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# Cotutelle PhD Thesis

**"Ecologia Microbica e Patologia Vegetale"**
Settore scientifico disciplinare: Area 07 Agr/12
and
"Aspects moléculaires et cellulaires de la biologie"

# Reverse genetic studies of *Benyvirus – Polymyxa betae* molecular interaction: Role of the RNA4-encoded protein in virus transmission

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# PhD Thesis Resume

## Introduction

*Beet necrotic yellow vein virus* (BNYVV), the leading infectious agent that affects sugar beet, is included within viruses transmitted through the soil from plasmodiophorid as *Polymyxa betae*. BNYVV is the causal agent of Rhizomania, which induces abnormal rootlet proliferation and is widespread in the sugar beet growing areas in Europe, Asia and America; for review see (Peltier et al., 2008). In this latter continent, *Beet soil-borne mosaic virus* (BSBMV) has been identified (Lee et al., 2001) and belongs to the benyvirus genus together with BNYVV, both vectored by *P. betae*. BSBMV is widely distributed only in the United States and it has not been reported yet in others countries. It was first identified in Texas as a sugar beet virus morphologically similar but serologically distinct to BNYVV. Subsequent sequence analysis of BSBMV RNAs evidenced similar genomic organization to that of BNYVV but sufficient molecular differences to distinct BSBMV and BNYVV in two different species (Rush et al., 2003). Benyviruses field isolates usually consist of four RNA species but some BNYVV isolates contain a fifth RNA. RNAs 1 contains a single long ORF encoding polypeptide that shares amino acid homology with known viral RNA-dependent RNA polymerases (RdRp) and helicases. RNAs 2 contains six ORFs: capsid protein (CP), one readthrough protein, triple gene block proteins (TGB) that are required for cell-to-cell virus movement and the sixth 14 kDa ORF is a post-translation gene silencing suppressor. RNAs 3 is involve on disease symptoms and is essential for virus systemic movement. BSBMV RNA3 can be trans-replicated, trans-encapsidated by the BNYVV helper strain (RNA1 and 2) (Ratti et al., 2009). BNYVV RNA4 encoded one 31 kDa protein and is essential for vector interactions and virus transmission by *P. betae* (Rahim et al., 2007). BNYVV RNA-5 encoded 26 kDa protein that improve virus infections and accumulation in the hosts.

We are interest on BSBMV effect on Rhizomania studies using powerful tools as full-length infectious cDNA clones. B-type full-length infectious cDNA clones are available (Quillet et al., 1989) as well as A/P-type RNA 3, 4 and 5 from BNYVV (unpublished). A-type BNYVV full-length clones are also available, but RNA-1 cDNA clone still need to be modified. During the PhD program, we start production of BSBMV full-length cDNA clones and we investigate molecular interactions between plant and Benyviruses exploiting biological, epidemiological and molecular similarities/divergences between BSBMV and BNYVV.

**Material and method**

Sugar beet plants were grown on BSBMV infected soil. Total RNA was extracted from infected sugar beet root using Trizol reagent. Virus RNA was synthesized by ImProm-II Reverse Transcriptase system and molecular cloning on bacteria has been used to obtained full length infectious cDNA clones. Using different viral replicons based on BNYVV RNAs (Rep3 or Rep5) we cloned nucleotide sequences of Benyviruses RNA4's ORFs combined with Flag or HA tags in order to investigate expression on different plants (*Chenopodium quinoa*, *Tetragonia expansa* and *Spinacia oleracea*)

During PhD we developed new transmission test on *Beta vulgaris* plants using avirulifer *P. betae* in order to investigate the essential role of BSBMV RNA-4 and p32 protein expression for BNYVV RNA1, 2 and 3 transmission by *P. betae*.

During molecular characterization of BSBMV p32 protein, we used agroinfiltration system on *Nicotiana benthamiana* to investigate protein subcellular localization fuse with GFP tag. We expressed BSBMV p32 fused with Halo tag on *E. coli* for it purification and antiserum production.

**Results and Discussion**

We obtained full length infectious cDNA clone of BSBMV RNA-1 and we demonstrate that they transcript is able to substitute BNYVV RNA-1 as it is replicated and packaged *in planta* in a chimeric viral progeny with BNYVV RNA-2. During full length infectious cDNA clone of BSBMV RNA-2 we identified, by 5'-RACE PCR from BSBMV total RNA extraction, the correct nucleotide sequence at 5' non coding region. We identified one amino-acid substitution in BSBMV RNA-2 encoded p13 movement protein ($M^{31}V$) that maybe prevents virus cell to cell movement in plant. We reverted this mutation without obtaining full-length infectious clone. Recently we investigated if p15 movement protein is correct expressed and we demonstrated that BSBMV p15 encoded by RNA2 can be complemented by BNYVV p15 or BSBMV p15 expressed by Rep5 viral replicon. Sequence analysis revealed one nucleotide deleted in our full-length cDNA clone of BSBMV RNA-2, PCR site mutagenesis of this nucleotide permitted to obtain a full-length infectious cDNA clone. We demonstrated that transcripts of BSBMV RNA-2 is able to substitute BNYVV RNA-2 as it is replicated and packaged *in planta* in a chimeric viral progeny with BNYVV RNA-1.

During BSBMV full-length cDNA clones production, unexpected 1,730 nts long form

of BSBMV RNA-4 has been detected from sugar beet roots grown on BSBMV infected soil. Sequence analysis of the new BSBMV RNA-4 form revealed high identity (~100%) with published version of BSBMV RNA-4 sequence (NC_003508) between nucleotides 1-608 and 1,138-1,734, however the new form shows 528 additionally nucleotides between positions 608-1,138 (FJ424610). Two putative ORFs has been identified, the first one (nucleotides 383 to 1,234), encode a protein with predicted mass of 32 kDa (p32) and the second one (nucleotides 885 to 1,244) express an expected product of 13 kDa (p13). As for BSBMV RNA-3 (Ratti et al., 2009), full-length BSBMV RNA-4 cDNA clone permitted to obtain infectious transcripts that BNYVV viral machinery (Stras12) is able to replicate and to encapsidate *in planta*. Moreover, we demonstrated that BSBMV RNA-4 can substitute BNYVV RNA-4 for an efficient transmission through the vector *P. betae* in *Beta vulgaris* plants, demonstrating a very high correlation between BNYVV and BSBMV. At the same time, using BNYVV helper strain, we studied BSBMV RNA-4's protein expression *in planta*. We associated a local necrotic lesions phenotype to the p32 protein expression onto mechanically inoculated *C. quinoa*. Flag or GFP-tagged sequences of p32 and p13 have been expressed in viral context, using Rep3 replicons, based on BNYVV RNA-3. Western blot analyses of local lesions contents, using FLAG-specific antibody, revealed a high molecular weight protein, which suggest either a strong interaction of BSBMV RNA4's protein with host protein(s) or post translational modifications. GFP-fusion sequences permitted the subcellular localization of BSBMV RNA4's proteins. Moreover we demonstrated the absence of self-activation domains on p32 by yeast two hybrid system approaches. We also confirmed that p32 protein is essential for virus transmission by *P. betae* using BNYVV helper strain and BNYVV RNA-3 and we investigated its role by the use of different deleted forms of p32 protein. Serial mechanical inoculation of wild-type BSBMV on *C. quinoa* plants were performed every 7 days. Deleted form of BSBMV RNA-4 (1298 bp) appeared after 14 passages and its sequence analysis show deletion of 433 nucleotides between positions 611 and 1044 of RNA4 new form. We demonstrated that this deleted form can't support transmission by *P. betae* using BNYVV helper strain and BNYVV RNA-3, moreover we confirmed our hypothesis that BSBMV RNA-4 described by Lee et al. (2001) is a deleted form. Interesting after 21 passages we identify one chimeric form of BSBMV RNA-4 and BSBMV RNA-3 (1146 bp). Two putative ORFs has been identified, the first one (nucleotides 383 to 562), encode a protein with predicted mass of 7 kDa (p7), corresponding to the N-terminal of p32 protein encoded by BSBMV RNA-4; the second one (nucleotides 562 to 789) express an expected product of 9 kDa (p9) corresponding to the C-terminal of p29 encoded by BSBMV RNA-3. We demonstrated that this chimeric RNA is replicated by BNYVV viral

machinery in the presence of BNYVV RNA-3, this results open the prospect to obtain a new viral vector able to to express different endogenous protein and escape from Rep5, Rep3 or RepIII (Ratti et al., 2009) competition for replication.

**Conclusion and prospect**

We demonstrated that transcript of full length infectious cDNA clone of BSBMV RNA1 can complement BNYVV RNA-1 for an efficient replication of BNYVV RNA-2 and that they can produce chimeric viral progeny *in planta*. This confirm a strong correlation between BNYVV and BSBMV. Moreover we demonstrated that just BSBMV p32 or BNYVV p31 protein expression can support BNYVV RNA-1, 2 and 3 transmission through vector *P. betae* on *Beta vulgaris* plants. We start molecular characterization of BSBMV p32 and our analysis revealed a complex expression profile, with glycosylation, myristoylation and maybe host protein(s) interaction that need to be better investigate in the future.

A paper concerning essential role of BSBMV RNA-4 p32 protein expression on vector transmission of the virus will be soon submitted for publication. Moreover results obtained by our research in this topic, opened new research lines that our laboratories will develop in a closely future. In particular BSBMV p32 and its mutated forms will be used to identify factors, as host or vector protein(s), involved in the virus transmission through *P. betae*. The new results could allow selection or production of sugar beet plants able to prevent virus transmission then able to reduce viral inoculum in the soil.

References

Lee, L., Telford, E.B., Batten, J.S., Scholthof, K.B., and Rush, C.M. (2001). Complete nucleotide sequence and genome organization of Beet soilborne mosaic virus, a proposed member of the genus Benyvirus. Arch Virol *146*, 2443-2453.

Peltier, C., Hleibieh, K., Thiel, H., Klein, E., Bragard, C., and Gilmer, D. (2008). Molecular Biology of the Beet necrotic yellow vein virus. Plant Viruses *2*, 14-24.

Quillet, L., Guilley, H., Jonard, G., and Richards, K. (1989). In vitro synthesis of biologically active beet necrotic yellow vein virus RNA. Virology *172*, 293-301.

Ratti, C., Hleibieh, K., Bianchi, L., Schirmer, A., Autonell, C.R., and Gilmer, D. (2009). Beet soil-borne mosaic virus RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in Beta macrocarpa. Virology *385*, 392-399.

Rahim, Andika I.B., Han C., Kondo H., Tamada R. (2007). RNA4-encoded p31 of Beet necrotic yellow vein virus is involved in efficient vector transmission, symptom severity and silencing suppression in roots. Journal of General Virology *88*, 1611-1619

# GENERAL INTRODUCTION

# GENERAL INTRODUCTION

## Sugar beet

Two plants are basically grown in the world for sugar production: sugarcane (*Saccharum officinarum*) in tropical and subtropical climates and sugar beet (*Beta vulgaris*) in temperate climates.

Sugar beet, one of the main industrial crop species, occupies globally a cultivated area of about 8.1 million hectares spread over 41 countries (Rush et al., 2006). The history of crop domestication has been characterized by a short, fast and interesting evolution with pronounced improvements in breeding and cultivation. The potential root sugar content has reached 15-20% throughout the years, today provides approximately 25% of world sugar consumption (FAO, 2009).

In the last years, an additional process of sugar beet cultivation found a great expansion in ethanol production. The recent interest for bioethanol production, as a replacement of fossil fuels in the transportation sector, has triggered significant research efforts in exploiting the crop's potential for production of biofuel and biogas as well.

The cultivated sugar beet is biennial: it develops the vegetative phase during the first year as a near-rosette plant and develops a large fleshy taproot that contains the nutrients reserve for the second year of growth. In the second year the plant begin the reproductive phase, after flowering induction by the combined exposure to low temperatures during winter and long photoperiods during the second year of growth (Milford, 2006).

### *1.1 Brief history of Sugar Beet cultivation*

Sugar beet belongs to the genus *Beta* of the *Chenopodiaceae* family morphologically characterized by inconspicuous, radially symmetric, petal less flowers and non-fleshy fruit (Francis, 2006). All cultivated beet species derive from sea beet (*Beta vulgaris* L. ssp. *maritima*). The genus *Beta* seems to be originated in a widespread area ranging from the British Isles and the North Atlantic coast across from the Mediterranean and the Black Sea as far as the Persian Gulf and the mouth of the Indus River (Biancardi, 2005).

Sea beet domestication is believed to have begun in prehistoric times, around the Persian Gulf as early as wheat and barley, about 12,000 years ago (Simmonds, 1976). Aristophanes (445-385 BC) and Euripides (480-406 BC) provided the earliest references of the beet plant. From Greece the plant spread in Italy where it was referred to by the Latin name beta, later used by Linnaeus to identify the taxonomic genus (Biancardi, 2005).

With the expansion of the Romans Empire, during the Middle Ages, beet cultivation was highly valued for its edible leaves and sweet-tasting root in particular from Northern Europe people. The history of sugar beet probably starts when the French botanist Olivier de Serres on 1590 extracted sweet syrup from beet roots (Dureau, 1886), however it was only during the second half of the eighteenth century, with Andreas Siegmund Marggraf, a Prussian chemist, that its potential as industrial sugar crop was discovered. He demonstrated that sugar crystals extracted from beet roots were exactly the same as those from sugar cane and his successor, Franz Carl Achard, increase sugar content of beets following several cycles of mass selection leading develop of "White Silesian", the ancestral variety of all modern sugar beet cultivars, whose sugar content ranged from 5 to 7% of total fresh root weight (Coons, 1936). For his results, Achard was supported, by king Frederick William III, to build the world's first sugar beet factory at Cunern in Silesia on 1801 (Winner, 1993).

On the history of modern sugar industry Napoleon played a key role. On 1806, the blockade of sugar cane British imports to France induced Napoleon to find a different way for the supply of sugar in France and he adopted a policy that encouraged the large-scale sugar beet cultivation for sugar production in a growing number of continental European countries. Within the end of the nineteenth century about 400 sugar factories were operating in France, Germany, Austria-Hungary, Bohemia, Czechoslovakia and Russia (Biancardi, 2005). In the middle of the twentieth century, crop productivity was significantly improved: Philippe Andre de Vilmorin introduced the progeny testing into breeding practice and achieved further sugar yield increase of "White Silesian" by means of mass (Coons, 1936).

In the American continent the first sugar beet factory was built in the United States in 1838, in Northampton, Massachusetts but was closed after its first campaign. After several failed attempts in various states, the first regularly operating factory was built in Alvarado, California. In the new century, sugar beet cultivation gradually spread to other countries: Italy, England, Ireland, Canada, Chile, Uruguay, Japan, Turkey, China etc. In the recent years, it has been introduced into warmer climates that are more suitable for sugar cane cultivation, such as Egypt, Morocco, Tunisia and Algeria (Biancardi, 2005).

Since 2009, within the European Union, the nearly two millions of hectares of sugar beet are mainly produced in Germany and France. Russian Federation, Ukraine and USA also cultivate nearly half million of hectares each year but production of roots per hectare is quite variable. France. Spain and Belgium, in fact, obtain the higher yield (93.7, 83.57 and 82.69 t/ha, respectively) and Ukraine and Russian Federation the lower (31.49 and 32,32 t/ha, respectively) as reported in Table 1 (FAO, 2009).

| Country | Area under cultivation (ha) | Root production (t) | Yield (t/ha) |
|---|---|---|---|
| European Union | 1,610,520 | 113,850,731 | 70.67 |
| Germany | 383,600 | 25,919,041 | 67.56 |
| France | 372,600 | 34,913,000 | 93.70 |
| Ukraine | 319,700 | 10,067,500 | 31.49 |
| Poland | 199,936 | 10,849,200 | 54.26 |
| United Kingdom | 140,000 | 8,330,000 | 59.5 |
| Netherlands | 72,700 | 5,735,000 | 78.88 |
| Belgium | 62,700 | 5,185,100 | 82.69 |
| Serbia | 61,399 | 2,797,596 | 45.56 |
| Italy | 60,600 | 3,307,700 | 54.58 |
| Spain | 49,700 | 4,153,900 | 83.57 |
| Czech Republic | 52,465 | 3,038,220 | 57.91 |
| Austria | 43,860 | 3,083,135 | 70.29 |
| Sweden | 39,800 | 2,405,800 | 60.44 |
| Russian Federation | 770,200 | 24,892,000 | 32.32 |
| United State of America | 464,827 | 26,779,190 | 57.61 |
| Turkey | 323,970 | 17,274,674 | 53.32 |
| China | 220,000 | 9,500,000 | 43.18 |
| Iran | 54,404 | 2,041,278 | 37.52 |
| Japan | 64,500 | 3,649,000 | 56.57 |
| Egypt | 115,000 | 5,133,513 | 44.64 |
| World | 4,323,671 | 229,490,296 | 53.07 |

Table 1. **World sugar beet production**. Cultivation area, roots production and yield of the most sugar beet productive country of the World.

## *1.2 Diseases of sugar beet*

A wide range of pathogenic agents (viruses, fungi, bacteria, nematodes and insects) commonly attack sugar beet culture, making in many cases its exploitation unprofitable due to the reduction of both taproot size and sugar content (Whitney and Duffus, 1986). All parts of the plant and all cultivation stages can be targeted by different pathogens. Consequently, diseases caused by pests of major importance have played a significant role in the current distribution of the crop and of the related sugar industry ((Cesarini, 1999); (Scholten and Lange, 2000)).

From viruses, rhizomania is the most dangerous and worldwide spread disease. This thesis focused on rhizomania disease and on rhizomania related viruses, in particular we compared molecular proprieties and protein functions of two *Benyviruses*: *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV).

| Disease | Causal agent |
|---|---|
| **Viruses** | |
| Rhizomania | *Beet necrotic yellow vein virus* (BNYVV) |
| Rhizomania related virus | *Beet soil-borne mosaic virus* (BSBMV) |
| | *Beet soil-borne virus* (BSBV) |
| | *Beet virus Q* (BVQ) |
| | *Beet oak leaf virus* (BOLV) |
| Beet mosaic | *Beet mosaic virus* (BtMV) |
| Beet yellows | *Beet yellows* (BYV) |
| | *Beet mild yellows* (BMYV) |
| | *Beet western yellows* (BWYV) |
| | *Beet chlorosis virus* (BChV) |
| Curly top | *Beet curly top virus* (BCTV) |
| **Bacteria** | |
| Bacterial vascular necrosis and rot | *Erwinia carotovora* spp. *betavasculorum* |
| Bacterial leaf spot or leaf blight | *Pseudomonas syringae* |
| Yellow wilt | Rickettsia-like organism |
| **Fungi** | |
| Cercospora leaf spot | *Cercospora beticola* |
| Alternaria leaf blight | *Alternaria alternata, Alternaria brassicae* |
| Powdery mildew | *Erysiphe betae* |
| Downy mildew | *Peronospora schachtii* (*farinosa*) |
| Fusarium yellows / Fusarium root rot | *Fusarium oxysporum* f. sp. *Betae* |
| | *Rhizoctonia solani* |
| Root rots | *Pythium* spp. |
| | *Phoma betae* |
| Southern sclerotium root rot | *Sclerotium rolfsii* |
| Black root / Black leg | *Aphanomyces cochlioides* |
| **Nematodes** | |
| Cyst nematode | *Heterodera schachtii* |
| Root-knot nematode | *Meloidogyne* spp. |

Table 2. **Principal sugar beet diseases**

# Rhizomania disease

The word 'rhizomània' is composed by the Latin parts 'rhizo' and 'man˘ıa', derived from the Greek 'ρίζ α' and 'μανία' meaning, respectively, 'root (radical)' and 'abnormal trend (madness)' (Biancardi, 2005). Rhizomania, meaning "crazy root" or "root madness" was initially describe by Canova (1959), during observations of sugar beet fields in Padan Plain, the border region of Po River, in the mid 50's and has since been reported in all sugar beet producing countries worldwide ((Tamada, 1999); (Lennerfors et al., 2005); (McGrann et al., 2009)).

Symptoms of the viral rhizomania disease are mainly localized on the lower part of the plant. Roots of infected beets appear "bearded" because of an excessive proliferation of lateral rootlets, which causes more or less taproot stunting (Fig. 1). Browning of the vascular system as well as constriction of the main root are visible on beets cross-sections (Fig. 1) ((Brunt and Richards, 1989); (Putz et al., 1990)). The size of the taproot can be strongly reduced, constricted and resembling the shape of a wine glass. Infected roots are inefficient in water and nutrient uptake and therefore the leaves commonly become pale yellow with long petioles and upright growth. BNYVV is rarely systemically spread to the leaves. When systemic spread occurs, it is mostly manifested by the necrosis and yellowing of the leave veins, that provided the name for the disease causal agent (Tamada, 1975). Disease responses at the physiology level include a reduced transpiration and $CO_2$ uptake, reduced content of nitrogen, chlorophyll and carotenoid and elevated content of amino nitrogen, sodium and potassium in the root sap ((Keller et al., 1989); (Královic and Králová, 1996); (Steddom et al., 2003).

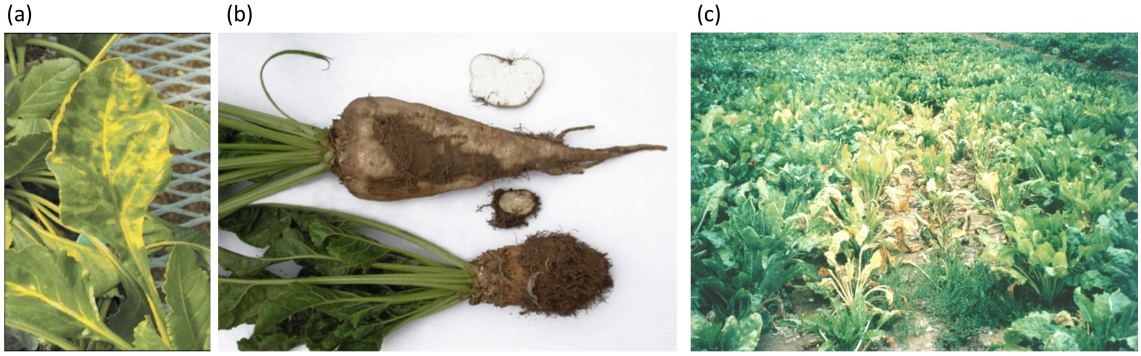



Fig. 1. Rhizomania symptoms caused by BNYVV infection on sugar beet. (a) yellowing and necrosis on the leaves veins; (b) lateral rootlets proliferation with browning of the vascular system and constriction of the main root; (c) symptoms on fields.

The disease causes severe economic losses as a consequence of a dramatic reduction in root yield, sugar content and purity, especially when infections occur early in the growing season, with sugar yield reduction commonly around 50-60% and sometimes up to 80% (McGrann et al., 2009).

## *2.1 The pathogen, Beet necrotic yellow vein virus*

Rhizomania disease is caused by the *Beet necrotic yellow vein virus* (BNYVV) (Tamada and Baba, 1973), the type member of *Benyvirus,* a genus remaining unassigned in terms of family classification (Rush, 2003). Benyviruses are all vectored by the plasmodiophorid protozoa/protist *Polymyxa betae* Keskin (Keskin, 1964) and are characterized by multipartite non-enveloped rod-shaped particles with a positive single strand RNA (ssRNA) harbouring a 5' Cap and 3' polyA.

BNYVV RNA-1 and -2 are essential and sufficient for viral multiplication when mechanically inoculated to the leaves of the *Chenopodiacea* plants like *B. macrocarpa*, *B. vulgaris*, *Chenopodium quinoa* and *Tetragonia expansa* ((Koenig et al., 1986); (Pelsy and Merdinoglu, 1996); (Tamada et al., 1989). These two RNAs carry "house-keeping" genes involved in RNA replication, assembly, cell-to-cell movement and suppression of post-transcriptional gene silencing (PTGS) ((Tamada, 1999); (Dunoyer et al., 2002). This characteristic allowed Quillet *et al*. (1989) to develop a replicative strain of BNYVV named "Stras12", by extracting total RNA from leaves infected with transcripts from full-length cDNA clones of B type BNYVV RNAs-1 and -2. However, the natural infection process requires the host-specific function of additional proteins encoded by RNAs-3 and -4, directly involved in pathogenesis and vector transmission for the efficient production of typical rhizomania symptoms and virus propagation between plants ((Lemaire et al., 1988); (Koenig et al., 1991)). Field isolates consist of 4 or 5 genomic RNAs whereas laboratory isolates maintained in the greenhouse by repeated mechanical inoculations, often develop internal deletions within RNAs -3 and -4 or may entirely loose these small genome segments (Bouzoubaa et al., 1985); (1991)).

RNA-1 (6,746 nucleotides, nts) contains a single large open reading frame (ORF) that encodes a 237 kDa polypeptide containing the conserved motifs of methyltransferase (MTR), helicase (HEL) and RNA-dependent RNA polymerase (RdRp, POL), the viral RNA replicase which is essential for virus replication (Bouzoubaa et al., 1987). Following translation, p237 is processed, by an autocatalytic cleavage that gives rise to p150 and p66

proteins (Hehn et al., 1997). The first one contains the MTR and HEL motifs and RdRp motif is present in the p66 protein. This proteolytic cleavage of the replicase distinguishes the *Benyviruses* from all other viruses with rod-shaped particles, which have their replication-associated proteins encoded on two ORFs.

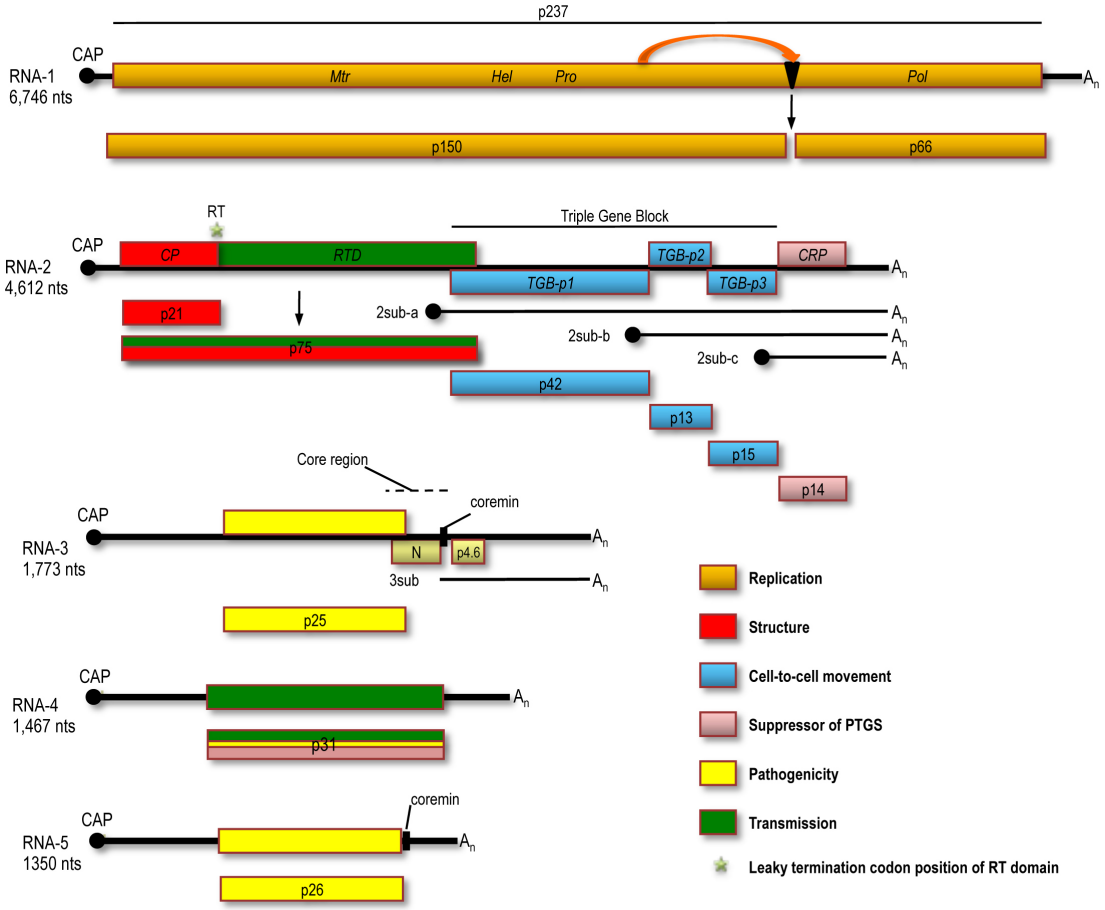



Fig. 2. Schematic representation of the BNYVV multipartite genome composed of four to five single-stranded positive-sence RNAs. All RNAs are 5'-capped and 3'-polyadenylated. Viral replicase contains conserved methyltransferase (MTR), helicase (HEL) and RNA-dependent-RNA polymerase (POL) motifs. The coat protein (CP) and a read-through (RT) domain are encoded by RNA-2 as well as the Triple Gene Block (TGB) proteins. Subgenomic RNAs are formed on RNA-2 and RNA-3. The molecular mass is indicated for each protein in kilodalton (kDa) inside the corresponding ORF. Function(s) attributed to each protein or nucleotide sequence is/are specified through a color code in or outside the boxes, respectively.

RNA-2 (4,609 nts) contains six ORFs. The first ORF encodes the 21 kDa viral Coat Protein (CP), followed by an in-frame region of 54 kDa read-through (RT) domain. CP is fused to RT during translation when the leaky UAG stop codon is bypassed by ribosomes that produce then a 75 kDa CP-RT ((Ziegler-Graff et al., 1985); (Niesbach-Klosgen et al., 1990)). The N-terminal part of the RT domain is involved in viral assembly (Schmitt et al., 1992) whereas the C-teminal part is essential for the viral transmission by *P. betae* (Tamada and Kusume, 1991). The next three partially overlapped ORFs, expressed by two subgenomic

RNAs, encode three protein products (p42, p13, p15) that exhibit typical motifs of the "triple gene block" (TGB) movement proteins, also found among other genera such as *Potexvirus*, *Carlavirus*, *Pomovirus*, *Hordeivirus*, *Allexivirus*, *Foveavirus* and *Peduvirus* (Morozov and Solovyev, 2003) (Verchot-Lubicz et al., 2010), thus facilitating virus cell-to-cell movement (Gilmer et al., 1992). The last ORF is also expressed from a subgenomic RNA (Gilmer et al., 1992) that encodes a cysteine-rich protein (p14), which possesses a suppressor activity of post-transcriptional gene silencing (PTGS) (Dunoyer et al., 2002).

RNA-3 (1,774 nts) encodes for two pathogenicity-associated proteins of 25 kDa (p25) and 6.8 kDa (N) and, putatively, for a third 4.6 kDa protein. Expression of p25, which has been serologically detected in the cytosolic fraction of infected *C. quinoa* leaves (Niesbach-Klosgen et al., 1990), is associated to the appearance of bright yellow local lesions on *C. quinoa* ((Tamada et al., 1989); (Jupin et al., 1992)), to the development of rhizomania symptoms in sugar beet roots ((Tamada et al., 1990); (Koenig et al., 1991)) and its expression induces abnormal root branching in transgenic *Arabidopsis thaliana* (Peltier et al., 2010). Variability on BNYVV RNA 3-encoded p25 protein, especially at amino acid positions 67-70 (Schirmer et al., 2005) has been recently associated with an increased pathogenicity and resistance breaking in resistant cultivars. Schirmer *et al*. (2005) suggested the tetrad motif $aa_{67\text{-}70}$ as being responsible for variable virulence with the isolates belonging to the p25-I and p25-II groups presenting the higher variability and pathogenicity rate. Moreover, Acosta-Leal et al., (2008) reported an additional variability in aa position 135 and hypothesized a possible correlation of $V_{67}L_{68}E_{135}$ residues with the ability of these isolates to overcome rhizomania resistance conferred by the *Rz1* resistance gene, derived from "Holly" source ((Lewellen et al., 1987); (1988)). In addition, the aa composition at positions 129 and 179 has been also correlated with variable pathogenicity and resistance breaking incidence ((Chiba et al., 2008); (Koenig et al., 2009). The functions of the additional two ORFs (N and 4.6) present in RNA-3 are not well documented. Jupin *et al.* (1992) reported that ORF N (1052 – 1231 nt), which overlaps the 3' terminal portion of the p25 ORF, has the potential to encode a protein of 6.8 kDa that is not detectably expressed from full-length RNA-3 but by spontaneous or experimental deletion of the upstream portion of the p25 ORF. When this protein is express, it induces necrotic local lesions on test plants as *Tetragonia expansa* and *Chenopodium quinoa.* The other 4.6 kDa protein has never been detected.

Lauber *et al*. (1998) identified on BNYVV RNA-3 a nucleotide sequence that is essential for long-distance movement (systemicity) of the virus in *Beta macrocarpa*, called "Core region" (nt 1033-1257).

RNA-4 (1,467 nt) is important for efficient transmission of the virus by the plasmodiophorid vector (Tamada and Abe, 1989). Furthermore, Rahim et al. (2007) confirmed that p31 protein expression is required for efficient vector transmission but demonstrated that p31 protein is also involved in enhanced symptom expression in host-specific manner. Indeed, p31 is able to enhance the ability of BNYVV to suppress silencing in roots of *Nicotiana benthamiana* plants (Andika et al., 2005); (Rahim et al., 2007).

When present, RNA-5 encodes for another pathogenicity-associated protein of 26 kDa (Kiguchi et al., 1996). Its function relates to the enhancement of virus transportation through the vascular bundles and an increased symptom severity. Due the aggressiveness of isolates containing RNA 5 (Tamada et al., 1996), it has been proposed that p26 probably acts in a synergistic manner with RNA 3-encoded p25 (Link et al., 2005).

## *2.2 The vector: Polymyxa betae*

The endoparasite *P. betae* is an eukaryotic protist belonging to the *Plasmodiophoridae* family, formerly considered as a fungi group but reclassified as protozoa in the Cercozoa group ((Barr and Asher, 1992); (Braselton, 1995)). *P. betae* infects mainly roots of *Chenopodiaceae* but practical experience suggests that it causes limited damage to the sugar beet crop. However, some differences in virulence between *P. betae* isolates have been reported upon the reduction of roots growth on the sugar beet plant ((Gerik and Duffus, 1988); (Blunt et al., 1991); (Kastirr et al., 1994)). More in general, five forms of another Polymyxa species, *P. graminis*, are also reported to vector many viruses in both tropical and temperate regions, in particular on cereals ((Legreve et al., 2000); (Legreve et al., 2002); (Kanyuka et al., 2003); (Dieryck et al., 2009)).

Life cycles of both *Polymyxa* species are highly similar and *P. betae cycle* is outlined on Fig. 2. Clusters of thick-walled resting spores, termed cystosori (Fig. 3), are released into the soil during senescence and decline of infected plant roots. In the presence of a susceptible host, warm and near-saturated soil moisture conditions, resting spores germinate and release primary zoospores, a motile spore that encyst on rootles and during infection inject their cytoplasmic content inside root cells inducing the formation of a multinucleate plasmodium. During this stage virus particles can be transferred to the host or acquired by the vector. Plasmodium can be differentiates in a zoosporangium or sporosorus. The sporangial phase leads to immediate production of secondary zoospores leading new infection cycle, whereas the sporogenic phase ensure cystosori release with long-term

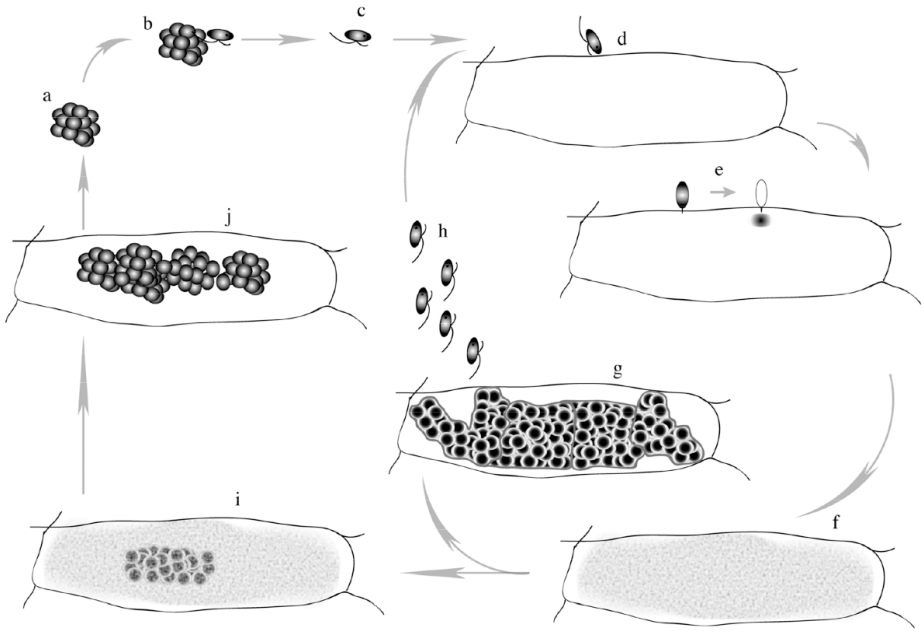
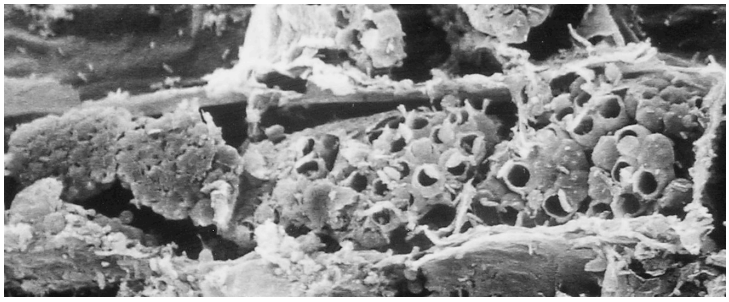



Fig. 2. Schematic representation of the *P. betae* life cycles and its developing states. (a) cystosori also called sporospore; good condition induce germinating of zoospore (b); swimming zoospores (c) is positively attracts by cortical or epidermal cells (d); (e) the zoospore encyst on the cell and injects its contents through the cell wall and the cellular membrane inducing plasmodium develop (f) that will ten to a zoosporangium (g) that will issue either (h) the secondary zoospores (h) able to infect new cells or (i) to the sporogenous plasmod (j) leading to new cystosori that will be further released in soil after root decomposition (Adapted from Peltier *et al.*, 2008).



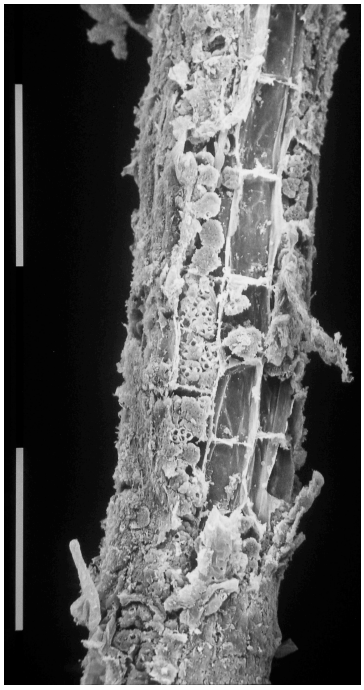
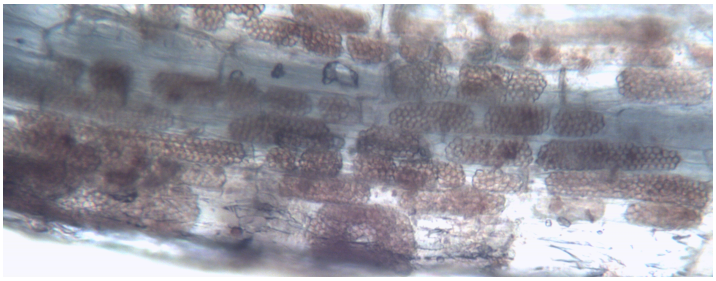


Fig. 3. Scanning Electron Microscope (SEM) ((a) and (b)) and optical microscope (c) images of *P. betae* resting spores in sugar beet cortical cells roots. (c) Optical microscope images of *P. betae*



(c)

persistence in the soil (20 – 25 years). Under optimal conditions, soil pH between 6.0 and 8.0, high water content and a temperature of + 25°C, the infection cycle is completed within 60 hours during the sporangial phase. One or the other phase is preferred depending on the external conditions or the host type ((Keskin, 1964); (Asher and Blunt, 1987)). Vectoring the rhizomania-responsible viruses, *P. betae* allows the dissemination of one of the worst viral disease of beet. Recently, Lubicz *et al*. (2007) have provided proof that BNYVV accumulates in resting spores and zoospores of its vector. Moreover, associations of the viral replication and movement proteins with sporangial and sporogenic stages of the plasmodiophorid vector led these authors to conclude that the virus resides inside its vector for more than a life cycle and to further suggest that *P. betae* besides being a vector may have an additional role as a host.

*P. betae* is also the vector for other soil-borne sugar beet viruses as BSBMV, BSBV and BVQ that can be transmitted in a single or mixed infection often associated to BNYVV.

## *2.3 BNYVV variability*

The study of BNYVV genome by restriction fragment length polymorphism (RFLP), single-strand conformation polymorphisms (SSCP) or sequence analysis of reverse transcription and polymerase chain reaction (RT-PCR) products from RNA-1 to -5, allowed to identify three major types of the virus named A, B, and P ((Kruse et al., 1994); (Koenig et al., 1995). Afterwards, partial or complete sequencing combined with phylogenetic analysis allowed the classification of BNYVV isolates also according to their pathogenicity, sequence diversity and geographic origin ((Koenig and Lennerfors, 2000); (Meunier et al., 2003); (Schmidlin et al., 2005)).

Types A and B are distributed worldwide and contain only four RNA species. Currently, type A is widespread in most European countries, the USA, China and Japan. Type B has a limited spread and has been found in Germany, France, UK, Belgium, Sweden, China, Japan, Lithuania, The Netherlands and Iran ((Miyanishi et al., 1999); (Lennerfors et al., 2000); (Sohi and Maleki, 2004); (Ratti et al., 2005))

Variations from 3% to 6% have been observed between nucleotide sequences of A and B types but a sequence identity of more than 99% suggests a highly conserved genome among isolates within A and B types ((Koenig and Lennerfors, 2000); (Meunier et al., 2003)). To date, the molecular discrimination between A and B types does not seem to be related to different pathogenicity in contrast with the P type that is known to be

responsible for severe rhizomania symptoms even in resistant varieties ((Heijbroek et al., 1999)). Type P has been identified in France (near Pithiviers) and contains 5 RNAs ((Kruse et al., 1994); (Koenig et al., 1995)) but isolates containing the fifth RNA have been reported earlier from Japan ((Tamada et al., 1989); (Kiguchi et al., 1996)) and later from China (Dawei et al., 1999), Kazakhstan (Koening and Lennerfors, 2000), UK ((Harju et al., 2002); (Ward et al., 2007)), Germany, where an Asian-like BNYVV isolate containing 5 RNAs has been recently identified (Koenig (Koenig et al., 2008) and more recently in Iran (Mehrvar et al., 2009).

According to nucleotide sequence analysis the P type isolates result related more closely to the A type than the B type isolates. Moreover sequence comparisons of RNA 5-encoded p26, led to the establishment of three groups (I, II and III) of isolates with a fifth RNA species; most isolates from Japan and China belong to group I, two isolates from Japan constitute group II and the French isolates are included in group III (Miyanishi et al., 1999).

More recently Chiba *et al*. (2011) attempted to explain the evolutionary history and route of BNYVV spreading. The authors proposed and early divergence of the A and B lineages isolates followed by loss of RNA 5 genome component in all B lineage isolates and some A lineage population. Comparing nucleotide sequence of CP, p25 and p31 protein from 73 BNYVV isolates collected worldwide they identified eight clusters that are best distinguished by the p25 sequence, derived from four original BNYVV populations variant and their mixed infections.

## Beet soil-borne mosaic virus (BSBMV)

*Beet soil-borne mosaic virus* (BSBMV) is a member of *Benyvirus* genus (Lee et al., 2001). It was first identified in Texas as a sugar beet virus morphologically similar but serologically distinct to BNYVV (Liu and Duffus, 1988).

Rush et al. (1994) demonstrated that PCR primers designed for the 3' end of each BNYVV RNA species amplify homologous regions of BSBMV and when these BSBMV PCR products were used as probes in Northern blots (Heidel et al., 1997), they weakly hybridized with BNYVV. In contrast, PCR specific primers designed on the 5' end of BNYVV RNAs do not amplify BSBMV.

When BSBMV was for first time completely sequenced by Lee et al. (2001), it was determined that BSBMV and BNYVV have identical genomic organization but sufficient molecular differences to be distinguished in two different species.

RNA-1 is 6,683 nts long and contains a single long ORF encoding a 239 kDa product that shares amino acid homology with known viral MTR, HEL and RdRp.

RNA-2 is 4,615 nts long and contains six ORFs. The 21 kDa viral capsid protein (CP) ORF is located at the 5' terminus followed by a leaky UAG stop codon, whose suppression leads to the expression of the 74 kDa RT protein. The next three BSBMV RNA-2 ORFs have typical motifs of TGB and the sixth 14 kDa protein is also similar to the cysteine-rich protein of BNYVV, which regulates RNA-2 and CP accumulation ((Hehn et al., 1995); (Lee et al., 2001)).

The 1,720 nts BSBMV RNA-3 encodes the 29 kDa ORF that shares 23% amino acid sequence identity with the 25 kDa ORF of BNYVV RNA-3. The role of BSBMV RNA-3 on symptoms determination on *C. quinoa* plants has been recently described (Ratti et al., 2009).

Lee et al. (2001) described a single putative ORF on the 1,203 nts BSBMV RNA-4 with a predicted mass (13 kDa) considerably smaller than the BNYVV RNA-4 31 kDa product.

Sequence analyses confirmed that specific regions of the BSBMV genome exhibited high degrees of nucleotide homology with BNYVV, whereas other regions were quite different. The predicted ORFs on BNYVV and BSBMV reveal 23% (RNA-3 ORF) to 83% (RNA-1 ORF) amino acid identity and the nucleotide identity score range between 35% and 77%. This results show that BNYVV and BSBMV viruses are distinct, yet more closely related to each other than to any of the other rod-shaped multipartite viruses with "fungal" vectors from the original genus *Furovirus* (Lee et al., 2001).

BSBMV is widely distributed only in the United States and, up to date, it has not been reported in other countries ((Rush, 2003); (Ratti et al., 2009)). Roots of BSBMV-infected sugar beets are in general asymptomatic, but when the virus systemically spreads to the leaves, foliar symptoms may be seen including yellow vein-banding, mottling or slightly disordered growth (Heidel and Rush, 1994). Pre-infection with BSBMV reduced significantly BNYVV titre (Mahmood and Rush, 1999). Moreover studies on separate and mixed infections of BSBMV and BNYVV have revealed that, upon single infection, BSBMV causes a reduction in fresh root weight whereas in mixed infections with BNYVV its pathogenic effect is less pronounced due to a suppressive action by the latter (Wisler et al., 2003).

# Aim of the study

*Beet necrotic yellow vein virus* (BNYVV) and, *Beet soil-borne mosaic virus* (BSBMV) belong to the *Benyvirus* genus and both vectored by *P. betae*. BSBMV is widely and essentially distributed in the United States and has never been reported in others countries yet.. Sequence analysis of BSBMV RNAs evidenced similar genomic organization to that of BNYVV but sufficient molecular differences to distinct BSBMV and BNYVV in two different species not serologically related.

The studies performed during my PhD have been focused on BSBMV and thus I started BSBMV studies using powerful tools as full-length infectious cDNA clones that allowed investigation of molecular interactions between plant and Benyviruses exploiting biological, epidemiological and molecular similarities/divergences between BSBMV and BNYVV, using different viral replicons based on BNYVV and BSBMV RNAs (Rep3, Rep5 and RepIII) and tagged protein expression in different plants (*Chenopodium quinoa*, *Tetragonia expansa* and *Spinacia oleracea*).

Experiments performed and results obtained during my PhD are described in chapter 1, 2 and 3 of this thesis. In chapter 1 production of BSBMV full-length cDNA clones, sequence comparison and function complementation between other *Benyvirus* are reported and then discussed. Chapter 2 is presented as a paper project where identification, role on long distance movement, symptoms expression and vector interaction of a new form of BSBMV RNA-4 are described. Chapter 3 is dedicated to molecular and functional characterisation of BSBMV RNA-4 expressed protein.

Finally a general discussion and conclusion will describe results and scientific benefits obtained. I would also discuss future prospects to continue my work and increase knowledge on the virus transmission of BSBMV and BNYVV.

Acosta-Leal, R., Fawley, M.W., Rush, C.M., 2008. Changes in the intraisolate genetic structure of Beet necrotic yellow vein virus populations associated with plant resistance breakdown. Virology 376, 60-68.
Andika, I.B., Kondo, H., Tamada, T., 2005. Evidence that RNA silencing-mediated resistance to Beet necrotic yellow vein virus is less effective in roots than in leaves. Molecular Plant-Microbe Interactions 18, 194-204.
Asher, M.J.C., Blunt, S.J., 1987. The ecological requirements of Polymyxa betae. In: Proc 50th IIRB cong, Brussels, 45-55.
Barr, K.J., Asher, M.J.C., 1992. The host range of Polymyxa betae in Britain. Plant Pathology 41, 64-68.
Biancardi, E., 2005. Brief history of sugar beet cultivation. In Biancardi E, Campbell LG, Skaracis GN & de Biaggi (eds.) Genetics and breeding of sugar beet. Science Publishers, Inc., Enfield, NH, USA, 3-9.
Blunt, S.J., Asher, M.J.C., Gilligan, C.A., 1991. Infection of sugar beet by Polymyxa betae in relation to soil temperature. Plant Pathology 40, 257-267.
Bouzoubaa, S., Guilley, H., Jonard, G., 1985. Nucleotide sequence analysis of RNA-3 and RNA-4 of beet necrotic yellow vein virus, isolates F2 and G1. Journal of General Virology 66, 1553-1564.
Bouzoubaa, S., Niesbach-Klosgen, U., Jupin, I., Guilley, H., Richards, K.E., Jonard, G., 1991. Shortened forms of beet necrotic yellow vein virus RNA-3 and -4: Internal deletions and a subgenomic RNA. Journal of General Virology 72, 259-266.
Bouzoubaa, S., Quillet, L., Guilley, H., 1987. Nucleotide sequence of beet necrotic yellow vein virus RNA-1. Journal of General Virology 68, 615-626.
Braselton, J.P., 1995. Current status of the plasmodiophorids. CRIT. REV. MICROBIOL. 21, 263-275.
Brunt, A.A., Richards, K.E., 1989. Biology and Molecular Biology of Furoviruses. Adv. Virus Res. 36, 1-32.
Canova, A., 1959. Appunti di patologie della Barbabietola. Informatore Fitopatologico 9, 390-396.
Cesarini, B., 1999. Le annersità: loro natura, prevenzione e lotta. In La barbabietola negli ambienti mediterranei. Casarini B, Biancardi E & Ranalli P (eds.) Edagricole, Bologna, Italy, pp. 273-421., 273-421.
Chiba, S., Kondo, H., Miyanishi, M., Andika, I.B., Han, C., Tamada, T., 2011. The evolutionary history of beet necrotic yellow vein virus deduced from genetic variation, geographical origin and spread, and the breaking of host resistance. Mol. Plant-Microbe Interact. 24, 207-218.
Chiba, S., Miyanishi, M., Andika, I.B., Kondo, H., Tamada, T., 2008. Identification of amino acids of the beet necrotic yellow vein virus p25 protein required for induction of the resistance response in leaves of Beta vulgaris plants. J Gen Virol 89, 1314-1323.
Coons, G.H., 1936. Improvement of the sugar beet. In: Yearbook of Agriculture, USDA, Washington, DC pp. 625-656, 625-656.
Dawei, L., Jialin, Y., Chenggui, H., Tao, L., Shucai, Q., Koenig, R., Yi, L., 1999. Detection and nucleotide analysis of RNA5 of beet necrotic yellow vein virus isolated in China. Chin J Biotechnol 15, 253-257.
Dieryck, B., Otto, G., Doucet, D., Legreve, A., Delfosse, P., Bragard, C., 2009. Seed, soil and vegetative transmission contribute to the spread of pecluviruses in Western Africa and the Indian sub-continent. Virus Res 141, 184-189.
Dunoyer, P., Pfeffer, S., Fritsch, C., Hemmer, O., Voinnet, O., Richards, K.E., 2002. Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. Plant Journal 29, 555-567.
Dureau, G., 1886. Traitè de la culture de la beterave. (2nd edn) Bureaux du J. des Fabricantes du Sucre, Paris, France.

FAO, 2009. FaoSTAT Agricolture Statistics Database. Pagina Web: http://faostat.fao.org/site/567/default.aspx - ancor.
Francis, S.A., 2006. Development of sugar beet. In Sugar beet, pp. 9-29. Edited by A. P. Draycott. Oxford: Blackwell Publishing Ltd, 9-29.
Gerik, J.S., Duffus, J.E., 1988. Differences in vectoring ability and aggressiveness of isolates of Polymyxa betae. Phytopathology 78:1340-1343 78, 1340-1343.
Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K.E., Jonard, G., 1992. Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA 2. Virology 189, 40-47.
Harju, V.A., Mumford, R.A., Blockley, A., Boonham, N., Clover, G.R.G., Weekes, R., Henry, C.M., 2002. Occurrence in the United Kingdom of Beet necrotic yellow vein virus isolates which contain RNA 5. Plant Pathology 51, 811.
Hehn, A., Bouzoubaa, S., Bate, N., Twell, D., Marbach, J., Richards, K.E., Guilley, H., Jonard, G., 1995. The small cysteine-rich protein P14 of beet necrotic yellow vein virus regulates accumulation of RNA 2 in cis and coat protein in trans. Virology 210, 73-81.
Hehn, A., Fritsch, C., Richards, K.E., Guilley, H., Jonard, G., 1997. Evidence for in vitro and in vivo autocatalytic processing of the primary translation product of beet necrotic yellow vein virus RNA 1 by a papain-like proteinase. Arch Virol 142, 1051-1058.
Heidel, G.B., Rush, C.M., 1994. Distribution of beet necrotic yellow vein virus, beet distortion mosaic virus, and un unnamed soilborne sugar beet virus in Texas and New Mexico. Plant Disease 78, 603-606.
Heidel, G.B., Rush, C.M., Kendall, T.L., Lommel, S.A., French, R.C., 1997. Characteristics of beet soilborne mosaic virus, a furo-like virus infecting sugar beet. Plant Disease 81, 1070-1076.
Heijbroek, W., Musters-van Oorschot, P.M.S., Schoone, A.H.L., 1999. Variation in pathogenicity and multiplication of beet necrotic yellow vein virus (BNYVV) in relation to the resistance of sugar-beet cultivars. European Journal of Plant Pathology 105, 397-405.
Jupin, I., Guilley, H., Richards, K.E., Jonard, G., 1992. Two proteins encoded by beet necrotic yellow vein virus RNA 3 influence symptom phenotype on leaves. EMBO J 11, 479-488.
Kanyuka, K., Ward, E., Adams, M.J., 2003. Polymyxa graminis and the cereal viruses it transmits: a research challenge. Mol Plant Pathol 4, 393-406.
Kastirr, U., Pfelistetter, E., Burgermeister, W., 1994. Virus content and virulence of Polymyxa betae Keskin isolates obtained from different regions in Europe. Journal of Phytopathology 141, 369-374.
Keller, P., Lüttge, U., Wang, X.C., Büttner, G., 1989. Influence of rhizomania disease on gas exchange and water relations of a susceptible and a tolerant sugar beet variety. Physiological and Molecular Plant Pathology 34, 379-392.
Keskin, B., 1964. Polymyxa betae n.sp., ein Parasit in den Wurzeln von Beta vulgaris Tournefort, besonders während der Jugendentwicklung der Zuckerrübe. Archiv. Mikrobiol. 49, 348-374.
Kiguchi, T., Saito, M., Tamada, T., 1996. Nucleotide sequence analysis of RNA-5 of five isolates of beet necrotic yellow vein virus and the identity of a deletion mutant. J Gen Virol 77 ( Pt 4), 575-580.
Koenig, R., Burgermeister, W., Weich, H., Sebald, W., Kothe, C., 1986. Uniform RNA Patterns of Beet Necrotic Yellow Vein Virus in Sugarbeet Roots, but Not in Leaves from Several Plant Species. J Gen Virol, 2043-2046.
Koenig, R., Jarausch, W., Li, Y., Commandeur, U., Burgermeister, W., Gehrke, M., Luddecke, P., 1991. Effect of recombinant beet necrotic yellow vein virus with different RNA compositions on mechanically inoculated sugarbeets. J Gen Virol 72 ( Pt 9), 2243-2246.
Koenig, R., Kastirr, U., Holtschulte, B., Deml, G., Varrelmann, M., 2008. Distribution of various types and P25 subtypes of Beet necrotic yellow vein virus in Germany and other European countries. Arch Virol 153, 2139-2144.

Koenig, R., Lennerfors, B.L., 2000. Genus Benyvirus. In van Regenmortel MHV, Fauquet CM & Bishop DHL (eds.) Virus Taxonomy. 7th Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, CA, pp., 917-922.
Koenig, R., Loss, S., Specht, J., Varrelmann, M., Lüddecke, P., Demi, G., 2009. A single U/C nucleotide substitution changing alanine to valine in the Beet necrotic yellow vein virus P25 protein promotes increased virus accumulation in roots of mechanically inoculated, partially resistant sugar beet seedlings. Journal of General Virology 90, 759-763.
Koenig, R., Luddecke, P., Haeberle, A.M., 1995. Detection of beet necrotic yellow vein virus strains, variants and mixed infections by examining single-strand conformation polymorphisms of immunocapture RT-PCR products. Journal of General Virology 76, 2051-2055.
Královic, J., Králová, V., 1996. Relations among Rhizomania disease, root content of potassium, sodium, α-amino nitrogen and crop yield of sugar beet. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 103, 561-570.
Kruse, M., Koenig, R., Hoffmann, A., Kaufmann, A., Commandeur, U., Solovyev, A.G., Savenkov, I., Burgermeister, W., 1994. Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existence of two major strain groups of beet necrotic yellow vein virus. J Gen Virol 75 ( Pt 8), 1835-1842.
Lauber, E., Guilley, H., Tamada, T., Richards, K.E., Jonard, G., 1998. Vascular movement of beet necrotic yellow vein virus in Beta macrocarpa is probably dependent on an RNA 3 sequence domain rather than a gene product. J Gen Virol 79 ( Pt 2), 385-393.
Lee, L., Telford, E.B., Batten, J.S., Scholthof, K.B., Rush, C.M., 2001. Complete nucleotide sequence and genome organization of Beet soilborne mosaic virus, a proposed member of the genus Benyvirus. Arch Virol 146, 2443-2453.
Legreve, A., Delfosse, P., Maraite, H., 2002. Phylogenetic analysis of Polymyxa species based on nuclear 5.8S and internal transcribed spacers ribosomal DNA sequences. Mycological Research 106, 138-147.
Legreve, A., Vanpee, B., Delfosse, P., Maraite, H., 2000. Host range of tropical and sub-tropical isolates of Polymyxa graminis. European Journal of Plant Pathology 106, 379-389.
Lemaire, O., Merdinoglu, D., Valentin, P., Putz, C., Ziegler-Graff, V., Guilley, H., Jonard, G., Richards, K.E., 1988. Effect of beet necrotic yellow vein virus RNA composition on transmission by Polymyxa betae. Virology 162, 232-235.
Lennerfors, B.-L., Lindsten, K., Koenig, R., 2000. First record of A and B type Beet necrotic yellow vein virus in sugar beets in Sweden. European Journal of Plant Pathology 106, 199-201.
Lennerfors, B.-L., Savenkov, E.I., Mukasa, S.B., Valizadeh, J.P.T., 2005. Sequence divergence of four soilborne sugarbeet-infecting viruses. Virus Genes 31, 57-64.
Lewellen, R.T., 1988. Selection for resistance to rhizomania in sugar beet. In Proceedings of the 5th International Congress Plant Pathology. Kyoto, Japan, pp. 455. 1988, 455.
Lewellen, R.T., Skoyen, I.O., Erichsen, A.W., 1987. Breeding sugarbeet for resistance to rhizomania: Evaluation of host-plant reactions and selection for and inheritance of resistance. In Proceedings of the 50th Congress of the IIRB. International Institute for Beet Research, Brussels, Belgium, pp. 139-156., 139-156.
Link, D., Schmidlin, L., Schirmer, A., Klein, E., Erhardt, M., Geldreich, A., Lemaire, O., Gilmer, D., 2005. Functional characterization of the Beet necrotic yellow vein virus RNA-5-encoded p26 protein: Evidence for structural pathgenicity determinants. Journal of General Virology 86, 2115-2125.
Liu, H., Duffus, J.E., 1988. The occurrence of a complex of viruses associated with rhizomania of sugarbeet. Phytopathology, 1583.
Lubicz, J.V., Rush, C.M., Payton, M., Colberg, T., 2007. Beet necrotic yellow vein virus accumulates inside resting spores and zoosporangia of its vector Polymyxa betae BNYVV infects P. betae. Virol J 4, 37.

Mahmood, T., Rush, C.M., 1999. Evidence of cross-protection between beet soilborne mosaic virus and beet necrotic yellow vein virus in sugar beet. Plant Disease 83, 521-526.
McGrann, G.R.D., Grimmer, M.K., Mutasa-Göttgens, E.S., Stevens, M., 2009. Progress towards the understanding and control of sugar beet rhizomania disease. Mol Plant Pathol 10, 129-141.
Mehrvar, M., Valizadeh, J.P.T., Koenig, R., Bragard, C., 2009. Iranian beet necrotic yellow vein virus (BNYVV): pronounced diversity of the p25 coding region in A-type BNYVV and identification of P-type BNYVV lacking a fifth RNA species. Arch Virol 154, 501-506.
Meunier, A., Schmit, J.-F., Stas, A., Kutluk, N., Bragard, C., 2003. Multiplex reverse transcription-PCR for simultaneous detection of beet necrotic yellow vein virus, Beet soilborne virus, and Beet virus Q and their vector Polymyxa betae KESKIN on sugar beet. Appl Environ Microbiol 69, 2356-2360.
Milford, G.F.J., 2006. Plant structure and crop physiology. In Sugar Beet pp. 30-49. Edited by A. P. Draycott. Oxford: Blackwell Publishing Ltd 2006, 30-49.
Miyanishi, M., Kusume, T., Saito, M., Tamada, T., 1999. Evidence for three groups of sequence variants of beet necrotic yellow vein virus RNA 5. Arch Virol 144, 879-892.
Morozov, S.Y., Solovyev, A.G., 2003. Triple gene block: Modular design of a multifunctional machine for plant virus movement. Journal of General Virology 84, 1351-1366.
Niesbach-Klosgen, U., Guilley, H., Jonard, G., Richards, K.E., 1990. Immunodetection in vivo of beet necrotic yellow vein virus-encoded proteins. Virology 178, 52-61.
Pelsy, F., Merdinoglu, D., 1996. Identification and mapping of random amplified polymorphic DNA markers linked to a rhizomania resistance gene in sugar beet (Beta vulgaris L.) by bulked segregant analysis. Plant Breeding 115, 371-377.
Peltier, C., Schmidlin, L., Klein, E., Taconnat, L., Prinsen, E., Erhardt, M., Heintz, D., Weyens, G., Lefebvre, M., Renou, J.-P., Gilmer, D., 2010. Expression of the Beet necrotic yellow vein virus p25 protein induces hormonal changes and a root branching phenotype in Arabidopsis thaliana. Transgenic Res.
Putz, C., Merdinoglu, D., Lemaire, O., Stocky, G., Valentin, P., 1990. Beet necrotic yellow vein virus, causal agent of sugar beet rhizomania. Plant Patholofy 69, 247-254.
Quillet, L., Guilley, H., Jonard, G., Richards, K.E., 1989. In vitro synthesis of biologically active beet necrotic yellow vein virus RNA. Virology 172, 293-301.
Rahim, M.D., Andika, I.B., Han, C., Kondo, H., Tamada, T., 2007. RNA4-encoded p31 of beet necrotic yellow vein virus is involved in efficient vector transmission, symptom severity and silencing suppression in roots. J Gen Virol 88, 1611-1619.
Ratti, C., Clover, G.R.G., Rubies Autonell, C., Harju, V.A., Henry, C.M., 2005. A multiplex RT-PCR assay capable of distinguishing beet necrotic yellow vein virus types A and B. J Virol Methods 124, 41-47.
Ratti, C., Hleibieh, K., Bianchi, L., Schirmer, A., Rubies Autonell, C., Gilmer, D., 2009. Beet soil-borne mosaic virus RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in Beta macrocarpa. Virology 385, 392-399.
Rush, C.M., 2003. Ecology and epidemiology of benyviruses and plasmodiophorid vectors. Annu Rev Phytopathol 41, 567-592.
Rush, C.M., French, R.C., Heidel, G.B., 1994. Differentiation of two closely related furoviruses using the Polymerase Chain Reaction. Phytopathology 84, 1366-1369.
Rush, C.M., Liu, H.Y., Lewellen, R.T., Acosta-Leal, R., 2006. The continuing saga of rhizomania of sugar beets in the United States. Plant Disease 90, 4-15.
Schirmer, A., Link, D., Cognat, V., Moury, B., Beuve, M., Meunier, A., Bragard, C., Gilmer, D., Lemaire, O., 2005. Phylogenetic analysis of isolates of Beet necrotic yellow vein virus collected worldwide. J Gen Virol 86, 2897-2911.

Schmidlin, L., Link, D., Mutterer, J., Guilley, H., Gilmer, D., 2005. Use of a Beet necrotic yellow vein virus RNA-5-derived replicon as a new tool for gene expression. J Gen Virol 86, 463-467.
Schmitt, C., Balmori, E., Jonard, G., Richards, K.E., Guilley, H., 1992. In vitro mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA 2: Evidence that a domain of the 75-kDa readthrough protein is important for efficient virus assembly. Proc Natl Acad Sci USA 89, 5715-5719.
Scholten, O.E., Lange, W., 2000. Breeding for resistance to rhizomania in sugar beet: A review. Euphytica 112, 219-231.
Simmonds, N.W., 1976. Evolution of Crop Plants. Longman, London, UK.
Sohi, H.H., Maleki, M., 2004. Evidence for presence of types A and B of beet necrotic yellow vein virus (BNYVV) in Iran. Virus Genes 29, 353-358.
Steddom, K., Heidel, G.B., Jones, D., Rush, C.M., 2003. Remote detection of rhizomania in sugar beets. Phytopathology 93, 720-726.
Tamada, T., 1975. Beet necrotic yellow vein virus. 144.
Tamada, T., 1999. Benyviruses. In Webster RG & Granoff A (eds.) Encyclopedia of Virology (2nd edn). Academic Press, London, UK, 154-160.
Tamada, T., Abe, H., 1989. Evidence that beet necrotic yellow vein virus RNA-4 is essential for efficient transmission by the fungus Polymyxa betae. Journal of General Virology 70, 3391-3398.
Tamada, T., Baba, T., 1973. Beet necrotic yellow vein virus from rhizomania-affected sugar beet in Japan. Ann Phytopathol Soc Japan, 325,Äì332.
Tamada, T., Kusume, T., 1991. Evidence that the 75K readthrough protein of beet necrotic yellow vein virus RNA-2 is essential for transmission by the fungus Polymyxa betae. J Gen Virol 72 ( Pt 7), 1497-1504.
Tamada, T., Kusume, T., Uchino, H., Kiguchi, T., Saito, M., 1996. Evidence that beet necrotic yellow vein virus RNA-5 is involved in symptom development of sugar beet roots. In Sherwood JL & Rush CM (eds.) Proceedings of the 3rd Symposium of the International Working Group on Plant Viruses with Fungal Vectors. pp., 49-52.
Tamada, T., Saito, M., Kiguchi, T., Kusume, T., 1990. Effect of isolates of beet necrotic yellow vein virus with different RNA components on the development of rhizomania symptoms. In: Koenig, R. (ed). Proc. of the first symposium of the international working group on plant viruses with fungal vectors. Eugene Ulmer, Stuttgart, 41-44.
Tamada, T., Shirako, Y., Abe, H., Saito, M., Kiguchi, T., Harada, T., 1989. Production and pathogenicity of isolates of beet necrotic yellow vein virus with different numbers of RNA components. Journal of General Virology 70, 3399-3409.
Verchot-Lubicz, J., Torrance, L., Solovyev, A.G., Morozov, S.Y., Jackson, A.O., Gilmer, D., 2010. Varied movement strategies employed by triple gene block-encoding viruses. Mol. Plant-Microbe Interact. 23, 1231-1247.
Ward, L., Koenig, R., Budge, G., Garrido, C., McGrath, C., Stubbley, H., Boonham, N., 2007. Occurrence of two different types of RNA-5-containing beet necrotic yellow vein virus in the UK. Arch Virol 152, 59-73.
Whitney, E.D., Duffus, J.E., 1986. Biotic diseases and disorders – Disease development. In Compendium of beet diseases and insects. Edited by E. D. Whitney and J. E. Duffus. St Paul: The American Phytopathological Society.
Winner, C., 1993. History of the crop. In: D.A. Cooke and R.K. Scoot (eds.). The Sugar Beet Crop. Chapmann & Hall, London, pp. 1-35, 1-35.
Wisler, G.C., Lewellen, R.T., Sears, J.L., Wasson, J.W., Liu, H.Y., Wintermantel, W.M., 2003. Interactions between Beet necrotic yellow vein virus and Beet soilborne mosaic virus in Sugar Beet. Plant Disease 87, 1170-1175.

Ziegler-Graff, V., Richards, K., Guilley, H., Jonard, G., Putz, C., 1985. Cell-free Translation of Beet Necrotic Yellow Vein Virus: Readthrough of the Coat Protein Cistron. J. Gen. Virol. 66, 2079-2087.

# Chapter 1

# Construction and analysis of genomic, full-length infectious clones of *Beet soil-borne mosaic virus* (BSBMV)

# CHAPTER I

# Construction and analysis of genomic, full-length infectious clones of *Beet soil-borne mosaic virus* (BSBMV)

## Introduction: viruses strategy

Depending of the host cell for their multiplication and protein expression, viruses minimize the size of their functional genome. During co-evolution with host plants, they developed many different strategies for efficient regulation and expression of their own proteins.

The majority of plant viruses possess single-stranded RNAs of positive polarity, so they can be directly used by the ribosomes for protein synthesis. On the other hand, negative-stranded single-stranded RNA viruses should be first transcribed to produce the messenger molecule for that region, minus-sense RNA genomes are therefore associated with the viral polymerase to produce infectious viral particles. Moreover there are ambisense RNA viruses, like the Tenuivirus *Rice stripe virus* (RSV), where proteins may be encoded by both genomic RNA and its complementary strand (Mandahar, 2006).

Initiation of protein synthesis in eukaryotes occurs mainly at the 5'-end of an mRNA molecule and ribosomes generally find the start codon with linear scanning mechanism (Kozak, 1986, 1999). Translation is then initiated at the AUG start codon within the best context and terminates when encountering a UAA, UAG or UGA stop codon (Caskey et al., 1968; Kozak, 1986). In plants, the most frequent context is AAACA**AUG**GC (Fütterer and Hohn, 1996), which is related to the mammalian sequence GCCRCC**AUG**G (Kozak, 1999): the optimal translation initiation motif includes a purine (R) at position -3 and the G at position +4. During evolving mechanisms, many viruses have developed several strategies, to express their polycistronic mRNAs that represent exceptions to the first AUG rule.

In general four levels of gene regulation expression can occur:

- genome segmentation;
- transcriptional regulation;
- translational regulation;
- post-translational regulation.

## *1.1 Regulation of gene expression through the genome segmentation*

Many viruses split they genome in multiple DNAs or RNAs fragments (Mandahar, 2006). Fragmentation of the viral genome has two major advantages:

- Each genome segment can contain one or more ORFs but, usually, the coding sequences are more accessible for the translation by ribosomes;
- Multipartite genomes make finer the control of expression for genes which coding sequences are separated on different genomic segments.

As presented in the general introduction, Benyviruses (BNYVV and BSBMV) have multipartite genome composed by four to five RNAs.

## *1.2 Regulation of gene expression at the transcriptional level*

Viruses may specifically express one or another protein from a genomic molecule by controlling the messenger RNAs (mRNAs):

- **Splicing** of viral mRNAs generates new coding sequences in the mRNA by removing parts of it. This kind of regulation is well described for plant viruses with DNA genome. Splicing is essential for the replication of *Wheat dwarf virus* (WDV) (Schalk et al., 1989) and for infectivity of *Cauliflower mosaic virus* (CaMV) (Kiss-Laszlo et al., 1995).
- **Sub-genomic (sg) RNAs**. A large number of RNA viruses, whether of positive or negative polarity, can produce one or more sgRNAs species that derive from the genome by internal initiation of RNA synthesis on the complementary gRNA strand. SgRNAs correspond to the mRNAs of the 3' proximal genes on polycistronic viral RNAs and they make possible the translation of coding sequences downstream the 5' proximal ORFs that are not required for replication. This

strategy is widely used by plant viruses and allows, for instance, the expression of TGB proteins of BNYVV (Gilmer et al., 1992) and, putatively, BSBMV.

## *1.3 Regulation of gene expression at the translational level*

During the initiation step of plant viruses translation, trans-regulation can occur with viral encoded proteins that specifically enhance translation of a downstream ORF in a bicistronic or a polycistronic mRNA as reported for several Caulimoviruses.

During translation initiation **leaky scanning** allows the expression of proteins from ORFs in the case of polycistronic mRNAs:

- Two ORFs can be consecutive on the same mRNA, so termination of the translation of the 5' proximal ORF is followed by a re-initiation at the second ORF, presumably without the release of the 40S ribosomal subunit.
- Two ORFs with in-frame initiation codons. The second start codon is preferred to the first one that is bypassed by ribosomes because the latter is placed in a suboptimal context different from the aforementioned AACA**AUG**GC (in plants) or is a non-AUG alternative start codon ((Kozak, 1989); (Ryabova et al., 2006)).
- Overlapping ORFs are the most frequent mechanism where the AUG codon of the second ORF overlaps the UGA termination codon of the first ORF. TGB are a group of three proteins whose genes generally overlap on the genome and leaky scanning is proposed to allow the expression of the third gene from TGB (TGBp3) of plant viruses like BNYVV (Zhou and Jackson, 1996) (Verchot et al., 1998) (Gilmer et al., 1992).

In some cases, "jumping" of the ribosomal machinery, allows ribosomes to "ignore" particular regions within the leader sequence of an mRNA that can contain short ORFs and encode just the downstream longer ORF. This mechanism is referred as **shunting** and transfers the ribosome from a donor to an acceptor site on the same mRNA, without involvement of mRNA scanning between these two sites. This mechanism is favoured by a strong RNA secondary structure on the leader sequence, as for CaMV (Futterer et al., 1990). However, no plus-sense RNA virus has yet been reported to use such translation strategy (Mandahar, 2006).

During the elongation step of translation, viruses can use a **frameshift** strategy to synthetize two proteins that are identical in their N-terminal region up to the frameshift point, but differ in their C-terminal region. This strategy is the result of ribosomes switch

by one nucleotide towards the 5' (-1 nt) or 3' (+1 nt) extremity of mRNA. Essential signals in the RNA for frameshift are represented by an heptanucleotide (called slippery) sequence where frameshift occurs, separated by four to nine nucleotides from a downstream hairpin structure (Drugeon et al., 1999).

At the level of termination, regulation can occur by **readthrough** strategy where leaky terminators are recognized by tRNAs that add another amino acid instead of stopping translation and continue through next triplets to the final termination codon. This strategy is reported for many plant RNA viruses (Maia et al., 1996) and enables the expression of the BNYVV CP-RT proteins (Ziegler-Graff et al., 1985).

## *1.4 Regulation of gene expression at the post-translational level*

Proteolytic cleavage strategy can produce separate structural and non-structural proteins from a viral polyprotein. This strategy is well documented for the polyprotein of Potyviruses where three distinct proteinases, P1, HC-Pro and NIa can release nine different proteins from a single polyprotein as for *Tobacco etch virus* (TEV)(Allison et al., 1985) or for BNYVV replicase (Hehn et al., 1997).

## *1.5 Reverse genetic approach to study viral protein expression*

The term reverse genetics in virology refers to the use of recombinant DNA technology to convert viral genomes into (cDNA) and generate viruses from the cloned DNA. Since manipulating RNA genomes still remains cumbersome, genetic manipulation of RNA viruses has exclusively relied upon cDNA intermediates from which biologically active RNA molecules were generated. Usually for RNA viruses, full-length cDNA copies of each viral genomic RNA are flanked at 5' end with a transcription promoter (e.g. T7, T3 or SP6) and appropriated restriction enzyme at the extremity that allows, after RT-PCR, cloning in bacterial plasmid. Viral RNAs may be produced *in vitro* from these constructs by using the appropriate RNA polymerase. Biologically active transcripts produced *in vitro* RNAs from full-length cDNA clones with bacterial phage promoters have been first time reported for poliovirus (Racaniello and Baltimore, 1981). *In vitro* transcription was successfully employed to produce infectious transcripts from cloned cDNA of several plant viruses: *Brome mosaic virus* (BMV) (Ahlquist and Janda, 1984), *Tobacco mosaic virus* (TMV)

(Dawson et al., 1986), *Beet necrotic yellow vein virus* (BNYVV) (Quillet et al., 1989) and many others.

Reverse genetics has now entered in the virologist custom to study viral cycles for which full-length infectious clones constitute a very powerful tool. Benefits of such approach are manifold, in particular:

- Working with single viral transcripts from constructs can guarantee single-virus infection and then avoid frequent mixed-infections that occur with soil-borne beet's viruses as BNYVV, BSBMV, BSBV and/or BVQ (Ratti et al., 2005);
- Function of single RNA and/or proteins can be investigate to understand pathogenicity mechanism and biological functions of the virus using different kinds of plants (*C. quinoa, Beta macrocarpa*, *Tetragonia expansa* and *Beta vulgaris*).

The use of reverse genetics permitted the characterisation of proteins involved in BNYVV pathogenicity (Jupin et al., 1992), cell-to-cell movement (Gilmer et al., 1992), systemic movement (Lauber et al., 1998), transmission (Rahim et al., 2007) and BSBMV pathogenicity (Ratti et al., 2009).

For all these reasons we decided to produce full-length cDNA clones of all BSBMV genomic RNAs.

## Full-length cDNA clones of BSBMV isolate MRM06

*Beet soil-borne mosaic virus* (BSBMV) is one of the member of the *Benyvirus* genus and consists of four single stranded positive RNAs with CAP at 5' and poly-A at 3' end, packaged into rod-shaped particles (Lee et al., 2001). Recently Ratti et al. (2009) characterized the RNA-3 of a new BSBMV isolate (MRM06) and demonstrated that it is replicated and encapsidated by the BNYVV replication machinery (BNYVV RNA-1 and -2, also called Stras12). The symptoms induced by BSBMV RNA-3, together with Stras12 helper strain, on *C. quinoa* leaves are more similar to the necrotic local lesion caused by BNYVV RNA-5 p26 that to severe chlorotic local lesions, or yellow spot symptoms, induced by BNYVV RNA-3 encoded p25. Moreover, comparing amino acid sequence the authors reported that BSBMV RNA-3 p29 is much closer to BNYVV RNA-5 p26 (43%) than to BNYVV RNA-3 p25 (23%). Alignment of full-length BNYVV and BSBMV RNA-3 sequence revealed nucleotide identity of 61% and a 20 nts sequence called "coremin" (5'-GUCCGAAGACGUUAAACUAC-3'), conserved between both RNAs and also present in

BNYVV RNA-5 and BSBMV RNA-4. Kinetics of local and systemic symptoms appearance on *B. macrocarpa* plants inoculated by BNYVV Stras12 helper strain supplemented with BNYVV RNA-3 or BSBMV RNA-3 transcripts, showed less efficient and delayed systemic movement of Stras12 supplemented with BSBMV RNA-3 when compared with BSBMV wild type infection. Such a difference could be linked to the presence of two "coremin" motifs within BSBMV (RNA-3 and -4) and the high accumulation of BNYVV RNA-3 that could compensate the lack of a second coremin. (Ratti et al., 2009).

## *2.1 Synthesis of a full-length infectious cDNA clone of BSBMV RNA-1*

Sugar beet plants were grown on soil infested with *P. betae* carrying BSBMV isolate kindly supplied by Marc Richard-Molard (ITB, Paris). Total RNAs were extracted from infected sugar beet root using Trizol reagent (Invitrogen, Carlsbad, CA) and then used to obtain all full-length infectious cDNA clone of BSBMV.

BSBMV RNA-1 full length cDNA was synthesized by ImProm-II Reverse Transcriptase system (Promega, Madison, CA) using olido(dT) primer and then amplified by PCR using Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies Italia) with a sense primer (5'-AAA*GCGGCCGC*TAATACG-ACTCACTATAGAAATTCTTCCCATTCGCCATCATTGAATCGTT-3') containing a T7 RNA polymerase promoter (underlined) and an *Not*I restriction site (*italic*), coupled with the oligo(dT) reverse primer (5'-AAA*ACGCGT*$(T)_{25}$-3') containing *Mlu*I site (*italic*). Primers match the extremities of the only BSBMV RNA-1 reference sequence to date published on GenBank database (NC_003506). PCR product was digested by *Not*I and *Mlu*I restriction enzymes, gel purified using Wizard SV Gel and PCR clean-up system (Promega, Madison, CA) and then cloned into *Not*I – *Mlu*I digested pUC19 (Fermentas) to obtain LB106 clone which was transferred into the *Escherichia coli* strain JM109. Ligation was performed according to Rapid DNA Ligation Kit (Fermentas).
LB106 and full-length infectious BNYVV RNA-2 type B clone, called pB2-14 (Quillet et al., 1989) were linearized with *Mlu*I and *Sal*I, respectively, before run-off transcription with the RiboMAX large-scale RNA production system T7 (Promega, Madison CA) in the presence of Cap analogue $m^7G(5')ppp(5')G$. Equal amounts of the transcripts were used to rub-inoculate *C. quinoa* leaves. As previously reported, chlorotic spots appeared 7 days post inoculation (dpi) on *C. quinoa* leaves rub-inoculated with BNYVV RNA-1 and -2 (Stras12). The appearance of local lesions indicated effective replication and cell-to-cell

movement of the viral RNA-1 and -2. *C. quinoa* inoculated with LB106 and BNYVV RNA-2 transcripts didn't show symptoms even after 14 dpi.

Nucleotide sequences at 5' and 3' ends of LB106 were analysed looking for mutation putatively responsible for non-infectivity of full length BSBMV RNA-1 cDNA clone. Total RNA extraction from BSBMV-infected sugar beet roots was used to amplify two different amplicons performing RT-PCR reactions using primer pairs reported on Table 3:

- Amplicon A (5' terminal zone) was digested by *Not*I and *Xba*I restriction enzymes, then gel purified and cloned in *Not*I – *Xba*I digested pUC19 to obtain LB126 clone.
- Amplicon B (3' terminal zone) was gel purified and inserted into the pGEM-T vector (Promega) to obtain clone LB153.

**5' and 3' end cloning of BSBMV RNA-1**

| PCR Amplicon | Primers | Length (bp) | Restriction Enzyme | Clone |
|---|---|---|---|---|
| Amplicon A | BSBMV1 Not T7 F<br>BSBMV1 R10 | 1,334 | *Not*I - *Xba*I | LB126 |
| Amplicon B | BSBMV1 F11<br>OligodT Mlu R | 859 | *Spe*I - *Mlu*I | LB153 |

**Primers 5' Race on BSBMV RNA-1**

| Name | Sequence | Use |
|---|---|---|
| BSBMV1 R 294-275 | 5'-AGATCATGCTTCCAAATGGC-3' | RT |
| BSBMV1 R 195-176 | 5'-CGAAACAGCAAAACCTCCAT-3' | 5' Race PCR |

| | Primers | Length (bp) | Restriction Enzyme | Clone |
|---|---|---|---|---|
| Amplicon C | BSBMV1 Not T7 5'Race F<br>BSBMV1 R10 | 1,342 | *Not*I - *Xba*I | EUB02 |

Table 3. BSBMV RNA-1 primer: 5' and 3' end cloning or 5' Race pairs for obtain full

Sequences of clones were determined and compared with LB106 sequence. Three nucleotide substitutions were found in the ORF1 coding sequence ($G^{987}A$, $T^{6447}C$ and $T^{6450}C$) and one was found in the 3' UTR ($G^{6521}A$). LB126 was digested by *Not*I and *Xba*I and cloned in *Not*I-*Xba*I-digested LB106 to obtain LB128 where 5' terminal zone of BSBMV RNA-1 was replaced. Moreover LB153 was digested by *Spe*I and *Mlu*I and cloned in *Spe*I-*Mlu*I-digested LB128 to obtain LB158 where both 5' and 3' terminal zones of BSBMV RNA-1 were replaced.

No symptoms were detected 14 dpi on *C. quinoa* leaves rub-inoculated with transcripts obtained from LB158 and pB2-14 cDNA clones.

RNA extracts from infected BSBMV sugar beet roots was used to perform BSBMV RNA-1 5' RACE characterization according to a method adapted from (Bensing et al., 1996) using primers reported on Table 3. Nucleotide sequence of BSBMV RNA-1 obtained by 5' RACE PCR showed 5 extra nucleotides after 6th nucleotide $^{6}$GATCT$^{11}$ and three nucleotide substitution (T$^{18}$C, T$^{19}$C and G$^{21}$A), when compared with LB158 or published BSBMV RNA1 sequence (NC_003506). According to our 5' RACE results, new BSBMV RNA-1 5'-end primer was designed (BSBMV1 Not T7 5'Race F – 5'-AAA*GCGGCCGC*TAATACG-ACTCACTATAGAAATTC**GATCT**TTCCCA**CC**C**A**CCATCATTG-3') and amplicon C was obtained after RT-PCR reaction. Amplicon C was digested with *Not*I and *Xba*I restriction enzyme and cloned in *Not*I - *Xba*I digested LB158 cDNA clone to obtain a new full length BSBMV RNA-1 cDNA clone (pUC17) with 6680 nucleotides in length excluding the polyA.
Mixture of the *in vitro* transcripts produced from plasmids pUC17 (t17) and pB2-14 (t2-14) were rub inoculated on *C. quinoa* leaves and chlorotic spots appeared 7 dpi confirming that pUC17clone is a full length infectious cDNA clone of BSBMV RNA-1 that is able to trans-replicate BNYVV RNA-2.
Northen blot analyses were performed on chlorotic lesions from *C. quinoa* leaves inoculated with t17 + t2-14 and Stras12. Total RNAs were extracted with Trizol reagent RNA extraction (Invitrogen, San Diego, CA) and encapsidated RNAs were either obtained using Protocol TM (Jupin et al., 1990). Membranes were probed with $^{32}$P-labeled riboprobes specifically complementary to RNA-1 and -2 of BNYVV and BSBMV as reported in the appendix. Results demonstrated that transcripts obtained from BSBMV RNA-1 cDNA are fully functional as they insure the replication of BNYVV RNA-2 and are as well encapsidated.

Western blot analysis was performed on chlorotic lesions appeared on *C. quinoa* leaves inoculated with tUC17 + tBS2-14 and Stras12 using rabbit anti-BNYVV CP, as described in the appendix, raised against the BNYVV coat proteins. As showed in Fig. 7 BNYVV CP is specifically detected in both sample, confirming encapsidation of BSBMV RNA-1 by BNYVV Coat Protein.

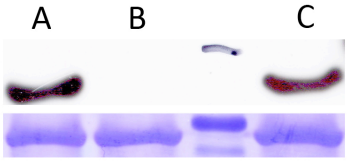



Fig. 7. Western Blot analysis with anti-BNYVV CP of different inoculated *C. quinoa* leaves spots: (A) Stras12 isolate; (B) Mock; (C) tUC17 + t2-14.

## *2.2 Synthesis of a full-length infectious cDNA clone of BSBMV RNA 2*

Full length BSBMV RNA-2 has been obtained from three PCR amplicons performed from the same cDNA using primer pairs reported in Table 4. PCR amplicons were cloned separately on pUC19 vector, after cleavage with the appropriated restriction enzyme:

- *Not*I – *Xma*I for amplicon D to obtain clone LB5
- *Xma*I – *Xba*I for amplicon E to obtain clone LB7
- *XbaI* – *Bgl*II for amplicon F to obtain clone LB9

**Full length BSBMV RNA-2 cDNA**

| PCR Amplicon | Primers | Length (bp) | Restriction Enzyme | Clone |
|---|---|---|---|---|
| Amplicon D (5' zone) | BSBMV2 NotI T7 F<br>BSBMV2 XmaI R | 1,843 | *Not*I - *Xma*I | LB5 |
| Amplicon E (middle zone) | BSBMV2 XmaI F<br>BSBMV2 XbaI R | 1,492 | *Xma*I - *Xba*I | LB7 |
| Amplicon F (3' zone) | BSBMV2 XbaI F<br>OligodT25 BglII R | 1,371 | *Xba*I - *Bgl*II | LB9 |

Table 4. BSBMV RNA-2 primer pairs used to obtain full-length cDNA clone.

After sub-cloning as described on Figure 1 we obtained a full length BSBMV RNA-2 cDNA clone, called LB38. This clone produced non infectious RNAs after *in vitro* transcription and co-inoculation with *in vitro* transcripts produced from pB15, the full-length infectious clone of BNYVV RNA-1 type B (Quillet et al., 1989).

As previously reported for BSBMV RNA-1 we decided to investigate the correct nucleotide sequence at 5' and 3' ends. Different RT-PCR amplicons were obtained using primer pairs reported on Table 5:

- Amplicon G was cloned into pGEM-T vector to obtain LB164
- Amplicon H was cloned into pGEM-T vector to obtain LB156

**5' and 3' end cloning of BSBMV RNA-2**

| PCR Amplicon | Primers | Length (bp) | Restriction Enzyme | Clone |
|---|---|---|---|---|
| Amplicon G (5' end) | BSBMV1 Nco F<br>BSBMV2 Xma R | 1,147 | *Nco*I - *Xma*I | LB164 |
| Amplicon H (3' end) | BSBMV2 F1<br>Oligo dT25 BglII R | 1,414 | *Xba*I - *Bgl*II | LB156 |

**Primers 5' Race on BSBMV RNA-2**

| Name | Sequence | Use |
|---|---|---|
| BSBMV2 384-365 | 5'-TTGAGGGTAACTGGAAACCG-3' | RT |
| BSBMV2 293-274 | 5'-TAACAGGTAAGCTTGCGGCT-3' | 5' Race PCR |

| | Primers | Length (bp) | Restriction Enzyme | Clone |
|---|---|---|---|---|
| Amplicon I (5' Race RT-PCR) | BSBMV2 Not T7 AA F<br>BSBMV1 Nco R | 681 | *Not*I - *Xma*I | EUB17 |

Table 5. BSBMV RNA-2 primer: 5' and 3' end cloning or 5' Race pairs used to obtain or characterize full-length cDNA clone.

Sequence analysis of LB156 and LB164 clones revealed several nucleotide substitutions when compared with LB38 sequence. Three nucleotide substitution were found in the ORF2 coding sequence ($C^{972}T$, $C^{1018}T$ and $G^{1080}A$), one was found in the ORF6 ($C^{4052}T$) and one in the 3'UTR ($C^{4613}T$) followed by insertion of two nucleotide ($^{4613}TG^{4615}$). LB156 was digested by *Xba*I and *Bgl*II and cloned in *Xba*I – *Bgl*II digested LB38 to obtain LB157. LB164 was then digested by *Nco*I and *Xma*I and cloned in *Nco*I – *Xma*I digested LB157 to obtain LB177.

Moreover, 5' RACE characterisation of BSBMV RNA-2 (see Table 3 for details) revealed that the first two GG nucleotides were incorrect and needed to be substituted by AA nts ($G^1A$ and $G^2A$). We corrected the sequence by designing a new primer on 5' BSBMV RNA-2 and performing RT-PCR from which the amplicon I was obtained and then cloned into pGEM-T vector to obtain EUB17. 5' BSBMV RNA-2 was extracted from EUB17 by *Not*I - *Nco*I digestion and cloned into *Not*I-*Nco*I digested LB177 to obtain a new full length BSBMV RNA-2 cDNA clone (EUB22).

The three full-length cDNA constructs in pUC19 (LB38, LB177 and EUB22) were linearized with *Bgl*II then run-off transcribed. All transcripts were separately rub-inoculated with pB15's transcripts onto *C. quinoa* leaves but no symptoms were detected 14 dpi.

### *2.2.1 Are TGBs proteins fully functional?*

As described by Guilley et al. (2009), Benyviruses need three main functions for successful multiplication on a plant host: (i) replication, provided by RNA-1 that encodes the viral RdRp; (ii) movement from the initial point of infection, provided by triple gene block (TGB) proteins expression; (iii) suppression of the host RNA silencing mechanism, provided by cysteine rich p14 protein. As RNA2 is involved in the last two functions virus

infection in *C. quinoa* leaves was successful obtained supplementing BNYVV RNA-1 with rep3-P30, encoding the *Tobacco mosaic virus* (TMV) movement protein, and Rep5-P19 encoding the *Tomato bushy stunt virus* (TBSV) silencing suppressor protein (Guilley et al., 2009).

In order to verify TGB and p14 proteins functionality of EUB22 clone its transcript has been supplemented by pB15 and Rep3-30 transcripts and rub-inoculated together on *C. quinoa* as described before. Chlorotic spots were observed on inoculated leaves 7 d.p.i. This result suggests that EUB22 is replicated by BNYVV RdRp and that its p14 protein is correctly expressed and full functional as observed chlorotic spots were identical to those induced by Stras12 isolate and no small necrotic local lesions as those induced by BNYVV RNA-1 and -2 carrying a non functional p14 protein (Gilmer et al., 1992). The most important information obtained from this results was that TMV p30 protein expression assisted cell-to-cell movement of the chimeric virus BNYVV RNA-1 and BSBMV RNA-2, suggesting that one, two or all TGB proteins carried by EUB22 clone were not functional.

Bleykasten-Grosshans et al. (1997) described three cDNA clones: Rep42, Rep1315 and Rep15 that can express, by BNYVV RNA 3-derived replicon (Jupin et al., 1990), first (TGBp1), second plus third (TGBp2 and TGBp3) or third (TGBp3) BNYVV's TGB proteins, respectively. Transcripts of pB15 and EUB22 clones have been rub-inoculated on *C. quinoa* together with transcripts obtained from Rep42, Rep1315 or Rep15 clones as described in Table 6. As *C. quinoa* symptoms analysis (yellow spots appearance) confirms complementation of movement proteins just using Rep3 p13-p15 clone (Table 6) we concluded that some problems occur, maybe on nucleotide sequence, in TGBp2 and TGBp3 of EUB22 clone and that TGBp1 of the same clone is fully functional.

| Inoculum | Symptoms (7 dpi) |
|---|---|
| Stras12 | YS |
| t15 + tEUB22 | --- |
| t15 + tEUB22 + tRep42 | --- |
| t15 + tEUB22 + tRep1315 | YS |
| t15 + tEUB22 + tRep15 | --- |

Table 6. Mechanical inoculation of *C. quinoa* plant, local symptoms after inoculation of BNYVV Stras12 helper strain or BNYVV RNA-1 transcripts (t15) and BSBMV RNA-2 transcripts (tEUB22) with three TGB proteins express in different viral replicon. The appearance of yellow spots (YS) indicates the correct replication and cell-to-cell movement of the virus.

cDNA from total RNA extraction from infective BSBMV sugar beet roots was used to performed PCR on BSBMV TGBp2 and TGBp3 using primer sense BSBMV p13 F (5'-AAA*CCATGG*ATGTCTAGAGAAATAAC-3', *Nco*I site in *italic*) and primer reverse BSBMV p15 R (5'-AAA*GGATCC*TTAACTATGATACCAAAAC-3', *Bam*HI site in *italic*) designed at 5' end of

TGBp2 coding sequence and 3' end of TGBp3 coding sequence, respectively. PCR amplicons were digested with *Nco*I and *Bam*HI and cloned into the BNYVV RNA-3 derived replicon (Rep3), and six different Rep3 BS p13-p15 cDNA clones (from I to VI) were selected during cloning procedure. Only Rep3 BS p13-p15 cDNA clone (IV) induced yellow spots symptoms 7 d.p.i. when inoculated together with pB15 and EUB22 transcripts. Comparison of Rep3 BS p13-p15(IV) and EUB22 sequences permitted to identified one mutation on EUB22 p13 ORF ($G^{3362}A$) that induce amino acid substitution ($V^{31}M$) and one nucleotide deletion on EUB22 p15 ORF ($^{3975}T^{3977}$) which is responsible for the production of a 11 amino acids shorter p15 (13,1 kDa instead of 14,6 kDa).

**PCR side-directed mutagenesis**

| BSBMV p13 ORF | | | BSBMV p15 ORF | | |
|---|---|---|---|---|---|
| | First PCR | | | First PCR | |
| | Primers | bp | | Primers | bp |
| 5' - end | BSBMV2 Xma F<br>BSBMV2 p13 Mut R | 1581 | 5' - end | BSBMV2 XbaI<br>BSBMV2 p15 Mut R | 750 |
| 3' - end | BSBMV2 p13 Mut F<br>OligodT25 BglII R | 1293 | 3' - end | BSBMV2 p15 Mut F<br>OligodT25 BglII R | 678 |
| | Second PCR | | | Second PCR | |
| | Primers | bp | | Primers | bp |
| Fusion | BSBMV2 XbaI<br>Oligo dT25 BglII R | 1372 | Fusion | BSBMV2 XbaI<br>Oligo dT25 BglII R | 1373 |

Table 7. PCR site-directed mutagenesis of p13 and p15 protein of BSBMV RNA-2.

PCR site-directed mutagenesis on EUB22 cDNA clones was performed using primer specifically designed on p13 ORF (reported on Table 7). Such modified amplicon was used to replace a DNA fragment on EUB22 cDNA clone using restriction enzymes *Xba*I and *Bgl*II to obtain MD231 clone. A second PCR site-directed mutagenesis on MD231 cDNA clone was performed to correct nucleotide deletion on p15 ORF using specific primer (Table 7) and then substitute the sequence, as described above, on MD231 cDNA clone to obtain pUC29.

The following rub-inoculations on *C. quinoa* leaves were performed, after in vitro transcription, as reported in Table 8:

| Inoculum | Symptoms (7 dpi) |
|---|---|
| Stras12 | YS |
| t15 + tEUB22 + Rep1315 | YS |
| t15 + tMD231 + tRep15 | YS |
| t15 + t29 | YS |

Table 8. Leaves symptoms 7 dpi on *C. quinoa* mechanical inoculated leaves combining BNYVV RNA-1 transcripts (t15) with BSBMV RNA-2 transcripts (tEUB22) and p13-p15 TGB proteins or tMD231 and p15 TGB proteins or full-length infectious BSBMV RNA-2 transcripts (t29).
YS = yellow spots

Yellows spots appeared 7 d.p.i. on all samples, allowing us to conclude that pUC29 is an infectious full length cDNA clones of BSBMV RNA-2 that is able to be trans-replicated by BNYVV RNA-1. Northern Blot and Western Blot analysis of chlorotic lesion appeared on *C. quinoa* confirmed that BSBMV RNA-2 is correctly replicated by BNYVV RNA-1 and the coat protein (encoded by RNA-2) is able to encapsidate both RNAs (Fig. 8).

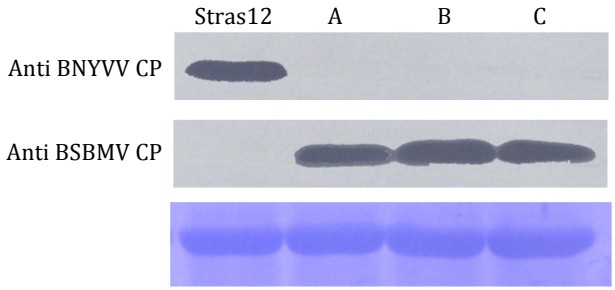



Fig. 8. Western Blot analysis with anti-BNYVV CP and BSBMV CP of different inoculated *C. quinoa* leaves spots: (A) Stras12 isolate; (B) t15 + tEUB22 + Rep1315; (C) t15 + tMD231 + tRep15; (D) t15 + tUC29.

## *2.3 Synthesis of a full-length infectious cDNA clone of BSBMV RNA 4*

Specific oligonucleotide primer, complementary to the 5' end of BSBMV RNA-4 sequence (NC_003508), was used for full length BSBMV RNA4 cDNA clone production. After total RNAs extraction from BSBMV infected sugar beet roots, RT reaction was performed with oligod$T_{25}$ primer followed by High Fidelity PCR amplification using primer BSBMV RNA-4 T7 F (5'-AAA*GCGGCCGC*TAATACGACTCACTATAG aaattcaaaactcaaaaatataattttgtatttccagttg-3', *Not*I restriction enzyme site is *in italic* and T7 nucleotides promoter is underlined) and primer oligod$T_{25}$ *Bgl*II R (5'-AAA*AGATCT*$(T)_{25}$-3', *Bgl*II site is in italic). PCR amplicon of full length BSBMV RNA-4 was then digested with *Not*I and *Bgl*II restriction enzyme and ligated into *Not*I-*Bgl*II digested pUC19 to obtain pUC47 clone.

Analysis of pUC47 nucleotide sequence revealed a 1,730 nts long BSBMV RNA-4, excluding the 3' poly(A) tail. pUC47 was linearized with *Bgl*II before run-off transcription as previous described obtaining the transcripts tUC47. tUC47 were then inoculated together with the BNYVV Stras12 helper strain on *C. quinoa* leaves and 7 dpi necrotic spots were observed confirming infectivity of full length BSBMV RNA-4 cDNA clone. Further experiments and results about BSBMV RNA-4 characterisation are described in Chapter 3.

## III. BSBMV MRM06 isolate: description and BNYVV comparison

The nucleotide sequence and genomic organization of new isolate BSBMV MRM06 were determined and compared to BSBMV EA isolate described by Lee et al. (2001).

BSBMV – MRM06 RNA-1 is 6,679 nt in length (accession number JF513082), excluding the 3' poly(A) tail, and contains a single ORF which encode a 238 kDa polypeptide with a putative MTR/HEL and RNA dependent RNA polymerases (RdRp) located in the N- and C-terminal parts, respectively. MRM06 RNA-1 shares 99.4% nucleotide identity with EA RNA-1, in particular, some nucleotide differences have been evidenced on MRM06 RNA-1 5' end (5'-AAATTC**GATCT**TTCCCACCCACC-3', where insertions are indicated in bold and mutations are underlined) that resulted essential for infectivity of the full length cDNA clone. Comparing MRM06 and EA amino acid sequence, 47 amino acids substitution are located in the MTR domain (major part between aa 110-190) liable of 96,6% identity and just 2 amino acids exchanges are located in the RdRp domain ($E^{1824}K$ and $I^{2058}M$) liable of 99,7% identity. Sequence motifs of a type 1 putative MTR domain have been identified at amino acid positions 218-228, 271-278 and 407-414 on MRM06 isolate according to amino acid positions 220-230, 273-280, and 407-420 reported for EA isolate by Lee (2001). The NTP-binding helicase motif (Gly-X-X-Gly-X-Gly-Lys-Ser, where X represents any amino acid), that is predicted to be involved in duplex unwinding during viral RNA replication and translation, is located at amino acid positions 944-951 or 941-948 on isolate EA or MRM06, respectively. Moreover, the motif GDD (Gly-Asp-Asp), characteristic of RdRp is located at amino acid positions 1939-1941 or 1936-1938 of isolate EA or MRM06, respectively. Finally, all the previous described sequence motifs are conserved

between RNAs-1 of BSBMV and BNYVV type A, B or P. BSBMV MRM06 RNA-1 and BNYVV RNA-1 share 77% nucleotides sequence identity leading to 84% and 87,5% on amino acids identity for MTR/HEL and RdRp domains, respectively. Amino acids identity and higher amino acids similarity (around 92 %) between BSBMV – MRM06 and BNYVV RNAs-1 ORFs are in accordance with capability of BSBMV RNA-1 to replicate BNYVV RNA-2. This result demonstrate the complementarity and the identical functions of polypeptides encoded by BSBMV and BNYVV RNA-1.

BSBMV – MRM06 RNA-2 is 4,615 nts in length (accession number JF513083), excluding the 3' poly(A) tail, and contains six ORFs that encode proteins involved in different functions: viral Capsid Protein (CP), Read-through (RT), Triple Gene Block protein (TGB1, TGB2 and TGB3) and a cysteine-rich (Cys-R) protein. High nucleotide identity (99,8%) has been obtained comparing MRM06 and EA RNA-2. MRM06 isolate showed 4 nucleotide substitutions ($A^{1}G$, $A^{2}G$, $G^{99}C$, $G^{119}C$) and one deletion ($^{93}G^{95}$) in the 5' UTR, 2 nucleotide substitution on RT protein ($A^{1567}G$ and $A^{2000}T$) translated, respectively, to $M^{475}V$ and $Q^{617}L$ amino acid mutation and one nucleotide substitution on Cys-R protein ($C^{4272}T$) which leads to a silent mutation. Amino acids sequence between BSBMV isolates MRM06 and EA is highly conserved within five proteins (CP, TGB proteins and Cys-R) with only two changes on RT protein. As reported in Table 9 comparison between MRM06 and BNYVV RNA-2 from type A, B and P showed high identity score within Triple Gene Block proteins (TGB1: 74.7 - 75%; TGB2: 81.4 – 82.2%; TGB3: 63.6 – 65.2%), medium identity score between CP (56 – 57.6%) and RT (58.3 – 58.7%) proteins and low identity score on Cys-R protein (32.6 – 34.6%). Similitude between proteins encoded by BSBMV and BNYVV RNAs-2 increase in terms of amino acid similarity suggesting a common function of each corresponding ORF product. Moreover, according to our results we can speculate about complementation of TGB2 and TGB3 proteins (p13 and p15 respectively) between BNYVV and BSBMV on cell-to-cell movement function. In other words, as BNYVV p13 and p15 proteins, expressed by viral vectors, are able to restore a defective cell-to-cell movement function of mutated, then not functional, BSBMV p13 and p15 proteins, we can conclude that TGB2 and TGB3 proteins from BNYVV and BSBMV clearly exert the same function during virus infection.

As previously reported by Ratti et al. (2009) BSBMV – MRM06 RNA-3 is 1,720 nts in length (accession number EU410955) and contains one ORF that encode a 29 kDa protein (p29) involved on virus symptoms. In this case, only 4 nts substitutions were detected when the

sequence was compared with the RNA-3 sequence of the EA BSBMV isolate described by Lee (NC_003507): one substitution was found in the 5' UTR ($G^{355}A$) and three along the p29 coding sequence ($T^{808}A$, $A^{887}G$ and $T^{1000}C$) which leads, respectively, to $D^{127}E$, $N^{154}D$ and a silent mutation that determine amino acid identity score of 99,2%. As shown in Table 9 BSBMV RNA-3 encoded p29 share around 23% of amino acid sequence identity with the 25 kDa ORF of BNYVV RNA-3.

BSBMV – MRM06 RNA-4 is 1,730 nts in length (accession number FJ424610) and show an high identity nucleotide score (99,4%) with RNA-4 described by Lee (NC_003508) between nucleotides 1-608 and 1,138-1,730 where we identified one nucleotide substitution ($T^{219}C$) and two nucleotides deletion ($^{269}C^{270}$ and $^{296}C^{297}$) on 5' UTR, one nucleotide substitution on ORF1 ($C^{521}T$) leading to silent mutation and three nucleotide substitutions on 3'UTR ($T^{1572}C$, $T^{1575}C$ and $G^{1594}T$). Lee et al. (2001) identified one ORF encoding a protein with predicted mass of 13 kDa that show 20% amino acid identity with p31 (31 kDa protein encoded by BNYVV RNA-4) increasing to 42% amino acid identity if just the N-terminal half of p31 is considered. However, the RNA-4 of MRM06 isolate shows 529 additionally nucleotides between positions 608-1,138, that determine a new BSBMV RNA-4 ORFs organization: two putative ORFs have been identified, the first one (nucleotides 383 – 1,234) encode a protein with predicted mass of 32 kDa (ORF1; p32) and the second one (nucleotides 885 – 1,244) express an expected product of 13 kDa (ORF2; p13). BSBMV RNA-4 p32 shows 49% amino acid identity and 68% amino acid similarity with p31 sequence.

In the table 9, we reported nucleotide and amino acid sequence identity analyses of the viral proteins of BSBMV isolate MRM06 compared with BNYVV type A, B and P and the *Rice stripe necrosis virus* (RSNV), a candidate member of Benyvirus genus (Lozano and Morales, 2009).

Table 9. Comparative nucleotide (a) and amino acid (b) sequence identity analyses of the viral proteins of *Beet soil-borne mosaic virus* (BSBMV) isolate MRM06 and *Benyvirus* viruses[a,b]

| **(a)** | **Nucleotide identity score** | | | |
|---|---|---|---|---|
| **BSBMV** – (MRM06) | RNA1 (****) | RNA2 (***) | RNA3 (****) | RNA4 (****) |
| **BSBMV** – (EA) | 99,4 % | 99,8 % | 99,8 % | 69,1 % |
| BNYVV – A type | 77,0 % | 66,5 % | 55,0 % | 56,1 % |
| BNYVV – B type | 72,8 % | 66,4 % | 55,4 % | 47 % |
| BNYVV – P type | 77,0 % | 66,6 % | 55,2 % | 55,7 % |
| RSNV | 53,8 % | 45,2 % | na | na |

| **(b)** | **Amino acid identity (and similarity) score** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **BSBMV** – (MRM06) | **RNA1 238 kDa** | | **RNA2** | | | | | | **RNA3** | **RNA4** |
| | **MeT/H[c]** | **RdRp[c]** | **CP (21 kDa)** | **RT (75 kDa)** | **TGB1 (42 kDa)** | **TGB2 (13 kDa)** | **TGB3 (15 kDa)** | **Cys-R (14 kDa)** | **29 kDa (ORF1)** | **32 kDa (ORF1)** |
| BSBMV – (EA) | 96,6 % (97,2 %) | 99,7 % (99,8 %) | 100 % | 99,7 % (99,9 %) | 100 % | 100 % | 100 % | 100 % | 99,2 % (99,6 %) | 31,4 % (33,6 %) |
| BNYVV – A type | 84,3 % (92,4 %) | 87,5 % (92,8 %) | 57,1 % (73,3 %) | 58,7 % (72,1 %) | 75 % (88,5 %) | 82,2 % (89 %) | 65,2 % (75,0 %) | 32,6 % (49,6 %) | 22,4 % (36,7 %) | 49,8 % (67,7 %) |
| BNYVV – B type | 85,0 % (92,9 %) | 87,6 % (92,7 %) | 57,6 % (72,8 %) | 58,3 % (71,7 %) | 75 % (88,5 %) | 81,4 % (88,1 %) | 65,2 % (75,0 %) | 34,6 % (50,4 %) | 22,8 % (35,9 %) | 49,8 % (68,4 %) |
| BNYVV – P type | 84,7 % (92,7 %) | 87,3 % (92,8 %) | 56 % (73,3 %) | 58,7 % (72,2 %) | 74,7 % (88,5 %) | 82,2 % (89 %) | 63,6 % (75,0 %) | 32,6 % (49,6 %) | 22,0 % (35,5 %) | 49,5 % (68,1 %) |
| RSNV | 37,5 % (55,3 %) | 55,4 % (70,5 %) | 29,8 % (44 %) | 20,7 % (34,2 %) | 34,7 % (52,5 %) | 41,5 % (56,9 %) | 24,1 % (44,5 %) | 15,6 % (29,9 %) | na[d] | na |

Data shown are expressed as percentages of nucleotide or amino acid identity and amino acid similarity in brackets.

[a]Using MacVector version 11.1

[b] The sequence of Benyviruses used for these analyses are available in the Genbank under the following accession numbers: *Beet soil-borne mosaic virus* (BSBMV), isolate MRM06 (JF513082, JF513083, EU410955, FJ424610) isolate EA (NC_003506, NC_003503, NC_003507, NC_003508); *Beet necrotic yellow vein virus* (BNYVV) A type (D84410, D84411, D84412, D84413), B type (X05147, X04197, M36894, M36897), P type (HM126464, HM117903, DQ682454, DQ682453); *Rice stripe necrosis virus* (EU099844, EU099845).

[c] The predicted ORF on RNA1 was divided on N-terminal 1500 amino acids and C-terminal 600 amino acids to compare the methyltransferase/helicase (Met/H) and RNA-dependent RNA polymerase (RdRp) regions, respectively. CP, coat protein; RT, read-through; TGB, triple gene block; Cys-R, cysteine-rich

[d]Not applicable or not present

Ahlquist, P., Janda, M., 1984. cDNA cloning and in vitro transcription of the complete brome mosaic virus genome. MOL. CELL. BIOL. 4, 2876-2882.
Allison, R.F., Sorenson, J.C., Kelly, M.E., 1985. Sequence determination of the capsid protein gene and flanking regions of tobacco etch virus: Evidence for synthesis and processing of a polyprotein in potyvirus genome expression. PROC. NATL. ACAD. SCI. U. S. A. 82, 3969-3972.
Bensing, B.A., Meyer, B.J., Dunny, G.M., 1996. Sensitive detection of bacterial transcription initiation sites and differentiation from RNA processing sites in the pheromone-induced plasmid transfer system of Enterococcus faecalis. Proceedings of the National Academy of Sciences of the United States of America 93, 7794-7799.
Bleykasten-Grosshans, C., Guilley, H., Bouzoubaa, S., Richards, K.E., Jonard, G., 1997. Independent expression of the first two triple gene block proteins of beet necrotic yellow vein virus complements virus defective in the corresponding gene but expression of the third protein inhibits viral cell-to-cell movement. Molecular Plant-Microbe Interactions 10, 240-246.
Caskey, C.T., Tompkins, R., Scolnick, E., Caryk, T., Nirenberg, M., 1968. Sequential translation of trinucleotide codons for the initiation and termination of protein synthesis. Science 162, 135-138.
Dawson, W.O., Beck, D.L., Knorr, D.A., Grantham, G.L., 1986. cDNA cloning of the complete genome of tobacco mosaic virus and production of infectious transcripts. PROC. NATL. ACAD. SCI. U. S. A. 83, 1832-1836.
Drugeon, G., Urcuqui-Inchima, S., Milner, M., Kadaré, G., Valle, R., Voyatzakis, A., Haenni, A.-L., Schirawski, J., 1999. The strategies of plant virus gene expression: models of economy. Plant Science 148, 77-88.
Futterer, J., Gordon, K., Sanfacon, H., Bonneville, J.-M., Hohn, T., 1990. Positive and negative control of translation by the leader sequence of cauliflower mosaic virus pregenomic 35S RNA. EMBO J. 9, 1697-1707.
Fütterer, J., Hohn, T., 1996. Translation in plants - Rules and exceptions. Plant Molecular Biology 32, 159-189.
Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K.E., Jonard, G., 1992. Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA 2. Virology 189, 40-47.
Guilley, H., Bortolamiol, D., Jonard, G., Bouzoubaa, S., Ziegler-Graff, V., 2009. Rapid screening of RNA silencing suppressors by using a recombinant virus derived from beet necrotic yellow vein virus. J Gen Virol 90, 2536-2541.
Hehn, A., Fritsch, C., Richards, K.E., Guilley, H., Jonard, G., 1997. Evidence for in vitro and in vivo autocatalytic processing of the primary translation product of beet necrotic yellow vein virus RNA 1 by a papain-like proteinase. Arch Virol 142, 1051-1058.
Jupin, I., Guilley, H., Richards, K.E., Jonard, G., 1992. Two proteins encoded by beet necrotic yellow vein virus RNA 3 influence symptom phenotype on leaves. EMBO J 11, 479-488.
Jupin, I., Richards, K.E., Jonard, G., Guilley, H., Pleij, C.W., 1990. Mapping sequences required for productive replication of beet necrotic yellow vein virus RNA 3. Virology 178, 273-280.
Kiss-Laszlo, Z., Blanc, S., Hohn, T., 1995. Splicing of cauliflower mosaic virus 35S RNA is essential for viral infectivity. EMBO J 14, 3552-3562.
Kozak, M., 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44, 283-292.
Kozak, M., 1989. Context effects and inefficient initiation at non-AUG codons in eucaryotic cell-free translation systems. MOL. CELL. BIOL. 9, 5073-5080.
Kozak, M., 1999. Initiation of translation in prokaryotes and eukaryotes. Gene 234, 187-208.

Lauber, E., Guilley, H., Tamada, T., Richards, K.E., Jonard, G., 1998. Vascular movement of beet necrotic yellow vein virus in Beta macrocarpa is probably dependent on an RNA 3 sequence domain rather than a gene product. J Gen Virol 79 ( Pt 2), 385-393.
Lee, L., Telford, E.B., Batten, J.S., Scholthof, K.B., Rush, C.M., 2001. Complete nucleotide sequence and genome organization of Beet soilborne mosaic virus, a proposed member of the genus Benyvirus. Arch Virol 146, 2443-2453.
Lozano, I., Morales, F., 2009. Molecular characterisation of Rice stripe necrosis virus as a new species of the genus Benyvirus. European Journal of Plant Pathology 124, 673-680.
Maia, I., Séron, K., Haenni, A.-L., Bernardi, F., 1996. Gene expression from viral RNA genomes. Plant Molecular Biology 32, 367-391.
Mandahar, C.L., 2006. Multiplication of RNA plant viruses. Springer, Netherlands.
Quillet, L., Guilley, H., Jonard, G., Richards, K.E., 1989. In vitro synthesis of biologically active beet necrotic yellow vein virus RNA. Virology 172, 293-301.
Racaniello, V.R., Baltimore, D., 1981. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc Natl Acad Sci USA 78, 4887-4891.
Rahim, M.D., Andika, I.B., Han, C., Kondo, H., Tamada, T., 2007. RNA4-encoded p31 of beet necrotic yellow vein virus is involved in efficient vector transmission, symptom severity and silencing suppression in roots. J Gen Virol 88, 1611-1619.
Ratti, C., Bianchi, L., Resca, R., De Biaggi, M., Henry, C.M., Jackeviciene, E., Cvjetkovic, B., Rubies Autonell, C., 2005. Incidence of sugar beet soil-borne viruses in sugar beet growing countries. Proceedings of the sixth Symposium of the International Working Group on Plant Viruses with Fungal Vectors, Bologna, Italy, September 5-7.
Ratti, C., Hleibieh, K., Bianchi, L., Schirmer, A., Rubies Autonell, C., Gilmer, D., 2009. Beet soil-borne mosaic virus RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in Beta macrocarpa. Virology 385, 392-399.
Ryabova, L., Pooggin, M., Hohn, T., 2006. Translation reinitiation and leaky scanning in plant viruses. Virus Research 119, 52-62.
Schalk, H.J., Matzeit, V., Schiller, B., Schell, J., Gronenborn, B., 1989. Wheat dwarf virus, a geminivirus of graminaceous plants needs splicing for replication. EMBO J 8, 359-364.
Verchot, J., Angell, S.M., Baulcombe, D.C., 1998. In vivo translation of the triple gene block of potato virus X requires two subgenomic mRNAs. J. Virol. 72, 8316-8320.
Zhou, H., Jackson, A.O., 1996. Expression of the barley stripe mosaic virus RNAβ "triple gene block". Virology 216, 367-379.
Ziegler-Graff, V., Richards, K., Guilley, H., Jonard, G., Putz, C., 1985. Cell-free Translation of Beet Necrotic Yellow Vein Virus: Readthrough of the Coat Protein Cistron. J. Gen. Virol. 66, 2079-2087.

# Chapter 2

# *Beet soil-borne mosaic virus* RNA-4 encodes a 32 kDa protein essential for virus transmission through *Polymyxa betae*

# *Beet soil-borne mosaic virus* RNA-4 encodes a 32 kDa protein essential for virus transmission through *Polymyxa betae*

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## Abstract

*Beet soil-borne mosaic virus* (BSBMV) together with *Beet necrotic yellow vein virus* (BNYVV) are members of the *Benyvirus* genus transmitted by *Polymyxa betae.* BSBMV has been identified in Texas and up to now is only reported in the United States and BNYVV has a worldwide distribution. BSBMV and BNYVV are morphologically similar but serologically distinct viruses and possess a similar genomic organization. RNAs -1 and -2 are essential for infection and replication while RNAs -3 and -4 play important roles on disease development and vector-mediated infection in sugar beet roots. However, sufficient molecular differences permit to separate BSMBV and BNYVV in two different species. Recently it has been demonstrated the ability of BNYVV helper strain (RNA-1 and -2) to replicate and encapsidate BSBMV RNA-3 suggesting a common and conserved viral RNA selection mechanism for both viruses. Here, we molecularly and functionally characterized a new 1,730 nucleotides long form of BSBMV RNA-4. We demonstrated that BSBMV RNA-4 is amplified by BNYVV viral machinery *in planta* and that it can substitute BNYVV RNA-4 for an efficient transmission through the vector *P. betae* in *Beta vulgaris* plants. Two putative ORFs have been identified on its sequence that could encode for a 32 kDa (p32) and a 13 kDa products. BSBMV p32 amino acid sequence is much closer to BNYVV RNA-4 p31 respect to BSBMV RNA-4 p13 protein previous described. Western blot analysis of FLAG-tagged p32 revealed a high molecular weight protein that suggests

BSBMV RNA-4 encoded protein post translation modifications or its strong interaction with host protein(s) that need to be better investigate. Moreover expression of BSBMV p32 protein by BNYVV RNA-5-based viral vector (Rep5) demonstrated that the p32 protein itself and not full-length RNA-4 is essential for the viral transmission.

# Introduction

*Beet soil borne mosaic virus* (BSBMV) is a member of *Benyvirus* genus together with *Beet necrotic yellow vein virus* (BNYVV), the casual agent of Rhizomania disease, which induce abnormal rootlet proliferation and is widespread in the sugar beet growing area in Europe, Asia and America (Peltier et al., 2008). *Rice stripe necrosis virus* (RSNV) has been also recently proposed as new member of *Benyvirus* genus (Lozano and Morales, 2009).

In nature BSBMV and BNYVV are vectored by the plasmodiophorid *Polymyxa betae* and have similar host range, particles number and morphology. BSBMV is widely distributed only in the United States and, up to date, it has not been reported in others countries. It was first identified in Texas in 1988 as a sugar beet virus morphologically similar but serologically distinct from BNYVV (Heidel et al., 1997).

Subsequent sequence analysis of BSBMV RNAs evidenced similar genomic organization to that of BNYVV but sufficient molecular differences to distinct BSBMV and BNYVV into two different species ((Lee et al., 2001); (Rush, 2003)).

Benyviruses field isolates usually consist of four RNA species but some BNYVV isolates contain a fifth RNA. RNAs -1 contains a single long ORF encoding polypeptide that shares amino acid homology with known viral RNA-dependent RNA polymerases (RdRp), methyltransferase (MTR) and helicases (HEL). RNAs 2 contains six ORFs: capsid protein is located at the 5'-terminus followed by a leaky UAG stop codon, whose suppression leads to the expression of the read-through translation protein (RT) involved on virus transmission; triple gene block proteins (TGB) that are required for cell-to-cell virus movement. The sixth 14 kDa ORF is a cysteine-rich protein with post-translation gene silencing suppressor activity (Dunoyer et al., 2002). RNAs -3 encodes a single protein with predicted masses of 25 kDa for BNYVV and 29 kDa for BSBMV that shares just 23% amino acid sequence identity. RNAs -3 are involved on disease symptoms and are essential for virus systemic movement. BNYVV RNA-4 encoded one 31 kDa protein that plays a multifunctional role in enhanced symptom expression, root-specific silencing suppression

and efficient vector interactions and virus transmission by *P. betae* (Rahim et al., 2007).

Lee et al. (2001) described a single putative ORF on the 1,203 nts long BSBMV RNA-4 with a predicted mass (13 kDa) considerably smaller than the BNYVV RNA-4 31 kDa (p31) protein. Some BNYVV isolates contain a fifth RNA, encoding 26 kDa protein that improve virus infections and accumulation in the hosts ((Heijbroek et al., 1999); (Link et al., 2005)). It has been recently observed that p29 encoded by BSBMV RNA-3 is much closer to the RNA-5 encoded p26 than to BNYVV RNA-3 encoded p25 (Ratti et al., 2009).

Studies of interaction between BNYVV and BSBMV infecting the same beet plants, through mechanical inoculation, showed a high degree of reciprocal cross-protection, a phenomenon that usually occurs between virus strains, suggesting a close relation between the two species (Mahmood and Rush, 1999). However, the absence of immunological cross-reaction between the two viruses, and the susceptibility to BSBMV of plants resistant to BNYVV ((Lee et al., 2001); (Wisler et al., 2003)) indicated that the two viruses are distinct. Ratti et al. (2009) demonstrated that BSBMV RNA-3 can be trans-replicated and trans-encapsidated by the BNYVV helper strain (RNA-1 and -2) but such replication does not occur in the presence of BNYVV RNA-3 due a replication competition, confirming the strong relation between the two viruses.

In our study, we reported a new form of BSBMV RNA-4. We confirmed the capability of BNYVV helper strain to replicate, encapsidate and spread BSBMV RNA-4 *in planta*. Using BNYVV helper strain, we also demonstrated that BSBMV RNA-4 could substitute BNYVV RNA-4 for an efficient transmission through the vector *P. betae* in *B. vulgaris* plants. However frame shift experiment on BSBMV RNA 4's ORFs and BSBMV p32 protein expression by BNYVV RNA-5-based viral vector (Rep5) demonstrated, for the first time, that just p32 protein expression is essential for an efficient transmission of the virus.

# Materials and methods

## *2.1 Synthesis of a full-length infectious cDNA clone of BSBMV RNA 4*

Sugar beet plants were grown on BSBMV infected soil kindly supplied by Marc Richard-Molard (ITB, Paris). Total RNAs were extracted from infected sugar beet roots using Trizol reagent (Invitrogen, Carlsbad, CA). RNA-4 cDNA was synthesized by Improm-II Reverse Transcriptase system (Promega, Madison, CA) using oligo(dT) primers and then amplified by PCR using Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies Italia). The

forward primer BSBMV4 NotI T7 and reverse primer OLIGOdT25 BglII R were used. PCR products were digested by *Not*I and *Bgl*II and cloned in *Not*I-*Bgl*II-digested pUC19 (Fermentas) to obtain a full length cDNA clone, pUC47 (GenBank accession no. FJ424610). Full-length pUC47, linearized by *Bgl*II, was used to synthetize BSBMV RNA-4 infectious run-off transcripts, named tUC47. In a similar way BNYVV RNA-4 full-length cDNA was obtained by RT-PCR from BNYVV B-type infected sugar beet roots and cloned into pUC19 between *Not*I and *Bgl*II restriction sites to obtain pUC-4B clone.

| Primer | Sequence (5' - 3') | Restriction enzyme |
|---|---|---|
| BSBMV4 NotI T7 F | AAA*GCGGCCGC*TAATACGACTCACTATAGAAATTCAAA ACTCAAAAATATAATTTTGTATTTCC | *Not*I |
| OLIGOdT25 BglII R | AAAA*GATCT*$T_{(25)}$ | *Bgl*II |
| BSBMV Flag-p32 NcoI | AAA*CCATGG*ACTACAAGGACGACGACGACAAG**CCAGGA** GCCGATGTGGAGATTTGCCG | *Nco*I |
| BSBMV p32 BamHI | AAA*GGATCC*TCACTGAAAATCTTGTTCGAAACAAAAC | *Bam*HI |
| BSBMV Flag-p13 NcoI | AAA*CCATGG*ACTACAAGGACGACGACGACAAG**CCAGGA** TGGATCAGTATACCCTTCCCTC | *Nco*I |
| BSBMV p13 BamHI | AAA*GGATCC*TCACAAATAATCACTGAAAATCTTG | *Bam*HI |
| BNYVV FlagP31 XmaI F | AAA*CCCGGG*ATGGACTACAAGGACGACGACGACAAG**CC AGGA**GCTGATGGAGAGATATGTCGGTG | *Xma*I |
| BNYVV p31 SalI BamHI R | AAA*GTCGACGGATCC*CTAATCGTGATAAAAGACAAAC | *Sal*I and *Bam*HI |
| FS P32 F NcoI | AAA*CCATGG*ACTGGCCGAT*TGGAGATTTG | *Nco*I |
| FS P32 F1 | TCACTATAGATGCTGGATCAG | --- |
| FS P32 R1 | TATACTGATCCAGCATCTATAGTG | --- |
| FS P32 F2 | CCCTCTCAATGAGAATGGTTGTGC | --- |
| FS P32 R2 | TCATTGAGAGGGAAGGGTATACTG | --- |
| FS P32 F3 | ACGATTTGTGTAACGTGGTTTGGTTGGAG | --- |
| FS P32 R3 | ACCACGTTACACAAATCGTTGAGTGG | --- |

Table 1. **BNYVV and BSBMV RNA-4 primers**. Restriction enzyme sequences appear in italic, whereas, Flag and T7 promoter sequences are underlined and Gly and Pro amino acids codon are in bold. The Oligo(dT) tract is represented by $T_{(25)}$. Mutated nucleotides are in red and * is G deletion on p32 nucleotide sequence.

Putative ORF sequences encoding 32 kDa and 13 kDa proteins were amplified by PCR using a sense primer (BSBMV Flag-p32 NcoI for ORF1 and BSBMV Flag-p13 NcoI for ORF2, Table 1), containing *Nco*I restriction site and a FLAG epitope sequence, and an antisense primer (BSBMV p32 BamHI and BSBMV p13 BamHI, respectively for ORF1 and ORF2) carrying a *Bam*HI site.

The PCR-amplified fragments were cloned into Rep3 viral vector (Bleykasten-Grosshans et al., 1997) using *Nco*I and *Bam*HI restriction enzyme obtaining Rep3-Flag-p32, Rep3-Flag-p13 and clones. Similarly, BNYVV FlagP31 XmaI F and BNYVV p31 SalI BamHI R primers pair (Table 1) were also used to amplify the BNYVV RNA-4 p31 sequence further cloned into Rep3 viral vector, between *Xma*I and *Bam*HI sites to obtain Rep5-Flag-p31 construction. A new set of sense primers depleted of the FLAG nucleotide sequence from primers described above was used to obtain Rep5-p32, Rep5-p13 Rep5-p31 clones

carrying wild type p32, p13 and p31 proteins into Rep5 viral vector (Schmidlin et al., 2005), respectively. FLAG epitope was also inserted in frame within p32 sequence (between amino acids 202 and 203) in pUC47 to produce BSBMV RNA-4-FlagIN.

Frame shift of both BSBMV RNA-4's ORFs was obtained with PCR site directed mutagenesis modifying ORFs sequences by four nucleotides insertion ($^{1}$C$^{2}$, $^{505}$C$^{506}$, $^{529}$C$^{530}$ and $^{689}$TAA$^{690}$) and one nucleotide deletion ($^{10}$G$^{10}$). Three different PCR reaction were necessary to obtain the final frame shift PCR amplicon that was cloned, using *Nco*I and *Bam*HI restriction sites, into Rep5 viral vector obtaining Rep5-FSp32p13 clone.

All constructs and PCR fragments were characterized by restriction fragment analysis and sequencing.

## *2.2 Viral inoculations*

Run-off transcripts were produced and inoculated together with the helper strain Stras12, containing BNYVV RNA-1 and -2 (Quillet et al., 1989), onto *Chenopodium quinoa* leaves, as previously described by Klein et al. (2007).

A transmission test, adapted from Koenig and Stein (1990), has been developed on *B. vulgaris* plants using aviruliferous *P. betae*. Seeds of rhizomania-susceptible sugar beet cultivars were planted on sterile sand. To produce Sap inoculum, *C. quinoa* leaves were rub-inoculated using BNYVV Stras12, supplemented with BNYVV RNA-3 *in vitro* transcripts and with transcripts derived from tUC47 and constructs described above. Mechanical inoculations of beet's roots were performed on ten days old sugar beet seedling using sap (inoculum) of *C. quinoa* infected leaves grounded in 50 mM $KH_2PO_4$ buffer containing 0.8% of macaloid. A set of 10 young sugar beet plants were placed in a glass tube with a diameter of 2 cm together with 3 ml of inoculum containing 0.09 g of 400 mesh carborundum powder and then mixed for 1 minute. Inoculated sugar beet seedlings were planted in a sterile sands box (10x10cm) and inoculated, one week later, with aviruliferous *P. betae* zoospore suspension derived from protozoa-infected sugar beet roots floated in a water solution for 2 hours. After a week, new sugar beet seeds were sow in the same box. Two months later roots from non inoculated sugar beet plants were collected, dried and then pulverized into new sterile sands where new sugar beet seeds were sow. Finally, three week later, roots were analysed by Transmission Electron Microscopy (TEM), RT-PCR, Western blot and Northern blot. All plants were grown in a greenhouse at 25 °C with a photoperiod of 16h.

## *2.3 Analysis of infection products*

Total RNA contents were isolated from analysed samples using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Encapsidated RNAs were obtained using Protocol TM (Jupin et al., 1990). BSBMV RNA-4 northern blot analyses were performed using random $^{32}$P-labeled DNA probes corresponding to nucleotides 6 to 603 for 5' probe and nucleotides 926 to 1728 for 3' probe whereas BNYVV RNAs analysis was performed as previously described ((Link et al., 2005); (Schmidlin et al., 2005)). Virus and *P. betae* infections were detected by RT-PCR as described (Ratti et al., 2005). Viral proteins were detected by western blotting after SDS-PAGE separation of total protein extracts from symptomatic samples and whole asymptomatic leaves or roots as described by Link et al. (2005) using rabbit anti-BNYVV CP and anti-BSBMV CP raised against the BNYVV or BSBMV coat protein, respectively. Rabbit Anti-Flag polyclonal IgG and mouse Anti-Flag monoclonal antibody or Anti-Flag-Peroxidase monoclonal antibody (Sigma-Aldrich, MO, USA) were also used.

## *2.4 Sequence analyses*

Sequences were treated with Vector NTI advance 11.5 software (Invitrogen Corp., Carlsbad, CA) and/or MacVector 11.1 software.

# Results

## *3.1 BSBMV RNA-4 full length clone*

An unexpected 1,730 nucleotides long BSBMV RNA-4 (FJ424610) has been detected from roots of different sugar beets grown on BSBMV infested soil. We obtained a full-length BSBMV RNA-4 cDNA clones sequence analysis of which revealed high identity (~100%) with the published BSBMV RNA-4 sequence (NC_003508) between nucleotides 1-608 and 1,138-1730 with 5 nts substitutions and 2 nts deletion. One nucleotide substitutions ($T^{219}C$) and two C nucleotides deletion ($^{269}C^{269}$ and $^{297}C^{297}$) were found in the 5' UTR, one nucleotide substitution was found in the ORF1 ($C^{521}T$) leading a silent mutation and finally three nucleotides substitution were found in the 3' UTR ($T^{1572}C$, $T^{1575}C$ and $G^{1594}T$).

However, the new BSBMV RNA-4 form presented 529 additionally nucleotides between positions 608 – 1,138 (Figure 1).

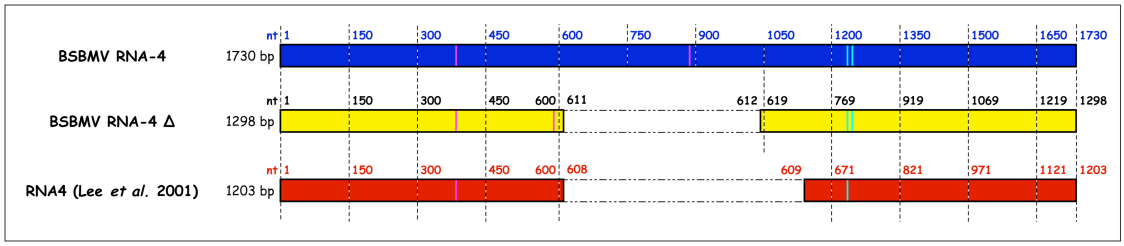


Figure 1. BSBMV RNA-4 comparisons . New BSBMV RNA-4 form is compared with RNA sequence described by Lee *et al.* (2001) and with an RNA4 deleted form obtained after 14 serial mechanical inoculations experiments. Magenta lines indicate ORFs start codons and cyan lines indicate stop codons.

Two putative ORFs have been identified, the first one (nucleotides from 383 to 1,234), encodes a protein with a predicted mass of 32 kDa (p32) whereas the second (nucleotides 885 - 1,244) putatively codes a 13 kDa protein (p13).

*In vitro* transcripts produced from the full length infectious BSBMV RNA-4 cDNA clone pUC47 (t47) were rub-inoculated, together with BNYVV Stras12 helper strain (Quillet et al., 1989), onto *C. quinoa* plant leaves. Typical chlorotic spots appeared 7 days post inoculation (d.p.i.) on leaves inoculated with Stras12 helper strain but local necrotic spots appeared when tUC47 transcripts were added (Figure 2). We confirmed that such appearance of necrotic spots corresponded to the effective replication of BSBMV RNA-4 and expression of encoded protein using northern blot analysis. We performed such

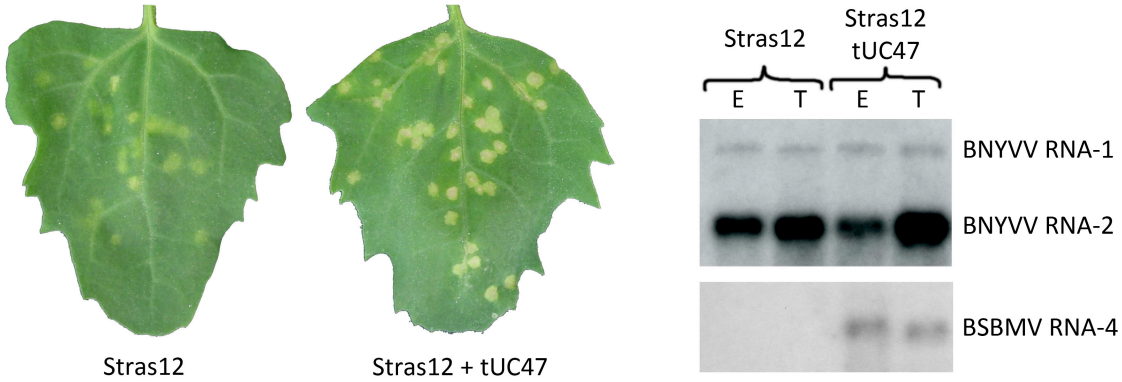


Figure 2. Symptoms on *C. quinoa* leaves obtained 7 dpi after rub inoculation of BNYVV RNA-1 ad -2 (left) supplemented with full-length transcripts of BSBMV RNA-4 (tUC47) that induces necrosis spots (right).

Figure 3. Northern blot analyses of *C. quinoa* lesion contents 7.d.p.i. induced by Stras12 helper strain alone (lanes 1 and 2) or supplemented with transcripts of BSBMV RNA-4 (tUC47; lanes 3 and 4). Two different RNAs protocol extraction were used: (T) Trizol protocol or (E) TM protocol. RNAs were detected using riboprobes complementary to BNYVV RNA-1 and -2 (upper panel) or to 5' end of BSBMV RNA-4 (lower panel).

hybridization on both total RNAs and encapsidated viral RNA, using BNYVV RNA-1 and -2 and BSBMV RNA-4 specific radiolabelled probes (Figure 3). BSBMV RNA-4 progeny was indeed detected within different inoculated plants as *Tetragonia expansa* and *Beta macorcarpa* (data not shown), indicating, as for BSBMV RNA-3 (Ratti et al., 2009), the efficient replication of BSBMV RNA-4 by BNYVV RNA-1 and -2 complex. Encapsidation of BSBMV RNA-4 by the BNYVV coat protein was confirmed by TM extraction protocol, (Jupin et al., 1990) and virus purification (Tamada et al., 1989).

## *3.2 Deleted forms of BSBMV RNAs*

Shortened forms of both BNYVV RNA-3 and RNA-4 have been reported after successive mechanical inoculation to *C. quinoa* leaves (Bouzoubaa et al., 1991). Sap of BSBMV infected sugar beet roots was used for the first mechanical transmission of a 7 days serial inoculation of the virus onto leaves of *C. quinoa* plants. At every passage, total RNA was extracted from a part of inoculated *C. quinoa* leaves from which cDNA was obtained using OLIGOdT25 Bgl II primer. The same primer was then coupled with specific forward primer designed at 5' end of BSBMV RNA-3 or BSBMV RNA-4 in a PCR reaction.

A deleted form of BSBMV RNA-4 (1298 bp) appeared after 14 passages (Figure 1) and its sequence analysis showed deletion of 433 nucleotides between positions 611 and 1044 of BSBMV RNA-4 new form (acc. no. FJ424610) and two nucleotides substitution in the p32 coding sequence ($C^{521}T$ and $T^{1130}G$) leading to silent and $W^{250}G$ mutations, respectively. Moreover BSBMV RNA-4 deleted form shows a single putative ORF that share N-terminal amino acid sequence (1 – 76 aa) and C-terminal amino acid sequence (221 – 283 aa) with p32 coding sequence. Interestingly, the putative ORF of BSBMV RNA-4 previously described (Lee et al., 2001) also share N-terminal amino acid sequence (1 – 75 aa) with p32 coding sequence but shows a completely different sequence in the last 33 amino acids of C-terminal part with 6.2% amino acids identity score.

A chimeric form of BSBMV RNAs -4 and -3 (1,142 bp, acc. no. JF513084) was as well detected after 21 serial mechanical inoculated passages and remained unaltered since fortieth passage (Figure 4). Sequence analysis of BSBMV Chimeric RNA revealed that nucleotides 1 to 557 were from BSBMV RNA-4 (1 – 557 nts of FJ424610) and nucleotides 557 to 1,142 were from BSBMV RNA-3 (1,138 – 1,720 nts of EU410955). Such chimeric RNA encodes putatively two ORFs . The first has a predicted size of 7kDa corresponding to the first 60 aa of BSBMV p32 (nucleotides 383 – 562), the second ORF (nucleotides 562 –

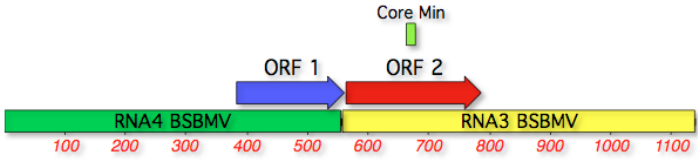


Figure 4. Schematic organization of Chimeric BSBMV RNA. ORF-1 and -2 have predicted masses of 7 and 9 kDa, respectively. Coremin sequence is present between nts 662 and 681.

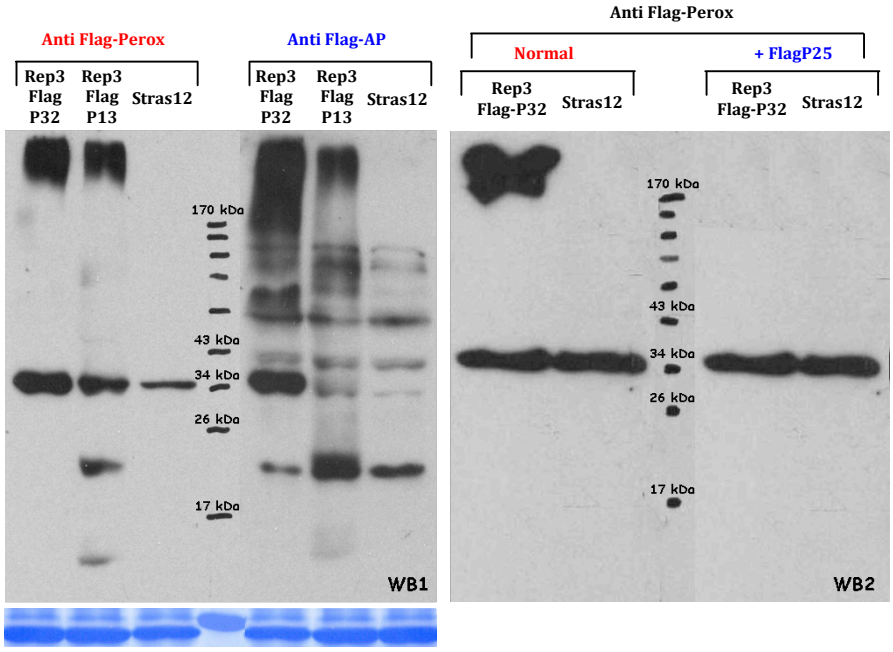


Figure 5. Western blot analyses of BSBMV RNA-4's ORFs expression. WB1: 7 d.p.i. *C. quinoa* lesions were analyzed using anti-Flag antibody conjugated with Peroxidase (on the right) or anti-Flag antibody conjugated with Alkaline Phosphatase (AP) (on the left). Same amount of proteins extract were loaded on gels. WB2: specific Flag competitor (FlagP25 protein) was added (on the right) or not (on the left – normal condition) during membrane incubation with anti-Flag conjugate with Peroxidase.

789) encodes a 9 kDa polypeptide corresponding to the 76 C-terminal amino acids of the second putative ORF of BSBMV RNA-3. Nucleotides sequence comparison of BSBMV RNA-3 and Chimeric RNA evidenced just one nucleotide substitution ($A^{1156}T$) leading to $K^{25}M$ mutation within the putative 9 kDa protein.

No deleted forms of BSBMV RNA-3 have been reported during each RT-PCR analyses performed until fortieth serial mechanical inoculation passage.

## *3.3 BSBMV RNA-4 encoded proteins*

Expression of the two putative proteins, p32 and p13, encoded by the new form of BSBMV RNA-4 were investigated *in planta*. The FLAG epitope sequence was fused in frame at the N-terminus of p32, p13 and BNYVV p31 proteins and cloned into Rep3 and Rep5 viral vectors. When inoculated onto *C. quinoa* leaves, together with Stras12 helper strain, the Rep3-Flag-p32 transcripts induced necrotic local lesions 7 d.p.i., identical to those induced by Stras12 + tUC47 (data not shown). Symptoms induced by Stras12 + Rep3-Flag-p13 transcripts, containing p13 sequence, did not differ from those induced in the absence of Rep-Flag-p13. Expression of BNYVV p31 protein using a replicon vector induced necrotic ring spot lesions comparable to those produced by full length BNYVV RNA-4 in *C. quinoa* leaves. Identical results were obtained performing the same experiments using viral vectors expressing p32, p13 and p31 proteins without FLAG epitope (data not shown).

When Rep5-FSp32p13 transcripts were used in the presence of BNYVV Stras12 helper strain, chlorotic local lesions were produced on *C. quinoa* leaves, indicating that the p32 expression was responsible for the necrotic local lesions induced by BSBMV RNA-4 or Rep5-p32 transcripts, thus excluding influence of RNA sequence.

Lesions from *C. quinoa* plants, inoculated with Stras12 supplemented with Rep3-Flag-p32 or Rep3-Flag-p13 transcripts, were analysed by western blot as previously described (Link et al., 2005). When FLAG-specific antibody conjugated with peroxidase was used, a protein of about 32 kDa was detected within all samples with different intensities. Such band appeared with a bigger intensity when Rep3-Flag-p32 was expressed. A band of about 13 kDa was also detected in sample infected by Stras12 + tRep3-Flag-p13 (Figure 5 WB1, left panel). A different chemistry was therefore used to detect flag tagged protein, using FLAG-specific antibodies conjugated with alkaline phosphatase. Such analysis permitted to conclude to the exclusive expression of a 32 kDa protein only in sample containing Rep3-Flag-p32 (Figure 5 WB1, right panel). Using such approaches we also

detected uncharacterized yet high molecular weight products from extracts containing viral vectors expressing Flag32 and Flag13 proteins.

Using BNYVV Flag-p25 competitor, we confirmed that the high molecular weight products were specific to the flag antibody (Figure WB2). We also proved that the 32 kDa protein detection corresponding to the presence of a plant peroxidase protein activity on the membrane did not disappeared in the presence of the competitor.

Chlorotic lesions appeared on *C. quinoa* leaves inoculated with Stras12 supplemented with BSBMV RNA-4-FlagIN transcripts. Western blot analysis, performed using FLAG-specific antibody conjugated with alkaline phosphatase, specifically revealed a protein of about 32 kDa (Figure 6). Similarly, western blot analysis of necrotic ring spot lesions induced by Stras12 + tRep5-Flag-p31, specifically detected the 31 kDa protein. In this case, a high molecular weight complex previously observed in Flag-p32-infected *samples* was also detected on Flag-p31 inoculated sample (Figure 6).

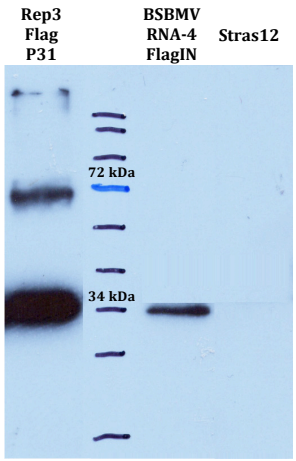


Figure 6. Western blot analysis using anti Flag conjugate with alkaline phosphatase on *C. quinoa* leaves inoculated with Stras12 + tRep5-Flag-p31 or tBSBMV RNA-4-FlagIN.

## *3.4 BSBMV RNA-4 doesn't affect long-distance movement of the virus in B. macrocarpa*

Ten days-old *B. macrocarpa* seedlings were inoculated with Stras12 helper strain supplemented with t35 (BNYVV RNA-3) or tUC31 (BSMV RNA-3) (Ratti et al., 2009) and tUC31 plus tUC47. Plants were also inoculated with total RNA extracted from BSBMV-infected sugar beet roots that were tested free from other beet soil-borne viruses by RT-

PCR multiplex analysis (Meunier et al., 2003). As reported in Table 2 yellow mosaic symptoms began to appear on systemic leaves 10-14 dpi for Stras12 supplemented with BNYVV RNA-3 and for wild type BSBMV isolate. No difference were observed on time necessary for systemic symptoms appearance (15-22 dpi) when Stras12 was supplemented with BSBMV RNA-3 or BSBMV RNA-3 plus -4, whereas no
symptoms nor viral RNA was detected 30 d.p.i. in the Stras12 inoculated plants. Therefore, the combination of two coremin sequences, supplied by BSBMV RNA-3 and -4, did not increased a systemic movement's efficiency as suspected (Ratti et al., 2009. Moreover, even if BSBMV RNA-4 contains a coremin sequence, such RNA species cannot replace RNA-3 for long distance movement on *B. macrocarpa* (Table 2).

| Inoculum | Symptoms (d.p.i.) | | Efficiency of systemic movement |
|---|---|---|---|
| | Local | Systemic | |
| Stras12 | 5-7 | - | 0 |
| Stras12 + t35 | 5-7 | 10-14 | >90 |
| Stras12 + tUC31 | 5-7 | 14-21 | >70 |
| Stras12 + tUC31 + tUC47 | 5-7 | 14-21 | >70 |
| Stras12 + tUC47 | 5-7 | - | 0 |
| BSBMV isolate | 5-7 | 10-14 | >90 |

Table 2. The Efficiency of viral systemic spread. Kinetics of *B. macrocarpa* local and systemic symptoms appearance after the inoculation of BNYVV Stras12 helper strain supplemented with BNYVV RNA-3 transcripts (t35), BSBMV RNA-3 transcripts (tUC31) alone or with BSBMV RNA-4 transcripts (tUC47) compared to wild type BSBMV infection. The efficiency of viral systemic spreading corresponds to the average of systemically infected plants versus total inoculated plants. 10 different *B. macrocarpa* plants were inoculated for each inoculum.
d.p.i. day post inoculation; - no symptoms detection

## *3.5 BSBMV RNA-4 p32 protein supports virus transmission through P. betae*

Considering high correlation between BNYVV and BSBMV viruses, demonstrated by capability of BNYVV viral machinery to replicate and encapsidate BSBMV RNA-3 (Ratti et al., 2009) and BSBMV RNA-4 (this study), the possible complementation between BSBMV and BNYVV RNAs-4 has been investigated for an efficient transmission of BNYVV RNAs through the vector *P. betae* in *B. vulgaris* plants.
Transmission experiments were performed using Stras12 isolate + t35 (called Stras123) as negative control, Stras123 in combinations with three different transcripts obtained from pUC47, deleted BSBMV RNA-4 and, as positive control, full length BNYVV RNA-4 (table3).

| | *C. quinoa* symptoms | TEM | mRT-PCR | | WB |
|---|---|---|---|---|---|
| | | | *P. betae* | RNA-2 | |
| Stras12 | CS | - | + | - | - |
| BSBMV wild type | NS | + | + | + | + |
| Stras12 + tUC47 | NS | + | + | + | + |
| Stras123 + tUC47 | NS | + | + | + | + |
| Stras12 + tUC47 Δ | CS | - | + | - | - |
| Stras123 + tUC47 Δ | CS | - | + | - | - |
| Stras123 | YS | - | + | - | - |
| Stras123 + tRep5-FSp32p13 | YS | - | + | - | - |
| Stras123 + tRep5-p32 | NS | + | + | + | + |
| Stras123 + tRep5-p31 | NR | + | + | + | + |

Table 3. Analysis of virus transmission through the vector *P. betae* performed with different viral infection combination from inoculated *C. quinoa* leaves. Test analyses were performed with Transmitted Electronic Microscope (TEM), multiplex RT-PCR and Western blot using anti BNYVV CP antibody.
CS = chlorotic spots; YS = yellow spots; NS = necrotic spots; NR = necrotic ring spots.

Stras123 + full length BSBMV or BNYVV RNAs-4 were successfully transmitted by *P. betae* to roots of healthy sugar beet plants on which rod-shaped viral particles were identified by TEM analysis, BNYVV coat protein expression was demonstrated by western blot and BNYVV or BSBMV RNAs replication was confirmed by northern Blotting (data not shown). No virus transmission occurred using Stras123 or Stras12 + deleted form of BSBMV RNA-4.

Previous analyses revealed competition between BNYVV and BSBMV RNAs-3 and RNA-3-derived replicons ((Lauber et al., 1999); (Ratti et al., 2009)). Therefore, Rep5 viral vector has been employed to express p32 or p31 proteins during transmission tests. We investigated the essential role of both protein in the efficient viral transmission by their expression out of their viral context. Transcripts from Rep5-p32, Rep5-p31, Rep5-FSp32 and from empty Rep5 viral vector were used together with Stras123 (Table 3). TEM, western blot analyses and RT-PCR confirmed that both p32 and p31 proteins could support *P. betae* transmission when expressed by Rep5 vector. Neither coat protein nor viral particles were detected or observed in the sample with p32 frame shift protein. (Figure 7)

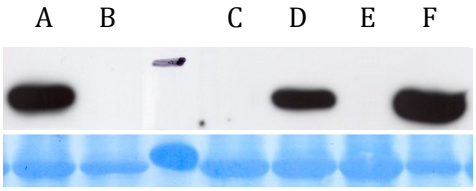


Figure 7. Western blot analysis using anti BNYVV CP antibody on sugar beet roots samples from transmission test. (A) Stras123 + tUC47; (B) Stras123 + tUC47 Δ; (C) Stras123; (D) Stras123 + tRep5-p32; (E) Stras123 + tRep5-FSp32p13; (F) Stras123 + tRep5-p31.

# Discussion

## *4.1 BSBMV RNA-4 is 1,730 nts long and it's replicated by BNYVV machinery*

Internal deletions or loss of smaller RNA species have been described for BNYVV isolates maintained by repeated mechanical inoculation of local lesion hosts (Hehn et al., 1994). Prolonged cultivation of field-infected plants especially when growth at high temperatures results also in the spontaneous deletion of part of *Soil-borne mosaic virus* RNA2 (Chen et al., 1995). Our study demonstrated that BSBMV RNA-4 is longer than the form previously isolated from leaves of field-infected sugar beet plants maintained in the greenhouse for indefinite period. The new BSBMV RNA-4 form shares 46 % nucleotide sequence identity with BNYVV RNA-4 instead of 55 % identity of BSBMV RNA-4 described by Lee *et al.*, (2001). Furthermore, shorter RNA-4 specie highly similar to the sequence described in 2001, appeared after several serial mechanical inoculations (Figure 1). According to sequence analysis we can speculate on the hypothesis that a spontaneous deletion mutant, similar to the BSBMV RNA-4 deleted form obtained during our study, was previously described by Lee *et al.*, (2001).

Moreover 3′ UTR sequence of the new full length BSBMV RNA-4, as well as all the other BSBMV RNAs, resulted highly conserved and comparable to the sequence of BNYVV RNAs. As previously reported viral RNA selection and replication mechanism appear to be conserved for both viruses. It has been demonstrated that compensatory mutations within 3′ UTR sequences of BSBMV RNA-3 validate the secondary structure essential for RNA replication making BNYVV RNAs-1 and -2 capable to replicate BSBMV RNA-3 (Ratti et al., 2009). Detection of BSBMV RNA-4 progeny, after the inoculation of BSBMV RNA-4 transcripts together with BNYVV helper strain, indicated that BNYVV RdRp can indeed replicate BSBMV RNA-4. Moreover, BSBMV RNA-4 was also encapsidated by BNYVV capsid proteins, as it was protected during the TM extraction and virus purification processes.

## *4.2 Recombination occur after several serial mechanical inoculations between BSBMV RNAs*

RNA recombination is a process that joins non-contiguous RNA segments together that plant RNA viruses are often subjected to. Viral RNA recombination is thought to occur when the viral replicase accidentally switches templates during complementary RNA synthesis ((Lai, 1992); (Nagy and Pogany, 2000)).

The resulting novel combinations of genes, sequence motifs, and/or regulatory RNA sequences could cause dramatic changes in the infectious properties of RNA viruses that can potentially lead to the emergence of new viruses or strains. Therefore, RNA recombination can help viruses to escape natural resistance mechanisms contributing to viral outbreaks (Cheng et al., 2006).

As described above appearance of BNYVV RNA-3 and -4 deleted forms has been reported after successive mechanical inoculation to *C. quinoa* leaves (Bouzoubaa et al., 1991) but, to our knoledge, no natural recombinant RNAs (Chimeras) have been described on viral species belonging to genus *Benyvirus*. In this paper we therefore report for the first time a natural Chimeric RNA derived from recombination process between BSBMV RNAs -4 and -3 that occurred during serial mechanical inoculation on *C. quinoa* leaves. Study of the chimeric RNA influence on BSBMV fitness was not part of the present study otherwise sequence analysis revealed two putative ORFs encoding small proteins which could be important for BSBMV adaptability. Interestingly, we demonstrated the ability of chimeric RNA to escape, during replication process, from competition with BNYVV or BSBMV RNA-3 (data not shown). This characteristic makes chimeric RNA the best candidate to become the basis of a new viral vector able to ensure co-expression of 3 different foreign proteins when used together with previous described Rep3 or RepIII and Rep5 replicons (Bleykasten-Grosshans et al., 1997);(Schmidlin et al., 2005); (Ratti et al., 2009).

## *4.3 BSBMV RNA-4 p32 encoded protein enhances symptoms expression*

BNYVV RNA-4 produced strong chlorotic lesions in *Tetragonia expansa* leaves and leaf stunting, curling and severe mosaic with leaf distortions on *Nicotiana benthamiana*. BNYVV p31 plays a multifunctional role in efficient vector transmission, enhanced

symptom expression and root-specific silencing suppression (Rahim et al., 2007). BSBMV RNA-4 encoded p32 share relevant amino acid identity (49.8 %) with B-type BNYVV p31 that increase to 68.4 % in terms of amino acid similarity suggesting a similar role of BSBMV RNA-4 on symptoms expression.

In our experiments full length BSBMV RNA-4, as well as p32 protein expressed by Rep3, have been associated, on *C. quinoa* leaves, to induction of necrotic spots identical to those induced by BNYVV p31 expressed by Rep3. Moreover yellow spots with necrotic ring appeared on *T. expansa* leaves inoculated with BSBMV RNA-4 (Data not shown).

It is known that viral proteins associated with symptom severity often show silencing suppression activity, as for BNYVV p31, (Rahim et al., 2007) a possible activity of p32 as a suppressor of RNA silencing cannot be excluded. A rapid screening of p32 RNA silencing suppressor activity has been performed according to the protocol developed by Guilley et al. (2009). No silencing suppression function was evidenced in such preliminary experiments (data not shown) but more studies are necessary to evaluate the same activity in a root context.

## *4.4 BSBMV RNA-4 is not involved on long distance movement*

In *B. macrocarpa*, BNYVV long distance movement is related to RNA-3 presence and involves a nucleotide sequence named "core" (Lauber et al., 1998). A conserved "coremin" sequence has been identified within core motif of BNYVV and BSBMV RNAs-3, in BNYVV RNA-5 and BSBMV RNA-4 as well as in other viral species belonging to the genus *Cucumovirus*. BSBMV RNA-3 was recently reported as less efficient element for long-distance movement of BNYVV RNA-1 and -2 in *B. macrocarpa* plants when compared to wild type BSBMV isolate (Ratti et al., 2009) then the presence of two motifs within BSBMV (RNA-3 and -4) was indicated as one possible explanation for this observation. As demonstrated by the results obtained during our experiments, the presence of a second coremin sequence is not able to influence, positively or negatively, the long-distance movement efficiency. A possible implication of other genomic RNA structural motifs present in *cis* (Miller and White, 2006) or in *trans* on BSBMV RNA-1 and/or RNA-2 may favour long distance movement.

## *4.5 BSBMV RNA-4 encoded p32 is essential for vector transmission*

Knowledge regarding benyviruses transmission through the vector *P. betae* has been up to now based on advances obtained by BNYVV studies. Previous study showed that wild-type (wt) BNYVV RNA-4 is required for efficient transmission of BNYVV by the plasmodiophorid *P. betae* (Tamada and Abe, 1989), moreover BNYVV RNA-4 deleted forms encoding truncated p31 protein are not able to transmit the virus (Rahim et al., 2007). No information was available about the role of BSBMV RNA-4. During our experiments we demonstrated that full length BSBMV RNA-4 is indeed essential for virus transmission through the vector. We also evidenced a complementation between BNYVV RNA-4 and BSBMV RNA-4 is possible for BNYVV RNAs -1, -2 and -3 acquisition and efficient transmission through *P. betae* in *Beta vulgaris* plants.

No data are available about ability of BSBMV RNA-4 described by Lee et al., (2001) to mediate virus transmission through the vector but we demonstrated that the presence of a deleted BSBMV RNA-4, obtained after several serial mechanical inoculation passages and highly similar to previous described BSBMV RNA-4, *P. betae* was unable to allow the transmission of BNYVV RNAs.

Finally, through expression of the BSBMV p32 and BNYVV p31 proteins with Rep5 viral vector, we demonstrated for the first time that the BSBMV p32 or BNYVV p31 proteins are definitively the transmission determinant for benyviruses.

More experiments will be then needed to better investigate the role of BSBMV p32 protein and understand the molecular mechanism occurring between virus and vector, in particular discover domain(s) involved on virus transmission can open the door for a new resistance strategy.

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References:

Bleykasten-Grosshans, C., Guilley, H., Bouzoubaa, S., Richards, K.E., Jonard, G., 1997. Independent expression of the first two triple gene block proteins of beet necrotic yellow vein virus complements virus defective in the corresponding gene but expression of the third protein inhibits viral cell-to-cell movement. Molecular Plant-Microbe Interactions 10, 240-246.

Bouzoubaa, S., Niesbach-Klosgen, U., Jupin, I., Guilley, H., Richards, K.E., Jonard, G., 1991. Shortened forms of beet necrotic yellow vein virus RNA-3 and -4: Internal deletions and a subgenomic RNA. Journal of General Virology 72, 259-266.

Chen, J., MacFarlane, S.A., Wilson, T.M., 1995. Effect of cultivation temperature on the spontaneous development of deletions in soilborne wheat mosaic furovirus RNA 2. Phytopathology 85, 299-306.

Cheng, C.P., Serviene, E., Nagy, P.D., 2006. Suppression of viral RNA recombination by a host exoribonuclease. Journal of virology 80, 2631-2640.

Dunoyer, P., Pfeffer, S., Fritsch, C., Hemmer, O., Voinnet, O., Richards, K.E., 2002. Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. Plant Journal 29, 555-567.

Guilley, H., Bortolamiol, D., Jonard, G., Bouzoubaa, S., Ziegler-Graff, V., 2009. Rapid screening of RNA silencing suppressors by using a recombinant virus derived from beet necrotic yellow vein virus. J Gen Virol 90, 2536-2541.

Hehn, A., Bouzoubaa, S., Jonard, G., Guilley, H., Richards, K.E., 1994. Artificial defective interfering RNAs derived from RNA 2 of beet necrotic yellow vein virus. Arch Virol 135, 143-151.

Heidel, G.B., Rush, C.M., Kendall, T.L., Lommel, S.A., French, R.C., 1997. Characteristics of beet soilborne mosaic virus, a furo-like virus infecting sugar beet. Plant Disease 81, 1070-1076.

Heijbroek, W., Musters-van Oorschot, P.M.S., Schoone, A.H.L., 1999. Variation in pathogenicity and multiplication of beet necrotic yellow vein virus (BNYVV) in relation to the resistance of sugar-beet cultivars. European Journal of Plant Pathology 105, 397-405.

Jupin, I., Richards, K.E., Jonard, G., Guilley, H., Pleij, C.W., 1990. Mapping sequences required for productive replication of beet necrotic yellow vein virus RNA 3. Virology 178, 273-280.

Klein, E., Link, D., Schirmer, A., Erhardt, M., Gilmer, D., 2007. Sequence variation within Beet necrotic yellow vein virus p25 protein influences its oligomerization and isolate pathogenicity on Tetragonia expansa. Virus Res 126, 53-61.

Koenig, R., Stein, B., 1990. Distribution of beet necrotic yellow vein virus in mechanically inoculated sugarbeet plantlets of cultivars with different degrees of rizomania resistance. Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors, Braunschweig, Germany, August 21-2., 87-90.

Lai, M.M.C., 1992. RNA recombination in animal and plant viruses. Microbiological Reviews 56, 61-79.

Lauber, E., Guilley, H., Tamada, T., Richards, K.E., Jonard, G., 1998. Vascular movement of beet necrotic yellow vein virus in Beta macrocarpa is probably dependent on an RNA 3 sequence domain rather than a gene product. J Gen Virol 79 ( Pt 2), 385-393.

Lauber, E., Jonard, G., Guilley, H., Gilmer, D., 1999. Effects of structural modifications upon the accumulation in planta of replicons derived from beet necrotic yellow vein virus RNA 3. Arch Virol 144, 1201-1208.

Lee, L., Telford, E.B., Batten, J.S., Scholthof, K.B., Rush, C.M., 2001. Complete nucleotide sequence and genome organization of Beet soilborne mosaic virus, a proposed member of the genus Benyvirus. Arch Virol 146, 2443-2453.

Link, D., Schmidlin, L., Schirmer, A., Klein, E., Erhardt, M., Geldreich, A., Lemaire, O., Gilmer, D., 2005. Functional characterization of the Beet necrotic yellow vein virus RNA-5-encoded p26 protein: Evidence for structural pathgenicity determinants. Journal of General Virology 86, 2115-2125.

Lozano, I., Morales, F., 2009. Molecular characterisation of Rice stripe necrosis virus as a new species of the genus Benyvirus. European Journal of Plant Pathology 124, 673-680.

Mahmood, T., Rush, C.M., 1999. Evidence of cross-protection between beet soilborne mosaic virus and beet necrotic yellow vein virus in sugar beet. Plant Disease 83, 521-526.

Meunier, A., Schmit, J.-F., Stas, A., Kutluk, N., Bragard, C., 2003. Multiplex reverse transcription-PCR for simultaneous detection of beet necrotic yellow vein virus, Beet soilborne virus, and Beet virus Q and their vector Polymyxa betae KESKIN on sugar beet. Appl Environ Microbiol 69, 2356-2360.

Miller, W.A., White, K.A., 2006. Long-distance RNA-RNA interactions in plant virus gene expression and replication, in: Alfen, D.B. (Ed.), pp. 447-467.

Nagy, P.D., Pogany, J., 2000. Partial purification and characterization of Cucumber necrosis virus and Tomato bushy stunt virus RNA-dependent RNA polymerases: Similarities and differences in template usage between tombusvirus and carmovirus RNA-dependent RNA polymerases. Virology 276, 279-288.

Peltier, C., Hleibieh, K., Thiel, H., Klein, E., Bragard, C., Gilmer, D., 2008. Molecular biology of the beet necrotic yellow vein virus. Plant Viruses 2, 14-24.

Quillet, L., Guilley, H., Jonard, G., Richards, K.E., 1989. In vitro synthesis of biologically active beet necrotic yellow vein virus RNA. Virology 172, 293-301.

Rahim, M.D., Andika, I.B., Han, C., Kondo, H., Tamada, T., 2007. RNA4-encoded p31 of beet necrotic yellow vein virus is involved in efficient vector transmission, symptom severity and silencing suppression in roots. J Gen Virol 88, 1611-1619.

Ratti, C., Clover, G.R.G., Rubies Autonell, C., Harju, V.A., Henry, C.M., 2005. A multiplex RT-PCR assay capable of distinguishing beet necrotic yellow vein virus types A and B. J Virol Methods 124, 41-47.

Ratti, C., Hleibieh, K., Bianchi, L., Schirmer, A., Rubies Autonell, C., Gilmer, D., 2009. Beet soil-borne mosaic virus RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in Beta macrocarpa. Virology 385, 392-399.

Rush, C.M., 2003. Ecology and epidemiology of benyviruses and plasmodiophorid vectors. Annu Rev Phytopathol 41, 567-592.

Schmidlin, L., Link, D., Mutterer, J., Guilley, H., Gilmer, D., 2005. Use of a Beet necrotic yellow vein virus RNA-5-derived replicon as a new tool for gene expression. J Gen Virol 86, 463-467.

Tamada, T., Abe, H., 1989. Evidence that beet necrotic yellow vein virus RNA-4 is essential for efficient transmission by the fungus Polymyxa betae. Journal of General Virology 70, 3391-3398.

Tamada, T., Shirako, Y., Abe, H., Saito, M., Kiguchi, T., Harada, T., 1989. Production and pathogenicity of isolates of beet necrotic yellow vein virus with different numbers of RNA components. Journal of General Virology 70, 3399-3409.

Wisler, G.C., Lewellen, R.T., Sears, J.L., Wasson, J.W., Liu, H.Y., Wintermantel, W.M., 2003. Interactions between Beet necrotic yellow vein virus and Beet soilborne mosaic virus in Sugar Beet. Plant Disease 87, 1170-1175.