. Production of RNA transcripts with T7 RNA polymerase

[1] Abbreviations used: BBMV (*Broad bean mottle virus*), BMV (*Brome mosaic virus*), CCMV (*Cowpea chlorotic mottle virus*), D-RNAs (defective RNA molecules), das (days after sowing), dpi (days post inoculation), LD (large D-RNAs), p.i. (post inoculation), PTGS (post-transcriptional gene silencing), RT-PCR (reverse transcription–polymerase chain reaction), SD (small D-RNAs), TBSV (*Tomato bushy stunt virus*).

and plant inoculation were done as described by Pogany *et al.* (1995) for BBMV, by Janda *et al.* (1987) for BMV and by Shang and Bujarski (1993) for CCMV. Plants were grown in a greenhouse (22 ± 5°C) or in a growth chamber with 16 h of light and a constant temperature of 12, 16, 20 or 24°C.

Inoculations

Host plants of BBMV (broad bean and peas), BMV (barley) and CCMV (cowpea and *N. benthamiana*) at the two-leaf stage were mechanically inoculated using 1 μg of viral RNA in an inoculation buffer (10 mM Tris pH 7.4, 1 mM EDTA, 0.2% bentonite and 0.2% celite). Viral RNA was obtained from plants inoculated with *in vitro* transcription of full-length cDNA clones of BBMV-Mo, BMV-M1, and CCMC-T.

Serial passages

Ten (BBMV), 15 (BMV) and 20 (CCMV) dpi, new plants were inoculated using a leaf extract of infected plants in phosphate buffer 10 mM (pH 7.0) and an abrasive (Carborundum 600 mesh, Prolabo). Plants were grown in a growth chamber at 12, 16, 20 or 24°C with 16 h day length or in a greenhouse (22 ± 5°C) for seven serial passages. For each serial passage viruses were analysed in 12 plants for each host. These experiments were repeated three times. For the experiments without passages, plants inoculated with the virus were kept in a greenhouse or in a growth chamber and analysed by D-RNA detection each week during the three months following inoculation.

Virus and D-RNAs analysis

To detect the virus and the presence of D-RNAs in the samples, the virus was purified from systemically infected leaves of each plant 2 weeks p.i., and the virion RNA was then extracted, following Romero *et al.* (1993). Virion RNAs were tested either by northern blot hybridisation (Kroner *et al.,* 1989) using digoxigenine-labeled RNA probe that was complementary to nt 39 to 461 of BBMV RNA2 or to the full length RNA3 of BMV and by a reverse-transcription polymerase chain reaction (RT-PCR) (Romero *et al.,* 1993). Oligonucleotide primers 37, 58, 90 and 170 for BBMV (Llamas *et al.*, 2004) or 5'-CGGAATTCCGATTCCGG CGAACATTCTATTTTACCAACATC-3' (primer B5, specific for BMV RNA3 with an *Eco*RI restriction site (underlined), corresponding to nt 25 to 57 in BMV RNA3) and 5'-CGGAATTCTGGTCTCTTTTAGAGATTTA-3' (primer B3; *Eco*RI site (underlined) corresponding to nucleotide positions 2117 to 2098 of BMV RNA3) for BMV were also used. Finally, the cDNA products were cloned and sequenced as described by Pogany *et al.* (1995).

Results

Characterization of DI-RNAs of BBMV generated without passages

We previously had demonstrated that BBMV D-RNAs could be formed *de novo* after several passages of BBMV infection through broad bean plants by using a high concentration of viral inoculum (Romero *et al.*, 1993; Pogany *et al.*, 1995). To continue these studies and to observe if broad bean plants could support the formation of D-RNAs without passages, broad bean seedlings were inoculated with BBMV RNA that was free of D-RNAs and the plants were grown in a greenhouse. At 30 dpi additional RNA bands, similar to D-RNA, emerged (data not shown). To determine the nature of this new RNA, it was amplified by RT-PCR with a pair of BBMV-RNA2-specific oligonucleotide primers 58 and 170, and the cDNA products were cloned and sequenced. These analyses showed that the additional RNA was a D-RNA derived from BBMV RNA2. The new accumulated D-RNA shared the characteristics of previously described BBMV D-RNAs: a large central deletion, conservation of the 5' and 3' extremes and maintenance of the open reading frame of protein 2a. These results demonstrate that in BBMV, D-RNAs can be formed *de novo* without passages.

We also had demonstrated that *de novo* formation or accumulation of BBMV D-RNAs preferably occurred at lower temperatures, reducing the number of passes necessary for their accumulation (Llamas *et al.*, 2004). To study the effect of temperature on the formation of D-RNAs without passages, broad bean plants inoculated with BBMV-RNA and free of D-RNA components were incubated in a growth chamber or in a greenhouse at temperatures of 12, 16 and 20°C. Samples of upper leaves were collected periodically to determine the presence of new RNA bands. D-RNA was observed

Table 1. Effect of temperature on the formation of BBMV D-RNAs without passages through broad bean plants[a]

Temperature (°C)	Days post-inoculation[b]	Size of D-RNA detected (kb)
12	36	1.9
16	38	1.9
20	40	1.9
Greenhouse	35	2.4

[a] Broad bean seedlings were inoculated with BBMV D-RNA- free and maintained in a greenhouse (22 ± 5°C) or a growth chamber at different temperatures. The viral RNA was purified periodically and analysed by electrophoresis and RT-PCR. [b] Numbers indicate days after inoculation at which D-RNAs were observed.

between 30 to 40 dpi (Table 1), giving no visible effect of the temperature on D-RNA formation under these conditions. The size of new D-RNAs was as expected for the effect of the growing conditions on accumulation of D-RNAs in broad bean (Table 1), as described by Llamas *et al.* (2004).

To test whether the growth state of broad bean plants had an effect in the formation and size of D-RNAs without passages, the assay was repeated using different growth stages of broad bean plants for inoculation with BBMV RNA free of D-RNA. Inoculated plants were grown in a greenhouse and the formation of D-RNA observed periodically. The experimental results showed that the later the inoculation was performed the faster D-RNA was formed (Table 2). Thus, in plants inoculated at 11 das, the D-RNA was formed 35 dpi, but in plants inoculated at 50 das, D-RNA was formed only 10 dpi; the size of the D-RNAs formed *de novo* was similar to that obtained in the passages experiments (Llamas *et al.*, 2004). Overall, the data demonstrated that the *de novo* formation or accumulation of BBMV D-RNA also occurred without passages.

Table 2. Effect of broad bean growth stage on the formation of BBMV D-RNAs without passages under greenhouse conditions[a]

Inoculation (das)	Detection of D-RNAs (dpi)	D-RNAs size (kb)
11	35	2.4 and 1.9
21	32	2.4
29	39	2.4 and 1.9
36	32	2.4 and 1.9
42	26	2.4 and 1.9
50	10	1.9

[a] Broad beans, at different growth stages, were inoculated with BBMV D-RNA-free and maintained in a greenhouse (22 ± 5°C). The viral RNA was purified periodically and analysed by electrophoresis and RT-PCR.

Types of BBMV D-RNA formed at different temperatures and in different hosts

To characterize the BBMV D-RNAs generated and accumulated *de novo* at different temperatures and in different hosts, a group of experiments, with serial passages of the virus, was made. Plants were incubated in a growth chamber at different temperatures or in a greenhouse and were inoculated with BBMV RNA free of D-RNA components. Passages through new plants were done at the same temperatures. The formation of D-RNAs was analysed every 10 d. D-RNAs were formed after 1 passage at 12°C in broad bean and pea, and in 7 passages in pea cv. Voluntario at 20°C as described by Llamas *et al.* (2004). Five different kinds of D-RNAs were identified, all of them with a single internal deletion of the RNA2 with sizes between 489 and 761 nt (Table 3). They had certain common characteristics at the ends of the deletions such as palindrome, repeated or similar sequences, or A, U rich regions (Fig. 1). From these results it is not possible to establish a clear relationship between the type of D-RNA generated, the plant host and the temperature at which the plants were grown.

Formation of D-RNAs of BMV by serial passages

The possible formation and accumulation of D-RNA in BMV, after several passages, was analysed. BMV infection was passaged seven times trough barley seedlings (cv. Scarlet) in a greenhouse. In parallel experiments, barley plants were incubated in growth chambers at 20, 16 and 12°C. Plants were inoculated with BMV RNA that was free of D-RNA components and the passages through new plants were done at the same temperatures. The first four passages, in a greenhouse, did not reveal the presence of D-RNA (Fig. 2A, lanes 1-4), however, after the fifth passage through barley additional D-RNA bands emerged (lanes 5 to 7). These bands were characterized by RT-PCR as D-RNAs derived from BMV RNA3, and their size was similar to that described by Damayanti *et al.* (1999) of 1.6 kb. When plants were grown at different temperatures,

Table 3. The location of crossover sites in five types of BBMV D-RNAs formed after serial passages in peas or broad bean plants maintained in a greenhouse or a growth chamber[a]

Clone	Position of crossovers[b]	Size of deletion (nt)	Source
DS1	1621-2382	761	Pea cvs. Froy and Voluntario, 12°C Pea cv. Froy, Greenhouse
DS4	1481-2224	743	Pea cv. Alderman, 16°C
DS5	1660-2197	537	Pea cvs. Froy and Voluntario, 16°C Pea cv. Froy, Greenhouse
DS6	1652-2176	524	Broad bean cv. Aguadulce, 16°C
DS7	1496-1985	489	Broad bean cv. Aguadulce, 16°C

[a] Peas and broad bean seedlings were inoculated with the D-RNA-free BBMV-RNA and maintained in a greenhouse (22 ± 5°C) or a growth chamber at different temperatures. Every 10 dpi, the virus was passed to a new plant. The virus and its RNA were purified after each passage and the D-RNAs were amplified by RT-PCR using the RNA2-specific primers 58 and 170. RT-PCR products were cloned and sequenced. [b] Numbers indicate the nt positions of the crossover sites (BBMV Mo RNA2, Acc. Nº U24496).

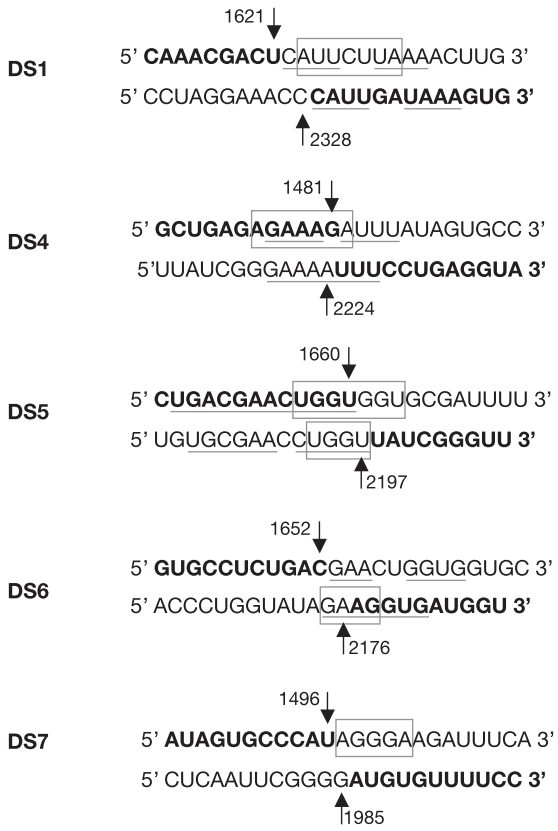

Figure 1. Schematic representations of crossover sites in five types of BBMV D-RNAs from pea or broad bean hosts. The upper chain indicates the 5' end of the deletion and the lower chain the 3' end. Conserved sequences are in bold. Underlined bases indicate repeated sequences on both edges and the box the palindrome sequences close to the deletion points. Numbers indicate the nucleotide positions of crossover sites.

D-RNAs were formed at 16 and 20°C, after five passages (Fig. 2B). However, at 12°C, formation of D-RNA was observed after the second passage (Fig. 2C). At 16 and 20°C and in the greenhouse only one type of D-RNA molecule of 1.6 kb was observed, but at 12°C two D-RNAs of 1.6 and 1.5 kb were formed (Fig. 2C). All of these D-RNAs were amplified, using the specific primers for BMV RNA3, cloned and sequenced, confirming that they were generated from deletions of BMV RNA3, maintaining the 3a protein open reading frame and the conservation of the 5' and 3' extremes (Table 4). The size and deletion points of these molecules were similar to the D-RNAs described by Damayanti *et al.* (1999).

Effect of the temperature on the formation of D-RNA in BMV without passages

Damayanti *et al.* (1999) observed the formation of D-RNA in barley 12 weeks after inoculation with BMV, without passages; this D-RNA was not detected after inoculation on new seedlings at 1-6 weeks p.i., but was detected after prolonged infections (≥ 8 weeks p.i.). To analyse external factors that could affect D-RNA formation and accumulation, barley plants inoculated with BMV RNA, D-RNA free were kept in a greenhouse or in a growth chamber at 12, 16 and 20°C. Samples of upper infected leaves were collected periodically to determine the presence of D-RNA. At 70 dpi, D-RNA was observed from greenhouse grown plants or when

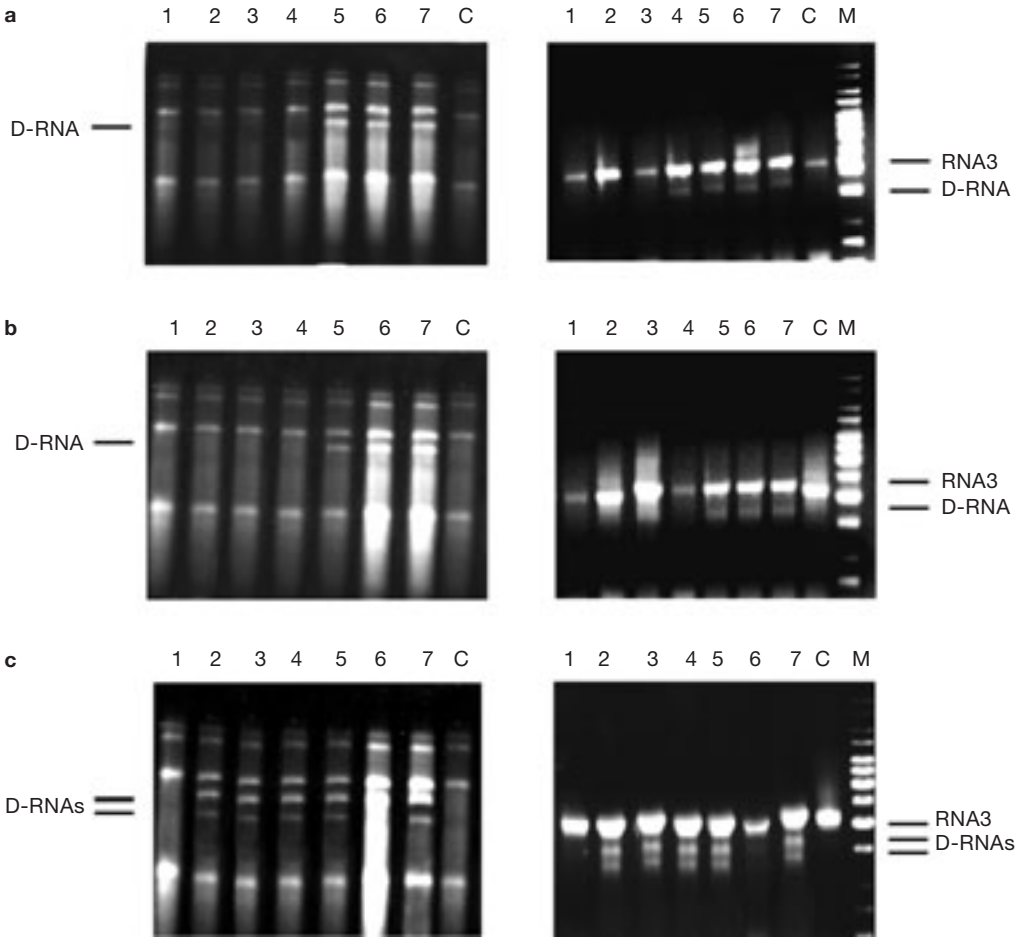

Figure 2. Detection of *de novo* generated BMV D-RNAs after serial passages in barley plants grown in a) a greenhouse, b) a growth chamber at 16°C and c) a growth chamber at 12ºC. Seedlings were inoculated with BMV-RNA free of D-RNA. Fifteen days after inoculation, the encapsidated viral RNA was purified and inoculated into new plants. Encapsidated RNA was analysed after each passage by electrophoresis in agarose gel (left half) and amplified by RT-PCR with a pair of BMV RNA3 specific oligonucleotide primers B5 and B3 (right half). Lanes 1 to 7 indicate the corresponding passages. C, BMV RNA without D-RNA. M, 1-kb ladder (Biotools, Madrid, Spain)

Table 4. The location of crossover sites in two types of BMV D-RNAs formed after serial passages in barley plants maintained in a greenhouse or a growth chamber[a]

Clone	Position of crossovers[b]	Size of deletion (nt)	Growth temperature
BS1	372-850	478	12, 16 and 20°C, Greenhouse
BS2	369-386 204-266	500 62	12°C

[a] Barley seedlings were inoculated with D-RNA-free BMV-RNA and maintained in a greenhouse (22 ± 5°C) or a growth chamber at different temperatures. Every 15 dpi, the virus was passed to a new plant. The virus and its RNA were purified after each passage and the D-RNAs were amplified by RT-PCR using the RNA3-specific primers B5 and B3. RT-PCR products were cloned and sequenced. [b] Numbers indicate the nucleotide positions of the crossover sites (BMV MI RNA3, Acc Nº V00099).

Table 5. Effect of temperature on the formation of BMV D-RNAs without passages through barley plants[a]

Temperature (°C)	Days post-inoculation[b]	D-RNA size (kb)
12	43	1.6 and 1.5
16	45	1.6
20	70	1.6
Greenhouse	73	1.6

[a] Barley seedlings were inoculated with D-RNA-free BMV and maintained in a greenhouse (22 ± 5°C) or a growth chamber at different temperatures. The virus and its RNA were purified periodically and analysed by electrophoresis and RT-PCR. [b] Numbers indicate the days after inoculation at which the D-RNAs were observed.

the growth temperature was 20°C (Table 5) and at 43-45 dpi in plants grown at 16 and 12°C. The *de novo* D-RNAs formed were similar to those described by Damayanti *et al.* (1999), D-RNA from two deletions was only found when plants were grown at 12°C (Table 4). Low temperature, as described by Llamas *et al.* (2004) in BBMV, also appears to improve D-RNA formation in BMV infections.

Formation of defective RNAs of CCMV

CCMV is a bromovirus closely related to BBMV and BMV. However, defective molecules have not been described for this pathogen. To determine if this virus could generate and accumulate D-RNAs, cowpea and *N. benthamiana* plants were inoculated with viral RNA, and kept in a greenhouse or in a growth chamber at different temperatures during seven serial passages. Twenty days p.i. upper infected leaves were collected to detect the possible presence of D-RNA. D-RNAs were not detected in either of the hosts, even after seven serial passages (Fig. 3). In another experiment cowpea and *N. benthamiana* plants, infected with the virus, were kept in a greenhouse for 100 d to determine if defective molecules could be generated without passages as in BMV and BBMV. None of the samples over this period showed the presence of D-RNAs. These results show that while BBMV and BMV form and accumulate D-RNAs under the above conditions, CCMV did not have the suitable conditions for D-RNA formation in the host plants that were used.

Discussion

Serial passaging in a viral infection, in whole plants, at high infection multiplicity, leads to the accumulation of D-RNAs (Burgyan *et al.*, 1991; Knorr *et al.*, 1991; Pogany *et al.*, 1995). However, D-RNAs have also been detected in protoplasts and plants infected with wild type *in vitro* transcripts of *Tomato bushy stunt virus* (TBSV) and BMV without serial passages (Law and Morris, 1994; Damayanti *et al.*, 1999). This study analysed generation of D-RNAs in BBMV without passages and found that BBMV generates and accumulates D-RNAs molecules with the previously described characteristics (Romero *et al.*,1993; Pogany *et al.*, 1995, 1997). It also tested whether growth stage in

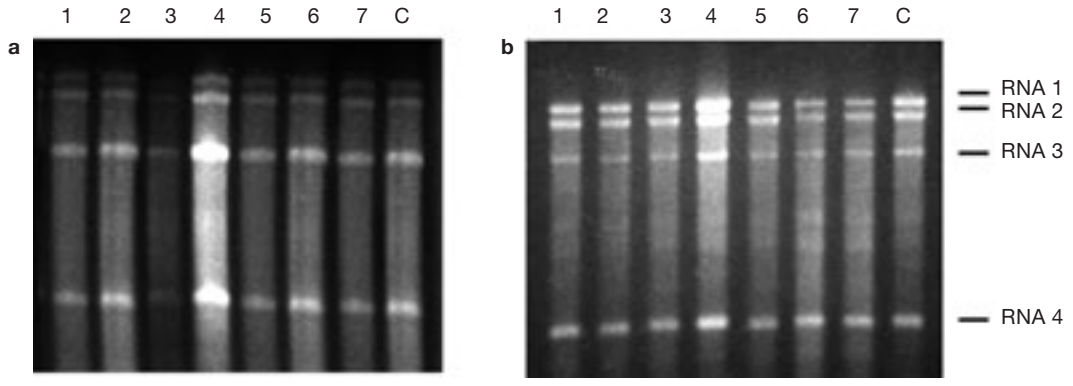

Figure 3. Electrophoresis analysis of CCMV RNAs after serial passages in *Nicotiana benthamiana* plants. Seedlings were inoculated with CCMV RNA and kept in a growth chamber at 16°C (a) or in a greenhouse (b). Twenty days after inoculation, encapsidated viral RNA was purified and inoculated into new plants. The encapsidated RNA was analysed, after each passage, by electrophoresis in denaturing agarose gel. Lanes 1 to 7 indicate the corresponding passages. C, CCMV RNA from plants inoculated with RNA transcripts.

broad bean plants had an effect on formation of D-RNAs without passages, and showed that the later the inoculation the faster D-RNAs formation occurred. The time was reduced to only 10 dpi for generation of D-RNAs in plants inoculated at 50 das. Law and Morris (1994) detected TBSV D-RNAs in protoplasts infected with virus transcripts, suggesting that passaging does not cause formation of these molecules but must provide conditions which promote their amplification and accumulation. In our case D-RNAs were probably replicated at low concentrations after inoculation and needed a longer period for detectable accumulation. The fast accumulation of D-RNAs observed when the inoculation was carried out during flowering could be caused by physiological changes in the host such as old broad bean leaves affecting the RNA polymerase. These changes could diminish the efficiency of the enzyme forcing it to make more errors during replication of the virus thus increasing formation of D-RNAs and its subsequent detection. Recently we reported that D-RNAs had important effects on productivity, reducing grain yield by an additional 25% compared to the loss in yield caused by the virus without D-RNAs (Sandoval *et al*., 2007). As formation of BBMV D-RNAs occurred without passages, early virus infection constitutes a major danger in the field infections.

Previously, we described that the lowering the temperature to 12°C, improved the formation and accumulation of BBMV D-RNAs, reducing the number of passages (Llamas *et al.*, 2004). In this work low temperature did not have any visible effect on D-RNA formation and accumulation without passages, probably because the formation of D-RNAs was slow and needed time for accumulation, while in experiments with passages, the inoculum pressure together with sub-optimal RNA polymerase functioning at low temperature give faster D-RNA accumulation.

The effect of the plant host on accumulation of D-RNAs has been well documented (Hillman *et al.*, 1987; Graves *et al.*, 1996; Inoue-Nagata *et al.*, 1998; Kaplan *et al.*, 2004). Further, we have recently demonstrated that the host can affect the size of defective RNAs (Llamas *et al.*, 2004). The present work confirms that the host affects D-RNAs even when their formation and accumulation were obtained without passages.

Five different kinds of BBMV-D-RNAs were formed when plants were grown in different hosts and at different temperatures; the lack of a visible effect of these external factors is the main characteristic of these molecules. However, some cultivars seem to have similar behaviour in the generation of D-RNAs, probably related to the presence of host proteins used for the recombination and formation of these molecules in the BBMV RNA2. The only observed effect was on D-RNAs size, in these conditions we found small (SD, 1900 nt) and large (LD, 2,300 nt) D-RNAs, as described by Llamas *et al.* (2004). At the borders of the deletions sites of the defective molecules, it is possible to distinguish features which favour their generation, such as conserved and palindrome sequences, secondary structures, and adenine-uracil rich regions (Chang *et al*., 1995; Pogany *et al.*, 1995; Hernández *et al.,* 1996; Damayanti *et al.,* 1999). These findings illustrate that the mechanism «copy-choice» proposed for the formation of D-RNAs have hot spots for their crossover sites (Shapka and Nagy, 2004).

BMV D-RNA has only been found during prolonged infections and could not be maintained, even in initially inoculated leaves (Damayanti *et al.*, 1999). The BMV D-RNAs observed after passages through new hosts were maintained in the subsequent passages. The only variation with the experiments carried out by Damayanti *et al*. (1999), was the type of barley cultivar used. Whilst they used barley cvs. Gose-shidaka and Hinode-hadaka we used cv. Scarlet. Damayanti *et al.* (1999) also detected replication and encapsidation of D-RNAs in protoplasts after co-inoculation with BMV genomic RNAs, but when virions containing or lacking D-RNAs were inoculated to new plants, D-RNAs were only detected at 8 weeks p.i.; Damayanti *et al.* (1999) explained this through a lack of cell to cell movement. However, in the present experiments D-RNAs accumulated in passages after having detected them. Under these conditions D-RNA moved systemically and was encapsidated as in the case of the auxiliary virus. The present data reveal that lowering the temperature to 12°C improved the formation or accumulation of BMV D-RNAs as only two passages were required to generate the *de novo* D-RNAs. In contrast five passages were required for the formation of BMV D-RNAs at 16 to 20°C, similar to the behaviour of BBMV previously described by Llamas *et al.* (2004). The role of temperature in generation of D-RNAs was also observed when formation of BMV D-RNAs occurred without passages. The observation of Szittya *et al.* (2003) that at low temperatures the efficiency of post-transcriptional gene silencing (PTGS), an antiviral defence mechanism in plants, was restricted could explain the increase in formation of D-RNAs. Damayanti *et al.* (1999) found two types of D-RNA molecules derived from BMV

RNA3, the first was generated by only one deletion and the second by two deletions; in our work we obtained D-RNA molecules with two deletions only when plants were grown at 12°C, It is possible that the lower temperatures preserved secondary structures of virus RNA templates and thus favoured the formation of D-RNAs.

The facility with which D-RNAs emerged *de novo* at low temperatures and the infection of *Arabidopsis thaliana* (L.) Heynh. by BMV (Dzianott and Bujarski, 2005) are a great methodological improvement to study mechanisms of D-RNA formation during BMV infection and to study the effect of these molecules on symptom development induced by the helper virus.

The other member of the *Bromovirus* genus, CCMV, did not form D-RNAs with or without passages in two different hosts assayed. Possibly it did not find an adequate host for generation of D-RNAs or CCMV RNAs did not have the appropriate structures to facilitate the crossover jumps of RNA polymerase during virus RNA replication. It thus appears that a variety of factors can contribute to the formation and accumulation of D-RNAs during viral bromovirus infection.

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