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Molecular Biology of *Raspberry ringspot nepovirus*

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ABSTRACT

The *Raspberry ringspot virus* (RpRSV), first reported in raspberries (*Rubus idaeus*) in Scotland, is also a causative agent in Germany of the grapevine fanleaf disease and the Pfeffinger disease of sweet cherries. The RpRSV belongs to the genus nepovirus in the *Comoviridae* family, has polyhedral particles and is transmitted in the soil via nematodes. Different strains of RpRSV have been identified, from different types of hosts, and different types of nematode vectors have been found for the different strains of RpRSV. Furthermore, the different strains of RpRSV induce different types of symptoms on herbaceous hosts. The complete nucleotide sequences of the genomic RNAs 1 and 2 of two isolates of RpRSV infecting grapevine (RpRSV-grapevine and RpRSV-cherry), as well as partial sequences of other isolates of RpRSV from grapevine, raspberry, blackberry, were determined. In this paper, we report sequence comparisons and analysis between the different available sequences of RpRSV in relation to known and putative biological functions, and try whenever possible to correlate molecular and biological data.

Keywords: artificial miRNAs, *Comoviridae*, non-coding regions, RNA 1, RNA 2, RNA silencing

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INTRODUCTION

The *Raspberry ringspot virus* (RpRSV)(Murant 1970), belonging to the genus nepovirus from the *Comoviridae* family, was first reported in raspberry plants (*Rubus ideaeus*) in Scotland (Cadman 1956). Since then, RpRSV has been reported in different countries and in different plants (Murant 1970). In Germany and in Switzerland, RpRSV is, along with *Arabis mosaic virus* (ArMV) and *Grapevine fanleaf virus* (GFLV), two other nepoviruses, a causative agent of grapevine fanleaf disease (Bercks 1968), one of the most widespread and damaging virus diseases affecting grapevine. Three important strains of RpRSV have been identified in England (Murant 1970, and references therein): the Scottish strain (S), the English strain (E) which is serologically distinct from the Scottish strain, and the Lloyd George yellow blotch strain (LG). The symptoms observed on infected raspberries vary according to the susceptibility of the cultivar and the virus strain. In very susceptible cultivars, part or all of the cane may die in winter. In less susceptible cultivars, symptoms are mild or even absent. The infected plants are usually patchily distributed in crops, reflecting the distribution of the vector. Additionally, two serologically distinct strains of RpRSV occur in German vineyards: the cherry strain which is also the causal agent of the Pfeffinger disease of sweet cherries (RpRSV-ch, Ebel *et al.* 2003 and references therein), and the grapevine strain (RpRSV-grapevine, Wetzel *et al.* 2006), RpRSV-ch being the most common one of these two strains. In Switzerland, a RpRSV isolate from grapevine (RAC815), serologically distinct from both the German cherry and grapevine isolates has been reported (Gugerli 1989). The symptoms induced by RpRSV-ch on sweet cherries can be very severe, even leading to the death of the tree (**Fig 1**), while on grapevines, the symptoms induced by RpRSV-ch are mild and barely affect the yield (**Fig 1**). On the other hand, RpRSV-grapevine induces symptoms similar to those observed with GFLV, which is the most damaging and widespread virus of grapevine. RpRSV-grapevine can produce severe yield reduction as only few berries reach maturity, and the longevity of the grapevine is greatly reduced (**Fig 1**). Routine detection of RpRSV in grapevine in Germany is mainly based on immunological tests (ELISA), with commercially available strain-specific antisera.

RpRSV is transmitted in the soil via nematode vectors, different serotypes of RpRSV being transmitted by different nematode species. The raspberry Scottish serotypes of RpRSV are transmitted by *Longidorus elongatus*, while the raspberry/blackberry English serotypes are transmitted by *Longidorus macrosoma* (Wellink *et al.* 2000, and references

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Invited Mini-Review

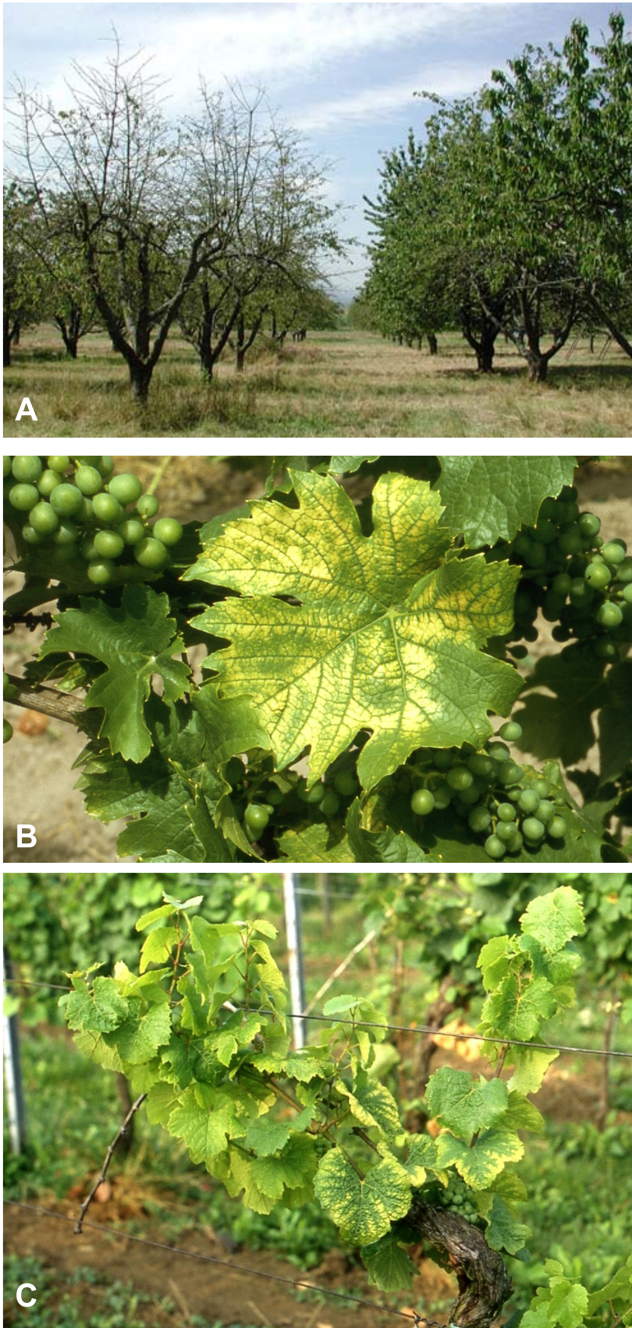

Fig. 1 RpRSV- induced symptoms in cherry trees and grapevine. (**A**) RpRSV-cherry-infected cherry trees (left) and healthy trees (right). (**B**) RpRSV-cherry-infected grapevine (Müller-Thurgau). A yellow mosaic can be observed on few leaves, but the grapes develop without visible symptoms. (**C**) RpRSV-grapevine-infected grapevine (Riesling) in Germany, showing reduced growth, leaf deformations, and with very few grapes.

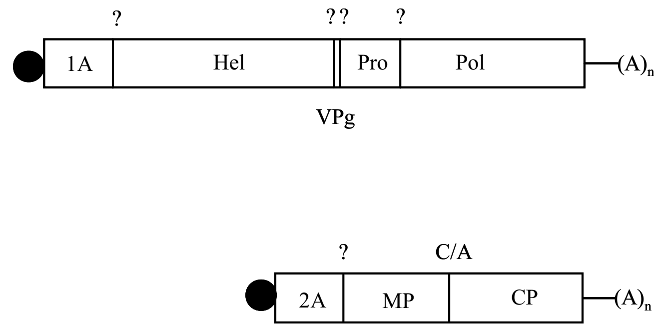

Fig. 2 Putative genomic organisation of RpRSV. Only the C/A cleavage site between the coat protein (CP) and movement protein (MP) has been determined (?: unidentified cleavage site). The genomic RNAs are linked at their 5′ end to a VPg (●), and are polyadenylated (A) at their 3′ end.

therein). However, the transmission of RpRSV-grapevine in German vineyards was shown to be mainly due to the nematode *Paralongidorus maximus* (Jones *et al*. 1994), while RpRSV-ch is transmitted by *Longidorus macrosoma* (Buser 1990). The nematode *L. macrosoma* was also found in fields infected with the RpRSV-RAC815 isolate in Switzerland (Gugerli 1989).

MOLECULAR BIOLOGY OF RpRSV

Nepoviruses have two positive-sense, single stranded genomic RNAs, which are polyadenylated at their 3′ end and have a covalently attached small genome-linked viral protein (VPg) at their 5′ end (for a review, see Mayo and Robinson 1996). The complete nucleotide sequences of RpRSV-ch and RpRSV-grapevine genomic RNAs 1 and 2, and of the genomic RNA 2 of a Scottish isolate infecting raspberry (RpRSV-S) have been reported (Blok *et al.* 1992; Ebel *et al.* 2003; Wetzel *et al.* 2006). Additional partial sequences of the RNA 2 of other RpRSV isolates are also available (Scott *et al.* 2000). The putative genomic organisations of RpRSV RNAs 1 and 2 (**Fig. 2**) are similar to that of GFLV (Mayo and Robinson 1996, and references therein). RNA 1 and RNA 2 are translated into polyproteins which are proteolytically matured by the RNA 1-encoded $1D^{pro}$ proteinase into final products referred to as 1A (of unknown function), $1B^{Hel}$ (Helicase), $1C^{VPg}$ (VPg), $1D^{pro}$ (proteinase) and $1E^{pol}$ (polymerase) for RNA 1, into final products referred to as 2A (involved in RNA 2 replication; Gaire *et al.* 1999), $2B^{MP}$ (movement protein) and $2C^{CP}$ (coat protein) for RNA 2. So far, a C/A cleavage site between the putative movement protein and the coat protein of RpRSV-S has been identified (Blok *et al.* 1992). The analysis of the polyproteins encoded by RpRSV RNAs 1 of the grapevine and cherry isolates showed that the characteristic motifs for the putative viral protease cofactor, the NTB-binding domain, the viral cysteine protease domain, and the RNA-dependant RNA polymerase core domain were all found (Ebel *et al.* 2003 and references therein; Wetzel *et al.* 2006).

Early experiments using hybrid isolates between the E, S and LG strains of RpRSV, obtained by combining the RNA 1 from one strain with the RNA 2 from another strain, revealed the location of the genetic determinants for some biological characters of RpRSV on one or the other or both RNAs (Harrison *et al.* 1972, 1974). The serological specificity, nematode transmissibility, and systemic yellowing symptoms on *Petunia hybrida* were determined by RNA 2. The severity of systemic symptoms in *Chenopodium quinoa* and other herbaceous hosts, the ability to infect Lloyd George raspberry and ability to invade the non-inoculated leaves of *Phaseolus vulgaris*, were all determined by RNA 1 (Harrison *et al.* 1972, 1974).

RpRSV 5′ and 3′ non-coding regions on RNAs 1 and 2

Conserved regions susceptible to form stem and loop structures were described in the 5′ non-coding regions of the RNAs 2 of *Tomato black ring virus* (TBRV) and *Grapevine chrome mosaic virus* (GCMV; Le Gall *et al.* 1995), ArMV and GFLV (Wetzel *et al.* 2001). These structures in GCMV were shown to induce a necrotic response in *Nicotiana* species when cloned in a viral vector derived from the *Potato virus X* (PVX) (Fernandez *et al.* 1999).

The analysis of the 3′ and 5′ non-coding sequences from both RNAs 1 and 2 of RpRSV highlighted several interesting features. While the respective lengths of the 3′ and 5′ non-coding regions were fairly similar between the different isolates, the lengths of the 3′ and 5′ non-coding regions differed markedly between RNAs 1 and 2 respectively. The 5′ non-coding regions of RNA 1 (145 nt and 136 nt for RpRSV-g and –ch respectively) are shorter than

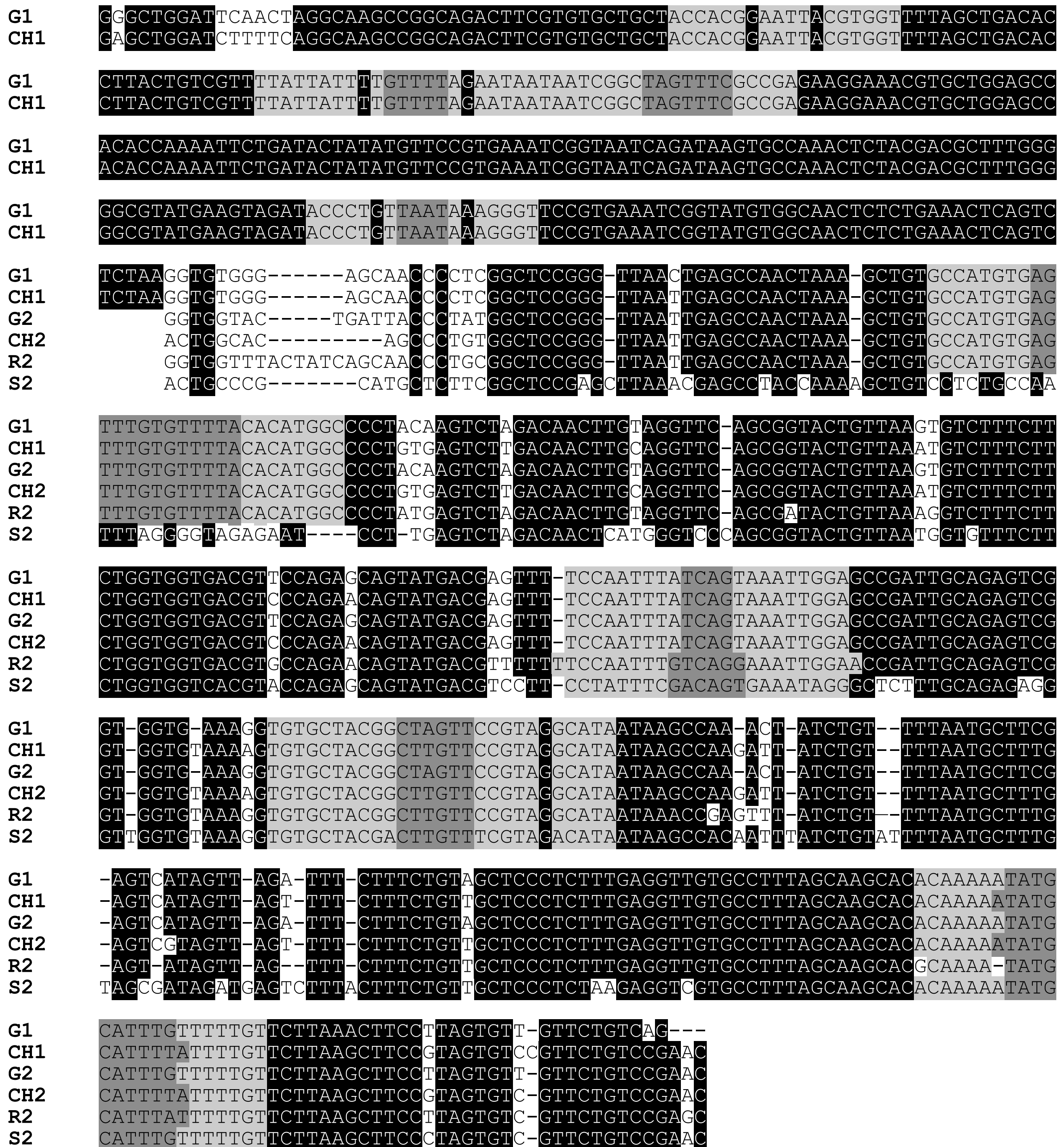

Fig. 3 3′ non-coding sequences of RpRSV RNAs 1 and 2. Conserved sequences are boxed in black. Putative conserved stem and loop structures between the different isolates are boxed in grey (the stem in light grey, the loop in dark grey). Dashes correspond to gaps introduced to optimise the alignments. G, CH, R, S: grapevine, cherry, RAC815, and Scottish isolates respectively; 1, 2: RNA 1 and 2 respectively.

the 5′ non-coding regions of their RNAs 2 (196 nt and 203 nt for RpRSV-g and –ch respectively, and 197 nt and 206 nt for RpRSV-RAC815 and –S respectively). Inversely, the 3′ non-coding regions of RNAs1 (689 nt and 695 nt for RpRSV-g and –ch respectively) are longer than the 3′ non-coding regions of their RNAs 2 (392 nt and 390 nt for RpRSV-g and –ch respectively, and 398 nt for both RpRSV-RAC815 and –S).

The 5′ non-coding regions of the RNAs 1 between RpRSV-g and –ch showed 77% identity. Identity levels between 65% and 73% were found in the 5′ non-coding regions of the RNAs 2 between the different RpRSV isolates. No conserved regions were found between the different isolates susceptible to form stem and loop structures similar to those described for the 5′ non-coding regions of the RNAs 2 of ArMV (Wetzel *et al.* 2001) or GCMV (Fernandez *et al.* 1999). The RNA 2 5′ leader of the *Blackcurrent reversion virus* (BRV) was shown to mediate efficient *in vivo* translation through an internal ribosomal entry site mechanism (Karetnikov and Lehto 2007). Regions complementary to a fragment of plant 18S rRNA were identified in the 5′ non-coding region of BRV RNA 2 and other nepoviruses including RpRSV-ch (Karetnikov and Lehto 2007). However, these sequences were only partially found in the sequences of the 5′ non-coding regions of the RNAs 2 of other RpRSV isolates, and in the 5′ non-coding regions of RpRSV RNAs 1.

The 3′ non-coding regions of RpRSV RNAs 1 were 95% identical between RpRSV-g and –ch, and the 3′ non-coding regions of RNAs 2 were 78% to 92% identical between the different RpRSV isolates. Several stem and loop structures were found in highly conserved regions in

the 3′ non-coding regions of both RNAs 1 and 2 (**Fig. 3**). In some of these structures, mutations and the corresponding compensatory mutations that restored the putative secondary structure were found between the sequences of the different isolates, which strongly suggests their involvement in one or another aspect of viral replication. The 3′ non-coding region of BRV was found to contain a cap-independant translational enhancer, which must base-pair with the 5′ non-coding region to be functional, and which provided a major contribution to translational efficiency (Karetnikov *et al*. 2006). Such complementary sequences between the 3′ and 5′ non-coding regions of the RNA 2 of BRV were also reported for other nepoviruses, including RpRSV-ch (Karetnikov *et al*. 2006). These sequences were however only partially conserved between the different RpRSV isolates.

RpRSV RNA 1-encoded proteins

The RNA 1-encoded proteins of RpRSV-grapevine and RpRSV-ch showed an overall level of identity of 91%. With the exception of the amino terminus of the protein which shows a higher degree of diversity (the first 400 amino acid show 81% identity between RpRSV-grapevine and RpRSV-ch), the differences between the sequences of two strains of RpRSV seem to be spread evenly over the rest of the genome. As no cleavage sites have been determined for the RNA 1-encoded protein of RpRSV, a comparison gene by gene is not possible. While the RNA 1 encodes all the genes necessary for the replication of the virus, it was also shown to be responsible for symptom severity on herbaceous hosts, and the ability to infect some herbaceous hosts (Harrison *et al.* 1974). RpRSV-grapevine and -cherry also produce different types of symptoms on *Chenopodium quinoas* (**Fig. 4**). Full-length infectious clones of the RNAs 1 and 2 of different isolates of the *Bean pod mottle virus* (BPMV), belonging to the comovirus genus of the *Comoviridae* family, were reported (Gu and Ghabrial 2005). The different isolates of BPMV induced symptoms of different severity, and the construction of chimeric clones between them allowed the identification of symptom severity determinants located on the RNA 1, corresponding to the coding regions of the protease cofactor and the C-terminal half of the putative helicase (Gu and Ghabrial 2005). It is tempting to postulate that either the 1A gene, which is the most divergent one and for which no function could be assigned yet, or the coding regions of the protease cofactor and the C-terminal half of the putative helicase as reported for the BPMV, would be involved in symptom severity or infectivity.

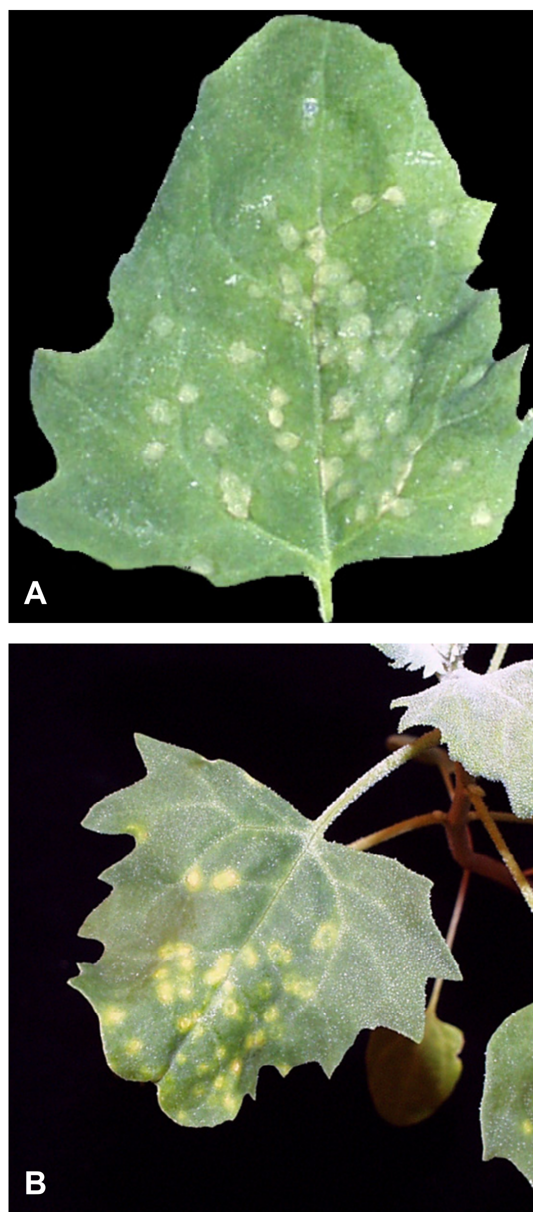

Fig. 4 RpRSV-induced symptoms on *Chenopodium quinoa*. (**A**) RpRSV-cherry-induced symptoms on a systemic *Chenopodium quinoa* leaf, showing necrotic lesions (**B**) RpRSV-grapevine-induced symptoms on a systemic *Chenopodium quinoa* leaf, showing chlorotic ringspots.

RpRSV RNA 2-encoded proteins

The RNA 2 of RpRSV encodes the protein 2A, the putative movement protein, and the coat protein. While 90-94% identity levels were found between the different isolates at the amino acid level in the 2A-movement protein region, the coat protein sequences proved to be the most divergent among the different isolates (Wetzel *et al.* 2006).

The RNA 2 of RpRSV was shown to carry the determinant(s) for the systemic yellowing symptoms observed on *Petunia hybrida* (Harrison *et al.* 1974). It is not possible however, from sequence comparisons alone between the different isolates, to determine which gene(s) on the RNA 2 of RpRSV is responsible of this function.

The coat protein of GFLV has been shown to be the sole protein involved in the transmission of the GFLV via its nematode vector *Xiphinema index* (Andret-Link *et al.* 2004). From this observation made on a similar virus, one can speculate that the coat protein of RpRSV is also solely responsible for the transmission of the virus by its nematode vector. A listing of amino acid differences between the English and Scottish serotypes, together with their exposure on the surface of the coat protein and therefore their susceptibility to be putatively involved in the nematode transmissibility has been published previously (Scott *et al.* 2000). From this list, differences in four positions (positions 381, 409, 439, 476) were found between the coat protein sequences of the three groups of isolates defined according to their nematode vector specificity (**Fig. 5**), which are located towards the carboxy-terminus of the protein. The amino acid at position 409 was however suggested to be buried (Scott *et al.* 2000) and therefore probably not directly involved in nematode transmission. The three other amino acids are exposed to the surface of the coat protein (Scott *et al.* 2000), and could be putatively involved in nematode transmission. It cannot be excluded, however, that other amino acids are involved in the nematode transmission of the virus.

CONTROL STRATEGIES OF THE VIRUS

RNA silencing-derived resistance

RNA silencing is a highly conserved defense mechanism in plants, triggered by double-stranded (ds) RNAs, leading to sequence-specific degradation of related RNAs in the cell. Most known plant viruses have RNA genomes and replicate via dsRNA intermediates. They can therefore act as

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Grapevine  AYEVDPLHLLYYASVDVPKDTLEGTRLARIDLRAKAQEMDSAVWRQWVKEGCMKPRIKIRISAAT

RAC815     AYEVDPLHLLYYESVNVPRDTLEGTRLARIDLRAKAQEMDSAVWRQWVKDGCMKPRIKVRISAAS
Cherry     AYEVDPLHLLYYELVNVPKDTLGGTLLTRIDVRAKAATFDSAVWRQWVRDGCLKPKIKMRITAAT
MX         AYEVDPLHLLYYEEVTVPKDTLGGALLARIDVRAKAATFDSAVWRQWVRDGCLKPRIKMRITAAT
E          AYEVDPLHLLYYEEVTVPKDTLDGTLLARIDVRAKAATFDSAVWRQWVRDGCLKPKIKVRITAAT

T          AYEVDPLHLLYYEEVTVPKDTLDGTLLARIDVRAKAATFDSAVWRQWVRDGCLKPRVKMRVTAAT
S          AYEVDPLHLLYYESVDVPKDTLAGTLLARIDVRAKAAIFDSAVWRQWVRDGCLKPKIKMRITAAT
LG         AYEVDPLHLLYYESVDVPKDTLAGTLLTRIDVRAKAATFDSAVWRQWVRDGCLKPKIKVRITAAT
Orr        AYEVDPLHLLYYESVDVPKDTLAGTLLTRIDVRAKAATFDSAVWRQWVRDGCLKPKIKVRITAAT
Shep       AYEVDPLHLLYYESVDVPKDTLAGTLLTRIDVRAKAATFDSAVWRQWVRDGCLKPKIKVRITAAT

Grapevine  SCFSGVVLGMCLDAYRRIPIMRDKGFSANLVTGLPNTMWATRTQAELEWDLDLSQECGHSFYALS

RAC815     SCFSGVVLGICFDAYRRIPVNRDKGFSANLITGLPNTLWATRNQSDLEWDLDLSQECGHSFYALS
Cherry     SCFSGIVLGACLDAYRRIPATTKTDFTASLVTGLPNIVWATRDTSEIEWDIDLAAVCGHTFFALE
MX         SCFSGIVLGACLDAYRRIPATTKADFTASLVTGLPNAMWATRDTSEIEWDIDLAAVCGHTFFALE
E          SCFSGIVLGACFDAYRRIPAATKGITASLVTGLPNTVWATRDTSEVEWDIDLAAVCGHTFFALE

T          SCYSGIVLGACLDAYRRIPATTKTDFTASLVTGLPNTMWAMRDTSEIEWDIDLAAVCGHTFFALG
S          SCFSGIVLGACFDAYRRIPAATKTGITASLVTGLPNTVWATRDTSEVEWDIDLAAVCGHTFFALE
LG         SCFSGIVLGVCFDAYRRIPAATKGITASLVTGLPNTIWATRDTSEVEWDIDLAAVCGHTFFALE
Orr        SCFSGIVLGACFDAYRRIPAATKGITASLVTGLPNTVWATRDTSEVEWDIDLAAVCGHTFFALE
Shep       SCFSGIVLGACFDAYRRIPAATKGITASLVTGLPNTVWATRDTSEVEWDIDLAAVCGHTFFALE

Grapevine  DTLGYMDFLIYVLRGNEMTAVADWSFYVAFYVDWSQESFTAMLAPTLKWPPTPGIISTFKEVRGP

RAC815     DALGYMDFIVYVLRGNELTAVADWTFYLAFYVDWAQESSTLLTPTLVWPPTPDIISTFREVRGP
Cherry     DTFGYMDFLVYVLRGNEVTAVADWSIYVSFHVDWTQESMSATLIPTFVWPPEPADISYFKEVWGP
MX         DTFGYMDFLIYVLRGNEITAVADWSMYVSFHVDWTQESMSATLIPTFVWPPKPSDIFLFKEVWGP
E          DTFGYMDFLIYVLRGNEITAVADWSIYVSFHVDWTQESMLATLIPTFVWPPKPTDISLFKEVWGP

T          DTFGYMDFLVYVLRGNEITAVADWSIYVSFHVDWTQESMLATLIPTFVWPPKPTDISLLKEVWGP
S          DTFGYMDFLIYVLRGNEITAVADWSIYVSFHVDWTQESMLATLIPTFVWPPKPTDISLLKEVWGP
LG         DTFGYMDFLIYVLRGNEITAVADWSIYVSFHVDWTQESMLATLIPTFVWPPKPTDISLLKEVWGP
Orr        DTFGYMDFLIYVLRGNEITAVADWSIYVSFHVDWTQESMLATLIRTFVWPPKPTDISVFKEVWGP
Shep       DTFGYMDFLIYVLRSNEITAVADWSIYVSFHVEWTQESMLATLIPTFVWPPKPTDISLFKEVWGP

Grapevine  YAFSLDGTKARLDFGFLPGVSLVEGSETVRTCPRVLASFYRSWTGKLRISIEEVSSIFLTGSYMV

RAC815     FSFSLDGTREQLDFGFLPGVPLVEGTTTVRTFHRVLANFYRAWTGKIRISIEEVSSIFLTGTYMV
Cherry     YHFTLDGTEAKESFSLMPGMAIPRGAQTVRTFPRVLAAHFRSWTGKVRMSIQEVSSIFLTGTYMV
MX         YHFSLDGTEAKVDLDIMPGMAIPRGHTTVRTFPRVVAAHFRSWTGKIKMSIQEVSSIFLTGTYVV
E          YRFTLDGTEAKESFAIMPGTAIPRGQQIVRTFPRVIAAHFRSWTGKIKMSIQEVSSIFLTGTYMV

T          YRFTLDGTEAKGSFAIMPGTAIPHGQQIVRTFPRVVAAHFRSWTGKVRMSIQEVSSIFLTGTYMV
S          YRFTLDGTEAKESFAIMPGTAILHGQQIVRTFPRVVAAHFRSWTGKVRMSIQEVSSIFLTGTYMV
LG         YRFTLDGTEAKGSFAIMPGTAIPHGQQIVRTFPRVVAAHFRSWTGKVRMSIQEVSSIFLTGTYMV
Orr        YHFTLDGTEAKESFVIMPGTAIPHGQQIVRTFPRVVAAHFRSWTGKVKMSIQEVSSIFLTGTYMV
Shep       YRFTLDGTEAKRSFAIMPGTAIPHGQQIVRTFPRVVAAHFRSWTGKVRMSIQEVSSIFLTGTYMV

Grapevine  GVAWNAGDDLGGITTRKHWMVKSGEIFDLDLYGPHGEYPTFAGKANGTPYIVVQKVGGIVGPKDS

RAC815     GVAWNATDSLGSIVSRRHWIVKSGEIFDLDLYGPYGEYPTFAGKQNGIPHIVVQKIGGIVGPKDS
Cherry     GVSWNATADLTDITTRKHWIVKSGEVFELDLYCPYGENPTFTGLVNGIPYIIVHRLGGIIGPKDS
MX         GVSWNVTADLADIVTRKHWIVKSGEIFELDLYCPYGKNPTFTGLANGKPYIIVHKLGGIIGPKDS
E          GVSWNATADLADIITRKHWIVKSGENFELDLYCPYGENPTFTGLANGRPHIIVHKLGGIIGPKDS

T          GVSWNATADLADIVTRKHWIVKSNEIFEVDLYCPYGENPTFTGQANGKPFIIVHKLGGIVGPKDS
S          GVSWNATADLADIVTRKHWIVKSNEIFEVDLYCPYGENPTFTGQANGKPFIIVHKLGGIVGPKDS
LG         GVSWNATADLADIVTRKHWIVKSNEIFEVDLYCPYGENPTFTGQANGKPFIIVHKLGGIVGPKDS
Orr        GVSWNATADLADIVTRKHWIVKSNEIFEVDLYCPYGENPTFTGQANGKPFIIVHKLGGIVGPKDS
Shep       GVSWNATADLADIVTRKHWIVKSNEIFEVDLYCPYGENPTFTGQANGKPFIIVHKLGGIVGPKDS

                                                                   381
                                                                    ↓
Grapevine  TGSFGFFLHIHGMTGIYKNPTLHSPERGQMHAWFRMNNIQVDNLSFSIPGRIEDMSALAGSYDIT

RAC815     TGSFGFFVHIHGMTGVYKNPTLHSSEKGQMHAWFRVHNILVDNLSFNIPGRIEDIRALAGSYDTT
Cherry     VGTFGFMIHLHGLTGVYKNPTLHSGDRSVGSAWFRVTNILDDNLVFNIPGRIEDMVAVAGKYEVT
MX         VGTFGFMIHIHGLTGMYKNPTLHSGDRSVGSAWFRITNIADDNLVFNIPGKIEDMAAVTGNYDVT
E          VGTFGFMIHIQGLTGMYKNPTLHSGDRSVGSAWFRISNIVNNNLVFNIPGKIENMVAVAGNYDVT

T          VGTFGFMIHIHGLTGVYRNPTLHSGDRSVGSAWFRINNIADDNLVFNIPGKIENIIAAAGNYDVT
S          VGTFGFMIHIHGLTGVYKNPTLHSGDRSVGSAWFRINNIADDNLVFNIPGRIEDIIAAAGKYDVT
LG         VGTFGFMIHIHGLTGVYRNPTLHSGDRSVGSAWFRISNIVDDNLVFNIPGRIEDIIAAAGKYDVT
Orr        VGTFGFMIHIHGLTGVYRNPTLHSGDRSVGSAWFRINNIADDNLVFNIPGRIEDIIAAAGEVDVT
Shep       VGTFGFMIHIHGLTGVYRNPTLHSGDRSVGSAWFRINNIADDNLVFNIPGRIEDIIAATGKYDVT
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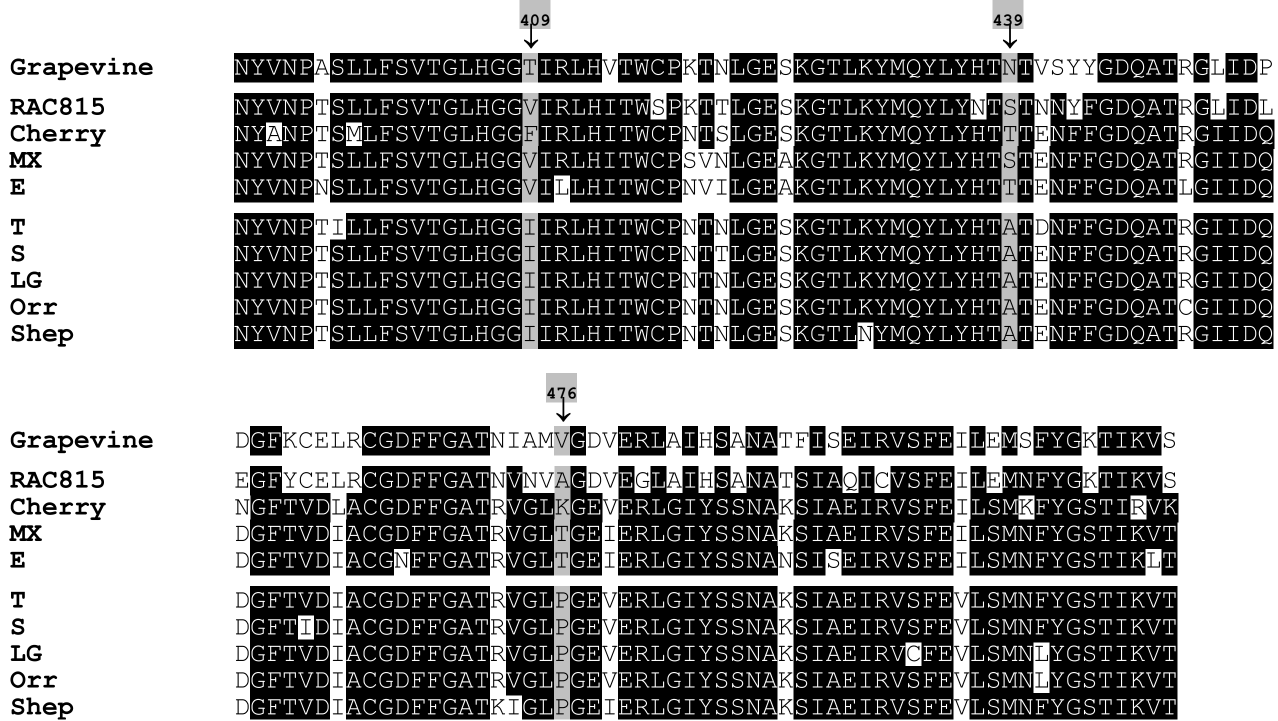

Fig. 5 Multiple alignment of the coat proteins of the different RpRSV isolates. A separation was introduced between groups of isolates transmitted via different nematodes species: *Paralongidorus maximus* (Grapevine isolate), *Longidorus macrosoma* (isolates RAC815, cherry, MX, E), and *Longidorus elongatus* (isolates T, S, LG, Orr, Shep). Amino acids found in at least 8 sequences out of ten are boxed in black. Amino acids differing between the three groups of isolates are indicated by arrows together with their position number (boxed in light grey).

inducer of the RNA silencing mechanism, and be silencing targets (for recent reviews, see Lecellier and Voinnet 2004; Voinnet 2005; Uhrig 2006; Wang *et al*. 2006). Transgenic plants carrying inverted repeat constructs (Smith *et al.* 2000) containing virus-derived sequences, which can form dsRNA structures and activate the silencing mechanism, have been used to induce virus resistance in plants. RpRSV-derived sequences have been used in different inverted repeat constructs, to produce transgenic *Nicotiana benthamiana* plants, in order to induce resistance against RpRSV, or multiple resistance against the viruses involved in the grapevine fanleaf disease in Germany (ArMV, GFLV, RpRSV; Winterhagen 2006), for which no natural resistance genes are known in grapevine. In preliminary experiments, a resistance against ArMV or GFLV could be detected in some transgenic lines of *N. benthamiana* plants carrying these constructs. However, the RpRSV-grapevine isolate managed in most cases to overcome the resistance and establish a viral infection (Winterhagen 2006). Furthermore, as RNA silencing is an antiviral mechanism, plant viruses have developed counter-defense strategies. Numerous viral-encoded proteins acting as suppressors of gene silencing and targeted against different components of the host gene silencing machinery have been identified (Voinnet 2005 and references therein). The small coat protein of *Cowpea mosaic comovirus*, belonging to the family *Comoviridae*, was also found to be a suppressor of gene silencing. However no gene silencing suppressor has been found for nepoviruses yet.

Artificial microRNA-derived resistance

Recently, microRNAs (miRNAs), which are generated from processing of longer pre-miRNAs precursors into products 20-24 nt in length (Bartel 2004), have been identified as important regulators of gene expression in both plant and animals, in a sequence-specific manner. It has also been shown that the alteration of several nucleotides within a miRNA 21 nt sequence does not affect its biogenesis (Vaucheret *et al.* 2004). The possibility to modify plant miRNA sequences to target specific sequences, originally not under miRNA control, has been investigated towards protecting plants against viruses (Niu *et al.* 2006; Simon-Mateo and Garcia 2006). Once mature, these artificial miRNAs (amiRNAs) target the genomic RNAs of the plant viruses against which they were designed, and the plants transformed with the recombinant miRNA precursor became specifically immune to infection with these viruses. This amiRNA-mediated approach allows therefore multiple virus resistance, and the resistance trait is maintained at 15°C, where resistance induced through RNA-mediated gene silencing can break down (Niu *et al.* 2006). So far, these experiments have been conducted only in *Arabidopsis thaliana* and tobacco plants (Qu *et al*. 2007), but any other crop of agronomical interest susceptible to transformation could benefit from this application.

CONCLUSION

Although very little is known about the molecular biology of RpRSV, the full-length sequences of the genomic RNAs 1 and 2 of both RpRSV-cherry (Ebel *et al.* 2003) and -grapevine strains (Wetzel *et al.* 2006), and full-length infectious clones of RpRSV-grapevine, constructed under the control of a double 35S promoter (Wetzel *et al.* unpublished results), are now available and constitute valuable tools to get deeper insights into the molecular biology of RpRSV.

Point mutations or exchange of fragments between coat proteins from different isolates in the infectious clone, together with nematode feeding experiments, could allow to determine more precisely the level of involvement of the coat protein in the transmission of RpRSV by nematodes, and the nematode specificity. Furthermore, a mapping on the RpRSV genome of the symptom determinants can now be undertaken, by swapping different fragments between the cherry and grapevine strains of RpRSV in the infectious clones, as well as the identification of a putative suppressor of gene silencing.

These experiments altogether will help to better understand the complex relationship between RpRSV and its

hosts, which will contribute to the design of new and more efficient strategies to control the virus.

DATABASE ENTRY

The full-length sequence of the RNA2 of the isolate RAC815 presented in this paper has been submitted to the Genbank/EMBL database and has been assigned the accession number EF534293

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