INTRODUCTION

Raspberry bushy dwarf virus (RBDV) is one of the most important viral pathogens of red raspberry (Rubus idaeus), occurring worldwide wherever raspberries are cultivated. Other Rubus species such as black raspberry (Rubus occidentalis), as well as blackberry and blackberry–raspberry hybrids, can also be infected with RBDV. Plants infected with RBDV may exhibit ‘crumbly fruit’ symptoms in which drupelet formation (raspberry fruits consist of a structured aggregation of fleshy drupelets) is severely affected, significantly reducing yield and fruit quality. In addition, when in combination with several other viruses, RBDV can cause stunting of plant growth and vivid ‘yellowing’ of the leaves (Jones et al., 1996). More recently, RBDV was shown to cause natural infection of grapevine (Mavrič et al., 2003). Yield effects of RBDV in this plant are not yet known, but the virus may be an economically significant pathogen also of this widely grown, high-value crop.

Currently, RBDV is the sole member of the virus genus Idaeovirus and has not been assigned to a family, although it has some similarities to ilarviruses from the family Bromoviridae (Jones, 2005). The virus genome consists of two positive-sense single-stranded RNAs; the larger RNA (RNA1) of about 5.4 kb encoding a putative polymerase protein, and the smaller RNA (RNA2) of about 2.2 kb encoding a putative cell-to-cell movement protein (MP) and the coat protein (CP). Virus particles, which are isometric in shape with a diameter of about 33 nm, contain two genomic RNAs as well as a third, subgenomic RNA (RNA3) of 946 nt that is not replicated but is the template for CP expression. RBDV is transmitted in the field by infected pollen and apparently without the intervention of any specific vector, although pollinating insects may be involved in the process (Murant et al., 1974). Once established in the crop, spread of the virus between plants can be very rapid and will threaten the entire plantation. Control of the disease can be achieved only by ensuring that nursery stocks are propagated from certificated virus-free plants, and by identifying cultivars that are resistant to the virus. One such resistance gene, Bu, was shown to operate in the cultivar Glen Clova; however, it is now apparent that Bu resistance-breaking isolates of RBDV do occur in the UK (Jones et al., 1982; Barbara et al., 1984). Testing new cultivars or different Rubus species to obtain new sources of resistance is a very time consuming and labour intensive process. Plants may be grown in the field in close proximity to other known infected individuals, and assessed for infection by RBDV on a yearly basis. Alternatively, plants can be grown in a glasshouse and grafted with cuttings from an infected plant, a process which is both complex and expensive. The presence of virus in the graft recipient plants can be confirmed by ELISA after a period of 3–12 months, depending on the time of year when the test was started. To overcome these limitations we sought to construct a set of infectious cDNA clones of RBDV that could be mechanically inoculated to
plants and might allow a more rapid and easy way to test for new sources of resistance to the virus. Initial results from this work have provided some insights into the RBDV infection process.

METHODS

RBDV isolates. D200 is a common or S-type isolate of RBDV as described by Barnett & Murant (1970), maintained in R. idaeus cv Autumn Bliss in an aphid-proof glasshouse at SCRI. The RB isolate was obtained from a Russian raspberry variety, Skromnitsa, generously provided by Dr Vicky Knight (East Malling Research Institute, East Malling, Kent, UK), and maintained at SCRI in the original source plant under licensed conditions. Both isolates are easily sap transmitted to *Nicotiana benthamiana* and *Chenopodium quinoa*, and remain viable when stored as freeze-dried samples.

Construction of infectious clones. Clones for infection by *Agrobacterium tumefaciens* were generated by in vivo transformation from cDNA clones of RBDV RNA1 and RNA2. Both RNAs were cloned into an intermediate plasmid, p0799, before being transferred into a binary vector for agroinfiltration of plants. The intermediate plasmid was constructed by PCR amplification of CaMV 35S promoter and NOS terminator fragments and insertion of multiple cloning site sequences using oligonucleotide primers into the high copy number plasmid pUC19. Thus, upstream of the 35S promoter, p0799 carries unique restriction sites for the enzymes *Asl* and *Asi*I. In addition, the 35S promoter carries a *Stu*I restriction site at the transcription start site followed by I restriction site at the transcription start site followed by 46 nt leader sequence derived mostly from the 5′ end of RNA1 and 1031 nt 3′ end with T7 RNA polymerase promoter (underlined); 5′-GGGGTTAC-

RNA2: 5′-ATATTTGG-

The RNA1 cDNA was cloned directly into p0799, and the 35S promoter was digested entirely into its 5′ terminus but should have 3′ terminus but have no additional, non-viral residues at the 3′ terminus. For construction of a subgenomic-like RNA3 clone, a full-length D200 RNA2 clone was digested with *XhoI* (cuts at nt 35) and *HindIII* (cuts at nt 1318), blunt-ended with Klenow polymerase and recircularized to produce clone p0852. This results in the complete deletion of the MP gene from RNA2 and provides the CP gene with a 46 nt leader sequence derived mostly from the 5′ non-coding region of genomic RNA 2.

Infection of plants. For agroinfiltration, binary constructs were transformed into A. *tumefaciens* strain AGL1. Cells were pelleted by centrifugation from liquid cultures, resuspended in infiltration buffer [10 mM MgCl₂, 10 mM MES (Sigma) pH 5.7, 150 μM acetyl-syringone] at an OD₅₉₅ of about 0.2 and incubated for 2–3 h at room temperature. Combustions of cultures were mixed in equal volumes and infiltrated using a plastic syringe through the lower surface of the leaves of *N. benthamiana* plants.

For infectious transcripts, the clones p0836, p0841 and p0852 were linearized by digestion with *Sma*I. Transcripts were synthesized using the MEGAscript T7 kit (Ambion) and capped with the ScriptCap kit (Epiconcept Biotechnologies), according to the manufacturer’s instructions. Leaves of small *N. benthamiana* plants were dusted with carboxylysid powder and inoculated by gentle rubbing with mixtures of RNA transcripts (mixtures contained equal amounts of each RNA, and approx. 0.5–1 μg of each RNA per leaf).

Infiltrated or inoculated leaves were sampled after 7 days and upper leaves were sampled after 12–14 days. RNA samples were extracted as described before (MacFarlane *et al.*, 1991) and examined by Northern blotting using standard procedures and RBDV-specific RNA probes labelled using the AlkPhos kit (GE Healthcare). Virus infection was also analysed using an ELISA assay to detect virus CP (Jones *et al.*, 2000).

Production of protoplasts. Protoplasts were isolated from fully expanded leaves of *N. tabacum* cv Samsun NN plants that had been kept in a growth room (16 h light, 19°C, 8 h dark, 16°C) for 7–10 days prior to collection. Leaves were abraded with carboxylysid and digested overnight at 25°C with 0.05% Macerozyme/0.25% cellulase (both Yakult Pharmaceuticals). Isolated protoplasts were collected by centrifugation onto a sucrose cushion, washed and resuspended in buffer (12% mannitol, 6 mM CaCl₂, 80 mM KCl pH 7.2), and for each experiment 2–3 x 10⁶ cells were electroporated with about 30 μg of mixed transcripts. The protoplasts were incubated in diffuse light at room temperature for 48 h before extraction of RNA. RNAs were examined by Northern blotting as described above.

Clones for the *in vitro* production of infectious RNA: the RBDV RNA2 was amplified by RT-PCR from RBDV RB-infected plant RNA as above using primers 1009 [5′ end with T7 RNA polymerase promoter (underlined): 5′-TTTTATACGACTCATATAGATA-TATTTCACAGTTACGTGTCG-3′] and 952 [3′ end with *Sma*I site (underlined): 5′-ATACCCTGGGTTTGCTCAACAAACCAACCCGCTA-

TG-3′]. RBDV RNA1 was amplified using the infectious RNA binary construct p0836 (above) as template using primers 1030 [5′ end with T7 RNA polymerase promoter (underlined): 5′-GGGGTAA-

TACGACTCATATAGATAATTGGTTGCTCAGCAAACAACGGCTA-

3′] and 1031 [3′ end with *Sma*I site (underlined): 5′-GGGCCCCGGG-

TTTGGCTGACAAAACTGCCGTAAGGAC-3′]. After amplification, the DNAs were A-tailed using *Taq* polymerase (Roche) and inserted into the pcR4-TOPO vector (Invitrogen) using the manufacturer’s instructions to produce clones p0863 (RNA1) and p0841 (RNA2). Transcripts were predicted to carry a single non-viral G residue at the 5′ terminus but have no additional, non-viral residues at the 3′ terminus.
Production of CP mutants. Portions of the CP gene from the RB strain of RBDV were amplified by PCR using primers shown in Supplementary Table S1 (available in JGV Online) to add 5' and 3' attB sequences that allow for recombinational cloning using the Gateway system (Invitrogen) either into the binary expression plasmid pMDC32 (for CP mutants lacking residues at the N terminus of the protein) or into pBAT-TL-K–RFP (Uhrig et al., 2004) to fuse portions from the N terminus of the CP in front of the monomeric red fluorescent protein (RFP). The recombination process inserted an additional nine non-viral amino acids (DPALFLYKVV) between the RBDV CP peptides and the RFP protein. The different CP constructs examined in this work are shown in Fig. 1.

RESULTS AND DISCUSSION

The presence of RNA3 enhances RBDV infection

Transcripts of RBDV RNA1, RNA2 and RNA3 were synthesized using the full-length cDNA clones as templates and some of each were subsequently capped. Mixtures of uncapped and capped RNAs (RNA1+RNA2, with or without RNA3) were assembled and manually inoculated onto small N. benthamiana plants (two leaves per plant). Analysis of total RNA extracted from inoculated leaves showed that viral RNAs did not accumulate when either uncapped RNA1 and RNA2 alone or uncapped RNA1 and RNA2 with uncapped RNA3 were used as inoculum (Fig. 2). However, addition of capped RNA3 to uncapped RNA1 and RNA2 led to the accumulation of viral RNA. Inoculation with capped RNA1 and RNA2 only led to the low level accumulation of viral RNAs; however, inclusion of RNA3 (either capped or uncapped) with capped RNA1 and RNA2 resulted in high level accumulation of viral RNAs. In upper, uninoculated leaves no viral RNAs were detected in plants inoculated with either uncapped RNA1 and RNA2 alone or uncapped RNA1 and RNA2 with uncapped RNA3. However, all other combinations of transcripts led to a detectable systemic infection, with levels of viral RNA being similar in all plants. These results show that capped RNA1 and RNA2 together can produce an infection, but that addition of RNA3 greatly increases the level of viral RNA in the initial, inoculated leaf.

RNA3 stimulates viral RNA replication

The increase caused by RNA3 in levels of accumulated viral RNA in infected plants could result from an enhancement of RNA replication and/or from improved virus movement. To examine these possibilities, infection experiments were done using isolated plant protoplasts. In these tests, no viral RNA accumulation was seen in protoplasts transfected with capped RNA2 only or with a mixture of RNA1 and RNA2 (either capped or uncapped) (Fig. 3). However, low level accumulation of viral RNAs occurred in protoplasts transfected with uncapped RNA1 and RNA2 together with capped RNA3, and much higher level accumulation of viral RNAs occurred in protoplasts transfected with capped RNA1 and RNA2 together with capped RNA3. These results show that the presence of RNA3 greatly stimulates viral RNA replication, although an additional effect of RNA3 on virus movement in whole plants cannot be discounted.

RNA3 is required for infection using agroinfiltration

Because of the speed and simplicity of plant inoculation using agroinfiltration we tested whether the binary constructs containing RBDV RNA1, RNA2 and the RBDV CP gene could produce an infection of plants. Infiltration with a mixture of agrobacteria harbouring strain D200 RNA1 and strain RB RNA2 binaries only did not produce detectable infection in either infiltrated or upper leaves (Fig. 4). However, infiltration with a mixture of RNA1, RNA2 and the RB strain CP gene led to the accumulation of high levels of viral RNAs (RNA1, RNA2 and sgRNA3, which is derived via replication of RNA2) in the infiltrated leaf and lower levels of all three viral RNAs in the upper leaves. The lack of detection of RNA1 and RNA2 in leaves not infiltrated with the CP gene demonstrate that the signal detected in the samples taken from leaves of plants infiltrated with all three binary constructs is derived from replicating virus and not from residual (non-replicating) binary plasmid DNA or mRNA.

Extracts of infiltrated [7 days post-infiltration, (p.i.)] and upper, non-infiltrated (14 days p.i.) leaves were also

Fig. 1. Diagram of CP constructs used in genome activation experiments. RBDV CP represented by the black bar, other virus CPs represented by the unshaded bar, mRFP represented by the grey-shaded bar. Bar lengths are in proportion to proteins sizes.
analysed by ELISA using antibodies specific for the RBDV CP. Extracts having absorbance readings of more than twice that produced by uninfected control samples are interpreted as showing a positive reaction (for detection of the CP) and being infected with RBDV. In these experiments, extracts from chronically infected red raspberry produced an absorbance value, after overnight incubation of the reaction, 25-fold higher than from extracts of uninfected *N. benthamiana*, whereas at 7 days p.i. leaves of *N. benthamiana* plants infiltrated with all three RBDV binaries produced an absorbance value of between 11- and 13-fold higher compared with uninfected plants (Supplementary Table S2 available in JGV Online). By contrast, plants infiltrated with binaries for only RNA1 and RNA2 produced a negative reaction (absorbance values of all infiltrated leaf samples similar to those of the healthy plant samples). Although absolute absorbance values differed between experiments, in nine different experiments infiltration with only RNA1 and RNA2 consistently failed to produce a positive-ELISA reaction (0/46 plants), whereas infiltration with RNA1 + RNA2 + CP gene (almost) always produced a positive reaction (45/46 plants).

These results demonstrate the requirement for the CP gene (as a source of CP, see below) to initiate infection using the infiltration approach, and show that ELISA is a suitable method to monitor the infection. In the experiments described above, both the D200 strain CP and the RB strain CP, which are 97% identical, were able to stimulate infection of a mixture of D200 RNA1 and RB RNA2.

### Genome activation by RBDV but not AMV and ilarvirus CP

To confirm that the RBDV CP is required for RBDV genome activation, we infiltrated plants with agrobacteria carrying binaries for RNA1, RNA2 and the CP gene in which the translation initiation codon was mutated (first base of AUG triplet removed). These plants did not become infected, showing that the CP itself rather than the CP coding sequence is responsible for genome activation (Supplementary Table S2, Fig. 5). Other studies have shown that replication of AMV and ilarviruses, such as TSV, is greatly stimulated (more than 1000-fold) by the presence of a few molecules of the CP or by the...
subgenomic RNA4 (that encodes the CP) (Neeleman et al., 2001; Bol et al., 1971; Bol, 2005), a process that is referred to as genome activation. Experiments have shown that the AMV CP and TSV CP can functionally substitute for one another. To examine the similarities in genome activation of RBDV, AMV and TSV, we tested whether expression of either the AMV, TSV or BCRV, a Rubus-infecting ilarvirus (Jones et al., 2006), CP gene from a binary plasmid could activate replication of agroinfiltrated RBDV RNA1 and RNA2. In each case, the CPs from these different viruses did not activate RBDV replication (Supplementary Table S2, Fig. 5).

Identification of CP sequences involved in RBDV genome activation

Although the AMV and TSV CPs have little primary amino acid sequence homology it has been found that both proteins can bind in a specific manner to the 3' UTRs of both AMV and TSV RNAs and activate replication of either set of viral genomic RNAs. The binding occurs in a region at the base of stem–loop structures that are separated by AUUG motifs, and involves a basic region near the N terminus of each of the CPs (Zuidema et al., 1983). The N-terminal regions of the AMV and TSV CPs are relatively rich in lysine (AMV) and arginine (TSV) residues. Within these, a moderately conserved motif located between residues 13–26 of the AMV CP and 39–57 of the TSV CP was found to be involved in virus RNA binding, and an absolutely conserved arginine residue (R17 in AMV CP) was shown by site-specific mutagenesis to be essential for RNA binding. Although the primary amino acid sequence of the RBDV CP is quite different from that of AMV and TSV, the N-terminal part of the RBDV CP is similarly rich in basic residues, predominantly lysines. To examine whether a similar situation exists with RBDV, two different sets of CP mutants were constructed and assessed for their ability to activate virus replication.

The first set comprised six mutants in which increasing portions of the N-terminal region of the RBDV strain RB CP were fused at their C terminus to the monomeric RFP (Fig. 1). Laser-scanning confocal microscopy of leaves that had been infiltrated with agrobacterium carrying the different CP–RFP binary plasmids confirmed that each construct was expressed (data not shown). A construct carrying RFP not fused to any other protein was used as a negative control and in two separate experiments, when co-infiltrated with RNA1 and RNA2 binaries, failed to activate replication (Supplementary Table S2). Mutants CPN145–RFP (145 N-terminal amino acids fused to RFP) and CPN105–RFP (105 N-terminal amino acids fused to RFP) activated replication (i.e. produced CP-specific ELISA values) to high levels, in infiltrated leaves greater than 65 % of that produced by full-length CP, leading to wild-type levels in systemic leaves (Supplementary Table S2, Fig. 5). Mutants CPN72–RFP (72 N-terminal amino acids fused to RFP) and CPN48–RFP (48 N-terminal amino acids fused to RFP) activated replication to low levels, whereas mutant CPN34–RFP (34 N-terminal amino acids of the CP fused to RFP) gave very low level replication activation in infiltrated leaves, with only three of the eight plants that were tested producing ELISA values greater than 65 % of that produced by full-length CP, leading to wild-type levels in systemic leaves (Supplementary Table S2, Fig. 5).
than twofold above background and which did not lead to systemic infection.

The second set of constructs comprised four mutants with sequentially larger deletions at the N terminus of the CP (Fig. 1). Mutant CPNA10 retains the first, methionine, residue but is deleted for aa 2–10. Likewise, mutant CPNA15 is deleted for aa 2–15, mutant CPNA20 is deleted for aa 2–20 and mutant CPNA26 is deleted for aa 2–26. When binaries carrying each of these mutant CPs were infiltrated into plants together with binaries for RNA1 and RNA2, CPNA10 activated replication in infiltrated leaves to greater than 80% of that of wild-type CP, and CPNA15 gave 35% activation (Supplementary Table S2, Fig. 5). CPNA20 activated replication only to a low level (14%), while for mutant CPNA26 activation was less than 10% and ELISA values never rose above 3.9-fold that of the uninfected, control plant raising doubt as to whether they were true positives. Samples from leaves infiltrated with the constructs encoding wild-type and mutant CPs were examined by Western blotting using an anti-RBDV antibody but, even when including the P19 silencing suppressor protein from tomato bushy stunt virus to boost protein expression (Voinnet et al., 2003), only the wild-type protein could be detected (data not shown). Thus, we were not able to determine whether mutant CPNA26 failed to activate virus replication because the protein itself was not stable in the plants. Nevertheless, removal of up to 15 aa from the N terminus of the RBDV CP did not prevent it from strongly activating virus replication, and removal of up to 20 aa from the N terminus still permitted low level activation.

**Apparent differences between infectious clone systems**

Our results suggested an apparent difference in the infectivity of the synthetic transcript constructs compared with the agroinfiltrated constructs. With capped transcripts, inoculation of plants using only RNA1 and RNA2 led to low level virus replication in inoculated leaves and wild-type infection levels in systemic leaves (Fig. 2), whereas infiltration of plants with binary constructs of RNA1 and RNA2 only did not produce detectable infection in inoculated or systemic leaves (Fig. 4). We assume that, for the synthetic transcripts, initial translation of RNA1 produces enough replicate protein to allow the synthesis of subgenomic RNA3 from the RNA2 transcript template and the subsequent production of CP to activate productive replication. A similar situation occurs with AMV where extremely low level replication of viral transcript RNAs can occur in the absence of added CP or CP mRNA (Houwing & Jaspars, 2000; Neeleman et al., 2001) but is increased 1000-fold by their addition to the inoculum. By contrast, the 35S promoter cDNA constructs (similar but not identical to our agrobacterium-delivered RBDV binary constructs) of AMV genomic RNAs 1, 2 and 3 were moderately infectious when inoculated to plants (but were also significantly enhanced by the addition of the CP) (Neeleman et al., 1993), which was rationalized by suggesting that such DNA constructs are relatively long lived in the inoculated cell and would produce large amounts of viral RNA to kick-start the infection.

Transcription of the binary constructs in planta is expected to add an unknown number of plasmid-encoded residues as well as a poly(A) tail to the 3’ end of the RBDV genomic RNAs 1 and 2. These residues may interfere with CP sgRNA synthesis and expression from the RBDV RNA2 binary plasmid transcript, making the system completely reliant on CP expressed from the third binary plasmid. This hypothesis could be tested by constructing new RNA1 and RNA2 binary plasmids in which a ribozyme sequence is inserted immediately downstream of the viral RNA, to produce RBDV transcripts with authentic 3’ termini.

**ACKNOWLEDGEMENTS**

We thank Dr Kath Wright (SCRI) for confocal microscopy of RFP-tagged constructs. This work was funded by the Scottish Government Rural and Environment Research and Analysis Directorate (RERAD).

**REFERENCES**


(RBDV) with those of three previously characterised RBDV isolates. 


