Eur J Plant Pathol (2008) 120:97–102
DOI 10.1007/s10658-007-9194-9

SHORT COMMUNICATION

RNA2 of *Soil-borne cereal mosaic virus* is detectable in plants of winter wheat grown from infected seeds

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Received: 24 January 2007 / Accepted: 19 July 2007 / Published online: 1 November 2007

Abstract *Soil-borne cereal mosaic virus* (SBCMV) causes a severe disease in susceptible cultivars of winter wheat. The virus is vectored by the soil-borne protist *Polymyxa graminis*. Experiments were conducted to investigate whether SBCMV RNA2 could persist in seed from SBCMV-infected susceptible cultivars of winter wheat. Over 7,000 seedlings were generated from seed collected from two cultivars of SBCMV-infected winter wheat. Seedlings were grown in a glasshouse compartment and batch tested for the presence of SBCMV using real-time RT-PCR. The majority of batches tested positive for SBCMV, indicating an RNA2 transmission rate of 1.8–9.4% in wheat. The presence of the virus was confirmed by amplifying and sequencing a larger (400 bp) fragment of viral RNA2 in a sub-set of the seedlings testing positive by real-time RT-PCR. Root extracts from this sub-set tested negative for *P. graminis* using real-time PCR. The implications for disease epidemiology of this virus are discussed.

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Keywords Soil-borne wheat mosaic virus · Susceptible · Cultivar · SBWMV · *Furovirus* · *Triticum* · Epidemiology

Soil-borne cereal mosaic virus (SBCMV) causes a damaging disease of winter wheat (*Triticum aestivum*) across Europe, with yield losses of 50% recorded in the UK (Clover et al. 1999; Koenig and Huth 2000). SBCMV is a member of the genus *Furovirus* and has a bipartite, positive sense RNA genome. It is well established that the primary means of transmission of SBCMV is by the soil-dwelling plant parasite *Polymyxa graminis*. Once land is contaminated, the only practical means of control is by using winter wheat cultivars showing field resistance to the virus. Preventative measures therefore revolve around limiting soil movement from infected farms. Seed transmission is known to occur in related viruses of the genus *Pecluvirus* (Delfosse et al. 2001). A small study has shown that SBCMV is transmitted through seed in rye grown in Poland (Garbaczewska et al. 1997; Jezewska 1995), although this work has been received with scepticism by some researchers. The possibility that SBCMV is seed-transmitted, albeit at a low level, has a huge implication for disease epidemiology, risk assessment and control strategies. A single infected site, used for seed production, could produce dozens of new outbreaks. This work aimed to establish whether RNA2 of SBCMV was capable of being transmitted in seed from wheat infected in a field containing a natural inoculum source of

SBCMV. In addition, where presence was confirmed, the experiment was designed to determine rate of transmission of RNA2 of SBCMV in wheat.

Seed from susceptible cvs. Tanker and Equinox were collected from crops grown on land containing a natural inoculum source of SBCMV in 2001–2002 (Budge et al. 2003). For direct seed testing, triplicate batches of 20 seed from each cultivar were washed with 0.026 M Na_3PO_4: 12 H_20 for 10 min to remove any virus capsid from the surface of the seed (Delfosse et al. 1999). RNA was extracted using a MagExtractor® Total RNA purification kit (Thermo-Labsystems) in combination with a Kingfisher ml magnetic particle separator (ThermoLabsystems) following manufacturer's protocol (Toyobo, Japan). RNA extractions were tested for the presence of the virus using SBCMV specific real-time reverse transcriptase polymerase chain reaction (RT-PCR) designed to detect a portion of the coat protein gene on RNA2 using primers *SBCWMVCPF/SBCWMVCPR* and fluorescent-labelled probe *SBWMV237F* (Ratti et al. 2004).

To determine whether the virus could propagate in the tissue of the progeny plants, replicate batches of 100 seedlings (51 for Tanker and 41 for Equinox) were pre-germinated by soaking overnight in a 0.6% Penicillin G sulphate (Sigma P3032) and 2.0% Streptomycin sulphate (Sigma S6501) solution and planted into sterile, autoclaved horticultural silver sand. In addition, commercial Equinox and Tanker seed were planted in sterile sand to act as negative control material. Plants were grown for 12 weeks in a temperature controlled glasshouse compartment set at 18°C with no supplemental lighting and fed weekly using one-fourth strength sterile Hoagland's nutrient solution (Adams et al. 1986).

For each batch of plants the RNA was extracted from a mixture of leaf and root material as described above, except a Guanidine-based buffer (8 M Guanidine hydrochloride; 20 mM EDTA; 25 mM citric acid; 2% polyvinylpyrrolidone (Sigma); 0.5% Triton X 100 made up to 1 l with distilled water) was used in place of the MagExtractor® Lysis Buffer supplied in the MagExtractor® Total RNA purification kit (Thermo-Labsystems). In addition, separate RNA extractions were made from leaf and root material from selected samples. All nucleic acid preparations were tested for the presence of SBCMV, and selected preparations tested for the presence of *P. graminis*, using specific real-time assays as described in Ratti et al. (2004). Several controls were included in the extraction process including three healthy wheat samples in addition to a buffer control containing no plant material.

In order to confirm the presence of other genomic areas of SBCMV, primer pair *RNA2F* (GAGTGCT CAGTGAAACTGCTAAC)/*RNA2R* (TGACGG CAACGACGTGTCT) was designed to amplify a 401 nucleotide fragment of the gene on RNA2 encoding the hypothetical cysteine rich (cys-rich) protein gene (Accession AJ298069). RT-PCR was performed on root samples from two batches of Equinox (batch numbers 11 and 26) and Tanker (batch numbers 7 and 11) using the ReddyMix™ Reverse-iT™ One-step RT-PCR kit (Abgene). Manufacturer's recommended protocols were followed, except reaction volumes were 25 µl. Samples were placed on a thermocycler (Biometra) and heated as follows: 47°C for 60 min, 94°C for 5 min, then 40 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, followed by 72°C for 5 min. PCR products were purified, cloned, sequenced and homologous sequences identified using the blastn search algorithm on the EMBL sequence database (Boonham et al. 2002).

The results from the direct seed testing indicated that 2/3 20 seed batches of Equinox and 3/3 20 seed batches of Tanker tested positive for SBCMV using real-time RT-PCR with C_Ts ranging from 33.18 to 37.41. Plants from both cultivars grew well for the duration of the experiment, although variable germination was a problem for both cultivars. Final mean batch size was 91±28.4 plants per batch for Tanker and 78±12.8 plants per batch for Equinox. Most batches of Equinox (38/41) and all batches of Tanker (51/51) tested positive for SBCMV RNA2 using real-time RT-PCR. However, none of the plants expressed symptoms of SBCMV infection. The negative controls from each stage of the experiment all gave negative results for SBCMV including; commercial batches of Equinox and Tanker grown alongside the seeds on test; three healthy wheat samples extracted together with the samples on test and a buffer control containing no plant material; and water controls for both real-time RT-PCR plates and conventional RT-PCR gels. Separate leaf and root samples were repeated for five selected samples for each cultivar. Leaf material for Tanker consistently tested negative for SBCMV (0/5) although, one sample of five Equinox

Table 1 Mean (of duplicate wells) real-time RT-PCR C_T values for testing tissue from plants grown from seed collected from susceptible winter wheat grown on land containing a natural inoculum source of SBCMV in the UK for SBCMV and *P. graminis*

Batch	SBCMV						*P. graminis*	
	Tanker			Equinox			Tanker	Equinox
	E	L	R	E	L	R	R	R
1	30.56	–	–	31.13	–	–	–	–
2	36.29	–	–	31.76	–	–	–	–
3	32.20	–	–	40.00	–	–	–	–
4	31.29	–	–	40.00	–	–	–	–
5	31.23	–	–	31.00	–	–	–	–
6	30.01	–	–	31.78	–	–	–	–
7	30.53	–	–	28.62	40.00	32.63	–	40.00
8	32.57	–	–	30.72	–	–	–	–
9	31.38	–	–	31.65	–	–	–	–
10	32.23	–	–	29.00	40.00	31.91	–	40.00
11	28.84	40.00	32.02	28.11	36.90	35.73	40.00	40.00
12	32.24	–	–	31.43	–	–	–	–
13	30.89	–	–	32.61	–	–	–	–
14	30.40	–	–	29.73	–	–	–	–
15	30.65	–	–	27.69	40.00	36.44	–	40.00
16	31.12	–	–	31.19	–	–	–	–
17	30.79	–	–	29.40	–	–	–	–
18	33.72	–	–	35.66	–	–	–	–
19	29.65	–	–	31.76	–	–	–	–
20	34.18	–	–	30.18	–	–	–	–
21	32.10	–	–	33.81	–	–	–	–
22	30.68	–	–	31.69	–	–	–	–
23	29.87	–	–	28.60	–	–	–	–
24	28.75	–	–	27.48	40.00	35.87	–	40.00
25	30.39	–	–	31.85	–	–	–	–
26	31.87	40.00	31.10	33.82	–	–	40.00	–
27	30.06	–	–	30.04	–	–	–	–
28	30.94	–	–	37.15	–	–	–	–
29	31.12	–	–	31.90	–	–	–	–
30	31.02	–	–	38.50	–	–	–	–
31	28.30	–	–	32.51	–	–	–	–
32	30.78	–	–	37.58	–	–	–	–
33	30.17	–	–	40.00	–	–	–	–
34	30.87	40.00	37.12	34.35	–	–	40.00	–
35	29.75	–	–	32.03	–	–	–	–
36	32.36	–	–	33.84	–	–	–	–
37	30.53	–	–	34.03	–	–	–	–
38	30.20	40.00	35.63	36.24	–	–	40.00	–
39	29.29	–	–	33.09	–	–	–	–
40	29.43	–	–	34.09	–	–	–	–
41	29.15	–	–	34.32	–	–	–	–
42	29.56	–	–	–	–	–	–	–
43	33.81	–	–	–	–	–	–	–
44	32.92	–	–	–	–	–	–	–
45	34.78	40.00	38.85	–	–	–	40.00	–
46	37.60	–	–	–	–	–	–	–
47	34.71	–	–	–	–	–	–	–

Table 1 (continued)

Batch	SBCMV						*P. graminis*	
	Tanker			Equinox			Tanker	Equinox
	E	L	R	E	L	R	R	R
48	34.52	–	–	–	–	–	–	–
49	34.61	–	–	–	–	–	–	–
50	33.78	–	–	–	–	–	–	–
51	36.12	–	–	–	–	–	–	–
Commercial seed batch	40.00	–	–	40.00	–	–	–	–
Healthy wheat 1	–	–	–	40.00	–	–	–	–
Healthy wheat 2	–	–	–	40.00	–	–	–	–
Healthy wheat 3	–	–	–	40.00	–	–	–	–
Positive control[a]	20.43	–	–	30.55	–	–	24.89	24.89
Water control 1	40.00	–	–	40.00	–	–	40.00	40.00
Water control 2	40.00	–	–	40.00	–	–	–	–

RNA extractions were prepared from a mixture of leaf and root tissue (E) or exclusively root (R) or leaf (L) tissue. A C_T value of 40 indicates a negative result. (–) Indicates no data collected. The results for a commercial seed batch, grown alongside the seeds on test; three healthy wheat samples extracted together with the samples on test; and a buffer control, containing no plant material are presented. Results for the positive and water control from the real-time RT-PCR testing are also presented.

[a] Different RNA extracts were used as positive controls when testing batches of Equinox and Tanker for SBCMV.

did test positive by real-time RT-PCR. Root samples from both cultivars consistently tested positive for SBCMV (10/10) but negative for *P. graminis* (0/10) using real-time RT-PCR and PCR, respectively (Table 1).

Transmission rates of SBCMV RNA2 were calculated assuming a binomial probability distribution for each cultivar (Banyai and Barabas 2002). To prevent underestimation, transmission rates were calculated using minimum and maximum batch sizes for each cultivar, rather than the mean. Upper confidence limits were generated using the minimum batch size, and the lower confidence intervals were generated using the maximum batch size. The upper and lower transmission rates for cv. Equinox were 1.8 and 6.4%, respectively. No upper transmission rate was calculated for Tanker due to all 51 batches testing positive, however the lower transmission rate was 2.1%. Assuming there was no difference in the transmission rate between each susceptible cultivar, data were combined to give an overall transmission rate in both susceptible cultivars, with respective lower and upper transmission rates of 1.8 and 9.4%.

A 400 bp band was generated for root material tested from two root extracts each from Equinox and Tanker using primer pair *RNA2F/RNA2R* (Fig. 1). Searches using the blastn algorithm confirmed that all fragments had a high sequence identity to a SBCMV isolate from Wiltshire, UK (accession number AJ298069).

Plants were raised from seed harvested from susceptible cultivars grown on land containing a natural inoculum source of SBCMV. A portion of the coat protein gene on RNA2 of SBCMV was consistently detected in tissue from these plants using real-time RT-PCR. Conventional RT-PCR primers, designed to amplify a region of the cys-rich protein gene on RNA2, also consistently amplified a 400 bp product in root extracts from these plants. The plasmodio-

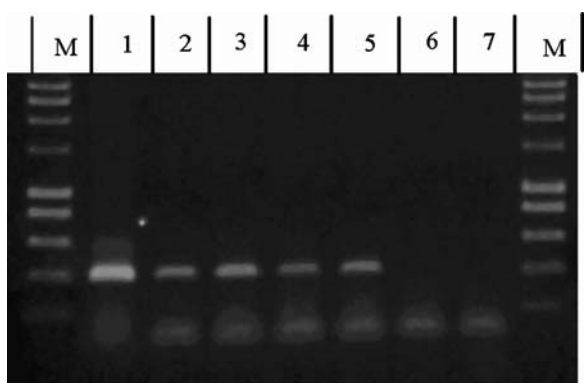

Fig. 1 Gel image showing 400 bp band of RNA2 from stem base extractions of Equinox (*lanes 2* and *3*) and Tanker (*lanes 4* and *5*) flanked by Hyperladder I (*M*). Other lanes: *1*=positive control; *6*=healthy wheat; *7*=water control

phorid vector *P. graminis* is the only accepted vector for SBCMV. However, no *P. graminis* was detected when root material was tested by real-time PCR. Therefore the data collected in this study suggest portions of RNA2 of SBCMV are capable of persistence in seed tissue and subsequent movement to the roots upon germination from infected seed. At no point in this study do we demonstrate the transmission of a complete RNA2; therefore it cannot be excluded that viral replicons, or truncated fragments of RNA2 capable of replicating in plant tissue, are being detected rather than the entire RNA2. Spontaneous deletion of fragments of RNA2 of *Soil-borne wheat mosaic virus* (SBWMV) have been reported for both natural infection and serial mechanical inoculation (Chen et al. 1995; Shirako and Brakke 1984). Transmission rates of the RNA2 detected in this study are difficult to estimate, due to the large number of positive results obtained. Assuming a binomial probability distribution, a transmission rate of SBCMV RNA2 in both susceptible cultivars ranges from 1.8 to 9.4%. This is the first study to indicate SBCMV RNA2 is capable of being vertically transmitted in field-grown winter wheat.

Symptoms are usually not seen in the UK until March or April. However, severe symptoms were evident in the crop from which the seed was harvested, as early as January during the 2001–2002 season (Budge et al. 2003). Studies of the inoculum dynamics of *Indian peanut clump virus* (IPCV) have shown early infection strongly correlates with increases in true seed transmission (Delfosse et al. 2002), indeed IPCV has been shown to be seed-transmitted in wheat at 0.5–1.3% (Delfosse et al. 1999). Work in Poland identified SBCMV virus particles in the mesophyll cells of rye seeds, suggesting the virus was transmitted at a rate of 3% in rye seed (Garbaczewska et al. 1997; Jezewska 1995). No seed-borne virus particles were observed in a small-scale study on 110 wheat seedlings using electron microscopy (Jezewska 1995). A study conducted in France found no seed transmission of SBCMV when 1,000 seed of susceptible cv. Soissons were grown and tested by enzyme-linked immunosorbent assay (ELISA) (J. Hariri, personal communication). It is accepted that real-time assays offer greatly increased sensitivity over conventional PCR and ELISA techniques (Mumford et al. 2000). The average threshold cycle (C_T) for the positives detected in this study was over cycle 30, therefore it might be expected that SBCMV would not be detectable using a serological method like ELISA, as used in the French study. Real-time assays have previously been shown to offer insight into epidemiological studies by lowering detection thresholds; for example Korimbocus et al. (2002), detected low levels of *Sugarcane yellow leaf virus* infecting root stocks of sugar cane, which other techniques, including ELISA, were unable to detect.

SBCMV is known to be present in the aerial parts of the wheat plant well beyond the heading stage. Indeed, virus particles have been detected in immature seed of durum wheat cv. Valnova, a known susceptible cultivar (Rubies-Autonell and Vallega 1991). Field observations have suggested the virus spreads more rapidly and over larger distances than can be explained by soil movement alone (Wiese 1987). Certainly it is difficult to explain the pattern of SBCMV spread in the UK assuming only the spread of infected soil (Fig. 2). Seed transmission could explain why SBCMV has been recorded in New Zealand, despite strict import restrictions on contaminating soil (L. Ward, personal communication).

For SBCMV to become established in a new field via seed transmission the root material of the infected plant would have to become infected by a strain of *P. graminis*, capable of encapsulating the virus within cystosori. This study does not present any information to demonstrate this; however, it has been shown that non-viruliferous *P. graminis* can acquire IPCV from

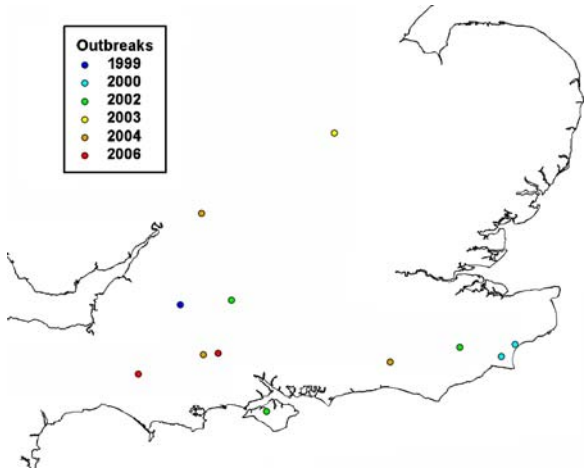

Fig. 2 A graphic showing the distribution of SBCMV in southern England from the first reported outbreak site in 1999 to the summer of 2006. Clear groupings are seen around Wiltshire and Kent, but outbreaks furthest north in Northampton and furthest south on the Isle of Wight seem sporadic

wheat plants infected by IPCV through seed-borne inoculum, and subsequently transmit the virus to plants grown in an automatic immersion system (Delfosse et al. 1999). Therefore the seed transmission of viable virus particles could theoretically produce new outbreak sites.

All data collected in this study suggests SBCMV RNA2, either in its entirety or in a truncated form, is transmitted via seed in winter wheat. However, the epidemiological significance of this finding is unclear. Many questions need to be answered before these results can be put into context. Is the transfer of SBCMV a regular occurrence or does it only occur in seasons were severe symptoms occur? Is SBCMV RNA2 transmission a peculiarity of the UK climate, or does it occur in other winter wheat producing countries, therefore potentially increasing the risk of importing infected seed? Are the transmitted SBCMV RNA2 particles whole and/or viable? Is SBCMV RNA2 accompanied by particles of RNA1? Are the transmitted viral particles capable of becoming encapsulated within cystosori of *P. graminis*, and thus form a new outbreak field? A more sustained study, over several seasons in several European locations, is essential to assist with the interpretation of these results.

Acknowledgements The authors wish to thank Richard Baker (CSL) for constructing the map of the outbreak sites and Theo Allnutt (CSL) for assistance with the calculations of infection rate. The authors would also like to thank the Plant Health Division of Defra for funding.

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