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Ophioviruses: State of the Art

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1. Introduction

As has happened in the last century with many plant diseases, the nature of the causal agents, particularly viruses, was not determined and studied until a few decades ago. Thus, an old disease named citrus psorosis was first described in 1891, but it was almost a century later when the viral agent was observed by immune electron microscopy as a novel spiral-filamentous particle (Derrick et al., 1988). In 1994, the real morphology of Citrus psorosis virus (CPsV) was observed by Robert G. Milne using negative staining electron microscopy; describing circular particles of different configurations resemble that of the tenuiviruses and the nucleocapsids of members of the family *Bunyaviridae* (Garcia et al., 1994). Due to the shape of the particles, they were called Ophiovirus, derived from the Greek "ophios", a serpent, referring to the snaky appearance of the virions (Figure 1, a).

Subsequently, in Japan, another ophiovirus is recognized in tulip, *Tulip mild mottle mosaic virus* (TMMMV) (Morikawa et al., 1995), and later, Robert G. Milne, a "virus hunter" (as once he called himself), found particles with similar morphology in diseased plants of lettuce and ranunculus (Milne, 2000). Thus began the study of the ophioviruses *Ranunculus white mottle virus* (RWMV) (Vaira et al., 1997), *Mirafiori lettuce big-vein virus* (MiLBVV) (Roggero et al., 2000), *Lettuce ring necrosis virus* (LRNV) (Torok et al., 2002, 2003) and *Freesia sneak virus* (FreSV) (Vaira et al., 2006). Most, if not all these viruses have been found around the world (Roistacher 1993; Navarro et al., 2004; Martin et al., 2006; Ghazal et al., 2008; Vaira et al., 2007, 2009; Plesko et al., 2009; Barcala Tabarrozzi et al., 2010).

1.1 Old diseases affecting major crops

In citrus: Citrus psorosis virus, the type member of the family

The first observation of symptoms of citrus psorosis disease was reported in 1891 (Swingle and Webber, 1896), and the first experimental evidence about that infectious disease transmitted by grafting in citrus trees was published in 1933 by H.S. Fawcett. Psorosis disease development is slow; it may take several years to manifest symptoms. Typical psorosis symptoms are bark-scaling of trunk and main branches, and more severe as rampant bark-scaling even on small limbs and twigs. Gum may accumulate below the bark scales and may impregnate the xylem producing wood staining and vessel occlusion. These symptoms have been used for field diagnosis of Psorosis (Roistacher, 1993). Chlorotic flecks

and spots on young leaves can be observed in spring time in the field, and in infected seedlings in the greenhouse. (For symptoms and diagnosis of psorosis disease, see an excellent review of Alioto et al., 2007). The disease has been reported from many citrus-growing areas all over the world (Roistacher, 1993). Trees affected with psorosis have been less productive causing damage to citrus industry in the Mediterranean basin, and in some areas of South America. In the ´80s, in Argentina and Uruguay it was a serious disease causing annual losses of about 5% of trees (Larocca 1985, Danós 1990) and the disease is still present as recently reported by Zanek et al (2006). There are reports of naturally spreading of psorosis in Argentina (Pujol and Beñatena, 1965), Uruguay (Campiglia et al., 1976), and in Texas, USA (Timmer and Garnsey, 1980). The suspected vector is unknown although the pattern of spread suggests an aerial vector (Beñatena and Portillo, 1984; Diamante et al., 1984). On the other hand, other ophioviruses are soil-transmitted by a root-infecting fungus from the *Olpidium* genus (see later in this section). Citrus psorosis virus has probably been vegetatively propagated for centuries around the world from citrus to citrus, and it could have lost any putative original capacity to be transmitted by *Olpidium*, and at the same time acquiring the ability of transmission by an aerial vector. Therefore, further studies are necessary to clarify this matter and to identify the natural vector of CPsV.

The first ophiovirus described was discovered in citrus, but most of them has been found in ornamental plants as ranunculus (dicotyledonous), freesia, tulips and lachenalia (monocotyledonous), and lettuce (dicotyledonous).

In ornamental plants: Tulip mild mottle mosaic virus, Ranunculus white mottle virus and Freesia sneak virus

Since 1979 the occurrence of mild mottle mosaic disease in tulip is described, and in 1989 it is reported for the first time in Japan by Yamamoto et al. (1989) as a virus-like disease of tulip, and as a soil-borne disease by Morikawa et al., (1993). In 1995, Morikawa and co-workers found a new virus recognized on tulip (*Tulipa gesneriana L.*, hybrids, *Liliaceae)* producing symptoms of venial chlorotic mottle mosaic on leaves and color-removing mottle on flower buds. They mechanically transmitted the virus in tulip and species as *Chenopodium quinoa*, *Tetragonia expansa*, *Nicotiana tabacum* and *Nicotiana benthamiana,* but it could not be back-inoculated from *C. quinoa* to tulip (Morikawa et al., 1995). The authors found that the disease spreads through bulbs of tulip and might be soil-borne. In 1998 Drs. T. Natsuaki and T. Morikawa (Utsunomiya University, Japan) have indicated that the vector of TMMMV is *Olpidium brassicae* (pers. comm.).

Other ornamental disease caused by an ophiovirus was found in ranunculus and anemone. In 1996 A.M. Vaira and co-workers described a new virus found in a plant of *Ranunculus* hyb. (cv. Grazia) collected in Liguria, Northern of Italy, in 1990. The symptoms described in ranunculus plants were mosaic, mottle and distortion of leaves and stems, giving the name *Ranunculus white mottle virus*. For years the virus was consistently isolated from plants and found in mixed infection with potyvirus (Vaira et al., 1997, 2009). They could mechanically transmit the virus to several herbaceous hosts (*N. benthamiana* and *N. clevelandii)*, and by EM, in negative stain the particle morphology appeared similar to CPsV, *Tenuivirus* and *Bunyaviridae* (Vaira et al., 1996, 1997). So far, there are no reports about a vector for RWMV.

A severe disease called freesia leaf necrosis (FLN) has been known in freesia cultures for forty years in Europe (Verbeek and Meekes, 2005) but its causal agent was not identified

until 2006 by Vaira et al., (2006). The authors found freesias (*Freesia refracta* hybrids, *Iridaceae*) with symptoms of FLN in the area around Sanremo (Italy), and later, in lachenalia cultivars (*Lachenalia* hyb., *Hyacinthaceae*) in South Africa (Vaira et. al, 2007). By electron microscopy the authors found an ophiovirus which is associated to this disease, which presents chlorotic inter-veinal lesions on the leaves, later coalescing and becoming sunken and necrotic. FLN is soil-transmitted as mild mottle mosaic disease in tulip (van Dorst, 1975; Vaira et al., 2006).

In lettuce: Mirafiori lettuce big-vein virus and Lettuce ring necrosis virus

Lettuce (*Lactuca sativa*) is other natural host for ophioviruses. In 1934 lettuce big-vein disease (BV) was described as possibly caused by a virus (Jagger et al., 1934). Big-vein is one of the most important diseases of lettuce crops worldwide. The symptoms, as the name refers are vein enlargement with chlorotic regions around the vascular tissue, making the plant no suitable for the market and producing important looses. The virus named Lettuce big-vein virus (LBVV), the type species of the genus *Varicosavirus* (van Regenmortel et al., 2000), was initially associated with big-vein disease (Kuwata et al., 1984). LBVV a rod-shaped virion transmitted by *Olpidium brassicae* (Kuwata et al., 1984; Vetten et al., 1987; Huijberts et al., 1990), but this varicosavirus had not been isolated or rigorously demonstrated to cause the disease. In big-vein affected lettuce the presence of unsuspected second virus with particles morphologically resembled those of ophioviruses was discovered by R. G. Milne and co-workers (Roggero et al., 2000). That ophiovirus was named *Mirafiori lettuce virus* (MiLV) since it was detected in Mirafiori, Turin (Italy). In 2002, Lot and co-workers demonstrated that the lettuce infected with MiLV alone consistently developed big-vein symptoms regardless of the presence or absence of LBVV (Lot et al., 2002). This important evidence showed that MiLV but not LBVV is the true causal agent of this disease, although both viruses are present in the diseased lettuce-plants. Later these viruses were renamed as *Mirafiori lettuce big-vein virus* (MiLBVV) and *Lettuce big-vein associated virus* (LBVaV) by the International Committee on Taxonomy of Viruses (ICTV). Recently, it has been determinate that both viruses are transmitted by *Olpidium virulentus*, a noncrucifer strain of *Olpidium brassicae* (Sasaya and Koganezawa, 2006).

Lettuce ring necrosis is still a serious disease producing coalescent necrotic rings and ring-like patterns on middle leaves of plants observed in greenhouses during winter and transmitted by the zoospores of *O. brassicae* (Bos et al., 1996). The disease was first described in The Netherlands and in Belgium as "kring necrosis" and observed in France where it was called "maladie des taches orangées".

As happened with LBVaV, a rod-shaped non-enveloped virus was tentatively named lettuce ring necrosis virus (LRNV) and both were closely associated to the diseases (Huijberts et al., 1990). In 2002, Torok et al. associated for the first time an ophiovirus with lettuce ring necrosis disease, and in 2003, the same authors published the molecular characterization of a this new ophiovirus (Torok et al., 2003). Later, the genome of LRNV was sequenced but no further analysis has been published so far.

1.2 Morphology of the ophiovirus particles – *In vitro* stability

Robert G. Milne described the particles as circles of at least two different contour lengths, the shortest length about 760 nm, and the largest about four times longer with 3 nm in

diameter when appear in a circular form (Figure 1,a) (Garcia et al., 1994). The circles can collapse to form pseudolinear duplex structures, coiled filamentous about 9-10 nm in diameter. The presence of this pseudolinear form seems to be associated with long incubation (one to several days) in vitro (Milne et al., 1996). Figure 1, b shows a model of different configurations the particles can adopt, resemble that of the tenuiviruses and the nucleocapsids of members of the family *Bunyaviridae* (Garcia et al., 1994, Milne et al., 1996) (see Vaira et al., (1997) for EM photos of different RWMV forms). Thin sections of *N. clevelandii* leaf tissue infected with RWMV were observed by Vaira et al., (1997) using EM immunogold against RWMV coat protein, but no inclusions neither enveloped particles were found. The label was abundant in the cytoplasm of parenchyma cells, but the nuclei, chloroplast, mitochondria and microbodies were unlabelled (Vaira et al., 1997). So far, that has been the unique observation of any ophiovirus in thin sections. Attempts have been done to see CPsV particles in different tissues but they resulted unsuccessful (R.G. Milne, Peña E. and Kitajima E., pers. communications).

Ophiovirus particles are unstable in CsCl and in phosphotungstate but not in 2% aqueous uranyl acetate. Besides, the particle structure remains intact in cesium sulphate (D. Alioto, E. Luisoni and R.G. Milne, unpublished data). In order to purify and separate the smaller from the larger particles, virions of CPsV can be ultracentrifuged in sucrose or cesium sulphate density gradients (Derrick et al., 1988; Garcia et al., 1991, Sanchez de la Torre et al., 1998). The buoyant density in cesium sulfate is 1.22 g/cm3 for RWMV and MiLBVV (Vaira et al., 1997; Roggero et al. 2000). The particles have limited stability at pH below 8 (Garcia et al., 1991), and the infectivity does not survive in crude sap held at room temperature for more than 2 hr or 12-24 hr at 4 °C in the case of CPsV (Garcia et al., 1991) and TMMMV (Morikawa et al., 1995). Particle structure survives limited treatment with organic solvents and nonionic or zwitterionic detergents (Garcia et al., 1991; Roggero et al., 2000).

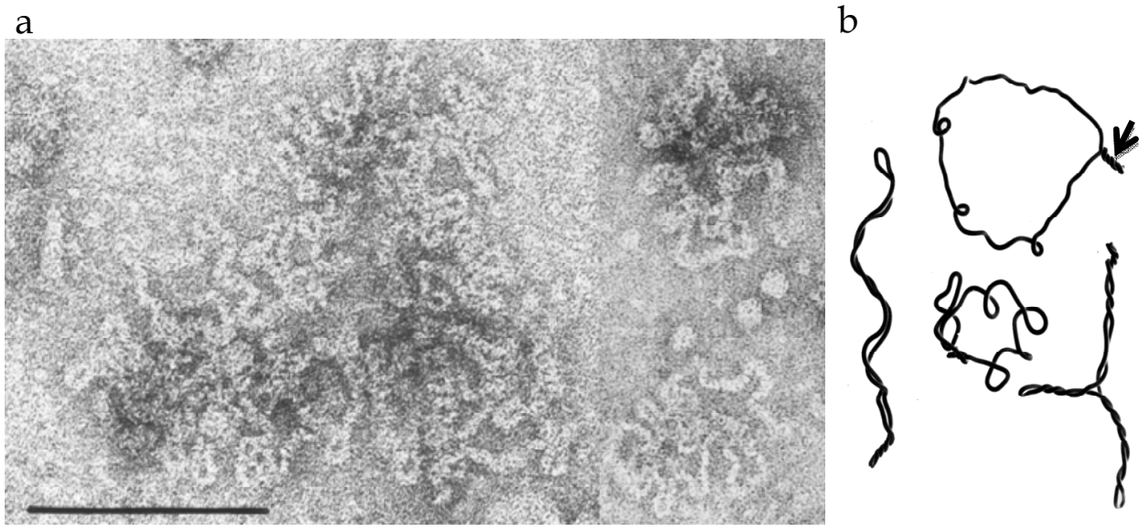

Fig. 1. a. Ophiovirus morphology: naked filamentous nucleocapsids. Circles of at least two different contour lengths, negatively stained in 1% uranyl acetate. Large (left) and small (right) particles. Bar = 100 nm. b. Wire models (not to scale) of possible forms for ophiovirus particles, representing larger and smaller particles in the circular and pseudolinear form. The putative "panhandle" structure is indicated by arrow (Milne et al., 1996 with modifications).

2. Genome organization, sequence analysis and putative proteins

Ophioviruses genome is divided into three or four individually encapsidated segments (Figure 2). CPsV, RWMV and FreSV have 3 RNAs (named as RNA 1, 2 and 3) and for MiLBVV and LRNV a fourth RNA has been reported (named as RNA 4).

The available information about ophiovirus genes and putative proteins are based on the sequences of CPsV, MiLBVV and LRNV, which are the ophioviruses completely sequenced so far. Partial sequences of RNA 3 of all ophiovirus species and the RNA-dependent RNA polymerase (RdRp) module of the RWMV RNA 1 are also available in database. Using ophiovirus-specific primers based on a highly conserved sequence of RNA 1, Vaira and co-workers (2003) amplified a 136 bp fragment detecting all ophiovirus species, making this RT-PCR the selected method to find new ophioviruses. All 136bp-fragment sequences are available in database.

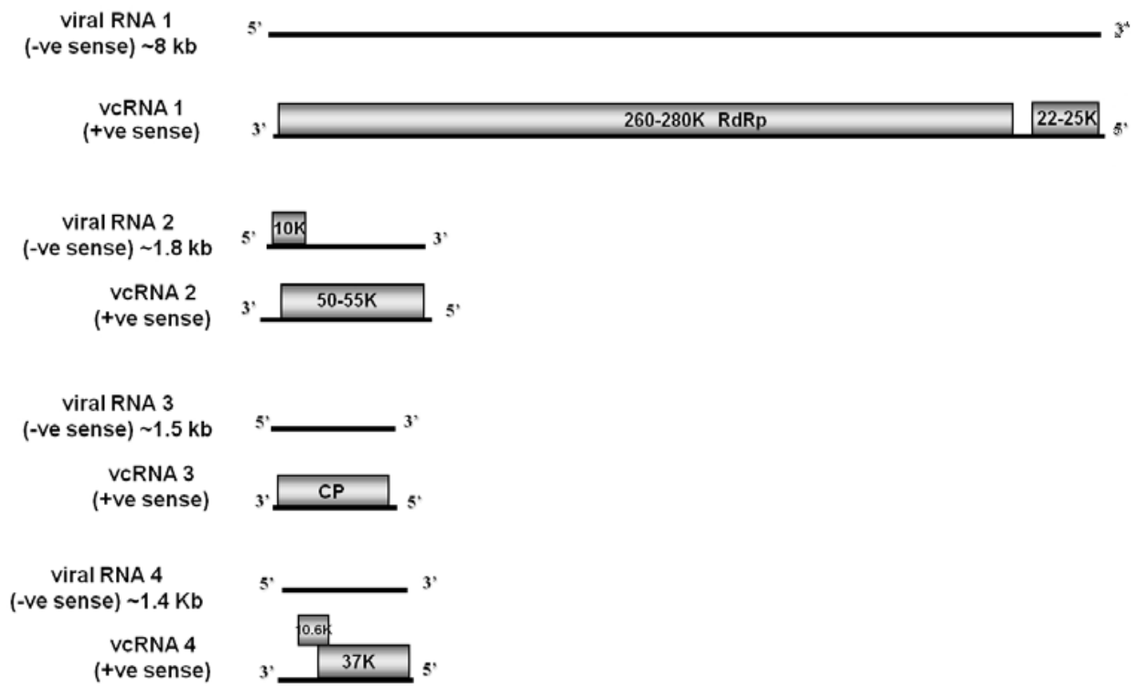

Fig. 2. Genome organization of ophiovirus. The length of the RNA segments and the predicted sizes of the ORF products are indicated. –ve sense: negative stranded RNA (viral RNA, vRNA), +ve sense: positive stranded RNA (viral complementary RNA, vcRNA). CPsV and RWMV have 3 RNAs. MiLBVV and LRNV contain 4 RNAs. The +ve sense of RNA 4 belongs to MiLBVV; LRNV contains only the 38K ORF (see the text). CP: coat protein. RdRp: RNA dependent-RNA polymerase.

In purified virus preparations the negative strand RNAs are the more abundant. The positive strands of all RNAs are also encapsidated although in much less amount. The size of RNA 1 is 8.2 kb for CPsV, 7.8 kb for MiLBVV, 7.6 kb for LRNV and 7.5 kb for RWMV (Naum et al. 2003; van der Wilk et al., 2002; Vaira et al., 1997; Torok et al., 2003). RNA 2 is

about 1.8 kb for RWMV, MiLBVV and LRNV, 1.7 for FreSV and 1.6 kb for CPsV, and the RNA 3 is 1.3-1.5 kb for all ophioviruses (Vaira et al., 1997; van der Wilk et al., 2002; Torok et al., 2003; Sanchez de la Torre et al., 2002). The fourth genomic RNA reported for MiLBVV and LRNV is about 1.4 kb (van der Wilk et al., 2002; Torok et al., 2003).

In the RNA 1, a protein of 22-25 kDa of unknown function is encoded in the 5´ region of the positive strand. Separated by an intergenic region of hundred nucleotides a large ORF of RNA 1 is encoded (see Figure 2). The 109-nt intergenic region observed for CPsV (isolate CPV 4 from Florida, USA) is rich in A-U (88.3%) and contains 18-nt sequence $(UUAAAA)_3$ that could form a hairpin loop. Near the end of the intergenic region, a typical AAUAAA polyadenylation signal is found 12 nt upstream of the putative CA start polyadenylation site (Naum et al., 2003). However, these sequences were not found for the CPsV Spanish isolate P-121 (Martín et al., 2005), neither for MiLBVV (intergenic region of 147 nt, 66% of A+U), and LRNV (intergenic region of 80 nt, 65 % of A+U), making it unlikely that were involved in conserved functions among ophioviruses.

The largest ORF of the RNA 1 encode a protein of 261K for LRNV, 263K for MiLBVV and 280K for CPsV containing the core polymerase module with the five conserved motifs of the RdRp active site (van der Wilk et al., 2002; Naum et al., 2003; Vaira et al., 2003). The ophiovirus RdRp are highly conserved among them, mainly in the module sequence (see section 4). Two regions of the RdRp may be regarded as a bipartite nuclear localization signal (NLS) in the CPsV (Naum et al., 2003; Martín et al., 2005), and at least one NLS was also found in MiLBVV and RWMV polymerases (van der Wilk et al., 2002; Vaira et al., 2003).

A protein about 50-55 kDa of unknown function is encoded by RNA 2 in the positive strand of the ophioviruses CPsV, MiLBVV and LRNV (Sanchez de la Torre et al., 2002; van der Wilk et al., 2002; Torok et al., 2003). The 54K protein of CPsV has been detected in infected tissue confirming its size and coding assignment (Peña E. J. unpublished results). It is probably involved in virion movement and suppression of post transcriptional gene silencing (PTGS), the antiviral defence mechanism of the plant (Robles Luna and Peña personal communication). These two mentioned functions seem to be shared with the 24K protein of CPsV. Sequence analyses of the 54K protein and the homologous 50K and 55K proteins from LRNV and MiLBVV contain a conserved NLS, as the RdRp of ophioviruses CPsV, MiLBVV and RWMV, suggesting that part of the cycle might occur in the nucleus. Similarity among 50-55K and 22-25K proteins is lower than that found among CPs, and is very high among the RdRp module sequence (see section 4).

In the viral complementary RNA 2 of MiLBVV an additional minor ORF encodes a putative protein of about 10kDa (see Figure 2), but its function is unknown. In the RNA 2 of CPsV and LRNV this small protein is absent, and so far, it is unknown whether this putative protein is present in RWMV and FreSV genomes. In case the 10kDa polypeptide were not present in these viruses, MiLBVV would be the unique ophiovirus with ambisense RNA 2.

Negative and positive stranded RNAs of ophioviruses are encapsidated in a single coat protein of 43-50 kDa (Garcia et al., 1991; Vaira et al., 1997; Barthe et al., 1998). Using antibody obtained against purified virion particles and expressing RNA 2 and RNA 3 coded proteins in *E. coli*, Sanchez de la Torre et al., (1998) demonstrated that the coat protein of CPsV is encoded in the RNA 3. The protein encoded by MiLBVV RNA3 has similar molecular mass and high sequence similarity (44.6%) with the coat protein of CPsV, thus, it

is presumed that coat protein of MiLBVV is also encoded by RNA 3 (van der Wilk et al., 2002). The CPs of MiLBVV and TMMMV are closer with 80% homology. Attempts to find similarities, with the exception of CPsV, some serological relationship between TMMMV and MiLBVV, and between RWMV and MiLBVV, have been found indicating that some epitopes in the capsid proteins among most of the ophiovirus are conserved (Roggero et al., 2000). In general, different isolates of the same ophiovirus specie showed highly conserved amino acid sequences in the coat protein as showed for CPsV (Martín et al, 2006) and MiLBV (Navarro et al., 2004), and less conserved among the different ophiovirus species.

Other than structural function of the coat protein can be assumed. Transmission facilitated by fungus zoospores has been reported for *Tombusviridae* family, involving coat protein (McLean et al., 1994), and even oligosaccharides as shown by Kakani et al, (2003). Rochon et al., (2004) proposed a model for the tombusvirus *Cucumber necrosis virus* transmission in which it binds to *Olpidium bornovanus* zoospores, showing that specific sites on the capsid as well as on the zoospore are involved. They also remark that the mechanism of the tombusvirus coat protein binding to the fungus is similar to poliovirus/host cell interactions and related viruses such as influenza, suggesting evolutionary conservation of functional features of plant and animal virus capsids.

MiLBVV and LRNV present a fourth RNA of negative polarity (van der Wilk et al., 2002; Torok et al., 2003, Torok et al., 2010). The RNA 4 of LRNV encodes a potential protein of 38 kDa, and the RNA 4 of MiLBVV one of 37kDa (p37). MiLBVV has an additional ORF of 10.6 kDa with a 38 nt overlapping sequence with the p37 (see Figure 2). This second ORF is proposed to be expressed by a +1 translational frameshift of p37, but lacks an initiation codon (van der Wilk et al., 2002). So far, the functions of these putative proteins are unknown.

3. The question about circular structures – A "panhandle" structure?

As mentioned before, virions of the ophioviruses appear circularized. The same morphology is observed for tenui- and phleboviruses particles, suggesting that ophiovirus can adopt a panhandle structure formed by the pairing of the conserved 5' and 3' ends of each genomic RNA (see Figure 1.b). Looking for this structure the 3´ and 5´ terminal sequences of CPsV, MiLBVV and LRNV have been checked (Figure 3). In CPsV, the first 12 nt of 5´ end of vcRNAs were found almost identical in the three RNAs, but unexpectedly no identity among the three RNAs at their 3´ ends was found (Figure 3.a), and were not able to form self-complementary panhandle structures between the 3´ and 5´ends of each RNA (Naum et al., 2003). Figure 3. b shows the alignment of the 3´ and 5´ends sequences of the four RNAs of LRNV presenting higher identities among the RNAs 1, 2 and 3, and less with the RNA 4. In the case of MiLBVV, both 5´and 3´ ends are conserved among the four viral RNAs. Both MiLBVV and LRNV do not anneal to perfect panhandle structures (Figure 3.c). Instead, van der Wilk et al., (2002) found that MiLBVV RNAs ends are able to fold into structures faintly resembling the "corkscrew" conformation of *Orthomyxoviridae* RNA termini. In the case of LRNV partial pairing of the conserved 5´ and 3' ends of genomic RNAs can be found and a "corkscrew" conformation can also be inferred. However, since this structure was not found for CPsV, alternative explanations for the circular structure of CPsV particles are required.

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RNA1   5´GAUACUUUUUUUCAAGGA-AAAGUAAUCAU
RNA2   5´GAUACUUUUUUUUUGUGAUUAAAGC-AUCAC
RNA3   5´GAUACUUUUUUUUUGUGGAAAAAGC-AUCAU
         ************    *    **     ****

RNA1      CGACAAGACGAUAAUCCUAAUUAGGAUACC 3´
RNA2      CACCCGUGGAAAUGUCACCAGGAUGACACC 3´
RNA3      UUUUAAAUAAACAAAAGUUCCCAAAUUCGG 3´
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(a) CPsV

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RNA1   5´UGAUAUAUUUCUAAAAUAUAUUACUGUUUC
RNA2   5´UGAUAAAUUUCUAUAAAAUUUAUCUGGCUU
RNA3   5´UGAUAGUUUUAUUUAAAGCUAUCAUGUUCA
RNA4   5´--AAAGUUGACUUUUUAAAUAAAGUAUCA-
           * *  *   *             *

RNA1   GAAACAGUAAUAUAUUUUAGAAAUA--UAUCA 3´
RNA2   -AAGCC-AGAUAAAUUUUAUAGAAAUUUAUCA 3´
RNA3   UGAACA-UGAUAG-CUUUAAAUAAAACUAUCA 3´
RNA4   -GAACU-UAAUAAUUUUUAUAAAAAAUUAUCA 3´
         * *    ***   **** * * *  *****
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(b) LRNV

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RNA1   5´GAUUAUUUU UUAAAAAUAUAACAAGUUCUC
RNA2   5´GAUUAUUUUAAUAUAUGCAAAACAGUUUUC-
RNA3   5´GAUUAUUUU-UUAAAAAUAUAACAAGCUCAU
RNA4   5´GAUUAUUUU-UUAAAAAAUAAACAAGUAUCU
         *********  ** *     ****

RNA1   UUAAUCAAAAUAUAUUU--UAAAAAUAUAUCA 3´
RNA2   UCAACAACAAUAUUUUU--AGAAAAAAUAUCA 3´
RNA3   UGAGCU--GAUAGUUUUAAUAAAAAAACUAUCA 3´
RNA4   UGAACA--GAUAUUUUUAAUAAAAAAAUAUCA 3´
       * *      ***  ***    ****  *****
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(c) MiLBVV

Fig. 3. Alignment of the 3´ and 5´ ends of positive stranded RNAs of CPsV, LRNV and MiLBVV. Thirty terminal nucleotides of 5´ and 3´ ends of vcRNAs (a) CPsV (b) LRNV and (c) MiLBVV. Identical nucleotides are denoted with asterisks. The conserved 5´ terminal sequences among the three ophioviruses and 3´end of MiLBVV and LRNV are underlined.

Comparing among RNA termini of these ophioviruses it is noted that 5´ terminal sequences GAUWNWUUUW (where N is any nucleotide and W is A or U) and 3´end of MiLBVV and LRNV (UAUCA 3´) are quite conserved (see Figure 3, underlined sequences).

4. The analysis of the putative RdRp – Taxonomical relationship with the negative-stranded RNA viruses

The aa sequence of the large ORF of RNA 1 ophioviruses was aligned with the RdRp aa sequences of members of the *Paramyxoviridae*, *Rhabdoviridae*, *Bornaviridae* and *Filoviridae*

families and *Varicosavirus* as reported by Naum et al, 2003, indicating that this protein is the putative RNA polymerase (Figure 4.a). The predicted 260-280K protein contains the core polymerase module with the five conserved motifs proposed to be part of the RdRp active site (Poch et al., 1989; Muller et al., 1994), and conserved residues recognized in all compared negative-stranded RNA viruses. Among the ophioviruses CPsV, MiLBVV, RWMV and LRNV, the aa sequences of the polymerase module is highly conserved (Figure 4.b). However, instead of the GDNQ of most of the non-segmented viruses present, the four ophioviruses have the SDD sequence in motif C, which is a signature for segmented negative-stranded RNA virus families *Orthomyxoviridae, Arenaviridae* and *Bunyaviridae*.

(a)

premotif A

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CDV    535 KEKEIKEVGRLFAKMTYKMRACQVIAENL  <95>
MEV    535 QEKEIKETGRLFAKMTYKMRACQVIAENL  <95>
NIV    542 KEKETKQAGRLFAKMTYKMRACQVIAEAL <147>
SEV    543 KEKEIKQEGRLFAKMTYKMRAVQVLAETL  <87>
MUV    553 KEKEIKATGRIFAKMTKRMRSCQVIAESL  <83>
NDV    543 KEKEVKVNGRIFAKLTKKLRNCQVMAEGI  <65>
HRSV   619 KERELS-VGRMFAMQPGMFRQIQILAEKM  <49>
TRTV   554 KERELS-VGRMFAMQPGKQRQVQILAEKL  <49>
MARV   556 KEKELN-IGRTFGKLPYRVRNVQTLAEAL  <47>
ZEBOV  553 KEKELN-VGRTFGKLPYPTRNVQTLCEAL  <47>
RYSV   537 KERELKIMARFFALLSFKMRLYFTATEEL  <41>
SYNV   567 KEREMKTKARFFSLMSYKLRMYVTSTEEL  <41>
NCMV   491 KEREMNPVARMFALMTLKMRSYVVITENM  <42>
LBVaV  518 KEREIKVAARMYSLMTERMRYYFVLTEGL  <39>
VSIV   530 KERELKLAGRFFSLMSWKLREYFVITEYL  <42>
VSNJV  530 KERELKIAGRFFSLMSWRLREYFVITEYL  <42>
BEFV   556 KERELKEEGRFFSLMSYELRDYFVSTEYL  <42>
RABV   543 KERELKIEGRFFALMSWNLRLYFVITEKL  <42>
BDV    326 KEKELKVKGRFFSKQTLAIRIYQVVAEAA  <38>
IHNV   494 KEMELKIKGRGFGLMTFMPRLLQVLRES-  <41>
MiLBVV 575 KEKEQKTAARFYGIASFKLKLWISSTMEM  <37>
RWMV     1 KEREQKIAARFYGIASFKLKLWISSTMEM  <37>
CPsV   635 KEKELKTEGRFYGVASFKLKIYISIIMEM  <37>
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Motif A **Motif B** **Motif C** **Motif D**

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CDV    FITTDLKKYCLNWR <57> IFIKYPMGGVEGYCQKLWTISTIPYL <12> SLVQGDNQTI <60> YDGMLISQSLKSI
MEV    FITTDLKKYCLNWR <57> IFIKYPMGGIEGYCQKLWTISTIPYL <12> SLVQGDNQTI <60> YDGLLVSQSLKSI
NIV    FLTTDLKKFCLNWR <57> IFIHYPKGGIEGYSQKTWTIATIPFL <12> AIVQGDNESI <60> YDGAVLSQALKSM
SEV    FLTTDLKKYCLNWR <57> IFIHNPRGGIEGYCQKLWTLISMSAI <12> AMVQGDNQAI <60> YDGKILPQCLKAL
MUV    FLTTDLTKYCLNWR <57> IFIVSPRGGIEGLCQKLWTMISISTI <12> SMVQGDNQAI <60> YKGRILTQALKNV
NDV    FITTDLQKYCLNWR <57> IYIVSARGGIEGLCQKLWTMISIAAI <12> CMVQGDNQVI <59> KDGAILSQVLKNS
HRSV   SIITDLSKFNQAFR <58> GLYRYHMGGIEGWCQKLWTIEAISLL <12> ALINGDNQSI <59> HNGVYYPASIKKV
TRTV   SIVTDLSKFNQAFR <57> GLYRFHMGGIEGWCQKMWTMEAISLL <12> SLLNGDNQSI <59> SEGVMYPAAIKKV
MARV   SFVTDLEKYNLAFR <57> NAYHYHLGGIEGLQQKLWTCISCAQI <12> SSVMGDNQCI <60> LNGVQLPQSLKTM
ZEBOV  SFVTDLEKYNLAFR <57> SSYRGHMGGIEGLQQKLWTSISCAQI <12> SAVMGDNQCI <60> LNGVQLPQSLKTA
RYSV   VINMDFVKWNQQMR <55> VCWIDDGAGKEGIRQKAWTIMTVCDI <12> LVGGGDNQVL <64> YKGVPLRSPLKQV
SYNV   SMNIDFSKWNQNMR <54> WSRTGDESGKEGLRQKGWTITTVCDI <12> LIGGGDNQVL <63> YSGVPLRGRLKVI
NCMV   CINMDFEKWNLNMR <56> KSYEGHIRGFEGLRQKGWTVFTVVLI <12> LMGQGDNQVL <64> YKGVPLCSSLKRI
LBVaV  NINIDFSKWNTNMR <54> MCYRGHLGGFEGLRQKGWTVATVCLL <12> LMGQGDNQII <64> LDGRQLPQWYKKT
VSIV   ANHIDYEKWNNHQR <56> VCWQGQEGGLEGLRQKGWTILNLLVI <12> VLAQGDNQVI <63> FRGVIRGLETKRW
VSNJV  ANHIDYEQWNNHQR <56> VCWNGQKGGLEGLRQKGWSIVNLLVI <12> VLAQGDNQVI <63> FRGVIRGLETKRW
BEFV   ANNIDYEKWNNYQR <56> VCWEGQKGGLEGLRQKGWSILNYLMI <12> ILAQGDNQTI <63> IEGTIKGLPTKRW
RABV   AFHLDYEKWNNHQR <58> TCWNGQDGGLEGLRQKGWSLVSLLMI <12> VLAQGDNQVL <63> FRGNILVPESKRW
BDV    VINLDYSSWCNGFR <54> TCAVGTKTMGEGMRQKLWTILTSCWE <12> ILGQGDNQTI <51> FRGVPVPGCLKQL
IHVN   NKSLDINKFCTSQR <67> GVFSGLKGGIEGLCQYVWTICLLLRV <12> ILAQGDNVII <63> CP-QHLTLAIKKA
MiLBVV SLFLDYSGHNTSQR <49> YISQGQLGAIEGWLGSLWGIQSQLMI <12> IGTTYSDDSC <50> YRGKPMDMSIKKM
RWMV   SLFLDYSGHNTSQR <49> YYSKGQLGAIEGWLGGLWGIQSQLML <12> IGTTYSDDSC <50> YKGIPIEMTLKKI
CPsV   TLFLDYSGHNTSQR <49> VWSKRQKGAIEGWFGPLWGIQSQLML <12> IGTTYSDDSC <50> YFDKPIDTSYKRI
```

(b)

```
                        Premotif A
                  ___________________________
MiLBVV <575> KEKEQKTAARFYGIASFKLKLWISSTMEMIKRAMKLLPGQMMTMTDDERRLIMFKMSEKL
RWMV         KERE QKIAARFYGIASFKLKLWISSTMEMVKRAMKLLPGQMMTMTDDERREIMFKMSEKL
CPsV   <635> KEKELKTEGRFYGVASFKLKIYISIIMEMIKRAMKLIPGQMMTMTEDERRTVMHRMSVML
LRNV   <475> KERE QKTSARFYGIASFKLKLWISSVMEMIKRAMKLLPGQMMTMTDDERREIMFKMSEKL
             **:* *  .****:******::**  ***:******:********:**** :*.:**  *

                     Motif A
                    _________
MiLBVV     LSKNSYSLFLDYSGHNTSQRPENTNFILEEIANMYGYYEGTPEFNELTSLSYVFSNINII
RWMV       LEEDTYSLFLDYSGHNTSQRPENTNFIMEEIANMYGYFEGSLEYEEFTSLPYVFSNIHLI
CPsV       EEKDAYTLFLDYSGHNTSQRPENNLFILEEIADMYGFEENSIERKRLIQVVYLFNELEIL
LRNV       MEKDAYSLFLDYSGHNTSQRPENCVFLLEEIANMYGFFEGSDQYREIVSLAYVFANIHVI
            .:::*:****************  *::****:***: *.: : ..: .: *:* ::.::

                              Motif B                          Motif C
                    ___________________________          ____________
MiLBVV     VEDSWSDYVYISQGQLGAIEGWLGSLWGIQSQLMIEDMFMQLGMNDYIGTTYSDDSCGVF
RWMV       VEDTWSDMIYYSKGQLGAIEGWLGGLWGIQSQLMLEDMFMQLGVKDFIGTTYSDDSCGVF
CPsV       YEHLFSDMVVWSKRQKGAIEGWFGPLWGIQSQLMLDDMMVSMGIEKYIGTTYSDDSCGVF
LRNV       VEDNWSDIVYYSHGQLGAIEGWLGGLWGIQSQLMLEDMFLQLGVKDYIGTTYSDDSCGVF
            *. :** :  *: * ******:* *********::**::.:*::.:**************

                                                              Motif D
                                                          ____________
MiLBVV     TQSSLDVHKLNGIIKNVQRYGEDMGLIVKLSQTQVTNGRCSMLKEHYYRGKPMDMSIKKM
RWMV       TQKSMDVQKLNGIIKNVQRCGEDMGLIVKLSQTQVTNGRCSMLKEHYYKGIPIEMTLKKI
CPsV       IKKNLDQEELNRMIEYIQSYSLKMGLLVKLSQTQITNGRCSMLKNHYYFDKPIDTSYKRI
LRNV       TQQKLNVSKLNDIVRNVQKYGEDMGLIVKLSQTQVTNGRCSMLKEHYYKGEPIDMTLKKM
            :..::  :** ::. :*  . .***:*******:*********:*** . *:: : *::
```

Fig. 4. **a.** Alignment of the core RdRp modules of some representative members of families *Borna-, Filo-, Paramyxo-* and *Rhabdoviridae*, and of the members of family *Ophioviridae* (CPsV, MiLBVV and RWMV). Conserved residues recognized previously in premotif A and motifs A, B, C and D, are shown in bold letters and additional strictly conserved residues are underlined. Numbers on the left of premotif A indicate the starting position, and numbers within brackets refer to the intervening sequences between motifs not represented. **b.** Alignment of the complete RdRp polymerase module of CPsV, MiLBVV, RWMV and LRNV ophioviruses. Identical residues are denoted with asterisks and similar residues by colons and dots. Virus acronyms are indicated in the legend of figure 5. Naum et al., (2003) with modifications.

These data support grouping these four viruses in the same genus, *Ophiovirus*, previously advanced on the basis of their similar virion morphology including TMMMV (Milne et al., 2000), and later FreSV (Vaira et al., 2011).

The amino sequence of the RdRp active site was exploited to study the phylogenetic relationships among the ophioviruses and other representative negative-stranded RNA viruses of families *Borna-, Filo-, Paramyxo-, Rhabdo-, Orthomyxo-, Bunya-* and *Arenaviridae*, and *Tenuivirus*. Figure 5 shows that ophioviruses CPsV, MiLBVV and RWMV appear as a monophyletic group that is separated from the other negative-stranded RNA viruses, reinforcing the taxonomic relatedness of the group.

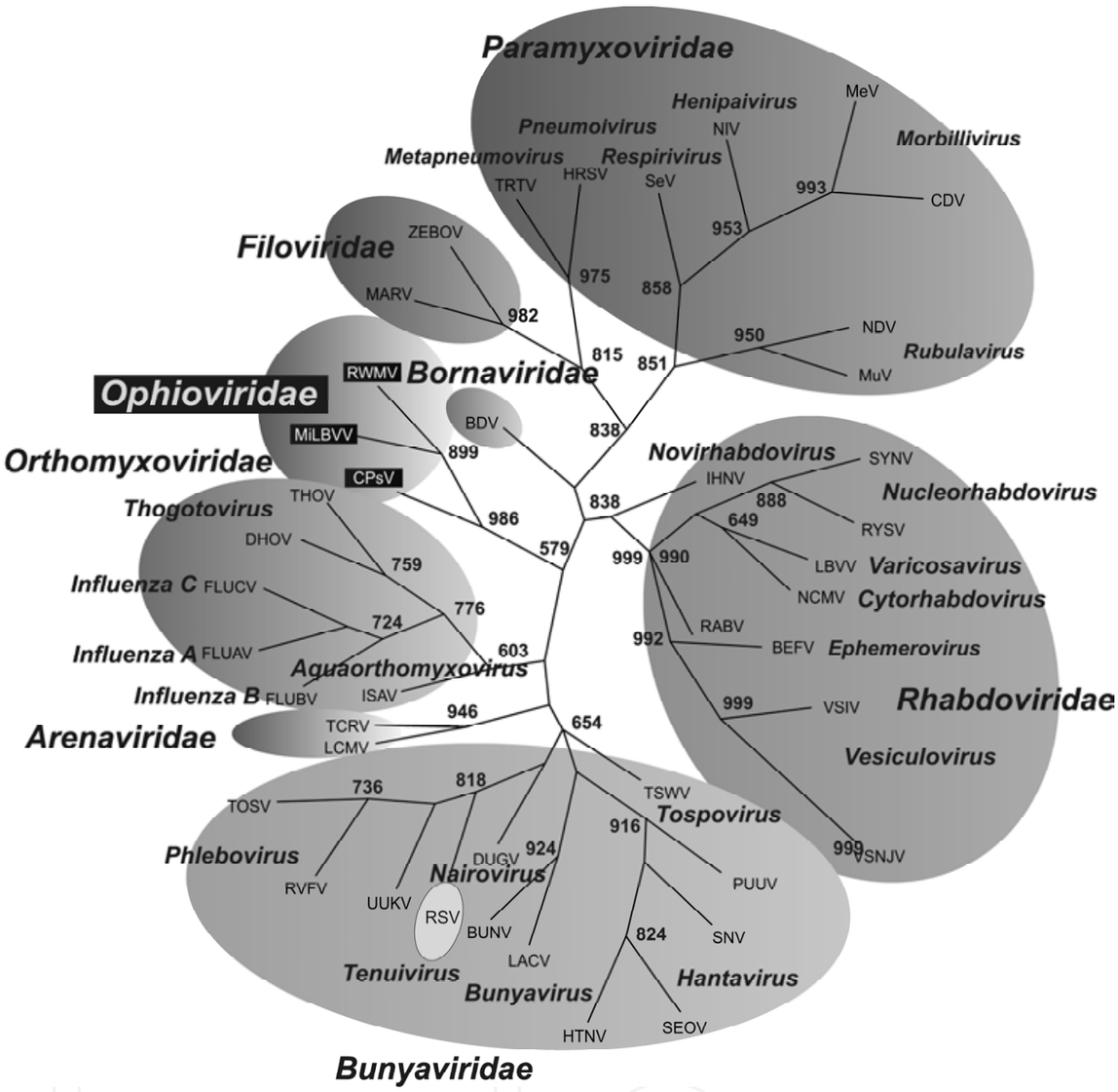

Fig. 5. Unrooted phylogenetic tree showing the relationship among representative negative-stranded RNA viruses, from the *Mononegaviales* order, *Tenuivirus, Arenavirus* genera and the ophiovirus CPsV, MiLBVV and RWMV, *Ophioviridae* family, based on their conserved RdRp modules (see Figure 4.a). Branch lengths are proportional to genetic distances between sequences. The tree was generated by the neighbor-joining method and bootstrap values (indicated for each branch node) were estimated using 100 replicas. Branch lengths are proportional to genetic distances between sequences and the scale bar represents substitutions per amino acid site. Borna disease virus (BDV), Marburg virus (MARV), Zaire Ebola virus (ZEBOV), Sendai virus (SeV), Mumps virus (MuV), Newcastle disease virus (NDV), Measles virus (MEV), Canine distemper virus (CDV), Nipah virus (NIV), Human respiratory syncytial virus (HRSV), Turkey rhinotracheitis virus (TRTV), Vesicular stomatitis Indiana virus (VSIV), Vesicular stomatitis New Jersey virus (VSNJV), Bovine ephemeral fever virus (BEFV), Infectious hematopoietic necrosis virus (IHNV), Lettuce necrotic yellows virus (LNYV), Northern cereal mosaic virus (NCMV), Sonchus yellow net virus (SYNV), Rice yellow stunt virus (RYSV), Lettuce big-vein associated virus (LBVaV),

Influenza A virus (FLUAV), Influenza B virus (FLUBV), Influenza C virus (FLUCV), Thogoto virus (THOV), Dhori virus (DHOV), Infectious salmon anemia virus (ISAV), Bunyamwera virus (BUNV), La Crosse virus (LACV), Hantaan virus (HTNV), Puumala virus (PUUV), Sin nombre virus (SNV), Seoul virus (SEOV), Dugbe virus (DUGV), Rift Valley fever virus (RVFV), Toscana virus (TOSV), Uukeniemi virus (UUKV), Tomato spotted wilt virus (TSWV), Rice stripe virus (RSV), Lymphocytic choriomeningitis virus (LCMV), and Tacaribe virus (TCRV). Naum et al., (2003) with modifications.

As mentioned, ophioviruses possess two ORFs in the same strand of RNA 1, with the RdRp located downstream the intergenic region (see figure 2), which is a distinct genomic structure of all segmented- and negative stranded RNA viruses.

Taken all these characteristics, the family *Ophioviridae* has been proposed to the ICTV Ninth Report (Vaira et al., 2011), with only one genus recognized, *Ophiovirus*, containing six species: CPsV, TMMMV, RWMV, FreSV and MiLBVV, without an order assigned. Moreover, the phylogenetic analysis done by Vaira et al., (2003) with the 45 aa strings derived from the 136nt fragment amplified from the available isolates of CPsV, RWMV, LRNV and FreSV supported the positions of these ophioviruses as distinct species, and a closer relationship between MiLBVV and TMMMV species.

5. Cultivars and transgenic lines resistant to ophiovirus

Since most of the ophiovirus are soil-transmitted, the cultivation of commercially important species addresses the challenge of the disease control searching for resistant cultivars economically important. In Japan, natural resistance has been found in tulip cultivars for tulip mild mottle mosaic disease. Bulb lots of 214 cultivars were tested, some of which were resistant to TMMMV, although resistance varies greatly among them (Morikawa et al., 2004). In Virginia, USA, Hansen el at., (2009) have been reported that in freesia cvs. 'Honeymoon'and 'Santana' mixed infection with the potyvirus Freesia mosaic virus, and the ophiovirus FreSV can be found and probably making more difficult to control the disease.

Big-vein diseased lettuce plants are infected with MiLBVV, and usually together with the varicosavirus LBVaV (Lot et al., 2002; Navarro et al., 2004; Plesko et al., 2009; Barcala Tabarrozzi et al., 2010). To control this disease resistant cultivars have been developed by conventional breeding method, like the cultivars Thompson and Pacific, using several resistant sources (Ryder 1981; Ryder and Robinson, 1991, 1995). However, although with some cultivars losses were reduced, do not exhibit high levels of resistance and do not eliminate the disease. More recently, partial big-vein resistance was identified in *Lactuca sativa* cultivars Great Lakes 65, Pavane, Margarita. In the same work, *Lactuca virosa,* which is not used in the market, was found ophio- and varicosavirus-free and big-vein symptomless (Hayes et al., 2006). Big-vein resistance breeding efforts using this line has been reported (Hayes et al., 2004) generating *L. virosa–L. sativa* hybrid but variation for the frequency of symptomatic plants was found.

In last 20 years, different strategies using transgenic plants have been developed successfully to gain virus resistance cultivars (Sudarshana et al. 2007; Prins et al., 2008). The most widely used have been protein-mediated (pathogen-derived resistance, PDR) and more recently RNAi-mediated resistance by post-transcriptional gene silencing (PTGS) mechanism (e.g. Jan et al., 2000; Shimizu et al., 2009; Fahim et al., 2010). Expression of the

coat protein gene of several RNA viruses were shown to confer virus resistance in experimental and natural hosts, and later, other virus-derived sequences in sense or antisense constructs carrying the movement protein or RdRp genes were expressed also conferring resistance against virus. Transgenic plants carrying the coat protein gene have resulted successes, which could indicate that the CP is involved in early events of virus infection (Reyes et al., 2011). However, it is not predictable which viral genes is the best to confer resistance (Morroni et al., 2008; Kamo et al., 2010).

The first attempt to get resistance against ophioviruses was done on lettuce (*Lactuca sativa* L) by agro-transformation expressing the coat protein gene of LBVaV in sense or antisense orientations. Interestingly, some of the lines were susceptible to LBVaV, but line A-2 was resistant to MiLBVV without big-vein symptoms regardless of the presence or absence of LBVaV (Kawazu and Fujiyama, 2006). In this line the LBVaV coat protein-mRNA derived from the transgene was not detected, probably due to RNA silencing. However, the mechanism by which line A-2 was resistant to MiLBVV is not clear, since there is no significant sequence homology between the transgene (LBVaV coat protein gene) and the MiLBVV coat protein gene. Later, lettuce was transformed with inverted repeats of a coat protein gene fragment of MiLBVV and two lines resulted resistant to this virus (Kawazu et al. 2009). These lines showed resistance to big-vein symptom expression but were susceptible to LBVaV. Moreover, MiLBVV was detected in roots but not in leaves of one of the lines after inoculation, suggesting that resistance to MiLBVV is less effective in roots than in leaves. Furthermore, T3, T4, and T5 generations showed high resistance to this ophiovirus and big-vein symptoms expression indicating that high resistance to lettuce big-vein disease is stably inherited (Kawazu et al., 2010).

Citrus plants do not present natural resistant species including oranges, mandarins and grapefruits, as well as hybrids and citrus relatives used as rootstock (Roistacher, 1993), promoting the generation of new alternatives of control. Transformation of woody plants present disadvantages as the time consuming in transformation procedure and multiplication. Lines are propagated by bud grafting onto seedlings used as rootstocks generating replicates of each line. For challenge, transgenic scions are infected by grafting using infective tissue. For citrus psorosis disease the first work was done by Zanek et al., (2008) producing 21 independent lines of transgenic sweet orange (cp-lines) expressing low and variable amounts of CPsV coat protein (isolate CPV4). In these lines no correlation between copy number and transgene expression was found and no significant differences were observed in the response to virus challenge among the lines or among the replicates. Although two different viral loads were evaluated to challenge the transgenic plants, no resistance or tolerance was found in any line after one year of continuous observations. An inherent difficulty in this assay is that as long the rootstock is susceptible to CPsV, the virus could move to the rootstock and replicate. Thus, viruses could be delivered to the scion as a continuous challenge overcoming the protection. Applying PDR strategy but using different viral genes, sweet orange transgenic lines were generated expressing the *54k* and *24k* genes of CPV 4 isolate (Reyes et al., 2011a). In these assays fourteen lines were selected including new cp-lines. These plants were evaluated for their acquired resistance against two isolates, PsA (CPV 4) and PsB (CPsV 189-34), which differ in symptoms severity. These lines were susceptible to both isolates when graft-infected, although one of them carrying the cp gene (CP-96 line) containing two copies of the transgene and expressing a low level of the coat protein showed a delay in symptom expression when inoculated with the PsB isolate.

Therefore, other transgenic approach was applied developing CPsV-resistant sweet orange plants. In order to trigger the PTGS prior to CPsV infection, transgenic sweet orange plants producing intron-hairpin RNA transcripts corresponding to *cp, 54k* or *24k* genes were generated. Lines carrying the ihpRNA derived from the *cp* gene (ihpCP, lines 10 and 15) provided a high level of virus resistance, but ihp54K and ihp24K lines resulted variable or highly susceptible to CPsV respectively (Reyes et al., 2011b). The siRNAs accumulation level was not directly correlated to the degree of the triggered virus resistance among the different lines, and no significant difference was observed between inoculated and non-inoculated ihpCP resistant lines, indicating that in these plants the virus has been controlled probably immediately after the virus enter to the cells. Moreover, these results support the idea that not all regions of the viral genome yield the same level of resistance applying pathogen-derived-resistance strategy (Valkonen et al., 2002). For negative-stranded RNA viruses as tospovirus, replication is regulated by the CP concentration at the point of switching from mRNA production to replication of the genome (Storms, 1998). In the case of CPsV and MiLBVV the coat protein could be early involved in these functions, and its absence could impede viral replication. Moreover, all these results indicate that pre-activation of the RNA-silencing machinery against the *cp* gene seems to be one alternative to prevent other ophiovirus infections.

6. Concluding remarks

Ophioviruses are the causal agent of old diseases as citrus psorosis and big-vein affecting major crops (citrus, ornamental plants and lettuce). Most of the ophioviruses are soil-transmitted by a root-infecting fungus *Olpidium*, and in the case of CPsV an aerial vector is also suspected, although there are no evidences so far. The virions are circles of at least two different contour lengths, particles can form pseudolinear duplex structures, and the coiled filamentous are about 9-10 nm in diameter. Ophiovirus genome is divided into three or four individually encapsidated segments. CPsV, RWMV and FreSV have 3 RNAs and MiLBVV and LRNV have a fourth RNA. In the RNA 1, a protein of 22-25 kDa of unknown function is encoded in the 5´ region of the positive strand. Separated by an intergenic region of hundred nucleotides, the large ORF of RNA 1 encodes the putative RNA-dependent RNA polymerase of about 260K- 280K. Two regions of the RdRp of CPsV may be regarded as a bipartite nuclear localization signal (NLS), one of them conserved in MiLBVV and RWMV. A protein about 50-55 kDa is encoded by vcRNA 2 of CPsV, MiLBVV and LRNV, probably involved in virion movement and suppression of post transcriptional gene silencing (PTGS). A conserved NLS sequence of the RNA 2 of CPsV, LRNV and MiLBVV is present, suggesting that part of the cycle might occur in the nucleus. In the vcRNA 2 of MiLBVV an additional putative protein of unknown function is encoded. Viral and viral complementary RNAs of ophioviruses are encapsidated in a single coat protein of 43-50 kDa, which is encoded in the RNA 3. RNA 4 of MiLBVV and LRNV encode a potential protein of 37-38 kDa, and an additional ORF in MiLBVV overlapping with the sequence of the p37, both of unknown function. The 5´and 3´ terminal sequences of MiLBVV are conserved among the four viral RNAs, but they do not anneal to perfect panhandle structures, which is expected to form according to the circular morphology. Instead, a "corkscrew" conformation similar to the *Orthomyxoviridae* RNA termini has been suggested. Similarly, for LRNV partial pairing of the 5´ with the 3' end sequences of genomic RNAs can be found and a "corkscrew" conformation could be inferred, but this structure was not found in CPsV.

Moreover, comparing among RNA termini among ophiovirus genomes, it is noted that among 5´ terminal sequences of CPsV, MiLBVV and LRNV, and between 3´ ends of MiLBVV and LRNV, the sequences are highly conserved. The amino sequence of the RdRp active site was exploited to study the phylogenetic relationships among the ophioviruses and other representative negative-stranded RNA viruses, forming a new family *Ophioviridae*, without order assigned, including five species.

For some cultivars of tulip and lettuce a natural resistance source has been used in breeding programs to gain resistant plants. However, that resistance has been variable and not enough to control these diseases. In the case of CPsV and MiLBVV pre-activation of the RNA-silencing machinery against the *cp* gene seems a good alternative to prevent ophiovirus infection in citrus and lettuce, and probably applicable in ornamental plants.

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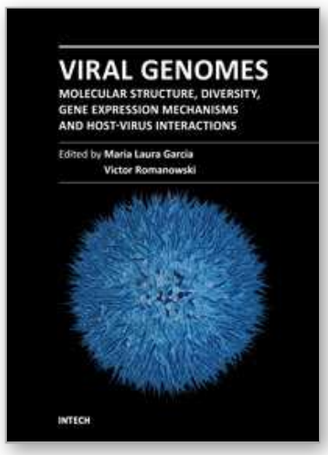

Viral Genomes - Molecular Structure, Diversity, Gene Expression Mechanisms and Host-Virus Interactions

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Viruses are small infectious agents that can replicate only inside the living cells of susceptible organisms. The understanding of the molecular events underlying the infectious process has been of central interest to improve strategies aimed at combating viral diseases of medical, veterinary and agricultural importance. Some of the viruses cause dreadful diseases, while others are also of interest as tools for gene transduction and expression and in non-poluting insect pest management strategies. The contributions in this book provide the reader with a perspective on the wide spectrum of virus-host systems. They are organized in sections based on the major topics covered: viral genomes organization, regulation of replication and gene expression, genome diversity and evolution, virus-host interactions, including clinically relevant features. The chapters also cover a wide range of technical approaches, including high throughput methods to assess genome variation or stability. This book should appeal to all those interested in fundamental and applied aspects of virology.

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