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Analysis of *Raspberry ringspot virus* in raspberry by amplification of RNA-2

Photo by Marcin Modestowicz, courtesy of morguefile.com

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Analys av *Raspberry ringspot virus* från hallonprover genom amplifiering av RNA-2

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Keywords: *Raspberry ringspot virus, nepovirus, raspberry, Rubus idaeus, PCR*

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Sammanfattning

Den forskning som hittills gjorts på virus i hallon är begränsad, trots att sjukdomarna som dessa virus orsakar resulterar i ekonomiska förluster för de som odlar hallon. *Raspberry ringspot virus* (RpRSV) är ett virus tillhörande familjen *Comoviridae* och släktet *Nepovirus*. Nepovirus karaktäriseras av sina ikosahedrala höljen och tvådelade genom av enkelsträngat (+) RNA (RNA-1 och RNA-2). Dessa virus sprids främst med hjälp av nematoder tillhörande släktet *Longidorus*. När nematoderna angriper växters rötter, sprider de via sin saliv viruset mellan plantor. RpRSV orsakar sjukdom hos hallon, vindruvor, jordgubbar och många andra växter. Syftet med det här projektet var att bekräfta infektion av RpRSV i hallonprover från Vitryssland genom amplifiering och sekvensbestämning av RNA-2. Syftet var även att karaktärisera virusisolaten från hallonproverna, eftersom det i nuläget finns mycket lite kunskap om nepovirus i hallon. RNA extraherades från stammaterial och cDNA syntetiserades genom omvänd transkription med slumpmässiga primers. cDNA-proverna användes sedan som templat vid PCR. Flera olika RpRSV-specifika primerpar testades i försök att amplifiera en del av RNA-2. Dessvärre lyckades inte amplifieringen med någon av de primerpar som testades. Ändring av bindningstemperaturen (annealing) och PCR-mixens sammansättning gav ingen förbättring. Detta tros bero på sekvensskillnader mellan isolaten från denna studie och de isolat som analyserats i tidigare studier, vilket gjort det svårt för primrarna att binda. För att kunna dra pålitliga slutsatser krävs dock mer omfattande analyser av dessa isolat.

Abstract

So far, only limited research has been carried out on viruses infecting raspberry, even though the diseases that these viruses cause result in economical losses for raspberry growers. *Raspberry ringspot virus* (RpRSV) belongs to the family *Comoviridae* and the genus *Nepovirus*. Nepoviruses are characterized by their icosahedral virus particles and bipartite genome of single-stranded (+) RNA (RNA-1 and RNA-2). These viruses are transmitted mainly by nematodes of the genus *Longidorus*, which feed on the roots of plants. RpRSV causes disease in raspberry, grapevine, strawberry and many other plants. The aim of this project was to confirm infection of RpRSV in raspberry samples from Belarus by amplification and sequencing of the *coat protein* gene. The aim was also to characterize the detected viruses in the samples, since there is little knowledge about nepoviruses in raspberry. RNA was extracted from raspberry stem tissue and cDNA was synthesized by reverse transcription with random primers. The cDNA samples were then used for PCR with different RpRSV-specific primers, in order to amplify parts of the RNA-2 component. However, there was no success in amplification of RpRSV RNA-2 for any of the primer pairs tested. Changing annealing temperature and reagent mixture composition did not improve the results. It is hypothesized that this can be due to sequence differences between these RpRSV isolates and previously studied isolates that prevent the primers from binding. However, more extensive analyses are needed to be able to make any reliable conclusions.

Contents

Introduction

Background

Red raspberry (*Rubus idaeus*) is grown in most parts of the world and grows wild mainly in temperate areas. This plant species grows seasonal woody shoots on a perennial root-system (Sønsteby and Heide, 2008). Raspberries are mostly propagated vegetatively from root buds, however, sexual reproduction can in the wild contribute to establishment of raspberries in new areas (Whitney, 1984). Raspberry plants are hosts to a number of plant viruses, which cause disease and sometimes death of the plant. The commercial trade of raspberry plants across the world contributes to the spread of viruses.

Plant-infecting viruses can be divided into about 50 families. One family is the *Comoviridae*. Members of this family have icosahedral virus particles containing a bipartite genome of (+) single-stranded (ss) RNA. These viruses infect fruit and vegetables such as raspberries, tomatoes, potatoes, squash and other crops but also a number of wild plant species are affected (Agrios, 2005).

Nepoviruses (nematode-transmitted viruses with polyhedral particles), which belong to the family *Comoviridae*, are characterized by two RNA molecules (RNA-1 and RNA-2) both polyadenylated at the 3' end and with a genome-linked viral protein (VPg) at the 5' end. Nepoviruses are divided into three subgroups (A, B and C) depending on the size of the RNA-2 molecule (Ebel et al., 2003). During infection, these viruses synthesize three types of particles. Each particle is named after its position (top, middle or bottom) during sedimentation through a sucrose gradient. Top particles contain no RNA, middle particles contain one RNA-2 molecule and bottom particles contain either one RNA-1 molecule or two RNA-2 molecules (Acosta and Mayo, 1990). These types of plant viruses are widespread in many countries where they destroy the vegetative parts of the plant and/or the fruit. The four species causing the most severe damage when infecting raspberry are *Arabis mosaic virus*, *Strawberry latent ringspot virus*, *Raspberry ringspot virus* and *Tomato black ring virus* (CSL, 2004).

Raspberry ringspot virus (RpRSV) is a nepovirus infecting mainly raspberries. The genome of RpRSV has been fully sequenced for a grapevine isolate (Ebel at al., 2003). The virus is transmitted by nematodes of the genus *Longidorus* which feed on the roots of the plants, though RpRSV in grapevine is manly transmitted by the nematode *Paralongidorus maximus* (Wetzel et al., 2005). Scott et al. (2000) suggested that an interaction between the viral coat protein (CP) and the feeding apparatus of the nematode plays an important role for viral transmission. Dispersal of the virus can also occur via infected seeds or mechanically. However, pollination of a plant with virus-infected pollen does not cause any infection (EPPO, 1997).

RpRSV is classified into subgroup A of nepoviruses, which means that the relative molecular mass (M_r) of RNA-2 is ± (1.3-1.5) x 10^6 for both the middle and bottom virus components (Digiaro et al., 2007). Blok et al. (1992) sequenced RpRSV RNA-2 from *Nicotiana*

clevelandii and found that it encoded a polypeptide of M_r 123508 kDa. The RNA-2 molecule translates as a single ORF and is then divided into two proteins. The products formed have been shown to be the CP and the putative movement protein (Scott et al., 2000).

RpRSV can be divided into different strains, according to geographical distribution, sequence similarity or serological differences. The two major types found in raspberry are the Scottish and the English strains, which each are transmitted by different *Longidorus* species. The Scottish strain is transmitted by *L. elongatus* and the English strain by *L. macrosoma*. Difference in CP amino acid sequence is believed to be the factor for nematode species specificity. A change (H to R) at position 219 in the CP sequences between Scottish and English RpRSV strains was observed. This change, which is located in a surface loop, might be directly or indirectly involved in binding of the virus to the nematode feeding apparatus (Scott et al., 2000). In grapevine, there are two serologically different strains found in German vineyards: cherry strain (ch) and grapevine (g) strain (Ebel et al., 2003). Also, RpRSV strains can be classified according to the sedimentation of the three viral particles (top, middle and bottom) typical for nepoviruses in a sucrose gradient. Different strains separate differently and with different proportions of the three particle types. The achieved pattern can be characteristic for one strain, but may also be host specific in the way that the sedimentation pattern will be different with a different plant host (Acosta and Mayo, 1990). This is because viruses in general use the host cells machinery for production of new viral particles, thus particles from the same virus will be slightly different when produced in different host species.

The same plant can be infected with several viruses at the same time. *L. elongatus* nematodes also transmit *Tomato black ring virus* (TBRV) and RpRSV is often found together with this virus. Symptoms of infection are for example brittle shoots, rolled leaves with yellowish-green rings or chlorotic spots (Figure 1) and later death of the plant for more susceptible cultivars. However, all cultivars can be infected without showing any symptoms due to e.g. seasonal variation in symptom expression. RpRSV causes serious damage on raspberries resulting in reduction of fruit production and killing of the plants, which leads to economical losses mainly in Germany and Russia, but also in other countries (EPPO, 1997). Since viral infections are less severe in raspberries than in wine, little research has been carried out on these types of viruses infecting *Rubus* plants. More research has been carried out on RpRSV in grapevine, where there is also more money to gain from the research.

Figure 1. Raspberry leaves with chlorotic spots caused by *Raspberry ringspot virus*. Photo used with permission from SCRI-Dundee Archive, Scottish Crop Research Institute, Bugwood.org.

In Sweden, there are no previous molecular studies on nepoviruses. In Finland, observations of RpRSV and other nepoviruses on currant (*Ribes* sp.), different weeds and a number of perennial plants have been made in the early and mid 1980's (Bremer, 1983; Tapio, 1985). In the study by Tapio (1985), it was shown that infection of RpRSV was rather prevalent in a number of plant species, but the presence of *Longidorus*-nematodes in the soil was low. According to the study, this points towards the conclusion that RpRSV was introduced to Finland by vegetative propagation.

Due to the perennial root system of raspberry plants, the virus can survive from one season to the next in the dormant roots. The virus can also survive in the soil when present in seeds, which can make virus elimination difficult. By sterilization of the soil using fumigants such as dazomet or dichloropropane-dichloropropene mixtures before planting, one can avoid virus infection (EPPO, 1997). An alternative method to fumigation can be crop rotation with plants that are not RpRSV hosts or suppressing the nematodes that transmit the virus (Pinkerton and Martin, 2005).

Aim of the project

The aim of this project was to verify previously made positive ELISA tests on samples from nepovirus-infected raspberry plants by RT-PCR and sequence analysis. The aim was also to identify the detected viruses and characterize them, since there is still little knowledge about nepoviruses in raspberry. The study will focus primarily on RpRSV.

Material and methods

RNA extraction from virus-infected plant material

RNA was extracted from infected raspberry stem tissue collected in Belarus (Table 1, Appendix) using Spectrum Plant Total RNA Kit (Sigma-Aldrich) according to manufacturer's instructions. Approximately 85 mg plant material was used for the extraction.

The RNA extract was visualized on a 1 % agarose gel together with MassRuler DNA ladder (Fermentas) as size marker. The concentration of RNA was also measured in a NanoVue spectrophotometer (GE Healthcare). Extracted RNA was stored at -70°C.

cDNA synthesis of extracted RNA

The extracted RNA was reverse transcribed to cDNA to be able to use it as template for PCR. For each RNA sample, the following mixture was prepared using SuperScript III first strand synthesis system for RT-PCR (Invitrogen): 8 µl DEPC-treated water (prepared with 0.1 % DEPC), 1 µl random primers (250 ng/µl), 1 µl dNTP (10 mM) and ~1 µg RNA. The reaction was incubated at 65°C for 5 min and then immediately put on ice for 10 min. Then, 4 µl 5 x First-Strand buffer, 2 µl DTT (0.1 M) and 1 µl SuperScript III Reverse transcriptase (200 U/µl) were added. The thermal profile for cDNA synthesis was as follows: 25°C for 5 min, 50°C for 60 min and 70°C for 15 min. For RNA samples with low concentration, no DEPC-treated water was added and 20 µl RNA extract was used instead of 3 µl.

Amplification of RpRSV coat protein cDNA sequence

The presence of RpRSV was tested by RT-PCR using primers specific for RpRSV RNA-2 and cDNA templates (Figure 2; Appendix Table 2). Cycling conditions and reagent mixture compositions used for the different PCRs are listed in the Appendix (Tables 3-10). The PCRs were run using a C1000 Thermal Cycler machine (Bio-Rad).

The primers RRV Forward/ RRV Reverse (forward: 5'- GCC GAC AAG GAA GCT CGG CA -3', reverse: 5'- CGA ACG TCG ATC CGA GCC AA -3') were tested first. Primers were designed using the DNAStar program Lasergene 6.0 (DNASTAR, Inc.) with the RpRSV RNA-2 sequence from a raspberry isolate (GenBank accession no. S46011.1) as source sequence and ordered from Invitrogen. The expected product size with these primers is 1184 bp. With these primers, a gradient PCR was also run with the aim to find the optimal annealing temperature. The annealing temperatures tested were 58-66°C and the other settings were the same as for the first PCR (Dream Taq DNA polymerase was used).

Primers, coded RRVF/ RRVR, were also tested (forward: 5'- CAA CAT CCC TGC ACT TTG TG -3', reverse: 5'- CAC AAG AGC ATC AAA AGC CA -3'). These primers were designed using NCBI Primer-BLAST (source sequence used was from a grapevine isolate, GenBank accession no. AY310445.1) and ordered from Primertech. The expected product size is 650 bp.

An alternative version, where forward primer RRVF and reverse primer RRV Reverse were used together (expected product size ~2 kb), was also tested. The following thermal profile was used: initial denaturation 95°C for 2 min, denaturation 95°C for 30 sec, annealing 50°C for 30 sec, elongation 72°C for 2 min and final elongation 72°C for 10 min. A gradient PCR was tried as well. The tested annealing temperature interval was 51-61°C and the final elongation time was 15 min, other settings were the same as for the PCRs above.

Another primer pair tested was RRV F2/ RRV R2 (forward: 5'- CTT GTG GAG GAG CAT TGG AT -3', reverse: 5'- GTT AGG TAG GCC CGT CAC AA -3'), which had an expected product size of ~1.5 kb and was designed using Primer3 software (Rozen and Skaletsky, 2000). The primers were ordered from Invitrogen. The RpRSV-ch RNA-2 sequence from a grapevine isolate (GenBank accession no. AY303788.1) was used as source sequence. For these primers, two different polymerases were tested: DreamTaq DNA polymerase and Taq DNA polymerase (Fermentas). A gradient PCR was run, testing the annealing temperatures 48.8-57°C. Other settings were the same as for PCR number 9 (thermal profile used: initial denaturation 95°C for 2 min, denaturation 95°C for 30 sec, annealing 55°C for 30 sec, elongation 72°C for 1 min 40 sec and final elongation 72°C for 10 min), except for the annealing time, which was increased to 45 seconds. Number of cycles was 40.

A pair of primers (CP-RRV Forward/ CP-RRV Reverse) made with a raspberry RpRSV isolate sequence as source sequence, GenBank accession no. S46011, was also tried out (forward: 5'- GCC TAT GAG GTA GAT CCA -3', reverse: 5'- GGT AGC ACC ATC AAA GTG ACG -3'). With these primers, the expected product size is ~ 1.5 kb. The primers were designed using the DNAStar program Lasergene 6.0 (DNASTAR, Inc.) and were ordered from Invitrogen. PCR number 10 was run with two different polymerases: DreamTaq DNA polymerase and Taq DNA polymerase (Fermentas). The CP-RRV primers were also tested in a gradient PCR with the annealing temperatures 48-60°C. For this PCR, the annealing time was increased to 45 seconds. All other settings were the same as for PCR number 9 (Table 7, Appendix). Two additional gradient PCRs were also run for the temperature interval 48.8-52.8°C, PCR settings were the same as before.

A new CP-RRV Reverse primer was designed, since the previous one was in the wrong direction (not written as antisense to the template sequence). The new primer sequence was 5'- CGT CAC TTT GAT GGT GCT ACC -3' and it was ordered from Invitrogen. With the new reverse and the above described CP-RRV Forward primer, a standard PCR and a gradient PCR were run. The gradient PCR tested annealing temperatures 45-55°C, other settings were the same as for PCR number 10 (thermal profile used: initial denaturation 95°C for 2 min, denaturation 95°C for 30 sec, annealing 50°C for 30 sec, elongation 72°C for 1 min 40 sec and final elongation 72°C for 10 min) except for the number of cycles: 34.

Primers designed according to Scott et al. (2000) were ordered from Invitrogen and tested; primer sequence forward (Scott F): 5'- GTC AAG ATT CCA TAC AGA GTA TGG -3', reverse (Scott R): 5'- TGC AGT GGA TCT ACC TCA TAG G -3'. The expected product size with these primers is ~1 kb. Gradient PCR experiments for the temperatures 45-55°C and 61-68°C were also carried out, using the same settings as for PCR number 10 (Table 7,

Appendix) except for the number of cycles, which was changed to 34. For PCR number 16, dilutions of template cDNA was made in order to test if there was a problem with inhibitors of PCR in the cDNA samples. The cDNA sample used for this PCR was diluted 1:10 and 1:20 and 1 µl of each dilution was used as template.

Also, a combination of Scott primers and the first primer pair (RRV Forward/ RRV Reverse) was tried out (see Appendix), as well as a combination of the primers Scott F and CP-RRV Reverse (expected product size ~2.4 kb).

The degenerate nepovirus primers (not shown in Figure 2) designed according to Digiaro et al. (2007) and coded Nepo-A F/ R were ordered from Invitrogen (forward: 5'- GGH DTB CAK TMY SAR RAR TGG -3', reverse: 5'- TGD CCA SWV ARY TCY CCA TA -3'). Standard IUPAC 1-letter DNA code applies here. With these primers the expected product size is 255 bp. For PCR number 15, dilutions of template cDNA was made in order to test if there was a problem with inhibitors of PCR in the cDNA samples. The cDNA sample used for this PCR was diluted 1:10 and 1:20 and 1 µl of each dilution was used as template.

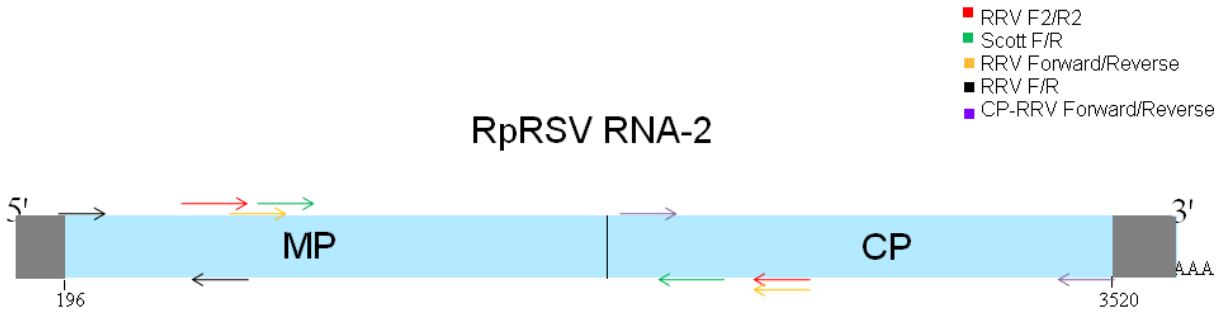

Figure 2. Schematic drawing of RpRSV RNA-2 based on information presented in Wetzel et al. (2006). Positions of the different primers used for amplification are shown by arrows. Grey areas are 5' and 3' untranslated regions.

All PCR products were visualized by gel electrophoresis using 1 % agarose gels stained with ethidium bromide (5 µl/ 100 ml), run in 0.5 x TBE buffer (Tris, Boric acid, EDTA) and with 6 x DNA Loading Dye (Fermentas). MassRuler DNA ladder mix (Fermentas) was used as size marker.

Results

Amplification of RpRSV coat protein cDNA sequence

From the first three PCRs with the primers RRV Forward/ Reverse (expected product size 1184 bp), only unspecific smaller products were formed or no products at all. In PCR number 1, samples 2, 4 and 15 showed unspecific products and no products were formed for the rest. However, there was also a product in the negative control (water instead of template) with the same size as the unspecific product for samples 2 and 4, which made these results unreliable (Figure 3). PCR 2 and 3 (annealing temperature 63°C and 62°C, respectively) did not succeed in amplification. The gradient PCR with sample 3 (58-66°C) failed in finding the optimal annealing temperature.

Products of PCR number 17 (annealing temperature 57°C), which was also run with the primers RRV Forward/ Reverse and sample 4, showed only primer-dimers on the gel.

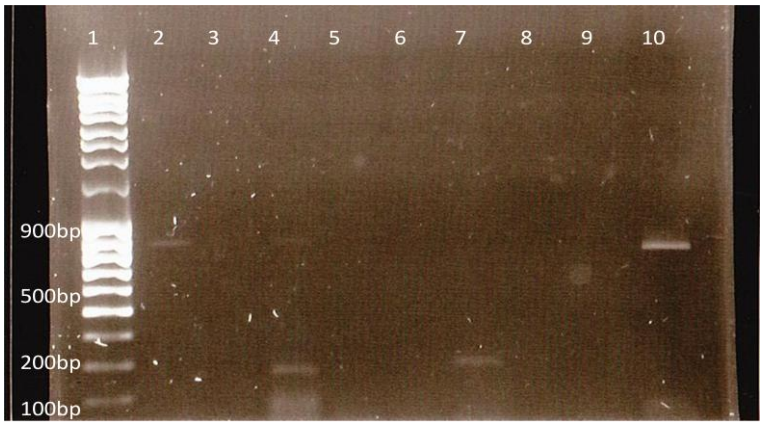

Figure 3. Gel picture showing the results from PCR no 1. Expected product size for successful amplification is ~1.1 kb. Lane 1 contains MassRuler DNA ladder; 2-9: cDNA samples number 2, 3, 4, 7, 14, 15, 17, 18, respectively, and lane 10 contains negative control.

PCR with the RRVF/ RRVR primers (expected product size 650 bp) gave similar results: unspecific amplification with annealing temperature 50°C and no product for all other temperatures that were tried (see Appendix for detailed information).

The PCR run with the primers RRVF and RRV Reverse (expected product size ~ 2 kb) resulted in unspecific amplification for sample 4 and no product for sample 9. The gradient PCR (51-61°C) with sample 4 was also unspecific in amplification and the same product was also found in the negative control.

PCRs (annealing temperature 50°C and 55°C) carried out with the primers RRV F2/ RRV R2 did not succeed in amplification of RpRSV RNA-2 when tested on cDNA samples 2, 4 and 15 with either of the polymerases (DreamTaq DNA polymerase and Taq DNA polymerase). No products were seen on the gels. Also, the gradient PCR made with sample 3 (48.8-57°C) showed no amplification.

With the primers CP-RRV Forward/ Reverse, unspecific amplification was obtained for sample 15 at 50°C for both of the polymerases tested. Sample 4 did not yield any amplified

product. The gradient PCR run with sample 15 (48-60°C) showed unspecific amplification at temperatures 48.8-52.7°C. The gradient PCR run with sample 8 for temperatures 48.8-52.8°C resulted in unspecific amplification for 48.8°C and no amplification for the rest of the temperatures. The gradient PCR, run with sample 3 and the same settings as the previous gradient PCR, showed unspecific amplification of several sizes for all annealing temperatures (Figure 4). However, none of the products were of the correct size.

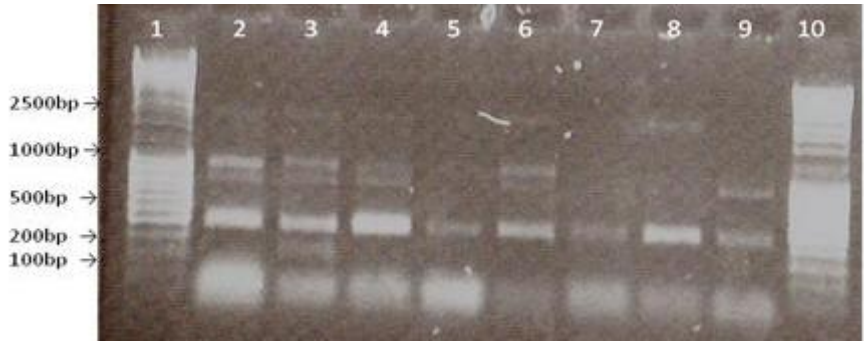

Figure 4. Gel picture showing the results from the gradient PCR run with sample 3 and the annealing temperatures 48.8-52.8°C. The expected product size for successful amplification is ~1.5 kb. Lane 1 and 10 contain MassRuler DNA ladder; 2-9: sample 3 with the annealing temperatures 48.8, 49.4, 49.9, 50.5, 51.1, 51.7, 52.2 and 52.8°C, respectively.

The PCR run with the new CP-RRV Reverse primer for samples 4, 14 and 17 (annealing temperature 50°C) did not succeed in amplification of RpRSV sequence, no products were formed. The gradient PCR made with sample 3 (45-55°C) had the same result.

PCR with primers designed according to Scott et al. (2000) and with the annealing temperature 55°C showed unspecific amplification for sample 14, however the same product was present in the negative control as well. For samples 4 and 17 no product was obtained. The gradient PCR (45-55°C) did not succeed in amplification for any of the temperatures. The PCR carried out with diluted template cDNA from sample 5 (annealing temperature 60°C) resulted in unspecific products, ~100 bp, for all template concentrations. However, the bands were stronger than for most of the previously run PCRs. The gradient PCR (61-68°C) made with sample 5 resulted only in primer-dimers.

The PCR run with either the primers RRV Forward + Scott R or Scott F + RRV Reverse (annealing temperature 60°C) did not succeed in amplification, only unspecific products of ~200 bp were formed.

For the PCR run with primers Scott F and CP-RRV Reverse (annealing temp 58°C), the result was an unspecific product of ~100 bp.

For the degenerate nepovirus-specific primers (Nepo-A F/ R), the PCR (annealing temperature 50°C) with different dilutions of sample 3 resulted in no amplification. Primer-dimers around 100 bp were the only bands on the gel.

Discussion

Amplification of RpRSV coat protein cDNA sequence

One aim of this project was to amplify the *CP* gene of RpRSV. Unfortunately, there were some problems with amplification of RpRSV RNA-2 from the raspberry samples. Several primer pairs were tried out with different PCR cycling conditions and different reagent mixtures.

For the primers designed with a grapevine isolate as source sequence (see Material and methods section), the problem could be that raspberry isolates of RpRSV differ too much in sequence from grapevine isolates. Mismatch between primer and template sequence could therefore prevent the primers from binding to the template cDNA. After a while it was discovered that the CP-RRV Reverse primer was designed in forward direction and not as antisense, which could be the reason why the amplification did not work for these primers. However, when a new correctly designed CP-RRV Reverse primer was used in PCR, there was still no amplification.

For the primers designed from the sequence of a raspberry isolate, one would expect the sequence to be similar enough to be able bind to the cDNA prepared from the samples used in this study. The amplification problem encountered could in this case be explained by e.g. differences to the English and Scottish strains. Sometimes, it is enough with one mismatching nucleotide to be unsuccessful in primer binding. Another possible explanation is that the raspberry samples, from which the RNA was extracted, did not contain RpRSV. Since the antibodies used in ELISA are more unspecific in recognition than primers it could be that the ELISA test showed false positive results. However, the antibodies had been tested to give reliable results and the plants, from which the material for the ELISA tests was collected, did show symptoms of infection. Nevertheless, the symptoms could have been caused by another virus such as TBRV, which was also found by ELISA in all samples except for samples 5 and 9 (Table 1).

The Scott F/ R primers used in this project were tested by Scott et al. (2000). In their study, the primers resulted in successful amplification of cDNA from Scottish and English RpRSV isolates grown in *Chenopodium quinoa*. However, in this study the primers did not work for any of the tested annealing temperatures. It is therefore hypothesized that the RpRSV present in the samples used here differs significantly in sequence from other previously studied isolates and that it might be a new strain of the virus.

A multiple alignment of five RpRSV CP amino acid sequences and one *Grapevine fanleaf virus* (GFLV) isolate present in the NCBI database (Figure 5, Appendix) show that even between isolates of the same strain, there are some difference in sequence. GFLV was included to compare the RpRSV sequences to another nepovirus. GFLV belongs to subgroup A and was chosen since members of the same subgroup share conserved sequence stretches. RpRSV has been shown to have a sequence identity of 39 % to GFLV when aligning RNA-2 polyprotein sequences (Blok et al., 1992; Digiaro et al., 2007). As expected, the sequences from the same strain (1 and 4) are the two most similar, which is shown by the highest

sequence identity (94 %). The greatest difference was between all five RpRSV sequences and the GFLV sequence. All RpRSV raspberry sequences were very similar to each other (sequence identities from 88 to 94 %). The fact that the alignment shows differences in amino acid sequence even for isolates of the same strain supports the idea that the amplification problems encountered here are due to differences in nucleotide sequence. However, since the multiple alignment was made at the end of the project, there was not enough time to design new primers towards a region with little sequence variation.

The presence of primer-dimers, which can be seen in Figures 3 and 4, indicates that the PCR overall was working as expected. The amount of primer-dimers is not so high that one can assume that they inhibit amplification of the viral sequence. Instead, they suggest that all ingredients were added properly since if something would have been missing, the gels would be completely blank.

The degenerate nepovirus primers (subgroup A specific) designed according to Digiaro et al. (2007) did not succeed in amplification of RpRSV RNA-2 which is in agreement with their results. Even though Digiaro et al. (2007) showed that they did not work for RpRSV, these primers were tested here in order to find out if they might work after all because the isolates used in this study appear to differ in sequence from any previously studied isolate.

Conclusion

To be sure why the amplification of RpRSV RNA-2 did not succeed, despite all attempts with different primer pairs and reagent mixtures, it is necessary to do more extensive analyses of the isolates used here. From the results gathered in this project, it is only possible to speculate about why it did not work. However, the most likely explanation is that the RpRSV sequence present in the raspberry samples is somehow different from previously studied sequences of this virus. This project showed that optimizing primer conditions can take a long time. The short time available also restricted the number of theories and primer pairs that could be tested. If there would have been more time for this project, it might have been possible to succeed with the amplification. There are still several theories and approaches left to be tested. For continued studies of these samples one could for example try to design a longer forward primer (~60 bp) against the 5’ end of the RNA-2 molecule and a reverse poly-T primer against the poly-A 3’ end of the RNA-2 molecule, which can sometimes help in amplifying a difficult sequence.

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Appendix

Tested cDNA samples for PCR

Table 1. Source information for the samples used for PCR.

Sample no	Raspberry cultivar	Origin of cultivar
2	Heracl	Russia
3	Abrikosovaya	Russia
4	Polana	Poland
5, 9	Balsam	Belarus
7	Meteor	Belarus
8, 14	Zolotye Kupola	Russia
15, 17	Alyonushka	Belarus
18	Zeva Herbsternt	Belarus*
21	Porana Rosa	Poland

Table 2. Overview of cDNA samples used for the different PCRs.

PCR	Sample number
1-2	2-4, 7, 14, 15, 17, 18
3 + gradient (58-66°C)	3
4-5	4
6-7	4, 5
8	4, 9
Gradient (51-61°C)	4
9	2, 4
10	4, 15
Gradient (48-60°C)	15
Gradient (48.8-52.8°C)	3, 8
Gradient (48.8-57°C)	3
Gradient (45-55°C)	3
11	4, 14, 17
12	4, 14, 17
13	3
14	3, 21
15	3
16	5
Gradient (61-68°C)	5
17	4

PCR settings for the first pair of primers (RRV Forward/ RRV Reverse)

Table 3. PCR programs used for the RRV Forward/ Reverse primers. Numbers in parenthesis show which PCR experiment the settings apply to.

PCR steps	(1) Temp, °C	Time	(2) Temp, °C	Time	(3) Temp, °C	Time
Initial denaturation	95	2 min	95	2 min	95	2 min
Denaturation	95	30 sec	95	30 sec	95	30 sec
Annealing	60	30 sec	*63*	*45 sec*	*62*	*45 sec*
Elongation	72	1 min 15 sec	72	1 min 15 sec	72	1 min 15 sec
Final elongation	72	10 min*	72	10 min	72	10 min
(Denaturation- Annealing- Elongation) x 40 cycles						

* = for gradient PCR this step was 15 min

Table 4. Reagents used for amplification in the first three PCR experiments (see number in parenthesis). The third PCR was run with either 2 or 3 µl template cDNA.

Reagents	(1) Volume, µl	(2) Volume, µl	(3) Volume, µl
Dream Taq buffer (10x)	5	5	5
dNTP (10 mM)	0.5	0.5	0.5
RRV Forward (10 µM)	1	*0.5*	1
RRV Reverse (10 µM)	1	*0.5*	1
Dream Taq DNA polymerase 5 u/µl (Fermentas)	0.25	0.25	0.25
MilliQ water	16.25	*17.25*	*15.25 and 14.25*
Template cDNA	1	1	*2 and 3 (two samples)*

The PCR with sample 4 (see PCR 17, Table 1) was run with the same setting as for PCR number 1, except for annealing temperature (57°C) and elongation time (1 min and 30 sec). The reagent mixture composition was the same as for PCR number 9 (Table 8).

PCR settings for the RRVF/ RRVR primers

Table 5. PCR programs for amplification using the RRVF/ RRVR primer pair. Numbers in parenthesis show which PCR experiment the settings apply to.

PCR steps	(4) Temp, °C	Time	(5) Temp, °C	Time	(6) Temp, °C	Time
Initial denaturation	95	2 min	95	2 min	95	2 min
Denaturation	95	30 sec	95	30 sec	95	30 sec
Annealing	50	30 sec	*60*	30 sec	*55*	30 sec
Elongation	72	1 min	72	1 min	72	1 min
Final elongation	72	10 min	72	10 min	72	10 min

PCR steps	(7) Temp, °C	Time
Initial denaturation	95	2 min
Denaturation	95	30 sec
Annealing	*52*	30 sec
Elongation	72	1 min
Final elongation	72	10 min

(Denaturation- Annealing- Elongation) x 40 cycles

For PCR number 4-6, the reaction mixtures were the same as for the first PCR (except for the primers). For PCR number 7, the primer volume was decreased to 0.5 µl.

PCR mixture used for the primer combination RRVF + RRV Reverse

Table 6. Reagents used for amplification.

Reagents	(8) Volume, µl
Dream Taq buffer (10x)	5
dNTP (10 mM)	0.5
RRVF (10 µM)	0.5
RRV Reverse (10 µM)	0.5
Dream Taq DNA polymerase 5 u/µl (Fermentas)	0.25
MilliQ water	17.25
Template cDNA	1

(Denaturation- Annealing- Elongation) x 40 cycles

PCR settings for the primer pair RRV F2/R2

Table 7. PCR programs used for the RRV F2/ RRV R2 primers. Numbers in parenthesis show which PCR experiment the settings apply to.

PCR steps	(9) Temp, °C	Time	(10) Temp, °C	Time
Initial denaturation	95	2 min	95	2 min
Denaturation	95	30 sec	95	30 sec
Annealing	*55*	30 sec	*50*	30 sec
Elongation	72	1 min	72	1 min
		40 sec		40 sec
Final elongation	72	10 min*	72	10 min
(Denaturation- Annealing- Elongation) x 40 cycles				

* = for gradient PCR this step was 15 min

Table 8. Reagents used for amplification with the primers RRV F2/ RRV R2 or CP-RRV Forward/ Reverse. For PCR with Taq DNA polymerase, extra $MgCl_2$ had to be added.

Reagents	**(9) Volume, µl**	**(10: Dream Taq) Volume, µl**	**(10: Taq DNA) Volume, µl**
Dream Taq buffer (10x)**	*2.5*	2.5	2.5
$MgCl_2$	-	-	1.5
dNTP (10 mM)	0.5	0.5	0.5
RRV F2 (10 µM)*	1	1	1
RRV R2 (10 µM)*	1	1	1
DNA polymerase (Fermentas) 5 u/µl	0.25	0.25	0.3
MilliQ water	18.75	18.75	17.2
Template cDNA	1	1	1

* = CP-RRV primers also used for PCR 10; ** = For PCR 10 with Taq DNA polymerase, 10x Taq buffer was used instead

PCR number 10 was made with four different combinations of primers and polymerase:

1. Dream Taq pol. & RRV F2/ RRV R2
2. Dream Taq pol. & CP-RRV primers
3. Taq DNA pol. & RRV F2/ RRV R2
4. Taq DNA pol. & CP-RRV primers

PCR settings for the primer pair CP-RRV Forward/ CP-RRV Reverse

For the CP-RRV primers, the same settings as displayed in Table 7 (PCR 10) apply for PCR 10 and gradient (48-60°C and 48.8-52.8°C). For reagent mixture compositions used for these PCRs, see Table 8.

Settings for PCR number 12, made with new CP-RRV Reverse primer, are shown in Table 9. The reagent mixture composition was the same as for PCR number 10 (Dream Taq polymerase), see Table 8.

Table 9. PCR program used for the new CP-RRV primers (with correct orientation of the reverse primer). The number in parenthesis shows which PCR experiment the settings apply to.

PCR steps	(12) Temp, °C	Time
Initial denaturation	95	2 min
Denaturation	95	30 sec
Annealing	*50*	30 sec
Elongation	72	*1 min 30 sec*
Final elongation	72	10 min

(Denaturation- Annealing- Elongation) x 40 cycles

PCR settings for the primer pair Scott F/ Scott R

PCR number 11 was run with the same settings as for PCR number 6 (Table 5). Standard (see PCR 9) reagent mixture composition was used.

PCR number 16 was run with the same settings as used for PCR number 5 (Table 5). Standard (see PCR 9, Table 8) reagent mixture composition was used, except for the dilution of template which is described in the material and methods section.

PCR settings for the primer combination Scott F/ R + RRV Forward/ RRV Reverse

For PCR number 13, the same conditions were used as for PCR number 5 (Table 5). Standard (see PCR 9, Table 8) reagent mixture composition was used. Two different primer combinations (A and B) were tried out in the following way:

A. RRV Forward and Scott R
B. Scott F and RRV Reverse

PCR settings for the primer combination Scott F and CP-RRV Reverse

Table 10. Program used for PCR with the primer pair Scott F/ CP-RRV Reverse. The number in parenthesis shows which PCR experiment the settings apply to.

PCR steps	(14) Temp, °C	Time
Initial denaturation	95	2 min
Denaturation	95	30 sec
Annealing	*58*	30 sec
Elongation	72	*2 min 30 sec*
Final elongation	72	10

(Denaturation- Annealing- Elongation) x 35 cycles

PCR settings for the degenerate primers Nepo-A F/ Nepo-A R

For PCR number 15 the same settings as for PCR number 10 were used, except for the elongation time which was 30 seconds (expected product size 255 bp). Standard (see PCR 9, Table 8) reagent mixture composition was used, except for the dilution of template which is described in the material and methods section

ClustalW multiple sequence alignment of translated RpRSV CP and GFLV CP sequences

```
CLUSTAL 2.0.12 multiple sequence alignment

gi|6650728|gb|AAF21986.1|AF116      ------------------------------------------------
gi|257078|gb|AAB23551.1|            MSQFWGEFPEKVIQTFQHLQVALIGDIKKCALSSPLFPELSKLDAHSQHH 50
gi|6650614|gb|AAF21937.1|AF111      ------------------------------------------------
gi|40254025|ref|NP_954617.1|       ------------------------------------------------
gi|34597328|gb|AAQ77239.1|          MSQFWGEFPEAVINTFQRLQIALIGDIKRCPLSSPLFPELSKLDAHSQHH 50
gi|99083473|gb|AAV68691.2|          ------------------------------------------------

gi|6650728|gb|AAF21986.1|AF116      ------------------------------------------------
gi|257078|gb|AAB23551.1|            LLASFELPRFGGVTPGVMEQLRDAESELAEAKQRLLRERLHAVANRQNIP 100
gi|6650614|gb|AAF21937.1|AF111      ------------------------------------------------
gi|40254025|ref|NP_954617.1|       ------------------------------------------------
gi|34597328|gb|AAQ77239.1|          LLASFELPKFGGVTPSVMEQLHDAESELAEAKARLLRERLHAVANKENIP 100
gi|99083473|gb|AAV68691.2|          ------------------------------------------------

gi|6650728|gb|AAF21986.1|AF116      ------------------------------------------------
gi|257078|gb|AAB23551.1|            YLGDCMYYDAPGISQEELLQAAFLEAPTPAWEHERIRPLWPKDEWFRDAR 150
gi|6650614|gb|AAF21937.1|AF111      ------------------------------------------------
gi|40254025|ref|NP_954617.1|       ------------------------------------------------
gi|34597328|gb|AAQ77239.1|          YLGDCMFYDAPGISQEELLQAAFLEAPTPEWESERIRPLWPKDEWFRDAQ 150
gi|99083473|gb|AAV68691.2|          ------------------------------------------------

gi|6650728|gb|AAF21986.1|AF116      ------------------------------------------------
gi|257078|gb|AAB23551.1|            QGPYLEDYGNIPLGDLDTLCLAFDALVEEHWMPIYLLISTFSMFQQYGTQ 200
gi|6650614|gb|AAF21937.1|AF111      ------------------------------------------------
gi|40254025|ref|NP_954617.1|       ------------------------------------------------
gi|34597328|gb|AAQ77239.1|          QGPYPEDYGSIPLGDIDTLCLAFDALVEEHWMPIYLMISTFATFQQYGTH 200
gi|99083473|gb|AAV68691.2|          ------------------------------------------------

gi|6650728|gb|AAF21986.1|AF116      ------------------------------------------------
gi|257078|gb|AAB23551.1|            PLLLECAQSAGSLIPACMMTDHHLEPTGDRQADKEARQDYADSQDSIQSM 250
gi|6650614|gb|AAF21937.1|AF111      ------------------------------------------------
gi|40254025|ref|NP_954617.1|       ------------------------------------------------
gi|34597328|gb|AAQ77239.1|          PLLLECVQSAGSLIPACMMTDHHLQPTGDRQADKEERQDYADSQDSIQSM 250
gi|99083473|gb|AAV68691.2|          ------------------------------------------------

gi|6650728|gb|AAF21986.1|AF116      ------------------------------------------------
gi|257078|gb|AAB23551.1|            GDFWKEFYTKDSGKKIPDSHKSRLANDPNKVGFTKSALFHKQPLSHSLAQ 300
gi|6650614|gb|AAF21937.1|AF111      ------------------------------------------------
gi|40254025|ref|NP_954617.1|       ------------------------------------------------
gi|34597328|gb|AAQ77239.1|          GDFWKEFYSKDSGKKIPDSHKSRLANDPNKVGFTKSALFHKQPLAHSLAQ 300
gi|99083473|gb|AAV68691.2|          ------------------------------------------------

gi|6650728|gb|AAF21986.1|AF116      ------------------------------------------------
gi|257078|gb|AAB23551.1|            TWANFRGTQDKADLVKVTMDMNIEKYTVRLPDAVRTTAGPLYIEWINLPR 350
gi|6650614|gb|AAF21937.1|AF111      ------------------------------------------------
gi|40254025|ref|NP_954617.1|       ------------------------------------------------
gi|34597328|gb|AAQ77239.1|          TWANFRGTQDKADLVKVTMDMNIEKYTVRLPDAVRTTAGPLYIEWINLPR 350
gi|99083473|gb|AAV68691.2|          ------------------------------------------------

gi|6650728|gb|AAF21986.1|AF116      ------------------------------------------------
gi|257078|gb|AAB23551.1|            MSENSARKLAEVGWNNADICGVDLAVKSHIAVGTPVRVIISLVDGACSDM 400
gi|6650614|gb|AAF21937.1|AF111      ------------------------------------------------
gi|40254025|ref|NP_954617.1|       ------------------------------------------------
gi|34597328|gb|AAQ77239.1|          MSENSARKLAEAGWNNADICGVDLAVKSHVAVGTPVRVIISLVDGACSDM 400
gi|99083473|gb|AAV68691.2|          ------------------------------------------------

gi|6650728|gb|AAF21986.1|AF116      ------------------------------------------------
gi|257078|gb|AAB23551.1|            PTATMCAFEVNLASQNNRSLNLPLLSLPFSRLLADLHDFQNRVKIACQFR 450
gi|6650614|gb|AAF21937.1|AF111      ------------------------------------------------
gi|40254025|ref|NP_954617.1|       ------------------------------------------------
gi|34597328|gb|AAQ77239.1|          PTATMCAFEVNLATQNNRSLNLPLLSLPFSQLLADLHDFQHRVKIACQFR 450
gi|99083473|gb|AAV68691.2|          ------------------------------------------------

gi|6650728|gb|AAF21986.1|AF116      ------------------------------------------------
gi|257078|gb|AAB23551.1|            DPEGFNVGTPMLSFSSLEFSELKQTAFERNSLLRDSWSEIEKRACHGGGR 500
gi|6650614|gb|AAF21937.1|AF111      ------------------------------------------------
gi|40254025|ref|NP_954617.1|       ------------------------------------------------
gi|34597328|gb|AAQ77239.1|          DPEGFNVGTPMLSFSSLEFSELKQTAFERKSLLRDSWSEIEKRACHGGGR 500
gi|99083473|gb|AAV68691.2|          ------------------------------------------------

gi|6650728|gb|AAF21986.1|AF116      ------------------------------------------------
gi|257078|gb|AAB23551.1|            CVASQGIVQTWEKEVNPPLKEYAPLVLPPVPQPKRNFIDQQTGEVVQSFM 550
gi|6650614|gb|AAF21937.1|AF111      ------------------------------------------------
gi|40254025|ref|NP_954617.1|       ------------------------------------------------
gi|34597328|gb|AAQ77239.1|          CIASQGIVQTWEKEINPPLKEYAPLVLPPVPQPRRNFIGQQSGEVVKPWL 550
gi|99083473|gb|AAV68691.2|          ------------------------------------------------

gi|6650728|gb|AAF21986.1|AF116      ------------------------------------------AYEVDPL 7
gi|257078|gb|AAB23551.1|            QKSRSMRFKSPSDLWSRPSVDGGSTSTQPPSKGSLRCENVPGCAYEVDPL 600
gi|6650614|gb|AAF21937.1|AF111      ------------------------------------------AYEVDPL 7
gi|40254025|ref|NP_954617.1|       ------------------------------------------AYEVDPL 7
gi|34597328|gb|AAQ77239.1|          PKSRSMRFKSPTDLWSRPSVDGGSTSTVVTGTEHLRCDDVPGCAYEVDPL 600
gi|99083473|gb|AAV68691.2|          ------------------------------------------------
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gi|6650728|gb|AAF21986.1|AF116      HLLYYEEVTVPKDTLDGTLLARIDVRAKAATFDSAVWRQWVRDGCLKPRV 57
gi|257078|gb|AAB23551.1|            HLLYYESVDVPKDTLAGTLLARIDVRAKAAIFDSAVWRQWVRDGCLKPKI 650
gi|6650614|gb|AAF21937.1|AF111      HLLYYEEVTVPKDTLGGALLARIDVRAKAATFDSAVWRQWVRDGCLKPRI 57
gi|40254025|ref|NP_954617.1|        HLLYYELVNVPKDTLGGTLLTRIDVRAKAATFDSAVWRQWVRDGCLKPKI 57
gi|34597328|gb|AAQ77239.1|          HLLYYASVDVPKDTLEGTRLARIDLRAKAQEMDSAVWRQWVKEGCMKPRI 650
gi|99083473|gb|AAV68691.2|          --------------------------------------------------


gi|6650728|gb|AAF21986.1|AF116      KMRVTAATSCYSGIVLGACLDAYRRIPATTKTDFTASLVTGLPNTMWAMR 107
gi|257078|gb|AAB23551.1|            KMRITAATSCFSGIVLGACFDAYRRIPAATKTGITASLVTGLPNTVWATR 700
gi|6650614|gb|AAF21937.1|AF111      KMRITAATSCFSGIVLGACLDAYRRIPATTKADFTASLVTGLPNAMWATR 107
gi|40254025|ref|NP_954617.1|        KMRITAATSCFSGIVLGACLDAYRRIPATTKTDFTASLVTGLPNIVWATR 107
gi|34597328|gb|AAQ77239.1|          KIRISAATSCFSGVVLGMCLDAYRRIPIMRDKGFSANLVTGLPNTMWATR 700
gi|99083473|gb|AAV68691.2|          ------------------------RITASADPVYTLSVPHWLIHHKLGT- 25
                                                **.   .   : .:   * :   .


gi|6650728|gb|AAF21986.1|AF116      DTSEIEWDIDLAAVCGHTFFALGDTFGYMDFLVYVLRGNEITAVADWSIY 157
gi|257078|gb|AAB23551.1|            DTSEVEWDIDLAAVCGHTFFALEDTFGYMDFLIYVLRGNEITAVADWSIY 750
gi|6650614|gb|AAF21937.1|AF111      DTSEIEWDIDLAAVCGHTFFALEDTFGYMDFLIYVLRGNEITAVADWSMY 157
gi|40254025|ref|NP_954617.1|        DTSEIEWDIDLAAVCGHTFFALEDTFGYMDFLVYVLRGNEVTAVADWSIY 157
gi|34597328|gb|AAQ77239.1|          TQAELEWDLDLSQECGHSFYALSDTLGYMDFLIYVLRGNEMTAVADWSFY 750
gi|99083473|gb|AAV68691.2|          ----FSCEIDYGELCGHAMWFKSTTFESPRLHFTCLTGNTKELAADWQAV 71
                                        .. ::* .  ***:::    *:    : .  * **    .***.


gi|6650728|gb|AAF21986.1|AF116      VSFHVDWTQESMLATLIPTFVWPPKPTDISLLKEVWGPYRFTLDGTEAKG 207
gi|257078|gb|AAB23551.1|            VSFHVDWTQESMLATLIPTFVWPPKPTDISLLKEVWGPYRFTLDGTEAKE 800
gi|6650614|gb|AAF21937.1|AF111      VSFHVDWTQESMSATLIPTFVWPPKPSDIFLFKEVWGPYHFSLDGTEAKV 207
gi|40254025|ref|NP_954617.1|        VSFHVDWTQESMSATLIPTFVWPPEPADISYFKEVWGPYHFTLDGTEAKE 207
gi|34597328|gb|AAQ77239.1|          VAFYVDWSQESFTAMLAPTLKWPPTPGIISTFKEVRGPYAFSLDGTKARL 800
gi|99083473|gb|AAV68691.2|          VELYAEL-EEATSFLGKPTLVFDPGVFNGKFQFLTCPPIFFDLTAVTALR 120
                                    * ::.:  :*:      **: : *          .  *  * * .. *


gi|6650728|gb|AAF21986.1|AF116      SFAIMPGTAIPHGQQIVRTFPRVVAAHFRSWTGKVRMSIQEVSSIFLTGT 257
gi|257078|gb|AAB23551.1|            SFAIMPGTAILHGQQIVRTFPRVVAAHFRSWTGKVRMSIQEVSSIFLTGT 850
gi|6650614|gb|AAF21937.1|AF111      DLDIMPGMAIPRGHTTVRTFPRVVAAHFRSWTGKIKMSIQEVSSIFLTGT 257
gi|40254025|ref|NP_954617.1|        SFSLMPGMAIPRGAQTVRTFPRVLAAHFRSWTGKVRMSIQEVSSIFLTGT 257
gi|34597328|gb|AAQ77239.1|          DFGFLPGVSLVEGSETVRTCPRVLASFYRSWTGKLRISIEEVSSIFLTGS 850
gi|99083473|gb|AAV68691.2|          SAGLTLGQVPMVGTTKVYNLNSALASCVLGMGGTIRGRVHICASIFYSIV 170
                                    .  :  *     *   * .  .:*:   .  *.::  :.  :*** :


gi|6650728|gb|AAF21986.1|AF116      YMVGVSWN-ATADLADIVTRKHWIVKSNEIFEVDLYCPYGENPTFTGQAN 306
gi|257078|gb|AAB23551.1|            YMVGVSWN-ATADLADIVTRKHWIVKSNEIFEVDLYCPYGENPTFTGQAN 899
gi|6650614|gb|AAF21937.1|AF111      YVVGVSWN-VTADLADIVTRKHWIVKSGEIFELDLYCPYGKNPTFTGLAN 306
gi|40254025|ref|NP_954617.1|        YMVGVSWN-ATADLTDITTRKHWIVKSGEVFELDLYCPYGENPTFTGLVN 306
gi|34597328|gb|AAQ77239.1|          YMVGVAWN-AGDDLGGITTRKHWMVKSGEIFDLDLYGPHGEYPTFAGKAN 899
gi|99083473|gb|AAV68691.2|          LWVVSEWNGTTMDWNELFKYPGVYVEEDGSFEVKIRSPYHRTPARLLAGQ 220
                                      *   ** .  *   : .     *:..  *::.:  *: . *:     :


gi|6650728|gb|AAF21986.1|AF116      GKPFIIVHKLGGIVGPKDSVGTFGFMIHIHGLTGVYRNPTLHSGDRSVGS 356
gi|257078|gb|AAB23551.1|            GKPFIIVHKLGGIVGPKDSVGTFGFMIHIHGLTGVYKNPTLHSGDRSVGS 949
gi|6650614|gb|AAF21937.1|AF111      GKPYIIVHKLGGIIGPKDSVGTFGFMIHIHGLTGMYKNPTLHSGDRSVGS 356
gi|40254025|ref|NP_954617.1|        GIPYIIVHRLGGIIGPKDSVGTFGFMIHLHGLTGVYKNPTLHSGDRSVGS 356
gi|34597328|gb|AAQ77239.1|          GTPYIVVQKVGGIVGPKDSTGSFGFFLHIHGMTGIYKNPTLHSPERGQMH 949
gi|99083473|gb|AAV68691.2|          SQRDMSSLNFYAIAGPIAPSGETARLPIVVQIDEIVRPDLSLPSLEDDYF 270
                                    .   :   .. .* **  . *  . : :  :  :  : :     .  ..


gi|6650728|gb|AAF21986.1|AF116      AWFRINNIADDNLVFNIPGKIENIIAAAGNYDVTNYVNPTILLFSVTGLH 406
gi|257078|gb|AAB23551.1|            AWFRINNIADDNLVFNIPGRIEDIIAAAGKYDVTNYVNPTSLLFSVTGLH 999
gi|6650614|gb|AAF21937.1|AF111      AWFRITNIADDNLVFNIPGKIEDMAAVTGNYDVTNYVNPTSLLFSVTGLH 406
gi|40254025|ref|NP_954617.1|        AWFRVTNILDDNLVFNIPGRIEDMVAVAGKYEVTNYANPTSMLFSVTGLH 406
gi|34597328|gb|AAQ77239.1|          AWFRMNNIQVDNLSFSIPGRIEDMSALAGSYDITNYVNPASLLFSVTGLH 999
gi|99083473|gb|AAV68691.2|          VWVDFSEFTLDKEEIEIGSRFFDFTSSTCRVSMG--ENPFAAMIACHGLH 318
                                    .*. ..::  *:  :.* .:: :: : :   .:    **   :::  ***


gi|6650728|gb|AAF21986.1|AF116      GGIIRLHITWCPNTNLGESKGTLKYMQYLYHTATDNFFGDQATRGIIDQD 456
gi|257078|gb|AAB23551.1|            GGIIRLHITWCPNTTLGESKGTLKYMQYLYHTATENFFGDQATRGIIDQD 1049
gi|6650614|gb|AAF21937.1|AF111      GGVIRLHITWCPSVNLGEAKGTLKYMQYLYHTSTENFFGDQATRGIIDQD 456
gi|40254025|ref|NP_954617.1|        GGFIRLHITWCPNTSLGESKGTLKYMQYLYHTTTENFFGDQATRGIIDQN 456
gi|34597328|gb|AAQ77239.1|          GGTIRLHVTWCPKTNLGESKGTLKYMQYLYHTNTVSYYGDQATRGLIDPD 1049
gi|99083473|gb|AAV68691.2|          SGVLDLKFQWSLNTEFGKSSGSITITQLVGDKAMG-LDGPSQVFAIQKLE 367
                                    .* : *:. *. .. :*::.*::.  * : ..      * . . .: . :


gi|6650728|gb|AAF21986.1|AF116      GFTVDIACGDFFGATR-VGLPGEVERLGIYSSNAKSIAEIRVSFEVLS-M 504
gi|257078|gb|AAB23551.1|            GFTIDIACGDFFGATR-VGLPGEVERLGIYSSNAKSIAEIRVSFEVLS-M 1097
gi|6650614|gb|AAF21937.1|AF111      GFTVDIACGDFFGATR-VGLTGEIERLGIYSSNAKSIAEIRVSFEILS-M 504
gi|40254025|ref|NP_954617.1|        GFTVDLACGDFFGATR-VGLKGEVERLGIYSSNAKSIAEIRVSFEILS-M 504
gi|34597328|gb|AAQ77239.1|          GFKCELRCGDFFGATN-IAMVGDVERLAIHSANATFISEIRVSFEILE-M 1097
gi|99083473|gb|AAV68691.2|          G-TAELLVGNFAGANPNTHFSLYSRWMAIKLDQAKSIKVLRVLCKPCPGF 416
                                    * . ::  *:* **.    :    . :.*   :*. *  :**  :    :


gi|6650728|gb|AAF21986.1|AF116      NFYGSTIKVT 514
gi|257078|gb|AAB23551.1|            NFYGSTIKVT 1107
gi|6650614|gb|AAF21937.1|AF111      NFYGSTIKVT 514
gi|40254025|ref|NP_954617.1|        KFYGSTIRVK 514
gi|34597328|gb|AAQ77239.1|          SFYGKTIKVS 1107
gi|99083473|gb|AAV68691.2|          SFYGRTSFPV 426
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Figure 5. Multiple alignment of the coat protein translation products of five *Raspberry ringspot virus* (RpRSV) isolates and one *Grapevine fanleaf virus* isolate (accession no: AAV68691.2). The RpRSV sequences were (from the top): Scottish strain (GenBank accession no: AAF21986.1; Scott et al., 2000), English strain (GenBank accession no: AAF21937.1; Scott et al., 2000), grapevine-ch strain (GenBank accession no: NP_954617.1; Ebel et al., 2003), Scottish strain (GenBank accession no: AAB23551.1; Blok et al., 1992), grapevine isolate (GenBank accession no: AAQ77239.1; Wetzel et al., 2006). The alignment was generated using the ClustalW program at www.ebi.ac.uk with standard settings. Identical amino acids for all sequences are marked by "*", conserved substitutions are marked by ":" and semi-conserved substitutions are marked by ".".

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