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Pomoviruses associated with the rhizomania syndrome: a reverse genetic approach of the genome organization and multiplication requirements *in planta*.

Thèse présentée par François Crutzen en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

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Abbreviations

Viruses

AbMV – *Abutilon mosaic virus*
BBMV – *Broad bean mottle virus*
BBNV – *Broad bean necrosis virus*
BGMV – *Bean golden mosaic virus*
BNYVV – *Beet necrotic yellow vein virus*
BSBV – *Beet soil-borne virus*
BSBMV – *Beet soil-borne mosaic virus*
BVQ – *Beet virus Q*
BWYV – *Beet western yellows virus* (now *Turnip yellows virus* – TuYV)
BYDV – *Barley yellow dwarf virus*
CaMV – *Cauliflower mosaic virus*
CMV – *Cucumber mosaic virus*
CWMV – *Chinese wheat mosaic virus*
EWMV – *European wheat mosaic virus*
FHV – *Flock house virus*
HIV – *Human Immunodeficiency virus*
OGSV – *Oat golden stripe virus*
PEMV – *Pea enation mosaic virus*
PLRV – *Potato leaf roll virus*
PMTV – *Potato mop-top virus*
PPV – *Plum pox virus*
PVX – *Potato virus X*
RCNMV – *Red clover necrotic mosaic virus*
RSV - *Rice stripe virus*
SBWMV – *Soil-borne wheat mosaic virus*
SrCSV – *Sorghum chlorotic spot virus*
TBSV – *Tomato bushy stunt virus*

TMV – *Tobacco mosaic virus*
TCV – *Turnip crinkle virus*

Other abbreviations

A.C.N. – Ante Christum Natum
BET – ethidium bromide
cDNA – complementary DNA
Ci – curie
cm – centimeter
CP – coat protein
CRP – cysteine-rich protein
DI – defective interfering
DNA – deoxyribonucleic acid
dNTP – deoxyribonucleotide triphosphate
Dpi – days post-inoculation
ds – double-stranded
DTT – dithiothreitol
EDTA – ethylene diamine tetra acetic acid
EGTA – ethylene glycol tetra acetic acid
ELISA – enzyme-liked immunosorbent assay
ESCRT – endosomal sorting complex required for transport
g – gram or relative centrifuge force
h – hour
HEL – helicase
HEPES – 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
ICTV – International committee on taxonomy of viruses
kb – kilobase
kDa – kilodalton
M – molar
MAb – monoclonal antibody
MET – methyl-transferase
µm – micrometer
min – minute

MM – molecular mass
mRNA – messenger RNA
MVB – multivesicular body
ng – nanogram
nm – nanometer
nt – nucleotide
O/N – over-night
ORF – open reading frame
pH – power of hydrogen
PAb – polyclonal antibody
PCR – polymerase chain reaction
PIPES – piperazine-1,4-bis(2-ethanesulfonic acid)
Poly-A – poly-adenines
PTGS – post-transcriptional gene silencing
PTM – post-translational modification
RACE – rapid amplification of cDNA ends
RdRp – RNA-dependant RNA polymerase
RNA – ribonucleic acid
rNTP – ribonucleoside triphosphate
rpm – rotation per minute
RT – read-through, reverse transcription or reverse transcriptase
Sat – satellite
SDS-PAGE – sodium-dodecylsulfate polyacrylamide gel electrophoresis
sgRNA – subgenomic RNA
siRNA – small interfering RNA
sp – species
ss – single-stranded
ssp – sub-species
T° – temperature
TGB – triple gene block
TLS – tRNA-like structure
TM – transmembrane motif
U – unit

UTR – untranslated region
UVs – ultraviolets
V – volts

GENERAL INTRODUCTION

Sugar beet, generalities

History and cropping

Beta vulgaris ssp. v*ulgaris*, which includes sugar beet, descends from *Beta vulgaris* ssp. *maritima* or *Beta maritima* (Francis, 2006). Originating from the Mediterranean shore, this beet subspecies was already cropped as vegetable five hundred years A.C.N. and its leaves were eaten following the example of spinach. Consumed as such until Middle-Ages, more than two thousand years have been necessary to develop a special interest for beets existing varieties and sweetness. Intensively grown in Europe to compete for sugar production with sugar cane coming from Southern colonies at the beginning of the 18th century, sugar beet is now well-established in fields from many countries with temperate climate from Europe but also Africa, America and Asia (Fig. 1) (Draycott, 2006).

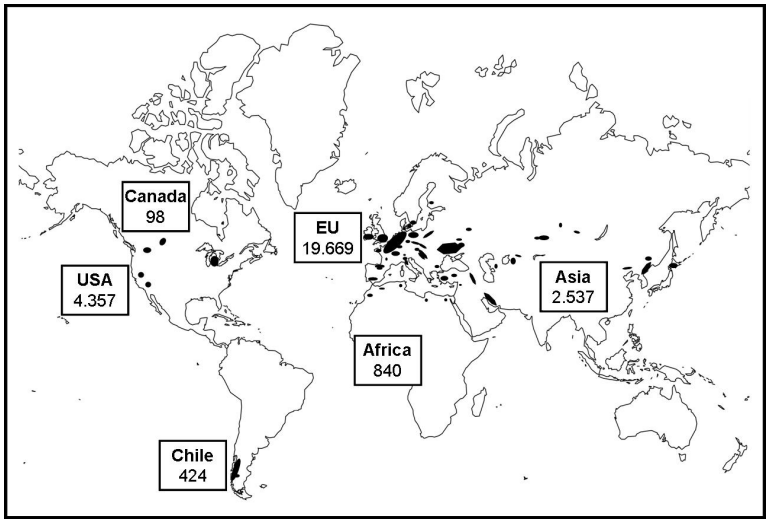

Fig.1. World map showing sugar beet growing countries and regions in black and the sugar production in thousands of tons (adapted from Draycott, 2006).

B. vulgaris is a halophytic plant – adapted to salty environment – belonging to the *Chenopodiaceae* family. Sowed in spring and harvested in late

autumn, sugar beets go firstly through a shoot growth, to favor secondly the taproot development and sugar storage (Milford, 2006). Nevertheless, beet cropping and therefore sugar production may be affected by various pathogens at the different growing stages.

The major diseases affecting sugar beet crops

Nematodes, fungi, bacteria and viruses are all microscopic pathogens that commonly attack sugar beet culture (Whitney and Duffus, 1986). However, as the development and severity of one or another disease is strongly governed by environmental conditions, different countries or even regions will be confronted with different pathogens combinations. By way of example, major diseases occurring in Belgian sugar beet fields are listed in Table 1 (adapted from Hermann and Wauters, 2002).

Table 1. Occurrence and severity of the major fungal, nematode and viral pathogens of sugar beet in Belgium (adapted from Hermann and Wauters, 2002).

Pathogen type	Disease	Pathogen name	Occurence	Severity
Fungus	Alternaria Leaf Spot	*Alternaria* spp.	+	-
	Beet[1] and Seedling[2] Rusts	[1]*Uromyces betae* & [2]*Puccinia subnitens*	+	++
	Cercospora Leaf Spot	*Cercospora beticola*	+	+++
	Damping-off	*Aphanomyces cochlioides*	+	++
	Powdery Mildew	*Erysiphe polygoni*	+++	+++
	Phoma Leaf Spot - Root Rot	*Phoma betae*	+	-
	Ramularia Leaf Spot	*Ramularia beticola*	+	+++
	Rhizoctonia Foliar Blight – Root Rot	*Rhizoctonia solani*	+	+++
	Verticillium Wilt	*Verticillium albo-atrum*	+	-
Nematode	Clover Cyst Nematode	*Heterodera trifolii*	+++	+++
Virus	Rhizomania	*Beet necrotic yellow vein virus*	+	+++
	Viral Yellowing	*Beet yellows virus* & *Beet mild yellowing virus*	++	+++

Amongst the fungal and viral pathogens that are predominant in Belgium, this thesis will focus on pomoviruses that are often found associated with the viral agent responsible for the rhizomania syndrome of sugar beet.

The rhizomania syndrome of sugar beet

General considerations

In 1952, severe problems of sugar beet growth were recorded in Padan Plain, the boarding region of Pô River that goes through North Italy from West to East. These observations led Antonio Canova, working at the "Istituto di Patologia Vegetale" of the Bologna University, to publish in 1959 the first report of rhizomania-like symptoms. Thirteen years later, the same author named the disease "rhizomania" (Canova, 1966), an Italian term meaning "root madness", as an extreme rootlets proliferation constitutes the main symptom. Shortly after Canova's communications, rhizomania syndrome has been identified in many other countries and sugar beet crops from all around the world are now under threat (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. 2a and b). Browning of the vascular system as well as constriction of the main root are visible on beets cross-sections (Fig. 2c). Necrosis and yellowing of the leaves veins (Fig. 2f), attributed to the presence of virus in the aerial parts, are rather rare as the viral pathogen remains in most cases confined into the roots system. Less specific to the syndrome but more frequent in infected crops is the leaves' yellowing (Fig. 2d). Foliar wilting may also be observed in some cases (Fig. 2e).

Economically, both the downsizing of the primary root and the reduced sugar content in infected beets are responsible for sugar yield decrease commonly around 50-60%, and sometimes up to 80% (Mc Grann *et al*., 2009).

Fig. 2. Rhizomania symptoms caused by BNYVV infection on sugar beet with (a) reduction of taproot size for varieties susceptible to BNYVV compared with a tolerant one, (b) lateral rootlets proliferation, (c) browning of the vascular system and constriction of the main root, (d) leaves yellowing for susceptible varieties, (e) foliar wilting and (f) yellowing as well as necrosis of the leaves veins (pictures kindly provided by Y. Galein).

The pathogen, *Beet necrotic yellow vein virus*

Identified as the causal agent of the rhizomania syndrome of sugar beet in the early seventies (Tamada and Baba, 1973), the *Beet necrotic yellow vein virus* (BNYVV) has since been characterized as belonging to the *Benyvirus* genus. Virions are rod-shaped and not enveloped by a biological membrane (Tamada, 1975). Particles of different sizes are visible under the electron microscope (Fig. 3), as four to five plus-sense single-stranded RNAs of different lengths compose the BNYVV genome (Fig. 4) (Putz, 1977; Bouzoubaa *et al*., 1985; Bouzoubaa *et al*., 1986; Bouzoubaa *et al*., 1987; Kiguchi *et al*., 1996; Koenig *et al*., 1997b).

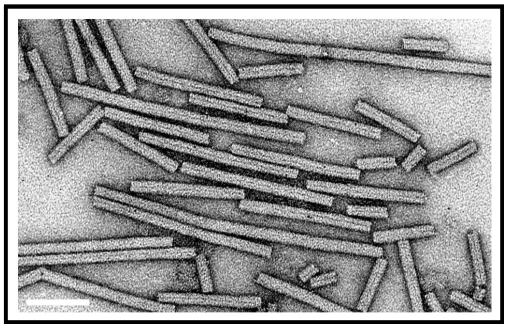

Fig. 3. Electron micrograph of BNYVV particles (kindly provided by T. Tamada). Rod size is correlated with the length of the encapsidated RNA. White bar represents 100 nm.

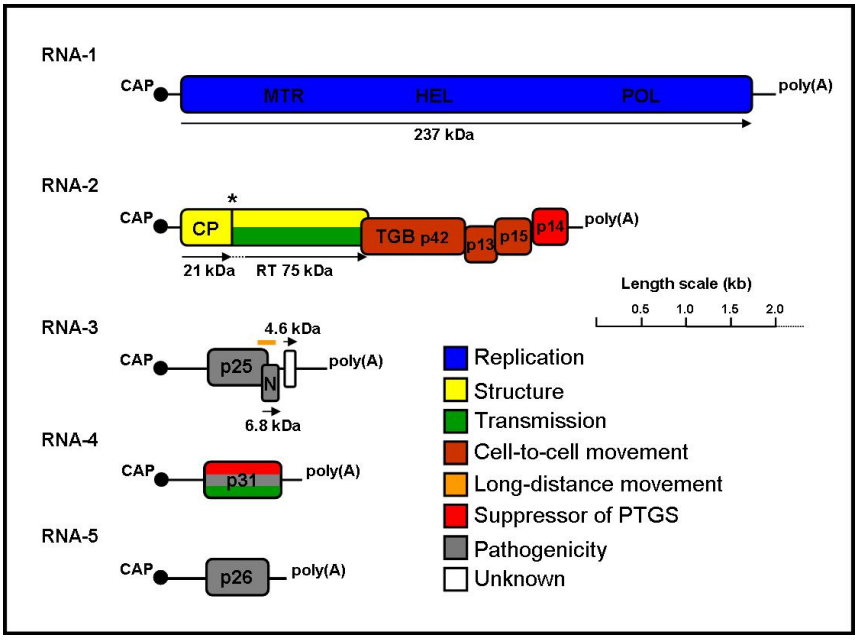

Fig. 4. Schematic representation of the BNYVV multipartite genome composed of four to five single-stranded positive-sense RNAs (from sequences of Bouzoubaa *et al*., 1985; Bouzoubaa *et al*., 1986; Bouzoubaa *et al*., 1987; Kiguchi *et al*., 1996). All RNAs are 5'-capped and 3'-poly-adenylated. Viral replicase contains conserved methyl-transferase (MTR), helicase (HEL) and RNA-dependant-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. The molecular mass is indicated for each protein in kilodalton (kDa) below, above or 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. The star indicates the leaky termination codon position of RT domain.

The viral replicase is encoded by RNA-1 (6.7 kilobases, kb). Following translation, the resulting 237 kilodalton (kDa) protein undergoes an autocatalytic cleavage that gives rise to proteins of 150 and 66 kDa (Hehn *et al*., 1997). The first one contains the methyl-transferase (MTR) and helicase (HEL) conserved motifs while RNA-dependant RNA polymerase (RdRp) motif is present in the last one. RdRp is generally involved in viral replication (Quadt and Jaspars, 1989).

RNA-2 (4.6 kb) first open reading frames (ORFs) encode for the 21 kDa coat protein (CP) and a 54 kDa read-through (RT) domain. This last is added to the CP during translation when the first UAG stop codon is bypassed by ribosomes that produce then the 75 kDa CP-RT (see chapter I, preliminary concepts) (Ziegler *et al*., 1985; Niesbach-Klösgen *et al*., 1990). The N-terminal part of the RT domain is involved in viral assembly (Schmitt *et al*., 1992) whereas the C-terminal part is essential for the viral transmission by the protist vector (see next sub-section) (Tamada and Kusume, 1991). Two transmembrane motifs (TM1 and TM2) predicted in the RT domain and their interaction in biological membranes, thought to be dependant on a KTER peptide motif (Lysine – Threonine – Glutamic acid – Arginine, respectively) (Tamada *et al*., 1996; Diao *et al*., 1999a; Adams *et al*., 2001) (Fig. 5), may be some crucial motifs involved in the virus transmission. Triple Gene Block (TGB) proteins, which coding sequences are located downstream that of CP-RT on RNA-2, allow BNYVV to move from cell to cell in the infected plant (Gilmer *et al*., 1992). Finally a 14 kDa cysteine-rich protein (CRP) suppressing post-translational gene silencing (PTGS) in plants (Dunoyer *et al*., 2002) is also expressed from BNYVV RNA-2 (Gilmer *et al*., 1992).

The third RNA encodes for two pathogenicity-associated proteins of 25 kDa (p25) and 6.8 kDa (N), p25 being largely responsible for the rhizomania symptoms in the fields (Jupin *et al*., 1992; Tamada *et al*., 1999; Klein *et al*., 2007). Another 4.6 kDa protein of unknown function may be expressed from RNA-3, but has never been detected. Long-distance movement – systemicity – of BNYVV in *Beta macrocarpa* has also been shown to rely on RNA-3, but rather on the nucleotide sequence than one of the encoded proteins (Tamada *et al*., 1989; Lauber *et al*., 1998).

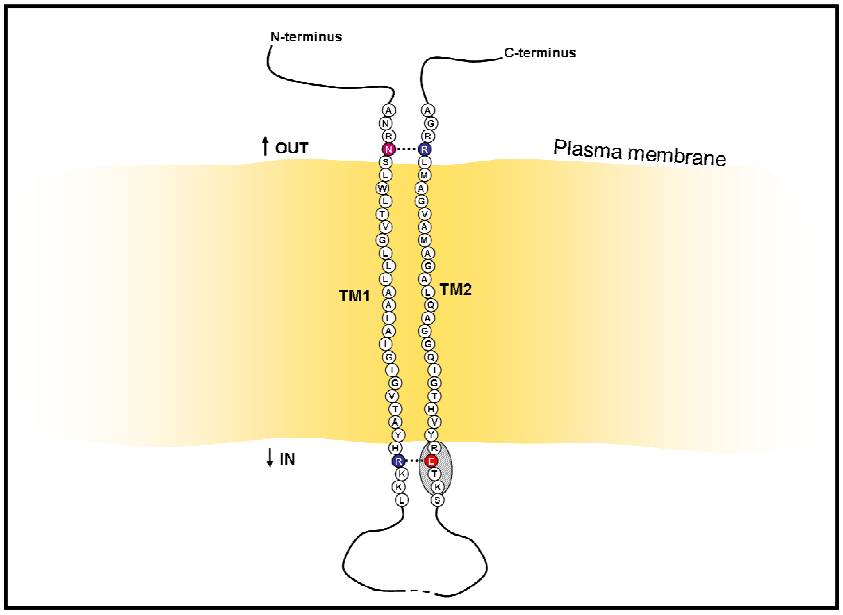

Fig. 5. BNYVV predicted TM1 and TM2 in the CP RT domain, interacting at the inner side of the plasma membrane through the KTER motif and an arginine residue (R). Potential asparagine(N)-arginine interaction is also represented at the outer side of the membrane. Adapted from Diao and co-authors (1999a) and Adams and co-authors (2001).

A 31 kDa protein (p31) involved in viral transmission and pathogenicity (Tamada and Abe, 1989; Rahim *et al*., 2007) is encoded by RNA-4. P31 has a PTGS suppressor activity in roots but not in leaves.

The fifth RNA encodes another pathogenicity-associated protein of 26 kDa (Link *et al*., 2005). However, viral isolates containing RNA-5 are reported only in Japan (Tamada *et al*., 1989), Pithiviers area of France (Koenig *et al*., 1997b), China (Li *et al*., 1999), Kazakstan (Koenig and Lennefors, 2000) and England (Harju *et al*., 2002).

Each RNA of BNYVV is capped and multiple adenosines form a Poly(A) tail at the 5' and 3' extremities, respectively (Putz *et al*., 1983). Cap-like structures consist of a 7-methylguanosine base linked to the 5' extremity of viral RNAs via their 5' hydroxyl group by a triphosphate bridge (Fig. 6) and can be represented as $m^7G^{5'}ppp^{5'}$. Mimicking 5'-capped and 3'-

polyadenylated mRNAs from eukaryotic cells, cap and Poly(A) structures of viral RNAs are supposed to protect them from degradation by cellular enzymes but also favor their translation and replication (Mandahar, 2006).

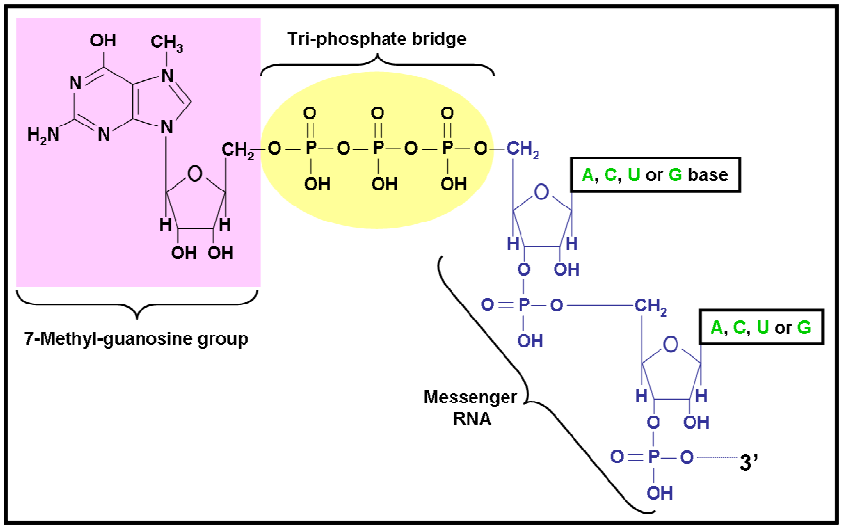

Fig. 6. Chemical structure of a 7-methyl-guanosine group or cap, linked to the 5' end of a mRNA by a triphosphate bridge (adapted from Banerjee, 1980).

BNYVV two first RNAs are essential and sufficient for viral multiplication when mechanically inoculated to the leaves of the *Chenopododiacea* plants *B. macrocarpa*, *B. vulgaris*, *Chenopodium quinoa* and *Tetragonia expansa* (Koenig *et al*., 1986; Kuszala *et al*., 1986; Tamada *et al*., 1989). They carry housekeeping genes necessary for local viral infection. This allowed Quillet and co-authors (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 BNYVV RNAs-1 and -2. However, RNAs-3 and -4 are necessary for the efficient virus propagation between plants and production of typical rhizomania symptoms (Lemaire *et al*., 1988; Koenig *et al*., 1991).

Among BNYVV populations infecting sugar beet, three types A, B and P are identified. These can be distinguished according to nucleotide and amino acid specificities, while differing only from 1 to 5% at the genomic level (Kruse *et al*., 1994; Koenig *et al*., 1995). Different geographical localizations and degrees of aggressivness are also attributed to these types of BNYVV. In Europe, whereas the A-type is rife in most countries growing sugar beet, the B-type stays rather confined to the central part – mainly Belgium, Germany and France (Koenig and Lennefors, 2000; Schirmer *et al*., 2005; Koenig *et al*., 2008). The P-Type, which induces more symptoms than A- and B-types in infected fields, is reported in France and the United-Kingdom (Koenig *et al*., 1997b; Schirmer *et al*., 2005; Ward *et al*., 2007).

The vector, *Polymyxa betae*

BNYVV is transmitted to beet by means of a soil-borne vector, *Polymyxa betae* (Tamada, 1975). This single-celled endoparasite is a eukaryotic protist belonging to the *Plasmodiophoridae* family, formerly considered as a fungi group but reclassified as protozoa in the Cercozoa group (Barr, 1992; Braselton, 1995). *P. betae* infects mainly roots of *Chenopodiaceae*. In addition, five special forms of another Polymyxa species, *P. graminis*, are also reported to vector many viruses in both tropical and temperate regions, mainly on cereals (Legrève *et al*., 2000; Legrève *et al*., 2002; Kanyuka *et al*., 2003; Dieryck *et al*., 2009).

Life cycles of both Polymyxa species are highly similar and that of *P. betae* is represented on figure 7. The primary roots infection begins with swimming biflagellate single-cells (2-4 µm diameter), the zoospores, which encyst on plant rootlets and inject their cytoplasmic content inside root cells. It develops first into a plasmodium stage which differentiates either in a zoosporangium or a sporosorus. The sporangial phase leads to immediate

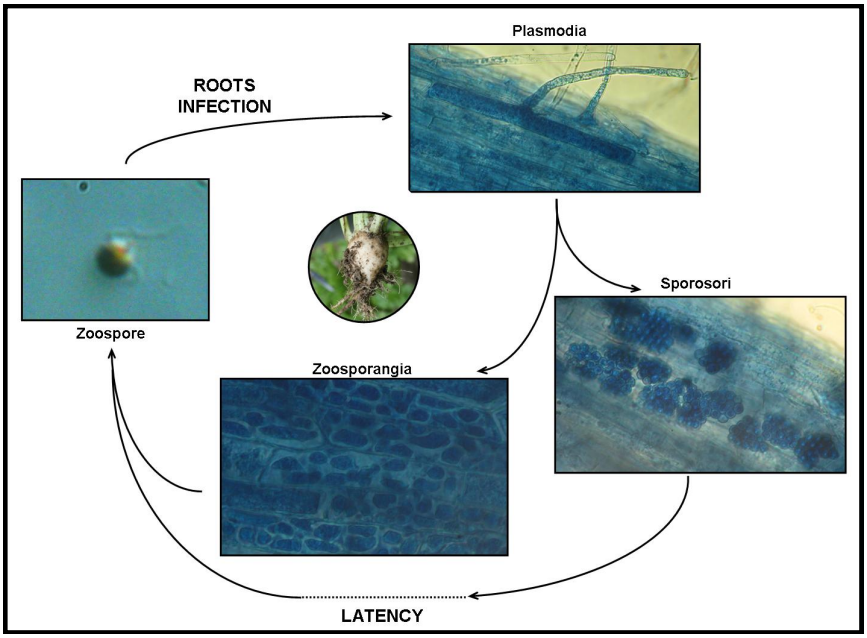

Fig. 7. Life cycle of *Polymyxa betae* in roots of *Beta vulgaris*, as described by Keskin (1964) (zoospore picture kindly provided by N. Desoignies).

production of zoospores and a new infection cycle, whereas the sporogenic phase ensures the long-term persistence in the soil and delayed production of zoospores. One or the other phase is preferred depending on the external conditions or the host type and new zoospores are released from sporosori when conditions are favorable again (Keskin, 1964; Keskin and Fuchs, 1969).

Whether the relation between *P. betae* and beets is harmful or not for the plant still remains to be determined. However, vectoring the rhizomania-responsible BNYVV, *P. betae* allows the dissemination of at least one of the worst viral disease of beet. Moreover, sporosori containing viral particle of BNYVV ensure the long-term persistence of the disease in soils (up to 15 years) (Abe and Tamada, 1986).

Two BNYVV-associated pomoviruses

Two other viruses, also thought to be transmitted to *Chenopodiaceae* plants by *P. betae* (Ivanovic *et al*., 1983), are frequently detected in beets infected with BNYVV (Meunier *et al*., 2003; Ratti *et al*., 2005). These are the *Beet soil-borne virus* (BSBV) and *Beet virus Q* (BVQ), belonging to the *Pomovirus*. The *Potato mop-top virus* (PMTV) constitutes the type-species of this viral genus.

The "Potato-mop top-like" viruses

Four virus species are listed in the *Pomovirus* genus, *Virgaviridae* family. The type species PMTV proved to share important similarities with the three other *Broad bean necrosis virus* (BBNV), BSBV and BVQ (Koenig and Lesemann, 2005). When visualized under the electron microscope, these viruses appear as rods of ~300, 150 and 70 nanometers (nm) with an axial canal (Fig. 8).

Fig. 8. Electron micrograph picture of PMTV particles (from Cowan *et al*., 1997, with agreement of the Society for General Microbiology). Rod size is correlated with the length of the encapsidated RNA. White bar represents 100 nm.

Virions are free of any enveloping membrane and contain three linear single stranded positive-sense RNAs of 6.0, 3.0-3.5 and 2.5-3.0 kb. RNAs are consensually numbered according to their size, from the biggest to the smallest one, as previously shown with BNYVV. However, a deleted form of PMTV was first described and RNAs-2 and -3 have been inverted compared to the three other pomoviruses. As RNAs numbers have nevertheless been conserved after the identification of common PMTV, RNAs-2 and -3 of this virus are rather referred according to the proteins they encode. Here is presented the particular genomic organization of the *Pomovirus* genus (Scott *et al*., 1994; Koenig *et al*., 1996; Koenig and Loss, 1997; Koenig *et al*., 1997a; Koenig *et al*., 1998; Savenkov *et al*., 1999; Sandgren *et al*., 2001) (Fig. 9).

The 6 kb long RNAs-1 encode putatively for two proteins. The smaller one, encoded by the 5' proximal ORF, comprises MTR and HEL conserved motifs in the N- and C-terminal parts, respectively. The larger protein is produced by addition of a read-through domain to the smaller one (see Chapter I, Preliminary concepts). This RT domain contains the RdRp motif. However the replication mechanisms of pomoviruses have never been investigated.

Structural proteins are presumably expressed from RNAs-2 (3.0-3.5 kb) that encode for the CP and a RT domain, following the example of BNYVV. Nevertheless, the size of the RT portion is rather variable when comparing between the four viruses, with BVQ presenting the smallest one. Studying PMTV, several functions have been attributed to the RT part of the CP. Using naturally occurring strains with shorter RT domains, Reavy and co-authors (1998) evidenced that the transmission of PMTV by its natural plasmodiophorid soil-borne vector, *Spongospora subterranea*, relies on the CP-RT C-terminal part. The transmission-related transmembrane motifs TM1 and TM2 are also predicted in the RT domain of the PMTV CP, like for BNYVV and other beny-, furo- and pomoviruses transmitted by *Polymyxa* sp. (Diao *et al*., 1999a; Adams *et al*., 2001). As evidenced for BNYVV (Haeberlé *et al*., 1994), the CP-RT of PMTV is localized at one extremity of the viral particles (Cowan *et al*., 1997). However, whereas the

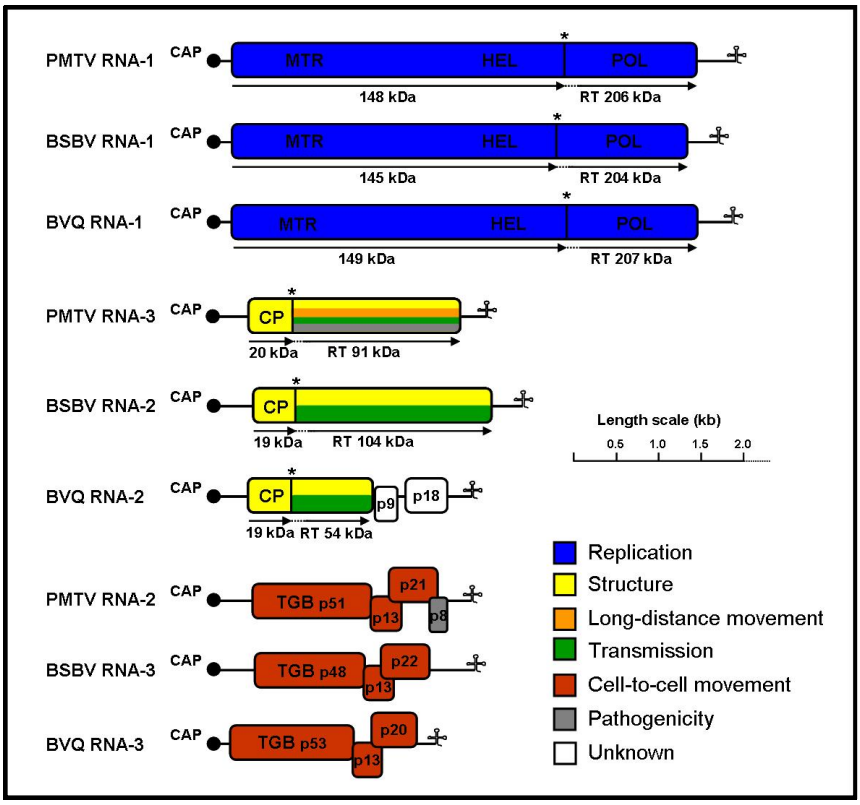

Fig. 9. Schematic representation of the BSBV, BVQ and PMTV genomes, composed of three single-stranded RNAs of positive polarity (from sequences of Scott *et al*., 1994; Koenig *et al*., 1996; Koenig and Loss, 1997; Koenig *et al*., 1997a; Koenig *et al*., 1998; Savenkov *et al*., 1999; Sandgren *et al*., 2001). 3' ends of all viral RNAs fold in tRNA-like structures whereas 5' ends are thought to be capped. RNAs-1 encoded replicases contain methyl-transferase (MTR), helicase (HEL) and RNA-dependant-RNA polymerase (POL) conserved motifs. The POL motifs are translated as read-through (RT) domains. Triple Gene Block (TGB) proteins are encoded by the smallest RNAs, RNA-2 for PMTV and -3 for BSBV and BVQ. PMTV RNA-2 comprises also a short cysteine-rich protein (CRP) encoding ORF, absent from corresponding RNAs-3 of the other two pomoviruses. Structural coat proteins (CP) and RT domains are expressed from PMTV RNA-3 and RNAs-2 of BSBV and BVQ. However, two additional small ORFs encoding for putative proteins of unknown functions are encoded by BVQ RNA-2. The molecular mass is indicated in kilodalton (kDa) for each protein under or inside the corresponding ORF. Function(s) attributed or suspected for each protein is/are specified through a color code. Leaky termination codons positions for RT domains are indicated by stars.

distal situation of the CP-RT in the benyvirus particles is supposed to promote assembly with the CP (Schmitt *et al*., 1992; Haeberlé *et al*., 1994), no such involvement in encapsidation is observed for the PMTV RT domain (Torrance *et al*., 2009). More recently, the long-distance movement of the CP-RT encoding RNA (RNA-CP) proved to be dependant on the RT C-terminal domain, whereas the two other RNAs moved regardless of the RT presence (Torrance *et al*., 2009). Finally, while the PMTV RNA-CP is known for some time to be necessary for the symptom induction on *Nicotiana benthamiana* (Savenkov *et al*., 2003), the involvement of the RT domain in virulence potentially through a subdomain 2 (SD2) has been evidenced only recently (Torrance *et al*., 2009). The large RT domain of PMTV CP might therefore be considered as a multifunctional operating system involved in functions as diverse as transmission, systemicity and symptom expression, through distinct domains.

Pomoviruses smallest RNAs (RNA-3, 2.5-3 kb) comprise at least three overlapping ORFs coding potentially for the famous movement-responsible TGB proteins. Viral cell-trafficking mediated by TGBp1, p2 and p3 has been widely studied for PMTV (Zamyatnin *et al*., 2004; Haupt *et al*., 2005; Tilsner *et al*., 2010) and contributed to better understand the underlying mechanisms of cell-to-cell movement for multipartite rod-shaped viruses. Finally, a fourth little CRP is encoded by PMTV RNA-TGB and that is absent from genomic RNAs of the three other BBNV, BSBV and BVQ. *In vivo*, this 8 kDa CRP is found associated with the endoplasmic reticulum membranes, potentially through a predicted transmembrane segment, and increases the symptoms severity (Lukhovitskaya *et al*., 2005).

Following the example of BNYVV, pomoviruses RNAs are thought to be 5'-capped. 3' ends, in turn, are not polyadenylated but rather predicted to fold in transfer RNA-like structures (TLSs) (Savenkov *et al*., 1999; Koenig *et al*., 1998) like other tymo-, furo-, peclu-, tobamo-, bromo-, cucumo- or hordeiviruses (Dreher, 2009). These viral TLSs are of crucial importance for the stability of viral RNAs and generally involved in encapsidation, translation or replication processes.

Beet soil-borne virus (BSBV)

The BSBV tripartite genome is organized as described in the previous section (Fig. 9) (Koenig *et al*., 1996, 1997a; Koenig and Loss, 1997). However, contrary to the well-studied PMTV, no functional study has been carried out on BSBV. Except for the CP that has been detected by Western blotting (Koenig *et al*., 1997a), neither the expression nor the function of the six other putative proteins have been investigated. Thus, even when two presumably transmission-related TM1 and TM2 are predicted in the BSBV CP RT domain (Adams *et al*., 2001), the involvement of the CP-RT in the transmission of this pomovirus by its vector *P. betae* has never been shown.

First identified in England (Ivanovic *et al*., 1983), BSBV is now reported in Germany and Sweden (Leseman *et al*., 1989), Finland (Bremer *et al*., 1990), France, Italy, Spain and The Netherlands (Lindsten, 1993), The United States (Lindsten and Rush, 1994), Belgium (Stas *et al*., 2001a), Iran (Farzadfar *et al*., 2002), Syria (Mouhanna *et al*., 2002), Bulgaria, Hungary, and Turkey (Meunier *et al*., 2003), Austria, Croatia, Lithuania, Japan and Switzerland (Ratti *et al*., 2005), Poland (Borodynko *et al*., 2006) and China (Wang *et al*., 2007). Transmitted by the same vector as the well-known harmful BNYVV (Ivanovic *et al*., 1983) and often found associated in the fields, the BSBV pathogenicity proved to be difficult to determine in the complex rhizomania syndrome. Mechanical inoculations of BSBV to leaves of different *Chenopodiacea* species and cultivars highlighted its ability to induce strong reactions on *Chenopodium* sp. but occasional or even no symptoms on *Beta* and *Spinacia* sp. (Henry *et al*., 1986). Mainly chlorotic or necrotic spots, rings and ringspots appearing as soon as five days post-inoculation (dpi) were recorded. Different approaches have also attempted to assess the damages due to an infection by BSBV on roots of *B. vulgaris*. Prillwitz and Schlösser (1992) opted for inoculating viruliferous sporosori of *P. betae* to healthy beets. They observed typical rhizomania-like symptoms on the roots, with abundant proliferation of lateral rootlets and browning of vascular rings, and recorded up to 40% losses of roots weight but no foliar symptoms. Moreover, pre-infection with BSBV reduced significantly the

titer of BNYVV, when this last was inoculated later. Such cross-protection against BNYVV has also been evidenced with the closely related *Beet soil-borne mosaic virus* (BSBMV) (Mahmood and Rush, 1999), another *Benyvirus* transmitted by *P. betae* and only reported in the United States (Wisler *et al*., 1994; Ratti *et al*., 2009). When mechanically inoculated to the beet roots (Kaufmann *et al*., 1993), BSBV has been shown to be responsible for tap-root weight reduction of 20% even if no foliar symptoms were visible. However, the variability between the different plants submitted to the same treatment was quite high. Finally, field observations in Sweden (Lindsten, 1993), the United States (Rush and Heidel, 1995) and Germany (Koenig *et al*., 2000) have evidenced no obvious impact of BSBV on the sugar yield and no clear symptoms, neither on the roots, nor on the leaves. In light of these observations, various experimental approaches have therefore shown mixed results, even diverging, with regards to the BSBV pathogenicity. Working with full-length infectious cDNA clones of BSBV has thus been proposed by Kaufmann and co-authors (1993) as a good alternative for testing the pathogenicity of a uniform viral population.

Beet virus Q (BVQ)

BVQ was previously considered as a one of the BSBV serotype because of the antisera cross-reactivity and their similar genomes. Only the sequencing of unpurified virus in the late nineties allowed the BVQ "Wierthe" serotype to be considered as a virus species distinct from the BSBV "Ahlum" serotype (Koenig *et al*., 1998). ORFs encoding for structural and non-structural viral proteins are organized similarly to those of BSBV and the other pomoviruses, except the RT domain of the CP (35 kDa) that is much shorter and lets place for two additional ORFs coding for putative proteins of 9 kDa and 18 kDa (Fig. 9). However, neither the *in vivo* expression of the BVQ putative proteins nor their respective functions have been investigated until now. Moreover, the short 35 kDa RT domain has been predicted to contain a single transmembrane helix, TM1, but no TM2 (Adams *et al*., 2001). This special organization of BVQ RNA-2 constitutes an exception among the *Pomovirus* genus and raises questions about the

BVQ transmissibility as both TMs are thought to be required for the viral transmission by its putative plasmodiophorid vector, *P. betae.*

BVQ has been identified in sugar beet crops from Belgium (Stas *et al*., 2001b), Bulgaria, France, Germany, Hungary and The Netherlands (Meunier *et al*., 2003), Iran (Farzadfar *et al*., 2005), Croatia, Italia, Lithuania, England (Ratti *et al*., 2005), Poland (Borodynko, 2006) and Spain (Rubies Autonell *et al*., 2006) but not in China (Wang *et al*., 2007). However, the incidence of BVQ may vary from 30% of the tested samples in Belgian sugar beet crops (Stas *et al*., 2001a) to 1.5% in Iranian ones (Farzadfar *et al*., 2005) and is always lower to that of BSBV, which is reported in 85% and 9.5% of the tested fields for the same countries, respectively. Moreover, mixed infections of BVQ with BNYVV and/or BSBV are mostly reported (Meunier *et al*., 2003; Ratti *et al*., 2005).

According to the different serotypes of BSBV recorded in the nineties, BVQ was still considered as belonging to the group 1 (Table 2). But studies on the BSBV pathogenicity used the viral serotypes -2 (Prillwitz and Schlösser, 1992) or –N (Henry *et al*., 1986), or detected BSBV using monoclonal antiboby (MAb) 2 specific for the Ahlum serotype (Kaufmann *et al*., 1993). It seems therefore that no mix-up has arisen between both pomoviruses and the pathogenicity of BVQ has never been investigated before 1998 (Koenig *et al*., 1998). From that time, BVQ has been reported to produce lesions that spread along the veins on *C. quinoa* leaves five dpi (Koenig *et al*., 1998; Rubies Autonell *et al*., 2006), but mechanical inoculation of beet roots and leaves have been unsuccessful (Koenig *et al*., 1998). The impact of an infection by BVQ on the beet root-weight has therefore never been tested and its involvement in the rhizomania disease remains a mystery.

Group	Name	Origin
1	Wierthe	Germany
	Dornheim	Germany
2	Ahlum	Germany
	86-109	Sweden
	1530	Belgium
	BSBV-N	England
3	Texas 7	USA
4	Spinach Spot	USA

Table 2. BSBV four serotypes according to Prillwitz and Schlösser (1992). Ahlum serotype corresponds to BSBV and the Wierthe one is now assimilated to BVQ. The group 3 has however since been attributed to another rod-shaped virus with multipartite genome, the BSBMV (Wisler *et al*., 1994; Heidel *et al*., 1997), and the group 4 should probably also be considered as such.

OBJECTIVES

Beet soil-borne virus and *Beet virus Q*, although being present in most of the countries growing sugar beet and frequently found associated with the rhizomania- responsible BNYVV, are poorly understood. As recently as four years ago, the full-length sequence available for each RNA of both viruses still derived from laboratory-maintained strains and studies of their genomic variability were restricted to the RNA-2 encoded coat proteins (Stas *et al*., 2001a; Lennefors *et al*., 2005) and the 3' extremity of BSBV RNA-3 (Koenig *et al*., 2000). No particular interest had also been taken in the potential proteins encoded by their tripartite genomes and their expression or function *in planta* had never been investigated. Finally, whereas the BSBV pathogenicity was much disputed depending on the experimental or observation approach, the BVQ one just remained a question mark. In light of this, we decided to get an insight into the particular biology and functioning of these two beet pomoviruses. In order to tackle with the multiplication dynamics of BSBV and BVQ, a reverse genetic approach – from gene to function, by contrast with the traditional forward genetic going from function to gene – was developed and both *in vivo* experiments and *in silico* analyses were performed.

On one hand, interest was taken in the potentially truncated CP RT domain of BVQ, encoded by genomic RNA-2. Systematic partial sequencing of BVQ strains from different parts of Europe was performed to determine the common arrangement of BVQ CP-RT coding sequence. By analyzing the genome organization of BVQ strains directly trapped in *Beta* bait plants that were grown in infested soil under controlled conditions, we made sure working on fresh BVQ strains. The variability between viral strains was also assessed at the nucleotide and amino acid levels to distinguish conserved regions from hot spots of diversity in both the genome and the potential gene product. Finally, predictions of transmembrane helices were carried out in

the collected sequences to search for new transmission-related motifs still lacking in BVQ CP RT domain. Rearrangements in BVQ RNA-2 and results from the *in silico* analyses are described in **chapter II**.

On the other hand, the behaviour of sugar beet pomoviruses was investigated *in planta*. Full-length cDNA clones were developed for all viral genomic RNAs of BSBV and BVQ to perform single- or mixed-infections, following the multiple virus associations observed in the fields. Multiplication requirements of viral transcripts from constructs were determined on lab host plants by intra- or inter-specific genome combinations. The infectiousness was assessed for the different transcripts arragements by detecting viral RNAs and structural proteins in the infection products and symptomatic reactions were analyzed to characterize one or another viral component as potential pathogenic factor. When the involvement in viral transmission of BVQ RT domain was investigated *in silico*, the detection of *in vivo* produced CP and CP-RT for both BSBV and BVQ might give further informations on their potential role in viral replication or symptom induction. **Chapters I** and **III** deal with the specific multiplication dynamics of BSBV and BVQ transcripts, respectively.

CHAPTER I

A full-length infectious clone of *beet soil-borne virus* indicates the dispensability of the RNA-2 for virus survival *in planta* and symptom expression on *Chenopodium quinoa* leaves

Opening comments

Reverse genetics has now entered the virologist custom for studying viral functioning and full-length infectious clones constitute very useful tools. For RNA viruses, full-length cDNA copies of each viral genomic RNA are flanked with a T7 transcription promoter at the 5' end of the coding strand following reverse transcription and polymerase chain reaction (RT-PCR). Amplicons are then stabilized by cloning in bacterial plasmid and viral RNAs may be produced *in vitro* from these constructs by using the RNA polymerase from phage T7 (Fig. 13). Benefits of such approach are manifold. Full-length cDNA clones make easy selecting, disrupting or even modifying genes of interest. This proved to be highly efficient to investigate the pathogenicity and particular biology of other BSBV- and BVQ-related viruses, e.g. the identification of proteins involved in BNYVV cell-to-cell movement (Gilmer *et al*., 1992), pathogenicity (Jupin *et al*., 1992) or transmission (Rahim *et al*., 2007) and in PMTV long-distance movement (Torrance *et al*., 2009) or virulence (Lukhovitskaya *et al*., 2005). Working with viral transcripts from constructs may also guarantee single-virus infection. In the context of frequent mixed-infections of beet with BNYVV, BSBV and/or BVQ (Meunier *et al*., 2003; Ratti *et al*., 2005), this avoids the hard and uncertain task of selecting specifically the desired virus from naturally infected plant. Moreover, preliminary viral multiplication on *C. quinoa* leaves before starting *in vivo* experiments may modify the viral genome, as discussed in chapter II, and affect the viral behaviour.

For these reasons, full-length cDNA clones were developed for the three BSBV genomic RNAs (cloning performed by Mohsen Mehrvar). RNAs carrying house-keeping genes necessary for BSBV to establish a local (a)symptomatic infection in leaves of *C. quinoa* and *B. macrocarpa* were then identified by means of different transcripts combinations. Such experimental approach was greatly favored by the multipartite genome of BSBV. The location of the putative replicase, structural and movement proteins encoding genes on separate RNAs made the selection or suppression of one or another function easier. Nevertheless, a clone mutated for the RT domain of the CP was used to differentiate the involvement of CP and CP-RT proteins in viral multiplication and symptom induction.

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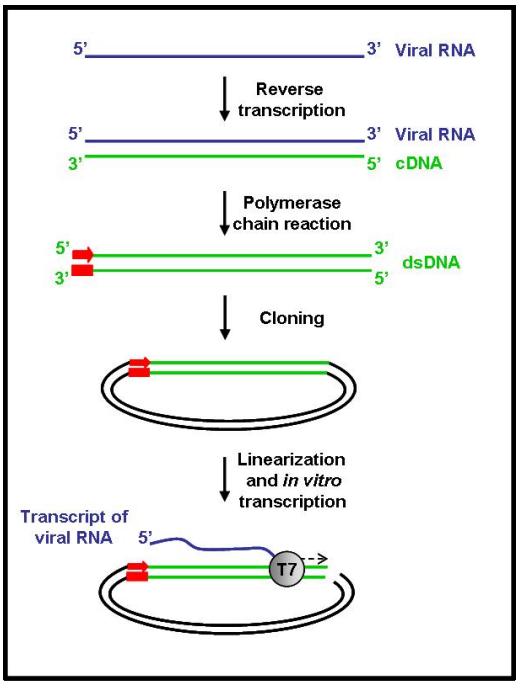

Fig. 13. Strategy for producing viral RNA transcripts *in vitro*. Complementary DNA (cDNA) is synthesized for the viral RNA of interest by reverse transcription. The cDNA is amplified in double-stranded DNA (dsDNA) flanked with a T7 transcription promoter (red arrow) at the 5' end of the coding-strand by means of PCR. Resulting amplicons are then cloned in a bacterial plasmid. Transcripts of viral RNA are finally produced by the T7 RNA polymerase enzyme from the DNA copies in linearized constructs.

Preliminary concepts

Depending on a host cell for their multiplication, viruses hijack cellular elements at their advantage and minimize the size of their functional genome. Viruses therefore developed many different strategies to enable but also regulate efficiently the expression of their own proteins from the compacted molecules composing their genomes. Moreover, the range of functions of viral proteins may be broadened by potential post-translational modifications (PTMs).

RNA genomes partitioned, better access to viral genes

While some viruses produce all their proteins from a single coding molecule, viral genomes may also be split in multiple DNAs or RNAs fragments (Mandahar, 2006). Such a fragmentation of the viral genome has two major advantages. On one hand, this makes coding sequences more accessible for the translation by ribosomes. As described hereafter, specific strategies of proteins expression are required when ORFs are in single queue on the same genomic molecule. On the other hand, multipartite genomes make finer the control of expression for genes which coding sequences are separated on different genomic segments. The replication and translation steps are finely regulated for each fragment through *cis*-acting sequences (on the same molecule). As presented in the general introduction, BNYVV, BSBV and BVQ genomes are all segmented, composed of four to five RNAs for the first and three for the lasts. Thus, the putative viral replicase, structural and movement proteins are encoded on separate RNA molecules for both the BSBV and BVQ.

Viral strategies to express compacted genomes

Most of the eukaryotic mRNAs carry a single ORF – they are called mono-cistronic – and are scanned by ribosomes from their 5' end toward the 3' extremity to find and translate the coding sequence (Kozak, 1999). Translation is then initiated at the AUG start codon within the best context – the most common one in plants being A**A**CAAUG**G**C, with nucleotides in

bold of high importance – and terminates when encountering a UAA, UAG or UGA stop codon (Caskey *et al*., 1968; Kozak, 1986; Fütterer and Hohn, 1996). Because of this major constraint that governs the proteins production in eukaryotes, viruses use multiple strategies to express their own proteins from compacted genomes. These can be divided into three categories, depending on whether transcription, translation or post-translation stage is concerned (Drugeon *et al*., 1999; Mandahar, 2006; Ryabova *et al*., 2006). Although presented separately in the following sections, several of these mechanisms may be combined by the same virus species.

At transcriptional level

Viruses may specifically express one or another protein from a genomic molecule by controlling the messenger RNAs (mRNAs) (Fig. 10).

While viral genomes composed of single-stranded RNAs with positive polarity can be directly used by ribosomes for synthesizing proteins, negative-stranded RNA should be first transcribed to produce the messenger molecule. Minus-sense RNA genomes, as those of rhabdoviruses, are therefore complexed with the viral polymerase to produce infectious viral particles. In addition, different proteins may also be encoded by both the genomic RNA and its complementary strand. Such **ambisense** RNAs are used by the tenuivirus *Rice stripe virus* (RSV), for instance (Ramirez and Haenni, 1994).

The **splicing** of viral mRNAs, which is reported only among plant viruses with DNA genomes such as *Geminiviridae* (Schalk *et al*., 1989; Drugeon *et al*., 1999), generates new coding sequences in the mRNA by removing parts of it.

Subgenomic (sg) RNAs are mRNAs produced by the viral polymerase from transcription promoters inside the non-coding strand. They make possible the translation of coding sequences downstream the 5' proximal ORF(s). This strategy is widely used by plant viruses and allows for instance the expression of TGB proteins as those of BNYVV (Gilmer *et al*., 1992), PMTV (Savenkov *et al*., 2003) and putatively BSBV and BVQ.

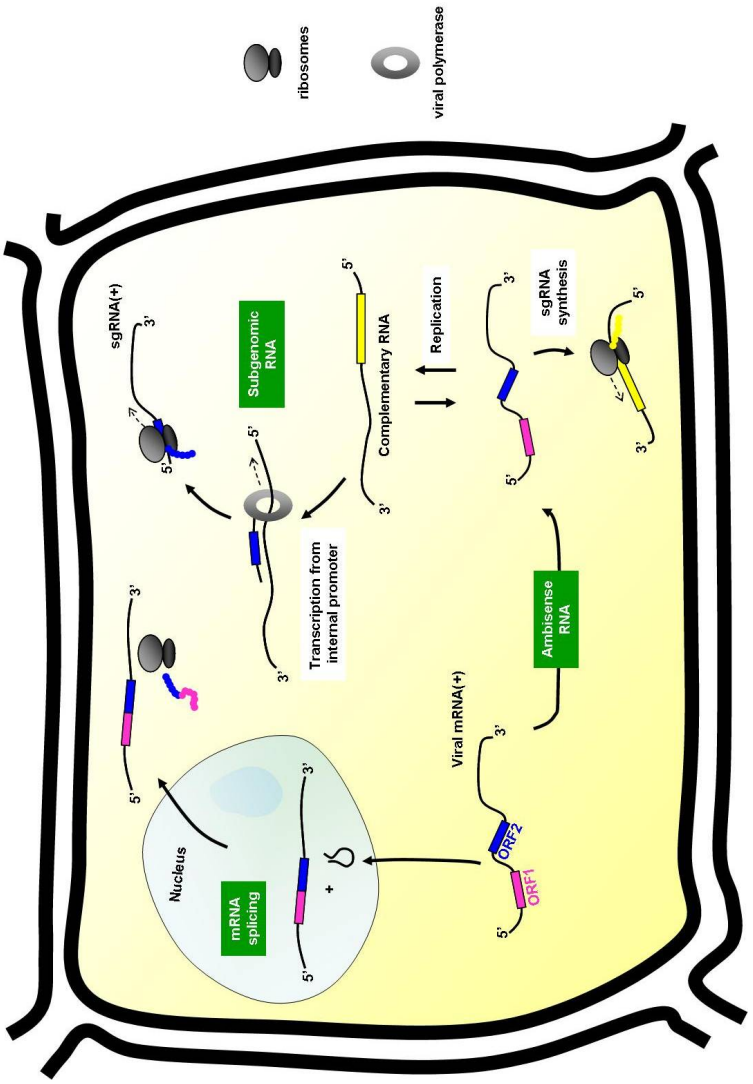

Fig. 10. Transcriptional strategies to express viral genes from compacted genomes inside the cytoplasm of a plant cell.

At translational level

When the mRNA carries multiple ORFs, those downstream the 5'-proximal one may be also used for the proteins production directly by ribosomes (Fig. 11). However, distinction should be made between the following mechanisms.

Ribosomes may pass through the first 5'-proximal start codon and begin translating at the second one. Such an unconventional **leaky scanning** allows the expression of proteins from ORFs that are consecutive and autonomous (one gene after the other) or in-frame (both genes are merged, but one is shorter than the other), or overlapping. 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 AACAAUGGC (in plants) or is a non-AUG alternative start codon (Kozak, 1989; Ryabova *et al*., 2006). Leaky scanning from overlapping ORFs is proposed to allow the expression of the third gene from TGB (TGBp3) of plant viruses (Zhou and Jackson, 1996; Verchot *et al*., 1998) like those of BNYVV (Gilmer *et al*., 1992), BSBV and BVQ.

After translation of the first ORF(s), ribosomes may also **reinitiate** translation with the following one(s). The efficiency of re-initiation is highly dependant on the length of the first ORF(s), usually short (sORF), and the distance between the two start codons (Ryabova *et al*., 2006). This termination-reinitiation process is reported for the *Cauliflower mosaic virus* (CaMV) (Fütterer and Hohn, 1991) but not for plant RNA viruses (Mandahar, 2006).

Jumping of the ribosomal machinery scanning the mRNA to a downstream coding sequence is referred as "**shunting**". In this case, the region between the take-off and landing sites is not scanned by ribosomes. Such jumps are favored by strong RNA secondary structures and were first described for CaMV (Fütterer *et al*., 1990). However, no plus-sense RNA virus is reported to use such translation strategy (Mandahar, 2006).

During the elongation step of translation, ribosomes may switch the reading frame by one nucleotide towards the 5' (-1nt) or 3' (+1nt) extremity of

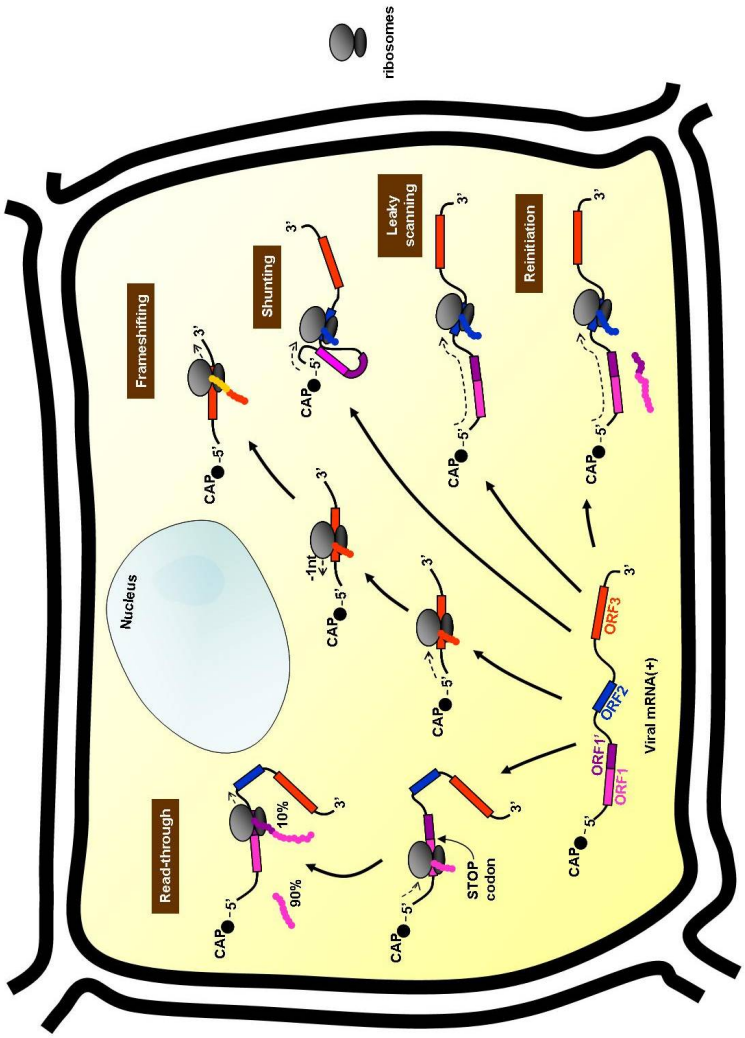

Fig. 11. Translational strategies to express viral genes from compacted genomes inside the cytoplasm of a plant cell.

mRNA. This **frameshift** leads to the production of two proteins that have similar N-terminal sequences but differ from the shift position to the C-terminal ends. A slippery sequence of seven nucleotides, separated by four to nine nucleotides from a downstream hairpin structure, constitutes the signal for +1 frameshifting, most common among plant RNA viruses of positive polarity (Drugeon *et al*., 1999).
Finally, the stop codon of some coding sequences may act as leaky terminators, i.e. once out of ten ribosomes can add another amino acid instead of stopping translation and continue to the next and final termination codon. Such a **read-through** mechanism therefore adds a read-through (RT) domain to the protein encoded by the ORF upstream the suppressible STOP codon. This strategy is reported for many plant RNA viruses (Maia *et al*., 1996) and enables the expression of the BNYVV CP-RT (Ziegler *et al*., 1985). The polymerases and CP-RTs of BSBV and BVQ are thought to be produced by read-through.

At post-translational level

For some viruses, the coding sequences are so compacted that several of them may be placed end to end, leading to the formation of (a) merged ORF(s). To produce separate structural and non-structural proteins, the merged protein(s) – or **polyprotein(s) –** undergo proteolytic cleavage. Examples of autocatalytic cleavages are shown for the famous polyprotein of potyviruses (Allison *et al*., 1986) (Fig. 12a) as well as the BNYVV replicase (Hehn *et al*., 1997) (Fig. 12b).

Post-translational modifications of viral proteins

Once translated from their mRNA, viral proteins may also undergo post-translational modifications (PTMs) different from the proteolysis discussed before. These subtle changes may in some cases greatly contribute to the proteins structure and function. **Phosphorylation** and **glycosylation** are widely reported as regulatory mechanisms affecting proteins enzymatic activity, folding, stability, subcellular localization or interactions (Dwek, 1996; Cohen, 2000). Whereas phosphorylation consists in adding a

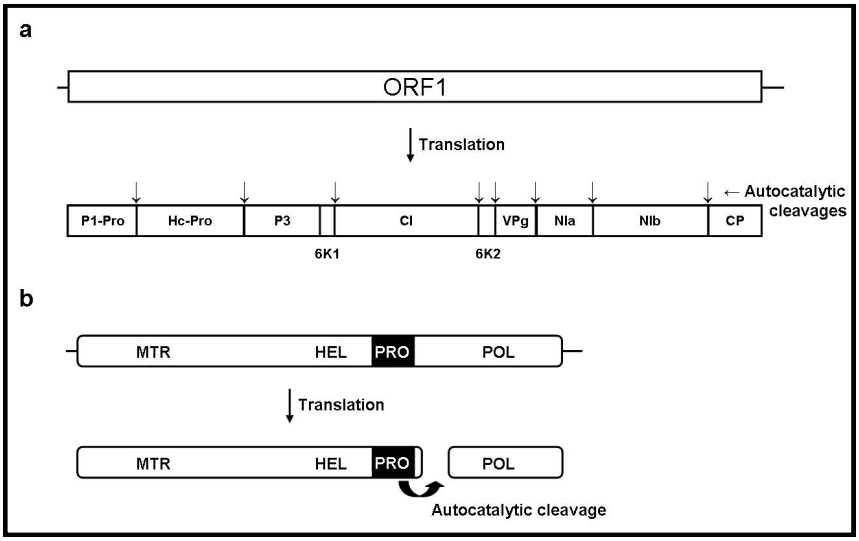

Fig. 12. Two types of polyproteins undergoing proteolytic cleavages are shown. (a) The *Tobacco etch virus* (TEV) monopartite genome encodes for a single polyprotein, subsequently cleaved in at least nine different proteins (adapted from sequence of Allison *et al*., 1986). Autocatalytic cleavage sites are indicated (↓). (b) BNYVV RNA-1 encoded replicase contains papain-like protease domain (PRO) responsible for autocatalytic processing of the polymerase (adapted from Hehn *et al*., 1997).

phosphate group (PO_4) to the lateral chain of an amino acid residue, glycosylation leads to the linkage of a glucidic molecule to an asparagine (N-glycosylation), serine or threonine (O-glycosylation). Commonly observed among eukaryotes and prokaryotes (Moens and Vanderleyden, 1997; Cozzone, 1998), viruses also resort to such PTMs to modulate the properties of their proteins. Some plus-sense RNAs viruses regulate the function of their replicase through their phosphorylated state (Jakubiec and Jupin, 2007). Glycosylation of the CP , for instance, is reported for *Plum pox virus* (PPV) and *Potato virus X* (PVX), although the functional role of such modification remains unclear (Fernández-Fernández *et al*., 2002; Baratova *et al*., 2004). Mostly involved in the cellular proteins degradation pathway but also in functions as diverse as cellular trafficking or gene expression (Marx, 2002), ubiquitination is used by viruses for their own advantage as well. The addition of 8.5 kDa ubiquitine on lysine residues has thus been shown to be

part of viral replication for *Tomato bushy stunt virus* (TBSV) (Barajas and Nagy, 2010) and is involved in budding of several enveloped viruses – virus release through the plasma membrane of the host cell (Isaacson and Ploegh, 2009).

As a matter of course, physico-chemical properties of proteins undergoing such PTMs are modified and these may be detected in experimental conditions.

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Short Communication

A full-length infectious clone of beet soil-borne virus indicates the dispensability of the RNA-2 for virus survival *in planta* and symptom expression on *Chenopodium quinoa* leaves

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For a better understanding of the functionality and pathogenicity of beet soil-borne virus (BSBV), full-length cDNA clones have been constructed for the three genomic RNAs. With the aim of assessing their effectiveness and relative contribution to the virus housekeeping functions, transcripts were inoculated on *Chenopodium quinoa* and *Beta macrocarpa* leaves using five genome combinations. Both RNAs-1 (putative replicase) and -3 (putative movement proteins) proved to be essential for virus replication *in planta* and symptom production on *C. quinoa*, whereas RNA-2 (putative coat protein, CP, and a read-through domain, RT) was not. No symptoms were recorded on *B. macrocarpa*, but viral RNAs were detected. In both host plants, the 19 kDa CP was detected by Western blotting as well as a 115 kDa protein corresponding to the CP–RT.

Beet soil-borne virus (BSBV) is a pomovirus transmitted to Chenopodiaceae by the protist *Polymyxa betae* (Ivanović *et al.*, 1983), which is also the vector of the aetiological agent of the rhizomania syndrome of sugar beet, beet necrotic yellow vein virus (BNYVV) (Tamada & Baba, 1973). Originally reported in Italy (Canova, 1959), rhizomania disease is now widespread in most countries where sugar beet is grown (McGrann *et al.*, 2009) and BSBV is often found in beet infected with BNYVV (Meunier *et al.*, 2003). However, the pathogenicity of BSBV and its contribution to the rhizomania syndrome remain unclear, with opinions still divided on this (Prillwitz & Schlösser, 1992; Kaufmann *et al.*, 1993; Lindsten, 1993; Rush & Heidel, 1995).

The BSBV genome consists of three single-stranded RNAs of positive polarity, packaged into rod-shaped particles (Koenig *et al.*, 1996, 1997; Koenig & Loss, 1997). RNA-1 (5.8 kb) encodes the putative viral replicase. The 19 kDa coat protein (CP) and a putative 85 kDa read-through (RT) domain are encoded by RNA-2 (3.5 kb). RNA-3 (3.0 kb) comprises three open reading frames (ORFs) encoding three putative proteins (48, 13 and 22 kDa) thought to be responsible for the viral cell-to-cell movement, resembling the well-known triple gene block proteins (TGBs) (Fig. 1).

In this study, the contribution of each RNA component to virus survival and symptom expression was investigated through the use of full-length cDNA clones on two host plants, *Chenopodium quinoa* and *Beta macrocarpa*. This last plant species was preferred to the natural host *Beta vulgaris* with a view of developing the basis for further molecular analysis of viral systemicity, following the example of previous studies on BNYVV (Lauber *et al.*, 1998).

Full-length cDNA sequences were generated from an Iranian BSBV isolate (Nyshabour, Khorasan Razavi Province), which was trapped from infested soil in roots of *B. vulgaris*. After total RNA extraction from sugar beet roots using the SV total RNA isolation kit (Promega), the three genomic RNAs were reverse transcribed and amplified by PCR (RT-PCR) using expand reverse transcriptase and the expand long template PCR System (Roche). Primers matching the extremities of the three viral RNAs were designed following the only references known to date (Koenig *et al.*, 1996, 1997; Koenig & Loss, 1997). Forward primers were flanked with a T7 promoter sequence (TAATACGACTCACTATAG) (Fig. 1). Ampli-

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are FN386612, FN386613 and FN386614.

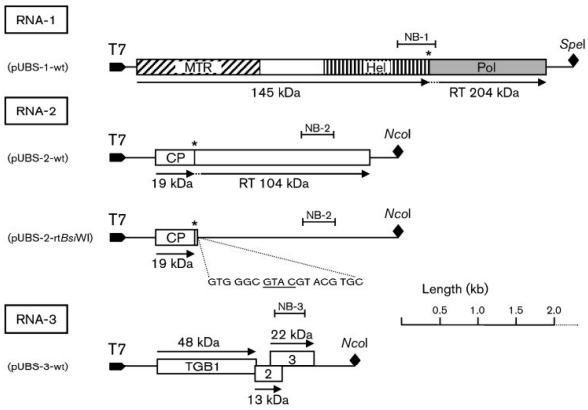

Fig. 1. Schematic representation of the BSBV tripartite genome: RNA-1 encodes the putative replicase with conserved motifs methyl-transferase (MTR), helicase (Hel) and RNA-dependent RNA polymerase (Pol) translated as an RT domain; RNA-2 encodes the putative CP and an RT domain; RNA-3 encodes the putative TGBs-1, -2 and -3. The asterisks (*) represent the RT stop codons. The molecular mass of the putative proteins is given beside each arrow beneath or above the corresponding ORF. The full-length cDNA clones (pUBS-1-wt, -2-wt, -2-rt*Bsi*WI, -3-wt; pUC19 backbone) are under the control of a T7 promoter and possess a unique restriction site (◆) for linearization and run-off transcription. pUBS-2-rt*Bsi*WI clone has a frameshift at the beginning of the CP–RT domain coding sequence as a result of the 4 nt insertion (underlined). The probes used for Northern blot detection of the three viral RNAs are positioned above the corresponding full-length clone (NB-1, -2 and -3).

cons were gel purified using QIAquick gel extraction kit (Qiagen) and inserted into the pGEM-T vector (Promega). Ligation products were transferred into the *Escherichia coli* strain JM109. Full-length cDNA clones were then sub-cloned into pUC19 and maintained in the *E. coli* strain DH5-α, to obtain the final constructs pUBS-1-wt (RNA-1), pUBS-2-wt (RNA-2) and pUBS-3-wt (RNA-3) (Fig. 1). A full-length cDNA clone of RNA-2 containing a frame shift mutation in the coding sequence for the putative CP–RT domain (clone pUBS-2-rt*Bsi*WI, Fig. 1) was obtained by filling a *Bsi*WI digestion (target site position 907–912) with DNA polymerase I Klenow fragment (Promega) followed by self-ligation with T4 DNA ligase (Roche).

The four full-length cDNA constructs in pUC19 were linearized with *Spe*I or *Nco*I (Fig. 1) before run-off transcription with the RiboMAX large-scale RNA production system - SP6 and T7 (Promega) in the presence of Cap analogue $m^7G(5')ppp(5')G$ (New England Biolabs). The transcripts were mechanically inoculated in a 50 mM KH_2PO_4 (pH 4.2) and 0.04 % bentonite buffer on both *C. quinoa* and *B. macrocarpa* leaves. *In vitro* transcript combinations of RNA-1 (1), -2 (2), -2 carrying a frame shift mutation in the RT domain coding sequence (2*) and -3 (3) were tested as follows: 1+2+3, 1+2*+3, 1+2, 1+3 and 2+3. Inoculation buffer alone was applied for the negative control. *C. quinoa* and *B. macrocarpa* were exposed to light for 16 h per day at 15–20 °C and 20–25 °C for night and day, respectively. The experiment was carried out on one *C. quinoa* plant (three leaves inoculated) and two *B. macrocarpa* plants (two leaves inoculated per plant) for each condition and repeated twice to ensure reproducibility.

Symptomatic lesions appeared on all the *C. quinoa* leaves inoculated with BSBV full-length transcripts of RNAs 1+2+3, 1+2*+3 or 1+3 at 3 days post-inoculation (p.i.). Small necrotic spots, necrotic ringspots (data not shown) and chlorosis were clearly developed 7 days p.i. (Fig. 2). However, the infection of the *B. macrocarpa* plants with the same three combinations of BSBV full-length transcripts did not produce any symptoms on the inoculated leaves (data not shown). These observations indicated that the presence of the BSBV RNA-2 was therefore not necessary for symptom expression, which occurred only on *C. quinoa*.

C. quinoa- and *B. macrocarpa*-inoculated leaves were harvested at 7 and 10–17 days p.i., respectively. The RNAs were extracted from the whole leaves using the polysomes extraction protocol (Jupin *et al.*, 1990) followed by phenol extraction and ethanol precipitation of the

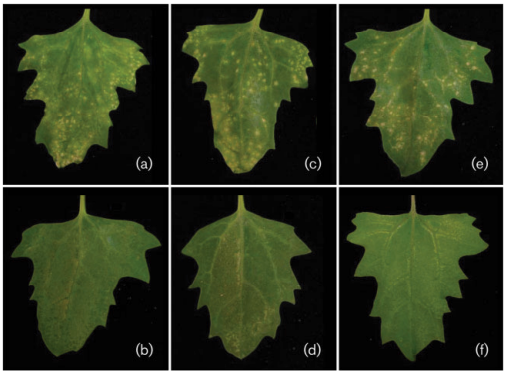

Fig. 2. *C. quinoa* leaves 7 days after inoculation with BSBV full-length transcript mixes 1+2*+3 (a), 1+2 (b), 1+2+3 (c), 2+3 (d) and 1+3 (e) and a negative control (f). Only genome arrangements 1+2*+3 (a), 1+2+3 (c) and 1+3 (e) provoked the appearance of symptoms on the inoculated leaves, with necrotic spots and ringspots (data not shown) and slight yellow chlorosis.

RNAs. The viral RNAs were detected by Northern blot with random DNA probes synthesized with the Prime-a-Gene Labelling System (Promega) and labelled with [α-^{32}P]dCTP (1.14×10^{14} Bq mmol^{-1}; Perkin Elmer). The probes matched nucleotide positions 3510–4015 (NB-1), 2281–2711 (NB-2) and 1930–2330 (NB-3) of RNAs-1, -2 and -3, respectively (Fig. 1).

Genomic combinations 1+2*+3, 1+2+3 and 1+3 resulted in corresponding viral RNA amplification on both host plants (Fig. 3a, b, lanes 1, 2 and 4), whereas 1+2 and 2+3 did not (Fig. 3a, b, lanes 3 and 5). The BSBV RNAs -1 and -3 full-length transcripts were therefore required for the efficient viral replication *in planta*, in contrast with BSBV RNA-2 that appeared dispensable for the viral RNA replication. However, even if no viral RNAs were detected in plants inoculated with the combinations 1+2 and 2+3 (Fig. 3a, b, lanes 3 and 5), a potential undetected infection cannot be ruled out for the mix 1+2. Lacking the putative cell-to-cell movement proteins encoded by RNA-3, the RNAs-1 and -2 might indeed replicate in the first inoculated cells but couldn't spread to the neighbouring ones (Hull, 2009). This will be further characterized using protoplast inoculation.

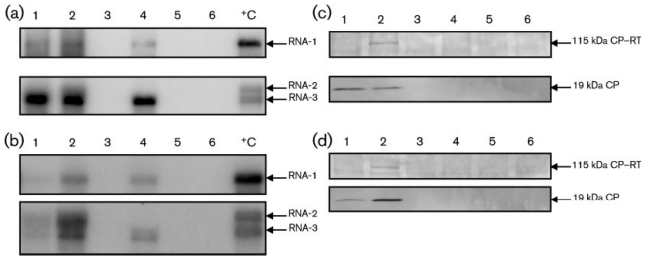

Fig. 3. Northern (a, b) and Western (c, d) blot analysis for the detection of BSBV RNAs and structural proteins in *C. quinoa* (a, c) and *B. macrocarpa* (b, d) leaves, 7 and 10–17 days p.i., respectively. Plant leaves were either inoculated with one of the five genome arrangements of BSBV full-length transcripts tested [combinations 1+2*+3 (lane 1), 1+2+3 (2), 1+2 (3), 1+3 (4) and 2+3 (5)] or mock-inoculated (6). The three BSBV genomic RNAs were detected in total RNA extracts using random DNA probes labelled with [α-^{32}P]dCTP and diluted transcripts constituted positive controls ($^{+}$C) (a, b). The 19 kDa CP and 115 kDa CP–RT proteins of BSBV were immuno-detected with primary polyclonal antibody directed against viral particles (c, d).

All three replicative combinations raised the infection rate by 100 % in both host plants, except the 1+2*+3 which infected half of the inoculated *B. macrocarpa*.

BSBV CP and CP–RT proteins were detected by Western blotting after SDS-PAGE separation of total protein extracts from symptomatic samples and whole asymptomatic leaves. Structural proteins were revealed using a polyclonal antibody directed against viral particles (DSMZ). The primary antibody was diluted 1 : 10 000 in TBS buffer containing 0.1 % Tween 20, 5 % powdered skimmed milk, and supplemented with total protein extracts from healthy *C. quinoa* or *B. macrocarpa*. Anti-rabbit alkaline phosphatase-conjugated secondary IgG (Sigma-Aldrich) was diluted 1 : 10 000 in TBS–Tween–milk buffer and the blots were revealed with premixed BCIP–NBT solution (Sigma-Aldrich).

A 19 kDa protein was detected in both *C. quinoa* and *B. macrocarpa* plants inoculated with the 1+2*+3 and 1+2+3 mixes of BSBV RNAs transcripts (Fig. 3c, d, lanes 1 and 2) but not when RNA-2 was omitted (combination 1+3; Fig. 3c, d, lane 4). Thus, the BSBV RNA-2 transcripts are indeed responsible for the expression of the 19 kDa protein *in planta*, which corresponds to the BSBV CP of the same predicted molecular mass that is encoded by the BSBV RNA-2 first ORF. Another protein with an estimated molecular mass of 115 kDa was also observed in the leaves of both plants inoculated with the genome combination 1+2+3 (Fig. 3c, d, lane 2) and not detected in the leaves inoculated with the BSBV RNA-2 transcripts mutated for the RT domain of the CP (genome combination 1+2*+3; Fig. 3c, d, lane 1). This product therefore corresponds to the predicted 104 kDa BSBV CP–RT protein. Transcript progeny analysis confirmed the stability of the frame shift mutation (data not shown).

According to the different genomic combinations of BSBV full-length transcripts inoculated, our study showed that both the putative replicase (RNA-1) and TGB proteins (RNA-3) of BSBV are essential and sufficient for the replication of such RNAs in the inoculated leaves of *C. quinoa* and *B. macrocarpa*. In addition, the symptom expression on *C. quinoa* did not depend on the presence of the BSBV RNA-2. The BSBV CP and CP–RT encoded by the BSBV RNA-2 are therefore not required for viral infection in the plant hosts or for symptom production on *C. quinoa* leaves. The dispensability of the CP for viral replication *in planta* is a common phenomenon, shared by the closely related pomovirus potato mop-top virus (PMTV) on *Nicotiana benthamiana* and *Nicotiana clevelandii* (McGeachy & Barker, 2000; Savenkov *et al.*, 2003), as well as other viruses such as the well-known tobamovirus tobacco mosaic virus (TMV) on *Nicotiana tabacum* (Siegel *et al.*, 1962). However, whereas symptom production due to TMV does not depend on the presence of the CP, no symptoms are expressed when PMTV lacks the CP and CP–RT. Thus, despite their taxonomic proximity and a highly similar genome organization, the BSBV and PMTV pomoviruses retain their own particularities. Nevertheless, it should be borne in mind that PMTV is a potato virus and symptom expression could be host-dependent.

It is also worth noting that, as the primers used to amplify full-length cDNA sequences of BSBV were designed according to a previously published nucleotide sequence of a German isolate, the biological properties of the resulting full-length cDNA clone might have been modified. However, viral terminal sequences are mostly highly conserved for a virus species as they are involved in viral replication (Duggal *et al.*, 1994; Koenig *et al.*, 2000), and the BSBV full-length clone replicated efficiently.

Consistent with earlier findings reporting on foliar symptoms on *Chenopodium* spp. and occasional lesions on *Beta* spp. leaves when inoculating BSBV from the crude sap of infected plants (Henry *et al.*, 1986), transcripts from the full-length clone of BSBV induced clear symptoms only on the *C. quinoa* leaves. Following the example of BSBV, the two first BNYVV RNAs, carrying the genetic material homologous to one of the three BSBV RNAs plus a cysteine-rich protein (p14) with PTGS suppressor activity (Bouzoubaa *et al.*, 1986, 1987; Dunoyer *et al.*, 2002), are also unable to induce a systematic symptomatic foliar reaction on *Beta* spp. However, lesions on *Beta* spp. leaves are clearly produced when at least BNYVV RNAs-1, -2 and -3 are present (Tamada *et al.*, 1989; Rahim *et al.*, 2007). Although BSBV and BNYVV both naturally infect sugar beet, BSBV lacks the genetic material necessary to induce clear symptoms on *B. macrocarpa* leaves whereas BNYVV has this through RNAs-3 and -4.

The use of a polyclonal antibody directed against BSBV viral particles allowed two proteins expressed from BSBV RNA-2 transcripts to be detected. As expected, the Western blot analysis showed the production of the BSBV 19 kDa CP in both *C. quinoa* and *B. macrocarpa*. However, whereas the expected molecular mass of the CP–RT is 104 kDa, a protein with an estimated molecular mass of 115 kDa corresponding to the CP–RT was identified in both host plants. It could be assumed that post-translational modifications of the CP–RT domain, such as phosphorylation or glycosylation events, are responsible for this 115 kDa observed molecular mass of CP–RT. Actually, post-translational modifications can reduce the protein's electrophoretic mobility in SDS-PAGE and many viral structural proteins are described as glycosylated and/or phosphorylated (Hahn & Shepherd, 1980; Seddas & Boissinot, 2006; Akamatsu *et al.*, 2007; Hafrén & Mäkinen, 2008; Zayakina *et al.*, 2008). Another hypothesis would involve the intrinsic properties of the BSBV CP–RT, knowing that highly hydrophilic domains containing many charged residues, such as arginine (Hu & Ghabrial, 1995), or the presence of transmembrane motifs (Rath *et al.*, 2009) in proteins might influence their migration on SDS-PAGE. This is supported by the presence in the RT domain of the BSBV CP of two predicted transmembrane helices, as is also reported for the closely related beet virus Q (BVQ)

(Crutzen *et al.*, 2009), which would be essential for viral transmission by the vector *P. betae* (Diao *et al.*, 1999; Adams *et al.*, 2001). Further experiments need to be performed to determine the specificity of CP–RT, from the post-translational modification or intrinsic properties, which influences the molecular mass of the protein on SDS-PAGE.

The rhizomania syndrome of sugar beet and its causal agent, BNYVV, have been widely studied since the 1970s because cropping losses, commonly about 50 % and sometimes up to 80 %, have been attributed to the disease (McGrann *et al.*, 2009). However, the involvement of BVQ and BSBV in the disease, often associated with beet infected with BNYVV, has never been clearly established and no particular interest in understanding their specific functionality was followed. Our study describing the first infectious full-length cDNA clone available for a beet *Pomovirus* therefore provides a useful tool for further investigating the pathogenicity of BSBV in the complex rhizomania syndrome, as well as its replication and infection mechanisms, and the potential viral interactions with other beny- and pomoviruses on both susceptible and rhizomania-resistant beets.

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CHAPTER II

The *beet virus Q* coat protein readthrough domain is longer than previously reported, with two transmembrane domains

Opening comments

Since the identification of BVQ as a distinct virus species and sequencing the entire genome of a BVQ strain maintained on *C. quinoa* leaves (Koenig *et al*., 1998), no further interest has been taken in this virus. However, the RNA-2 encoded 35 kDa CP RT domain of this BVQ strain is much shorter when compared with BBNV, BSBV and PMTV, as RT domains of 53, 85 and 71 kDa are described for these other pomoviruses, respectively (Table 3). Moreover, two small ORFs coding for putative proteins of 9 and 18 kDa of unknown function are located downstream of the CP-RT coding sequence. These little proteins display no homolog among furoviruses *sensu lato* (fungal-transmitted rod-shaped viruses, as plasmodiophorids were first considered as belonging to the fungi kingdom), but their coding sequences present similarities with internal parts of BSBV and PMTV RT domain (Koenig *et al*., 1998). Finally, the 35 kDa RT domain of BVQ is predicted to lack the transmission-related TM2 and this raises questions about the transmissibility by its putative vector, *P. betae*. Following these observations, it has been hypothesized that such special organization of BVQ RNA-2 resulted from deletion(s) event(s) in a longer RT domain like those of other closely related viruses (Koenig *et al*., 1998; Adams *et al*., 2001). To identify the common arrangement of the BVQ RT domain and get an insight into the transmission characters of this virus, interest was taken in RNA-2 organization of BVQ strains coming from different parts of Europe.

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Table 3. Listing of plant RNA virus species that possess a coat protein (CP) with read-through (RT) domain. Accession numbers are given for the CP-RT encoding RNAs. Only type-species are indicated for *Luteoviridae*-belonging viruses. Suppressible stop codons and molecular masses (MM) of the RT domains are indicated. Potential transmembrane motifs (TMs) predicted by Adams and co-authors (2003) for soil-borne viruses are mentioned. Vector types are also specified.

Virus	Accession number	Amber codon	CP-RT MM	TMs	Vector
Luteoviridae					
Enamovirus					
PEMV	L04573	UGA	33	-	
Luteovirus					Aphids
BYDV-PAV	D01214	UAG	50	-	
Polerovirus					
PLRV	D00530	UAG	57	-	
Benyvirus					Soil-borne
BNYVV	X04197	UAG	54	TM1 - TM2	
BSBMV	NC_003503	UAG	53	TM1 - TM2	
Virgaviridae					
Furovirus					
CWMV	AJ012006	UGA	65	TM1 - TM2	
EWMV	NC_002330	UGA	65	TM1 - TM2	
OGSV	AJ132579	UGA	57	TM1	
SBWMV	AB033690	UGA	64	TM1 - TM2	Soil-borne
SrCSV	AB033692	UGA	62	TM1 - TM2	
Pomovirus					
BBNV	NC_004424	UAA	53	TM1 - TM2	
BSBV	NC_003518	UAG	85	TM1 - TM2	
BVQ	NC_003511	UAG	35	TM1	
PMTV-Sw	NC_003724	UAG	71	TM1 - TM2	

Preliminary concepts

The CP-RT protein, a common organization

The addition of a RT domain to the viral CP is used by several single-stranded positive-sense plant RNA viruses belonging to different families and genera (Table 3). Soil-borne pomoviruses BBNV, BSBV, BVQ and PMTV encode for CP-RTs (Koenig *et al*., 1997a; Lu *et al*., 1998; Koenig *et al*., 1998; Reavy *et al*., 1998) as well as the other *Virgaviridae*-belonging *Chinese wheat mosaic virus* (CWMV), *European wheat mosaic virus* (EWMV), *Oat golden stripe virus* (OGSV), *Soil-borne wheat mosaic virus* (SBWMV), *Sorghum chlorotic spot virus* (SrCSV) from *Furovirus* genus (Diao *et al*., 1999a; Diao *et al*., 1999b; Shirako *et al*., 2000; Yamamiya and Shirako, 2000). These two last genera are transmitted by plasmodiophorids. CP-RTs coding sequences are also found on RNAs-2 of benyviruses, BNYVV and BSBMV (Ziegler-Graff *et al*., 1985; Niesbach-Klösgen *et al*., 1990; Lee *et al*., 2001), both vectored by the protist *P. betae* (Tamada, 1975; Wisler *et al*., 1994). Finally, RNAs-1 of aphids-transmitted viruses from *Luteoviridae* family are also reported to encode for a RT domain added to the CP. From the numerous virus species composing the *Luteoviridae* family, *Pea enation mosaic virus* (PEMV) (Demler *et al*., 1997), *Barley yellow dwarf virus* (BYDV) (Cheng *et al*., 1994) and *Potato leaf roll virus* (PLRV) (Bahner *et al*., 1990) type-species can be cited for *Enamo-*, *Luteo-* and *Polerovirus* genera, respectively. Two amber codons are mainly used to produce the RT domains of CPs. UAG leaky codon is found among luteo-, polero-, beny- and pomoviruses, whereas UGA is reported for enamo- and furoviruses. Another UAA suppressible codon is proposed for BBNV only. Molecular masses of the different RT domains range between 33 kDa for the shortest (PEMV) and 85 kDa for the longest one (BSBV).

Besides these plant viruses, the CP-RT organization is also reported for another bacteria-infecting virus. RNA phage Q beta, which parasites the well-known enterobacteria *Escherichia coli*, belongs to the *Allolevivirus* genus, *Leviviridae* family. Its monopartite single-stranded RNA genome

comprises 3 ORFs, of which the middle one encodes for the CP-RT or A1 protein (Kozlovska *et al*., 1993). The 14 kDa CP and a 22 kDa RT parts are shorter than those of the above-mentioned plant RNA viruses and the function of the RT domain is still not yet defined.

Although all aforesaid viruses produce structural CP-RTs, it is worth noting that, while benyviruses and virgaviridae are rod-shaped viruses with helical symmetry, virions of luteoviridae and alloleviviruses appear spherical with icosahedral symmetry. Therefore the CP-RT structure also varies depending on the virus genus.

Roles attributed to RT domains of viral CPs

As a structural protein being part of the viral particles, CPs RT domains of plant viruses have been shown to be essential for various functions at different stages of the viral cycle (Fig. 14).

Mutational analyses evidenced that CP RT domain may be involved either directly or indirectly in the **symptom induction**, as evidenced for the *Beet western yellows virus* (BWYV, *Luteoviridae*), PMTV or SBWMV (Chen *et al*., 1994; Bruyère *et al*., 1997; Torrance *et al*., 2009). For these viruses, this function is attributed to the C-terminal part of the protein. However, the RT nucleotide sequence might be involved in this phenotypic expression rather than the gene product. Targeted by innate defense mechanisms of the plant – RNA silencing machinery, the RT coding sequence could be cleaved in small interferering RNAs (siRNAs). Symptom production might then result from the interference of these siRNAs with plant mRNAs (Silhavy and Burgyán, 2004).

The N-terminal extremity of BNYVV CP RT domain, close to the linked coat protein, is known to be necessary for the viral **assembly** (Schmitt *et al*., 1992). In this case, virus encapsidation by the coat proteins is thought to be initiated by the formation of a nucleation complex, where CP-RT proteins interact with the viral RNAs extremity via the CP part and between themselves with the N-terminal portion of the RT domain (Fig. 14) (Haeberlé *et al*., 1994). However, virions formation is not always dependant on the RT part of the CP, as shown for the luteoviridae BWYV (Reutenauer

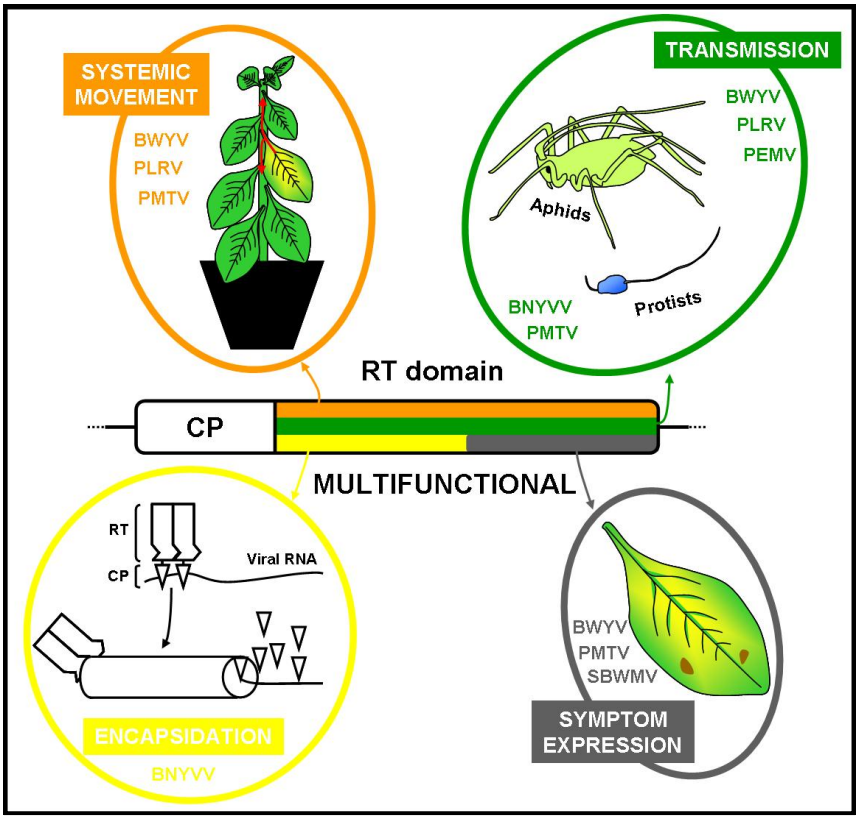

Fig 14. Mutlifunctional aspect of the BNYVV, BWYV, PEMV, PLRV, PMTV and SBWMV coat proteins (CP) read-through (RT) domains. For these viruses, RT part may be involved in viral transmission by aphids or plasmodiophorids vectors, symptom expression, virion formation (encapsidation model adapted from Haeberlé *et al*., 1994) or long-distance (systemic) movement in plants.

et al., 1993) and BYDV-PAV (Mohan *et al*., 1995), or PMTV (Torrance *et al*., 2009).

Systemic movement of the virus in the infected plant may also involve the CP RT domain, as reported for the BWYV, PMTV and PLRV (Mutterrer *et al*., 1999; Peter *et al*., 2008; Torrance *et al*., 2009). Nevertheless, the RT domains seem to be only part of the long-distance movement for these viruses, as they are not strictly essential for such purposes.

Finally, more common is the involvement of the RT domain in the viral **transmission** by means of aphid or protist vector. It is well-known that deletion events or amino acid changes in the CP RT domain of BNYVV, BWYV, PEMV, PMTV and PLRV affect greatly their transmissibility (Tamada and Kusume, 1991; Jolly and Mayo, 1994; Brault *et al*., 1995; Demler *et al*., 1997; Reavy *et al*., 1998). Nevertheless, whereas the N-terminal part of the RT is necessary for the BWYV and PLRV transmissibility by aphids (Bruyère *et al*., 1997; Peter *et al*., 2008), the C-terminal extremity of RT domains has been shown to be of crucial importance for the transmission of BNYVV and PMTV by their protist vectors (Tamada and Kusume, 1991; Reavy *et al*., 1998). Thus, the two transmembrane helices TM1 and TM2 predicted in the CPs RT domains of plasmodiophorid-transmitted viruses (Table 3) are proposed to facilitate the uptake of viral particles into the vector, as presented in the general introduction for BNYVV, PMTV, BSBV and BVQ (Diao *et al*., 1999a; Adams *et al*., 2001). However, no TMs2 are predicted in RT domains of BVQ and OGSV CPs (Adams *et al*., 2001).

Spontaneous deletion mutants for the CP-RT

To ensure continuous supply of fresh working material, laboratories use to multiply the viral strains of interest in lab host plants. In such cases, the natural propagation of the virus by its vector may be bypassed in order to optimize the virus production, for instance through mechanical inoculation of the leaves. However, aberrant genome arrangements appeared quickly because of such practices. In this way, spontaneous deletions have been reported in CPs RTs of BNYVV, BSBV, SBWMV and PMTV after serial passages on *T. expansa*, *C. quinoa*, *Triticum aestivum* or *Nicotiana* sp., respectively (Tamada and Kusume, 1991; Chen *et al*., 1995; Koenig *et al*., 1997a; Reavy *et al*., 1998; Koenig, 2000; Sandgren *et al*., 2001) (Fig. 15).
For these viruses, sequences deletions of 3 to 1030 nt are described in the C-terminal moieties of CPs RT domains, N-terminal portions being free of such deletion events. Moreover, according to whether the number of nucleotides removed is a multiple of 3 or not, the coding sequence following

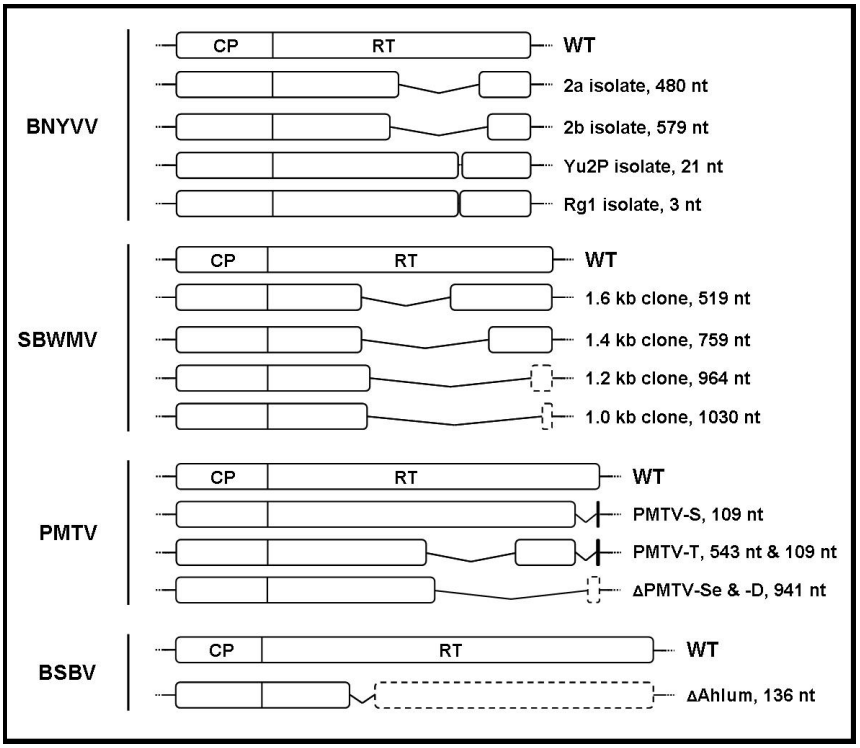

Fig. 15. Occurrence and positions of spontaneous deletions in sequences coding for read-through (RT) domains of coat proteins (CP) of BNYVV (Tamada and Kusume, 1991; Koenig, 2000), BSBV (Koenig *et al*., 1997a), PMTV (Sandgren *et al*., 2001) and SBWMV (Chen *et al*., 1995). Boxes represent ORFs that are translated (full lines) or not (dashed lines). Oblique bars between boxes indicate deletion position. The wild-type (WT) organization of CP-RT coding sequence is shown for each virus species. Names of deleted isolates and length of deleted parts are also specified.

the deleted part is in frame or not with the preceding one and RT domains undergo internal deletions or are truncated. Interestingly, these deleted strains replicate efficiently in the inoculated leaves in spite of the major rearrangement of their viral genome. Nevertheless, both BNYVV and PMTV deleted forms were not transmissible any more by their respective vectors (Tamada and Kusume, 1991; Reavy *et al*., 1998). A potential involvement of the RT domains deleted parts in natural viral transmission by the plasmodiophorid vector has therefore been hypothesized.

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Short Communication

The beet virus Q coat protein readthrough domain is longer than previously reported, with two transmembrane domains

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Ten beet virus Q (BVQ) strains from six different countries were sequenced to characterize the readthrough (RT) domain of the coat protein (CP). The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are FM244643–FM244652. With three nucleotide additions of 5, 285 and 1 nt, the common RT of 76 kDa was found to be longer than the single reference available to date (35 kDa). It is hypothesized that multiple inoculation cycles on *Chenopodium quinoa* were responsible for these three deletions in the C-terminal part of the BVQ RNA-2 previously described. Two putative transmembrane domains, TM1 and TM2, were predicted in the consensus amino acid sequence of the ten BVQ strains, and the putative BVQ TM2 was aligned with that of potato mop-top virus.

Beet virus Q (BVQ), first reported as one of the two beet soil-borne virus (BSBV) serotypes (Lesemann *et al.*, 1989; Barbarossa *et al.*, 1992), has been described as a distinct member of the genus *Pomovirus* as BSBV, potato mop-top virus (PMTV) and broad bean necrosis virus (BBNV) (Koenig *et al.*, 1998). Co-isolated with the causal agent of the rhizomania syndrome, beet necrotic yellow vein virus (BNYVV), the role of BSBV and BVQ in the disease remains a matter of debate (Prillwitz & Schlösser, 1992; Kaufmann *et al.*, 1993; Lindsten, 1993; Rush & Heidel, 1995). However, the almost ubiquitous association of at least one of these viruses in co-infections with BNYVV (Meunier *et al.*, 2003) raises questions about their role in the disease syndrome.

The tripartite genomic organization of the two pomoviruses is almost identical (Koenig *et al.*, 1998). However, there is a major divergence for RNA-2. The sequence coding for the coat protein readthrough (RT) domain is shorter for BVQ, with two additional ORFs encoding 9 and 18 kDa proteins (p9, p18) (Koenig *et al.*, 1998) (Fig. 1a). It has been hypothesized that this special genomic organization of BVQ RNA-2 might have resulted from deletion events from an original sequence comprising a longer RT protein, similar to that of BSBV (Koenig *et al.*, 1998).

This hypothesis was challenged by the systematic partial sequencing of RNA-2 for ten BVQ sources, where genomic RNAs were purified as described by Meunier *et al.* (2003). Soils producing rhizomania symptoms were collected in Belgium (Beclers, Mazy, Seneffe, Silly, Walviert), France (Pithiviers), Germany (Langendorf), Hungary (Tompaladony), Spain (Ciudad Real) and The Netherlands (Kamperland). RNA extracts containing BVQ particles were identified using multiplex RT-PCR (Meunier *et al.*, 2003). These extracts were then selected to amplify the BVQ RNA-2 region included between the coat protein stop codon and the RNA-2 3′ extremity using RT-PCR with Expand Reverse Transcriptase (Roche) and Expand Long Template PCR System (Roche). The ten amplified fragments were purified from the electrophoresis gel with a QIAquick Gel Extraction kit (Qiagen) and subsequently sequenced with four pairs of primers, forward and reverse. Sequencing was performed in a DNA sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and consensus sequences were obtained with ContigExpress and Vector NTI version 7.1 software (Invitrogen).

The sequencing results revealed a different arrangement for the coat protein RT domain of BVQ from the one described previously (Fig. 1a) (Koenig *et al.*, 1998). The sequenced RT was longer, with 291 nt within three distinct nucleotide additions, I1, I2 and I3, being responsible for this rearrangement (Fig. 1a). Five additional nucleotides were located at positions 864–868, almost 100 bp before the putative 35 kDa coat protein RT domain stop codon (975–977) (Fig. 1a, addition I1). A supplementary 285 nt were present at positions 1223–1507, that is, in the stop codon of the putative p9 coding sequence (Fig. 1a, addition I2). Finally, a single-nucleotide addition was noted at position 2043 (Fig. 1a, addition I3).

These three genetic modifications in the nucleotide sequence of BVQ RNA-2 induced major changes in the coding sequence length and amino acid composition of the

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are FM244643–FM244652.

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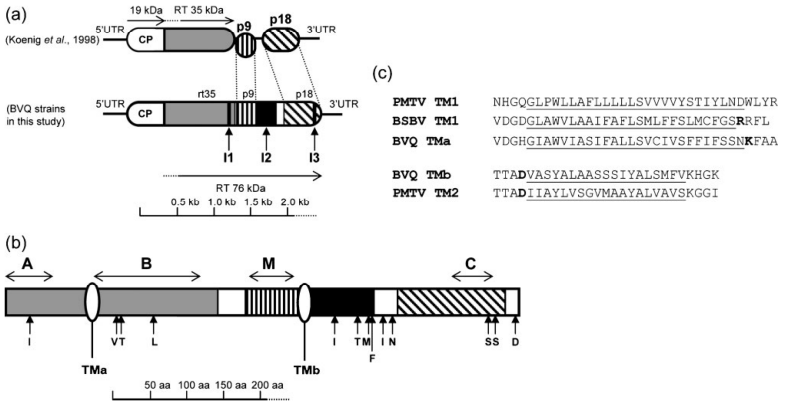

Fig. 1. (a) Schematic representation of the RNA-2 genomic organization for BVQ previously described (Koenig *et al.*, 1998) compared with the ten from this study that all contain three nucleotide additions of 5 (I1), 285 (I2) and 1 (I3) nt responsible for the longer putative RT domain of 76 kDa instead of 35 kDa. Nucleotide additions are represented by black boxes. The rt35 (grey box), p9 (vertical hatching) and p18 (diagonal hatching) nucleotide sequences conserved in the 76 kDa RT coding sequence are shown, as well as the untranslated regions (UTRs) and the coding sequences of the coat protein (CP) and the putative readthrough domains (RT). (b) Integrally and partially conserved rt35 (grey box), p9 (vertical hatching) and p18 (diagonal hatching) peptidic motifs as well as predicted transmembrane helices TMa and TMb are represented on the consensus amino acid sequence of the 76 kDa RT domain. The amino acid sequence coded by the I2 nucleotide addition is also indicated (black box). The A, B, M and C conserved domains are localized with horizontal arrows (↔). Variant amino acids are positioned with vertical arrows (↑). (c) Amino acid sequences of the two BVQ transmembrane domains TMa, aligned with TM1 of BSBV and PMTV, and TMb, aligned with TM2 of PMTV (Adams *et al.*, 2001). Transmembrane helices are underlined and amino acids of crucial importance for helices interaction are in bold.

coat protein RT domain (Fig. 1a and b). Indeed, nucleotide additions I1 and I2 suppressed putative 35 kDa RT and p9 stop codons, respectively, whereas the I3 moves the final stop codon from position 2105–2107 to 2098–2100. Reading frames between the I1 site and the beginning of the putative p9 sequence, and the I3 site and the final stop codon are moved by +1 and +2 nt, respectively. The expected molecular mass of the putative coat protein RT domain common for the ten sequenced BVQ strains is therefore 76 kDa instead of 35 kDa. This longer RT integrates the full amino acid sequence of the putative p9 but, because of the I1 and I3 nucleotide additions, the C-terminal extremities of amino acid sequences suggested for putative 35 kDa RT and p18 are modified (Fig. 1b)

The nucleotide and amino acid changes between all ten BVQ strains sequenced for the RT domain of the coat protein are listed and positioned (Tables 1 and 2). For a total length of 2097 nt and 699 aa, the ten BVQ strains had an identity of 99.05–100 % at the nucleotide level and 98.71–100 % at the amino acid level. This extremely low variability of the BVQ genome had been reported for other strains sequenced for the coat protein gene (Stas *et al.*, 2001; Lennefors *et al.*, 2005).

Three domains A, B and C are reported to be conserved between the RT domains of the three pomoviruses PMTV, BVQ and BSBV. A fourth one, M, is conserved only by the latter two (Koenig *et al.*, 1998). Despite the major rearrangement observed for the RT domain of the ten BVQ strains, these motifs were localized on the putative 76 kDa RT (Fig. 1b). Three varying amino acids were localized in domain B and only one in each domain A and C. Domain M was free of any amino acid mutation. The minimal variability within these four motifs A, B, M and C between the ten BVQ strains confirmed their importance for the virus.

A single transmembrane motif (TM1) is identified in the BVQ coat protein 35 kDa RT domain (Adams *et al.*, 2001). However, two complementary transmembrane domains, TM1 and TM2, are of crucial importance for the viral transmission by protist vectors such as *Polymyxa* and

Table 1. Varying residues in the nucleotide sequence of the coat protein RT domain for ten BVO strains, compared with the consensus sequence

| Source* | Position Number† | | |

Table 2. Varying residues in the amino acid sequence of the coat protein RT domain for ten BVQ strains, compared with the consensus sequence

Source*	Position number†												
	30	**151**	**155**	**199**	**447**	**477**	**494**	**497**	**512**	**526**	**655**	**665**	**693**
G-Langendorf (CAR91970.1)	**I**										**S**		
N-Kamperland (CAR91971.1)											**S**		
B-Beclers (CAR91969.1)			**T**									**S**	
B-Seneffe (CAR91968.1)			**T**									**S**	
B-Silly (CAR91965.1)			**T**	**L**					**I**			**S**	
F-Pithiviers (CAR91973.1)			**T**						**I**			**S**	
S-Ciudad Real (CAR91974.1)					**I**	**T**	**M**	**F**	**I**	**N**			**D**
B-Walviert (CAR91967.1)											**S**		
B-Mazy (CAR91966.1)		**V**									**S**		
H-Tompaladony (CAR91972.1)											**S**		
Consensus	M	I	M	R	T	I	I	L	V	D	A	N	N

*Origin of each strain with its corresponding accession number.
†+1 position begins at the first amino acid after the readthrough.

Spongospora (Diao *et al.*, 1999; Adams *et al.*, 2001). In this context, the identification of a new RT domain for the BVQ coat protein also provided new material for bioinformatic analysis. Predictions of transmembrane motifs with the consensus peptidic sequence of the RT domain were carried out using programs HMMTOP (Tusnády & Simon, 1998), TMHMM (Krogh *et al.*, 2001), TMPred (Hofmann & Stoffel, 1993) and TopPred (von Heijne, 1992). The results were correlated with the capacity of predicted motifs to form α-helices, which were proposed by GOR (Garnier *et al.*, 1996), Jpred (Cole *et al.*, 2008), PSIpred (Jones, 1999) and SOPMA (Geourjon & Deléage, 1995) analysis.

Two motifs appeared to form transmembrane helices, TMa and TMb (Fig. 1b and c). The first one corresponded to the TM1 already predicted for the shorter putative BVQ 35 kDa RT (Adams *et al.*, 2001) and was located at the N-terminal extremity of conserved domain B. The second transmembrane domain was specific to the putative 76 kDa RT domain and positioned on both the p9 peptidic motif C-terminal part and the N-terminal extremity of the peptidic sequence coded by the I2 addition (Fig. 1b). It is also important to note that, although BVQ TMa can be aligned on the TM1 of BSBV and PMTV, TMb was aligned only on TM2 proposed for PMTV (Fig. 1c) (Adams *et al.*, 2001).

Two amino acid residues from predicted TMa and TMb are also highlighted in Fig. 1(c). One is an aspartate (D) residue located at the TMb N-terminal extremity, whereas the other is a lysine (K) residue situated at the C-terminal extremity of predicted TMa. As suggested for some benyviruses, furoviruses and pomoviruses (Adams *et al.*, 2001), the electrostatic interaction between these basic (K) and acidic (D) residues at the inner side of the membrane is necessary for an efficient viral transmission by the protist vector. In that way, the D residue corresponds to the E residue from the KTER motif, described as essential for the transmission of BNYVV by *Polymyxa betae* (Tamada *et al.*, 1996). It is also worth noting that no amino acid changes were observed for these two motifs among all ten sequenced BVQ strains originating from six countries.

The sequencing of ten BVQ strains from Belgium, France, Germany, Hungary, Spain and The Netherlands seems to lift the veil on the genomic organization of RNA-2. Indeed, the shortness of a putative coat protein 35 kDa RT domain that allowed space for two small putative ORFs p9 and p18 was quite a striking arrangement for the RNA-2 of BVQ described to date, compared with other closely related viruses such as BSBV, PMTV and BNYVV. When first described, it had been already proposed that such a genomic arrangement might have resulted from deletion events in a longer sequence that originally resembled that of BSBV (Koenig *et al.*, 1998). This hypothesis is now confirmed with the identification of the common longer putative 76 kDa RT domain. Spontaneous deletions in the coat protein RT domain have been reported for other furoviruses *sensu lato* (Tamada & Kusume, 1991; Chen *et al.*, 1994; Tamada *et al.*, 1996; Reavy *et al.*, 1998) that, when mechanically inoculated in a repeated way, show some deletion events in the C-terminal part of the RT domain. Therefore, as the BVQ strain described in 1998 had been submitted to multiple mechanic inoculation cycles on *Chenopodium quinoa*, it can be assumed that three deletion events of 5, 285 and 1 nt occurred in genomic RNA-2. This triple deletion in the coat protein RT domain is, however, specific to BVQ, compared with BNYVV, PMTV and soil-borne wheat mosaic virus (SBWMV) where only single-deletion events have been reported (Tamada & Kusume, 1991; Chen *et al.*, 1994; Tamada *et al.*, 1996; Reavy *et al.*, 1998).

Although the RT domain of the coat protein was truncated in the originally sequenced BVQ strain, it affected neither viral replication in *C. quinoa* nor formation of viral particles, as those were purified from plants and used to produce antibodies (Koenig *et al.*, 1998). The deleted parts from the longer RT domain are therefore not required for viral encapsidation.

The lack of predicted TM2 for the putative 35 kDa RT raised some questions about the transmissibility of the virus by its plasmodiophorid vector. Indeed, it is suggested that the interaction between TM1 and TM2 is of crucial importance in facilitating the internalization of viral particles by the protist vector inside plant cells (Adams *et al.*, 2001). Therefore, the identification of two transmembrane helices TMa and TMb in the BVQ putative 76 kDa RT domain, corresponding to TM1 and TM2, respectively, clarifies the BVQ situation in terms of transmissibility.

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CHAPTER III

Rescue of *Beet virus Q* transcripts from a full-length cDNA clone by *Beet necrotic yellow vein virus* RNAs-1 and -2 in *Chenopodium quinoa* leaves

Opening comments

For the reason mentioned above in opening comments of chapter I, full-length cDNA clones were developed to investigate the BVQ multiplication dynamic *in planta*. Following the example of BSBV, attempts were made to amplify and clone BVQ RNAs in full-length pieces. However, no amplicon was obtained for RNA-1 and RNA-2 proved to be impossible to clone in a single piece. We then opted for an alternative strategy, aiming at amplify and clone piece by piece the whole BVQ genome, as described for PMTV (Savenkov *et al*., 2003). The different fragments were subsequently reassembled to get full-length cDNAs sequences of the three BVQ genomic RNAs. The infectiousness of BVQ transcripts from constructs was further tested on *C. quinoa*, like BSBV, but surprising multiplication requirements were evidenced.

This chapter is written as a project paper.

Table 4. Listing of synergisms and transcomplementations evidenced between plant viruses genera (reviewed by Latham and Wilson, 2008). Helper viruses genera and families are indicated on the left. Genome types (single- or double-stranded DNA or RNA, positive or negative) and viral particles shapes (bacilliform/bac, bullet-shaped/bu, filamentous/fil, isometric/isom, ribonucleoprotein/RNP, rod-shaped/rod or spherical/sph) specific to each family are specified according to the International Committee on Taxonomy of Viruses (ICTV). Dependant viruses genera are shown on the right.

Helper virus			**Dependant virus**
Family	**Organization**	**Genus**	
Geminiviridae	ssDNA isom	Begomovirus	Begomo-, Curto-, Nano-, Potex- and Tobamovirus
		Topocuvirus	Begomovirus
		Curtovirus	Begomo-, Curto-, Nano- and Tobamovirus
Caulimoviridae	dsDNA isom-bac	Caulimovirus	Caulimo- and Tobamovirus
Luteoviridae	ssRNA(+) isom	Luteovirus	Luteo- and Umbravirus
		Polerovirus	Clostero-, Potex-, Poty- and Umbravirus
		Enamovirus	Polerovirus
Tombusviridae	ssRNA(+) isom	Carmovirus	Carmo-, Clostero-, Potex- and Tombusvirus
		Tombusvirus	Clostero-, Cucumo- and Potexvirus
		Dianthovirus	Bromo-, Cucumo-, Hordei-, Potex-, Tobamo- and Tombusvirus
		Machlomovirus	Rymovirus
Sequiviridae	ssRNA(+) isom	Waïkavirus	Badna- and Sequivirus
Comoviridae	ssRNA(+) isom	Comovirus	Poty- and Tobamovirus
		Nepovirus	Como- and Tobamovirus
-	ssRNA(+) isom	Sobemovirus	Diantho-, Potex-, Sobemo- and Tobamovirus
Bromoviridae	ssRNA(+) isom	Bromovirus	Bromo-, Hordei- , Potex-, Sobemo- and Tobamovirus
		Cucumovirus	Begomo-, Bromo-, Clostero-, Como-, Cucumo-, Potex-, Poty-, Tobamo- and Tobravirus
		Alfamovirus	Alfamo-, Bromo-, Cucumo-, Ilar- and Tobamovirus
		Ilarvirus	Alfamovirus
-	ssRNA RNP	Umbravirus	Cucumo-, Enamo-, Polero-, Potex- and Tobamovirus
-	ssRNA(+) rod	Tobamovirus	Alfamo-, Begomo-, Beny-, Bromo-, Colimo-, Como-, Cucumo-, Diantho-, Hordei-, Nepo-, Potex- , Poty-, Sobemo-, Tobamo- and Tobravirus
-	ssRNA(+) rod	Hordeivirus	Bromo-, Hordei-, Potex-, Tobamo- and Tobravirus
	ssRNA(+) rod	Furovirus	Tobravirus
-	ssRNA(+) rod	Tobravirus	Polero-, Tobamo- and Tobravirus
-	ssRNA(+) rod	Pecluvirus	Benyvirus
Flexiviridae	ssRNA(+) fil	Vitivirus	Closterovirus
		Potexvirus	Clostero-, Como-, Hordei-, Polero-, Potex- and Tobamovirus
Closteroviridae	ssRNA(+) fil	Closterovirus	Clostero-, Hordei-, Potex- and Potyvirus
		Crinivirus	Potyvirus
Potyviridae	ssRNA(+) fil	Potyvirus	Clostero-, Como-, Cucumo-, Hordei-, Luteo-, Machlomo-, Polero-, Potex-, Poty- and Tobamovirus
		Rymovirus	Machlomovirus
Bunyaviridae	ssRNA(-) sph	Tospovirus	Tobamovirus
Rhabdoviridae	ssRNA(-) bul-bac	Rhabdovirus	Potexvirus

Preliminary concepts

Synergism and transcomplementation between viruses

The various interactions between viruses that multiply in the same host(s) should be distinguished according to whether competition, synergism or simple co-occurence occurs. Whereas competition is harmful for at least one of the viruses, synergism is defined as a relation that benefits for one or both viruses infecting the same plant simultaneously. In case of asymmetric synergy, i.e. different developments of the two viruses, one is considered as the "helper" of the other "dependent" one (Malyshenko *et al*., 1989; Latham and Wilson, 2008). In that context, the support or enhancement of a specific function of the dependent virus, relying on one or several protein(s) of the helper virus, is qualified as transcomplementation or heterologous complementation. The range of viral functions for which such a phenomenon has been reported covers many aspects of the viral cycle. Viral transmission by means of the vector (Rochow, 1970; Kassanis and Govier, 1971), seeds (Kuhn and Dawson, 1973) or mechanical inoculation (Mayo *et al*., 2000) is known to be enhanced or enabled through complementation. The host range may also be widen for some viruses to non-permissive plant species when another helper virus is present (Cohen *et al*., 1988), this kind of viral synergy being often due to cell-to-cell and long-distance movement permission (Fuentes and Hamilton 1991; Hacker and Fowler, 2000).

According to a recent work aiming to review this particular topic (Latham and Wilson, 2008), synergism and complementation are widely used among 69 virus species, belonging to 35 genera, and may occur between highly divergent viruses (Table 4). Such interactions have been evidenced between ambisense or negative-stranded RNA viruses and positive stranded RNA viruses (Huang *et al*., 2005; Lewandowski and Adkins, 2005), but also DNA and RNA viruses, as for instance the *Bean golden mosaic virus* (BGMV, DNA virus) and *Tobacco mosaic virus* (TMV, RNA virus) (Carr and Kim, 1983) or the *Abutilon mosaic virus* (AbMV, DNA virus) and *Cucumber mosaic virus* (CMV, RNA virus) (Wege and Siegmund, 2007). The kingdom barrier has even been crossed when an insect virus, the *Flock house virus*

(FHV), has been shown to move systematically in *N. benthamiana* plants expressing the movement proteins (MPs) of TMV or *Red clover necrotic mosaic virus* (RCNMV) (Dasgupta *et al*., 2001). Nevertheless, transcomplementation is mainly about viral movement, coat or PTGS proteins and less about replicases (Latham and Wilson, 2008).

Satellite and defective interfering (DI) particles

Definitions

Among all plant viruses, many are found associated with subviral agents that rely on the helper virus for their own replication but are absolutely unnecessary for the multiplication and natural propagation of the helper component. Depending on whether the subviral particle derives from its helper or if both are unrelated, they will be referred as defective (D) or satellite, respectively (Mayo *et al*., 2005). Defective particles are largely reported with animal and plant viruses. They constitute non-functional copies of the helper virus because of major rearrangements that occurred in the parental genome and affected some or all genes essential for their independent multiplication. However, sequences necessary for their replication by the helper virus are conserved. When affecting the helper virus multiplication, they are qualified as defective interfering (DI) (Simon *et al*., 2004). Satellites, in turn, are mostly associated to plant viruses (Table 5) and distinction is made between satellite viruses that encode their own coat protein and satellite RNAs or DNAs (satRNAs/DNAs), encapsidated by the helper virus structural protein. The major group of satssRNAs is further divided in larger satRNAs that may encode for functional protein(s) and the smaller ones that do not. The satellites length is generally comprised between 300 and 1800 nucleotides, and the smaller ones may be linear or circular (Roossinck *et al*., 1992; Mayo *et al*., 2005).

Table 5. Satellite viruses (encoding their own coat protein - CP) and nucleic acids (encapsidated in the helper's CP) classified according to their molecular features (listing from Mayo *et al*., 2005).

CLASS	GROUP	HELPER VIRUS	FEATURES
Sat **viruses**	*Chronic bee-paralysis virus* (CBPV) associated	CBPV sat	3 RNAs, 1 kb long each
	Tobacco necrosis sat virus-like	*Panicum mosaic virus* *Tobacco mosaic virus* *Tobacco necrosis virus* *Maize white line mosaic virus* *Cereal yellow dwarf –RPV virus*?	1 RNA, encoding for a 17-19 kDa protein
Sat **nucleic acids**	ssDNA sat	*Ageratum enation virus* *Ageratum yellow vein virus* *Bhendi yellow vein mosaic virus* *Chilli leaf curl virus* *Cotton leaf curl virus* *Eupatorium yellow vein virus* *Hollyhock leaf crumple virus* *Honeysuckle yellow vein virus* *Malvastrum yellow vein virus* *Papaya leaf curl virus* *Tobacco curly shoot virus* *Tomato leaf curl virus* *Tomato yellow leaf curl China virus*	Mostly circular, sat of DNAβ components from *Begomovirus* genus, 1.3 kb long
	dsRNA sat	*Saccharomyces cerevisiae L-A virus* *Trichomonas vaginalis T1 virus* *Ustilago maydis killer M virus ?*	0.5-1.8 kb long
	ssRNA sat, large	*Arabis mosaic virus* *Bamboo mosaic virus* *Beet ringspot virus* *Chicorey yellow mottle virus* *Grapevine Bulgarian latent virus* *Grapevine fanleaf virus* *Myrobalan latent ringspot virus* *Strawberry latent ringspot virus* *Tomato black ring virus* BNYVV sat-like RNA-5 ?	0.8-1.5 kb long, may encode a non-structural protein essential for their multiplication
	ssRNA sat, small linear	*Cucumber mosaic virus* *Cymbidium ringspot virus* *Groundnut rosette virus* *Panicum mosaic virus* *Pea enation mosaic virus* *Peanut stunt virus* *Tomato bushy stunt virus* *Turnip crinkle virus* *Robinia mosaic virus ?* *Tobacco necrosis virus ?*	Less than 0.7 kb long, no functional protein, only linear
	ssRNA sat, circular	*Arabis mosaic virus* *Cereal yellow dwarf virus-RPV* *Chicory yellow mottle virus* *Lucerne transient streak virus* *Solanum nodiflorum mottle virus* *Subterranean clover mottle virus* *Tobacco ringspot virus* *Velvet tobacco mottle virus* *Rice yellow mottle virus ?*	About 0.3 kb long, linear and circular

Symptom modulation and effects on the helper component

The simultaneous presence in a host of a subviral agent and its helper often modulates the infection by the viral pathogen. Most of the defective particles, both in an animal or vegetal context, strongly interfere with the helper virus accumulation and attenuate the symptom expression (Simon *et al*., 2004; Marriott and Dimmock, 2010). DI RNAs of *Broad bean mottle virus* (BBMV) and *Turnip crinkle virus* (TCV) constitute however exceptions that are reported to enhance the symptom severity (Li *et al*., 1980; Romero *et al*., 1993). Contrary to DI RNAs, the impact of satellites on the symptoms induced by their helper virus goes from alleviation to worsening, depending on the plant host, helper virus and satellite. Nevertheless, more common is the symptom attenuation, whereas exacerbation is rather rare (Roossinck *et al*., 1992).

Different types of interactions with the subviral agent have been evidenced to modulate the pathogenicity of the helper virus (Roossinck *et al*., 1992; Simon *et al*., 2004). Following the most intuitive explanation, the subviral particle and helper virus may **compete** for viral replication enzymes such as the RdRp. The symptom attenuation is then due to lower titer of the virus. Some sat/DI RNAs are also reported to target the post-transcriptional gene silencing pathway (**PTGS**), i.e. host defense mechanisms targeting specifically exogenous nucleic material. Depending on whether PTGS is activated or repressed, the helper accumulation and symptom induction will be reduced or enhanced. Finally, specific **interactions** between the sat/DI RNA and some viral components of the helper are proposed as well, leading to modulation of viral proteins expression or interactions. When these proteins are involved directly or indirectly in the pathogenicity, the subviral agent modifies the symptom expression. Nevertheless, the underlying molecular mechanisms that govern the particular relationship between the DI or satellite particles and their helper virus are still poorly understood and the interactions variability depending on the subviral agent-helper-host pathosystems doesn't make the job any easier (Roossinck *et al*., 1992; Simon *et al*., 2004).

Project paper

Rescue of *Beet virus Q* transcripts from a full-length cDNA clone by *Beet necrotic yellow vein virus* RNAs-1 and -2 in *Chenopodium quinoa* leaves

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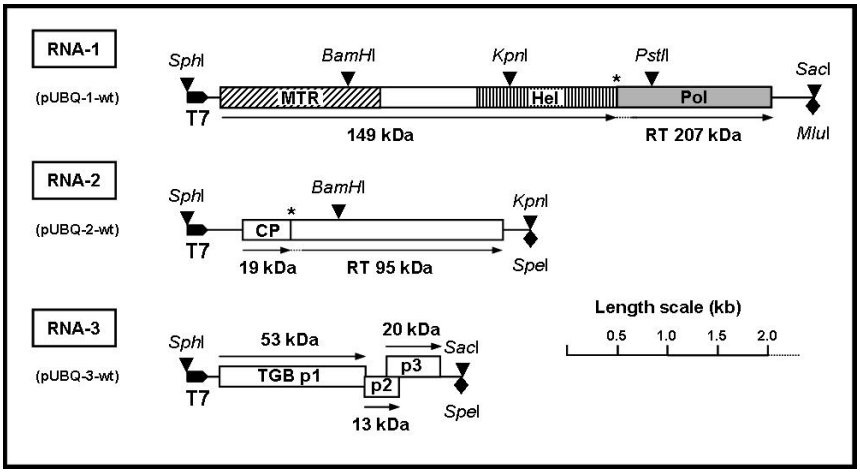
Abstract

Two pomoviruses, the *Beet soil-borne virus* (BSBV) and *Beet virus Q* (BVQ), are commonly found associated with the causal agent of the rhizomania syndrome of sugar beet, the *Beet necrotic yellow vein virus* (BNYVV). However, their pathogenicity and potential role in the disease remain unclear. In this study, a full-length cDNA clone was constructed for the tripartite genome of BVQ and the infectiousness of transcripts produced *in vitro* from constructs was tested on *Chenopodium quinoa* leaves. Surprisingly, transcripts of BVQ were not self-infectious but required the simultaneous presence of BNYVV RNAs-1 and -2 to multiply *in planta*. The rescue of BVQ by BNYVV had no impact on the accumulation of that last, but RNAs-3 and -4 of BNYVV affected the accumulation and the expression of BVQ RNAs and structural proteins, respectively. Nevertheless, such rescue was not observed when BVQ was co-inoculated with the closely related BSBV.

INTRODUCTION

Beet virus Q (BVQ) was first described in 1998 (Koenig *et al*. 1998) as a new virus species belonging to the *Pomovirus* genus. Previously considered as a serotype of the *Beet soil-borne virus* (BSBV), sequencing of non purified virus revealed that BVQ is equally distant from BSBV and another pomovirus of potato, the *Potato-mop top virus* (PMTV) (Koenig *et al*., 1998). Similarly to these viruses, the BVQ genome is organized in three single-stranded plus-sense RNAs (Fig. 1) (Koenig *et al*., 1998).



Project paper Fig. 1. Schematic representation of the BVQ tripartite genome and strategy of full-length cDNA clones construction. RNA-1 encodes for the putative replicase, with methyl-transferase (MTR, striped), helicase (HEL, hatched) and RNA-dependent RNA polymerase (Pol, grey shaded) conserved motifs. This last is translated as a read-through domain (RT). The coat protein (CP) and a RT domain are encoded by RNA-2. RNA-3 is composed of three open reading frames (ORFs) coding for the putative Triple Gene Block proteins (TGB-p1, -p2 and -p3). Asterisks (*) represent the read-through stop codons. The molecular mass of the putative proteins is associated to each arrow beneath or above the corresponding ORF. Full-length cDNA clones pUBQ-1-wt, -2-wt and -3-wt are under the control of a T7 promoter to allow transcripts production. Unique restriction sites used for the piecemeal cloning (▼) as well as linearization before run-off transcription (♦) are also indicated.

RNA-1 (6.0 kb) encodes for the putative viral replicase, with the methyl-transferase and helicase conserved motifs included in the major portion (149

kDa) and the RNA-dependant RNA polymerase (RdRp) part translated as a read-through (RT) domain of 58 kDa. The putative structural proteins, i.e. a 19 kDa coat protein (CP) and a 76 kDa RT domain, are encoded by RNA-2 (3.2 kb) (Crutzen *et al*., 2009a). Following the example of other beny-, furo- and pomoviruses transmitted by the plasmodiophorids *Spongospora subterranea* and *Polymyxa sp*. (Diao *et al*., 1999; Adams *et al*., 2001), the BVQ RT domain of the CP is thought to be involved in viral transmission by the putative soil-borne protist vector *Polymyxa betae* (Crutzen *et al*., 2009a). This last is also known to vector the closely related BSBV (Ivanović *et al*., 1983), as well as the *Beet necrotic yellow vein virus* (BNYVV), which causes the rhizomania syndrome of sugar beet (Tamada and Baba, 1973). Finally, three open reading frames (ORFs) coding for putative Triple Gene Block (TGB) proteins of 53 (TGBp1), 13 (TGBp2) and 20 kDa (TGBp3) are identified on BVQ RNA-3 (2.5 kb). Widespread among *Allexivirus*, *Benyvirus*, *Carlavirus*, *Foveavirus*, *Hordeivirus*, *Pecluvirus*, *Pomovirus* and *Potexvirus* genera, the TGB proteins have been shown to be required for viral movement from cell to cell (Morozov and Solovyev, 2003). The 8 kDa cysteine-rich protein (CRP) encoded by PMTV TGB-RNA (Scott *et al*., 1994) is however lacking in other pomoviruses genomes (Koenig *et al*., 1998; Lu *et al*., 1998).

Since its first characterization, BVQ has been identified in sugar beet crops from Belgium (Stas *et al*., 2001), Bulgaria, France, Germany, Hungary and The Netherlands (Meunier *et al*., 2003), Iran (Farzadfar *et al*., 2005), Croatia, Italia, Lithuania, England (Ratti *et al*., 2005), Poland (Borodynko, 2006) and Spain (Rubies Autonell *et al*., 2006) but not in China (Wang *et al*., 2007). Nevertheless, BVQ is seldom detected alone and rather found in association with BSBV and/or BNYVV (Meunier *et al*., 2003; Ratti *et al*., 2005). Responsible for one of the worst viral disease of sugar beet, BNYVV has been widely studied because of its dramatic impact in crops (Tamada and Baba, 1973; McGrann *et al*., 2009). Its multipartite genome is composed of four to five single-stranded RNAs of positive polarity, of which the two larger encode for proteins homologous to those putative of BSBV and BVQ, plus a CRP. Thus, BNYVV RNA-1 (6.7 kb) (Bouzoubaa *et al*., 1987)

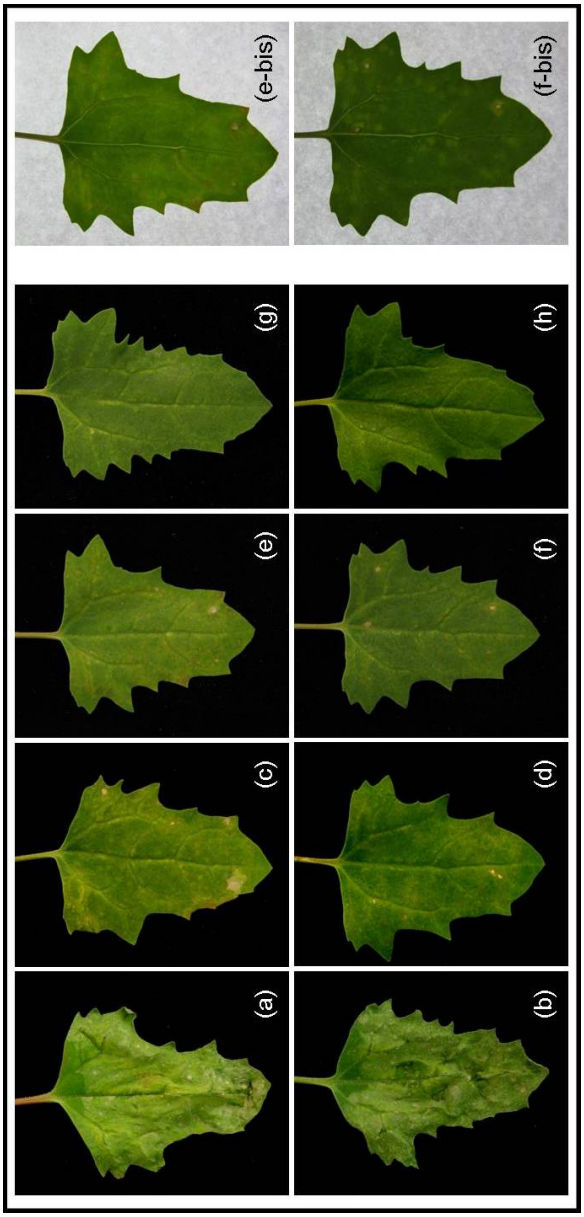
encodes also for the replicase whereas structural CP and CP-RT, TGB proteins and a CRP with post-transcriptional gene silencing (PTGS) suppressor activity are encoded by RNA-2 (4.7 kb) (Bouzoubaa *et al*., 1986; Dunoyer *et al*., 2002). These large RNAs, carrying the virus house-keeping genes, are necessary and sufficient for local infection *in planta* (Koenig *et al*., 1986; Quillet *et al*., 1989; Tamada *et al*., 1989). Regarding the smaller RNAs-3, -4 and -5, the first and the second are involved in virus long-distance movement (Lauber *et al*., 1998a) and transmission by *P. betae* (Tamada and Abe, 1989), respectively, but all encode for viral pathogenicity factors (Jupin *et al*., 1992; Link *et al*., 2005; Rahim *et al*., 2007).

When the aggressiveness of BNYVV in sugar beet crops needs no further proof, the pathogenic effect of BVQ remains a mystery. In addition, no functional study has been carried out on the particular genome of BVQ and no interest has been taken to investigate, nor the role, or the *in vivo* expression of its putative proteins. Being reported in most countries growing sugar beet, there is a real need for understanding the BVQ pathogenicity and possible involvement in the complex rhizomania syndrome. In light of this, a reverse genetic strategy was set up with full-length cDNA clones developed for each of the three genomic RNAs of BVQ. Their infectivity was tested on *Chenopodium quinoa* leaves and, surprisingly, mixed infections with BNYVV revealed to be of crucial importance for the BVQ transcripts multiplication and structural proteins expression *in planta*.

RESULTS

Symptom production on *C. quinoa* leaves

In order to assess the infectiousness of BVQ transcripts produced *in vitro* from full-length cDNA clones, these were inoculated on *C. quinoa* leaves. However, it turned out that no symptoms were produced following BVQ single inoculation (Fig. 2, picture g). When BVQ transcripts were combined with BSBV RNAs 1+2+3, BNYVV RNAs 1+2 or BNYVV RNAs 1+2+3+4, no dramatic symptoms worsening or decrease was observed. As expected, BSBV full-length transcripts induced many tiny necrotic spots, ringspots and



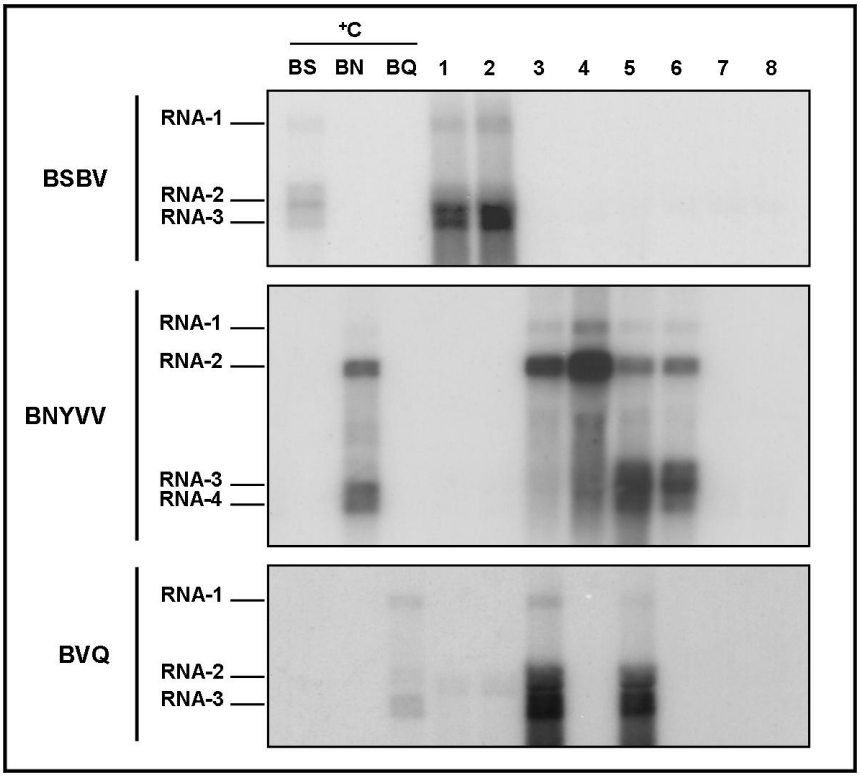
Project paper Fig. 2. Pictures of *C. quinoa* inoculated leaves 7 days p-i (BSBV RNAs 1+2+3 and BVQ RNAs 1+2+3 (a), BSBV RNAs 1+2+3 (b) and 10 days p-i (BNYVV RNAs 1+2 and BVQ RNAs 1+2+3 (c), BNYVV RNAs 1+2 (d), BNYVV RNAs 1+2+3+4 and BVQ RNAs 1+2+3 (e and e-bis), BNYVV RNAs 1+2+3+4 (f and f-bis), BVQ RNAs 1+2+3 (g) and negative control (h). All viral combinations induced the appearance of symptoms, except when BVQ was inoculated alone (g). Pictures e-bis and f-bis represent the same leaves from pictures e and f, respectively, but over white light to observe the symptoms change when BVQ is co-inoculated with BNYVV 1234.

slight yellowing of the *C. quinoa* inoculated leaves, but also wilting (Fig. 2, pictures a and b). BNYVV RNAs 1+2, in turn, produced extensive leaves yellowing and some necrotic and chlorotic spots (Fig. 2, pictures c and d). Similar symptoms were also observed on leaves inoculated with BNYVV RNAs 1+2+3+4, with less yellowing (Fig. 2, pictures e and f). Nevertheless, whereas localized yellow-green spots were clearly visible under white light for leaves inoculated with the four BNYVV RNAs (Fig. 2, picture f-bis), these spots disappeared when the BVQ RNAs 1+2+3 were co-inoculated and leaves yellowing appeared diffusely (Fig. 2, picture e-bis). The plant reaction was therefore slightly different, depending on whether the BVQ transcripts were present with BNYVV RNAs 1234 or not.

Multiplication of BVQ RNAs in presence of BNYVV but not BSBV

Viral multiplication in the inoculated leaves of *C. quinoa* was further tested by means of Northern blot. Such analyses evidenced that, contrary to the self-infectious BSBV and BNYVV transcripts, BVQ transcripts from full-length cDNA clone were not self-multiplicative in *C. quinoa* plants. None of the three genomic RNAs was detected in the asymptomatic leaves inoculated with BVQ transcripts only (Fig. 3a, lane 7). Nevertheless, a striking multiplication dynamic was revealed when mixed infections with BSBV or BNYVV were performed. While no RNA were found for BVQ when combined with BSBV transcripts (Fig. 3a, lane 1), BVQ RNAs-1, -2 and -3 were all detected in leaves co-inoculated with BNYVV transcripts (Fig. 3a, lanes 3 and 5). Multiplication of BVQ in *C. quinoa* leaves therefore relied on the simultaneous presence of BNYVV RNAs 1+2 or 1+2+3+4 but didn't take place in presence of BSBV RNAs 1+2+3.

It is also worth noting that the presence of BVQ transcripts did not affect the accumulation of BNYVV RNAs (Fig. 3a, lanes 3 to 6). The higher content of BNYVV RNAs-1 and -2, when they were inoculated alone (Fig. 3a, lane 4) compared to the combination with BVQ (Fig. 3a, lane 3), was not observed in the other tested leave. Nevertheless, the presence of BNYVV RNAs-3 and -4 had some negative impact on the accumulation of BVQ



Project paper Fig. 3a Northern blot detection of BSBV, BNYYV and BVQ in *C. quinoa* leaves inoculated with BSBV RNAs 1+2+3 and BVQ RNAs 1+2+3 (1), BSBV RNAs 1+2+3 (2), BNYVV RNAs 1+2 and BVQ RNAs 1+2+3 (3), BNYVV RNAs 1+2 (4), BNYVV RNAs 1+2+3+4 and BVQ RNAs 1+2+3 (5), BNYVV RNAs 1+2+3+4 (6), BVQ RNAs 1+2+3 (7) or mock-inoculated (8). Random DNA probes labeled with [α-^{32}P]dCTP were used to detect RNAs of the three viruses on separate blots. Diluted transcripts of BSBV (BS), BNYVV (BN) and BVQ (BQ) were used as positive controls ($^{+}$C).

RNAs (Fig. 3a, lane 5), as their amount was higher when mixed only with BNYVV RNAs-1 and -2 (Fig. 3a, lane 3). This was observed in all inoculated and tested leaves.

The slight bands observed on figure 3a, lanes 1 and 2, BVQ detection, are expected to be background due to probe aspecificity and partial hybridization on BSBV RNA-3.

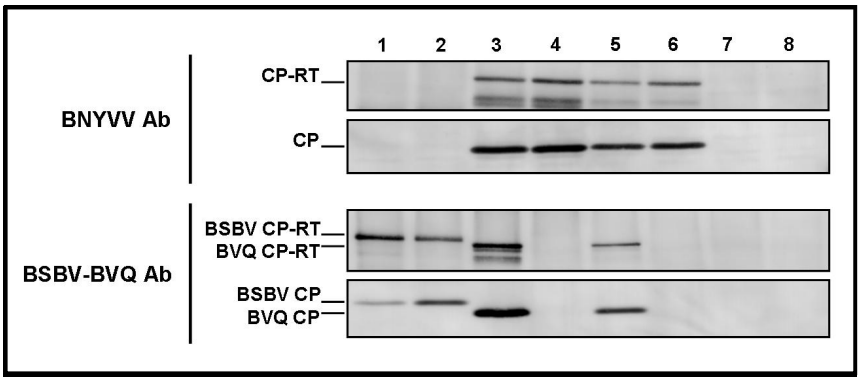
BVQ structural proteins produced *in vivo*

As shown on figure 3b, the CP and CP-RT were detected for the three viruses by Western blot. Consistently with the results from Northern blot, the putative BVQ CP and CP-RT encoded by RNA-2 were only detected in mixed infections with BNYVV (Fig. 3b, lanes 3 and 5). This observation therefore confirmed that BVQ transcripts were dependant on the presence of BNYVV, partial (RNAs-1 and -2) or complete (RNAs-1, -2, -3 and -4) genome combination, to multiply in *C. quinoa* and express their structural proteins. Nevertheless the expression level of BVQ CP and CP-RT was higher when BNYVV RNAs-3 and -4 were absent (Fig. 3b, lane 3). BNYVV CP and CP-RT were, in turn, similarly expressed in all *C. quinoa* leaves inoculated with transcripts 1+2 and 1+2+3+4, with and without BVQ transcripts (Fig. 3b, lanes 3 to 6).

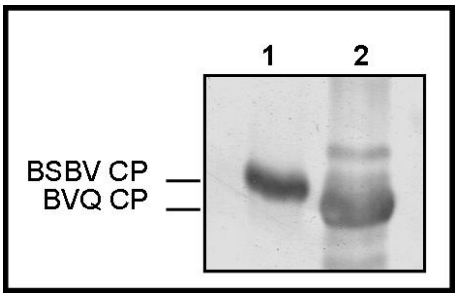
As a first report of the *in vivo* expression of BVQ CP and CP-RT, it has also to be noted that the estimated molecular masses (MM) of both proteins in SDS-PAGE conditions did not correspond to the predicted ones.
With an expected MM of 19 kDa, the BVQ CP migration pattern should be identical to the 19 kDa CP of BSBV. But, as clearly shown on figure 3b, the BVQ CP migrated faster than that of BSBV and its MM was estimated to 16.5-17 kDa. The same difference of MM was observed between the BSBV and BVQ CPs when these proteins were expressed in *E. coli* from cloned coding sequences (Fig. 4) or when BVQ CP was expressed in TNT® coupled Wheat Germ Extract System (Promega) (data not shown).
Conversely, the BVQ CP-RT MM in SDS-PAGE conditions was estimated to 107-110 kDa, therefore superior of the 95 kDa predicted one. Interestingly, a similar gel-shifting of 10-15 kDa is also reported for the CP-RT of BSBV (Crutzen *et al*., 2009b).

BVQ RNAs-2 and -3 are capped, with potential CCA motif in 3'

RACE of BVQ RNAs-2 and -3 by means of RNAs circularization confirmed that no mutation was inserted inside terminal sequences of pUBQ-2wt and



Project paper Fig. 3b Western blot detection of BSBV, BNYYV and BVQ in *C. quinoa* leaves inoculated with BSBV RNAs 1+2+3 and BVQ RNAs 1+2+3 (1), BSBV RNAs 1+2+3 (2), BNYVV RNAs 1+2 and BVQ RNAs 1+2+3 (3), BNYVV RNAs 1+2 (4), BNYVV RNAs 1+2+3+4 and BVQ RNAs 1+2+3 (5), BNYVV RNAs 1+2+3+4 (6), BVQ RNAs 1+2+3 (7) or mock-inoculated (8). Primary polyclonal antibodies, directed against BNYVV or BSBV viral particles, allowed the detection of the CP and CP-RT of these last viruses as well as those of BVQ, because of BSBV-PAb cross-reactivity. BSBV and BVQ structural proteins were therefore detected on the same blot.



Project paper Fig. 4. Western blot detection of BSBV and BVQ CPs, expressed in *E. coli* from pBS-cp (1) and pBQ-cp (2), respectively.

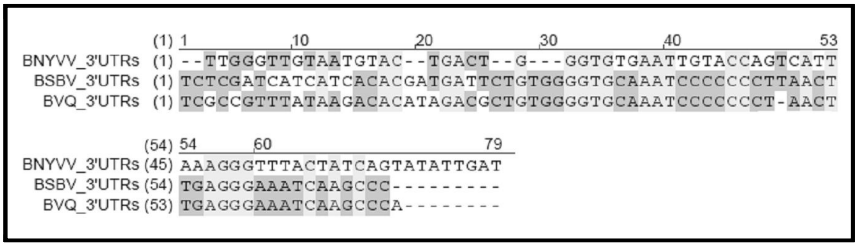
pUBQ-3wt clones through the primers used. However, an extra cytosine was found at pUBQ-3-wt 3' end (…AAATCAAGCCCC) when compared with the RNA-3 terminal sequence of the BVQ strain used for constructing the full-length cDNA clone (…AAATCAAGCCC). This supplementary nucleotide being present at the end of viral transcripts from BVQ RNA-3, it is not expected to disturb upstream secondary structures essential for viral replication (Koenig *et al*., 1998). An additional adenine was also found between 5' and 3' ends of circularized RNAs and that might probably be

added after the cytosine triplet (…AAATCAAGCCC**A**). Such "CCA" motif is reported for other plant RNA viruses, terminating their 3' terminal sequences folded in tRNA-like structures and being potentially involved in the aminoacylation of the viral RNA extremity (Dreher, 2009). Although not reported for the initially described strain, this CCA motif would confer the BVQ RNAs 3' termini the necessary sequence to fold in valylated tRNA-like structures (Koenig *et al*., 1998). *Mlu*I (**A**CGCGT) and *Spe*I (**A**CTAGT) restriction sites being directly adjacent to the pUBQ-1wt, -2wt and -3wt 3' "…CCC" extremities, "CCA" motif is present in BVQ transcripts.
Circularization of viral RNAs proved to be dependant on TAP treatment, whereas the CIP one only increased the specific ligation of capped RNAs. BVQ RNAs, at least the smaller ones, were therefore shown to be 5'-capped like BNYVV (Putz *et al*., 1983). If this was expected for BVQ, it was still to be determined experimentally.

DISCUSSION

Curiously, diverging results are evidenced for the infectiousness of BVQ depending on whether the viral inoculum consists of crude sap from infected plant or is produced *in vitro* by reverse genetic. Although BVQ particles from sap of beet roots have been reported to be self-infectious in *C. quinoa* leaves (Koenig *et al*., 1998; Rubies-Autonell *et al*., 2006), the present study evidenced the inability of BVQ transcripts to multiply alone in this host plant.

It has been described recently that BNYVV is able to trans-replicate the RNA-3 of the closely related BSBMV (Ratti et al., 2009). These two benyviruses share high similarities of sequence and structure in both the 5' and 3' untranslated regions (UTRs) and BSBMV RNA-3 is considered by BNYVV as one of its own RNAs. However, the 3' nucleotide sequences of BNYVV genomic RNAs are much different from those of BSBV and BVQ, which on the contrary present a high degree of sequence similarity (Fig. 5).



Project paper Fig. 5. Alignments of BNYVV, BSBV and BVQ consensus sequences for the last 70 nt of 3' untranslated regions (UTRs) using AlignX (Invitrogen). Nucleotides conserved in two sequences are pointed out in dark-grey boxes and light-grey boxes indicate nucleotides that are common for the three consensus sequences.

Morevover, while the 3' extremities of BNYVV genomic RNAs are characterized by the addition of a poly-A tail (Putz *et al*., 1983), those of BSBV and BVQ fold in tRNA-like structures with the same pseudoknots, stem-loops, hairpins and valine anticodon positions (Koenig *et al*., 1998). Because of these similarities shared by the BVQ and BSBV, a potential trans-replication of BVQ by BSBV could have been expected. But mixed infections evidenced that BVQ transcripts multiplied only in presence of the more distant BNYVV, and not with the closer BSBV. In this latter situation, strong competition between the two higly similar BVQ and BSBV might have occured, as also reported between BNYVV and BSBMV RNAs-3 (Ratti *et al*., 2009). BVQ and BNYVV being only distantly related, such competition woudn't take place. However, in light of the dissimilarities between BVQ and BNYVV UTRs, trans-replication of BVQ transcripts by BNYVV seems unlikely. BVQ transcripts would then rely on BNYVV for another vital function. Following the example of the closely related pomoviruses BSBV and PMTV, the BVQ RNA-2 might be dispensable for the viral multiplication in plant leaves (Savenkov *et al*., 2003; Crutzen *et al*., 2009b). There would be no need of complementation for the CP and CP-RT for the BVQ survival *in planta*. More probable is the complementation of the BVQ cell-to-cell movement, which is potentially ensured by the RNA-3-encoded TGB proteins. These are known to be of crucial importance for the virus multiplication in plants, as shown for BNYVV and BSBV (Gilmer *et*

al., 1992; Crutzen *et al*., 2009b). It has been demonstrated for many viruses such as the *Tobacco mosaic virus* (TMV) (Atabekov *et al*., 1999) or BNYVV (Lauber *et al*., 1998b) that TGBs from unrelated viruses can be exchanged and still ensure the viral movement. Such trans-complementation for the viral cell-to-cell movement between BNYVV and BVQ might have therefore saved this last from a certain death if its own TGB was deficient. Finally, no CRP-encoding gene is identified in the BVQ genome. Although the PTGS suppressor activity might be ensured by BVQ replicase, coat or movement proteins as reported for other viruses (Voinnet *et al*., 2005), BNYVV might have provided this crucial function to BVQ transcripts through p14.

While BVQ is reported to produce lesions spreading along the veins on *C. quinoa* leaves (Koenig *et al*., 1998; Rubies Autonell *et al*., 2006), the contribution of BVQ transcripts to the symptom expression was mitigated. In view of the slight modification of the chlorosis pattern when BVQ transcripts were co-inoculated with the four RNAs of BNYVV, the presence of BVQ led to an overall reaction of the leave rather than localized spot-shaped reactions. As no symptom changes were recorded on leaves co-inoculated with BVQ and BNYVV RNA-1 and -2, the symptom modification would not be due directly to BVQ potential pathogenic determinants but rather to synergistic effects between BVQ transcripts and BNYVV, changing the behaviour of this last.

The Northern and Western blot detection of viral components evidenced no difference in the accumulation of BNYVV RNAs or structural proteins whether the BVQ transcripts were present or not (Fig. 3a and b, lanes 3 to 6). The vital complementation of BVQ by BNYVV therefore didn't affect the replication nor the CP and CP-RT expression of this last. Nevertheless, the presence of BNYVV RNAs-3 and -4 reduced the accumulation of BVQ RNAs and structural proteins. In such situation, both viruses might thus compete for cellular factors necessary for their multiplication in *C. quinoa* leaves and some kind of cross-protection could occur, as reported between

BNYVV and *Beet soil-borne mosaic virus* (BSBMV) (Mahmood and Rush, 1999).

CONCLUSIONS

Given these contradictory results on the BVQ infectiousness, further investigations should be carried out to identify the underlying mechanisms responsible for the rescue of BVQ transcripts by BNYVV RNAs-1 and -2. Thus, the ability of BVQ transcripts to self-replicate should be first tested in protoplasts as viral replication and movement cannot be distinguished following leaves inoculation. The BVQ function requiring complementation could then be evidenced by mixing partial combination of BVQ transcripts with BNYVV RNAs-1 and -2 or even co-inoculating BVQ transcripts with other unrelated viruses like TMV.

MATERIALS AND METHODS

Full-length infectious clones of BVQ, BSBV and BNYVV

In this study, full-length cDNA clones of BSBV pUBS-1-wt, pUBS-2-wt and pUBS-3-wt (Crutzen *et al*., 2009b) and BNYVV pB15, pB2-14, pB35 and pB45 (Ziegler-Graff *et al*., 1988; Quillet *et al*., 1989) were used.

The BVQ isolate used to construct full-length cDNA clones of the three genomic RNAs originated from a Belgian sugar beet crop (Mazy city) showing rhizomania symptoms. *Beta vulgaris* was used as bait plant and extractions of total RNAs from roots were performed using FastRNA® Pro Green Kit (Qbiogene). BVQ RNAs were then piecemeal reverse transcribed and amplified by polymerase chain reaction (RT-PCR) using Expand Reverse Transcriptase and Expand Long Template PCR System (Roche). Four and two cDNA fragments were generated for RNAs-1 and -2, respectively, and RNA-3 was amplified as a single-piece (Fig. 1). Two silent mutations were inserted in the RNA-1 coding sequence at positions 1422 and

2991 to create unique restriction sites *BamH*I and *Kpn*I, respectively. Each amplicon, delimited by two restriction sites (Fig. 1), was gel-purified using the QIAquick Gel Extraction Kit (Qiagen) and first inserted into the pGEM-T® vector (Invitrogen). One out of two to four clones was then selected as a consensus sequence for each of the seven fragments. Full-length cDNA sequences were reassembled in pUC19 and restriction sites *Mlu*I or *Spe*I were inserted at 3' ends using PCR to obtain the final constructs pUBQ-1-wt (RNA-1), pUBQ-2-wt (RNA-2) and pUBQ-3-wt (RNA-3). *E. coli* strain DH5-α was used to maintain all constructions. Primers matching the extremities of the three viral RNAs were designed following the only full-length sequences known to date for BVQ (Koenig *et al*., 1998), and the forward ones were flanked with a T7 promoter sequence (TAATACGACTCACTATAG) (Fig. 1).

In vitro transcriptions and mechanical inoculation of *C. quinoa*

Before run-off transcription, the full-length cDNA constructs were linearized. Those of BSBV were *Spe*I- (pUBS-1-wt) or *Nco*I-treated (pUBS-2-wt and pUBS-3-wt) (Crutzen *et al*., 2009b) whereas pB15, PB2-14, pB35 and pB45 from BNYVV were all *Hind*III-restricted (Ziegler-Graff *et al*., 1988; Quillet *et al*., 1989). pUBQ-wt full-length cDNA clones were linearized with *Mlu*I or *Spe*I restriction enzyme (Fig. 1). Transcripts were subsequently produced for the three viruses and mechanically inoculated on *C. quinoa* leaves as described by Crutzen and co-authors (2009b), following seven viral combinations: BSBV RNAs 1+2+3 with BVQ RNAs 1+2+3, BSBV RNAs 1+2+3, BNYVV RNAs 1+2 and BVQ RNAs 1+2+3, BNYVV RNAs 1+2, BNYVV RNAs 1+2+3+4 with BVQ RNAs 1+2+3, BNYVV RNAs 1+2+3+4, BVQ RNAs 1+2+3. Inoculation buffer without any viral transcript constituted the negative control. For each condition, two leaves per plant were inoculated. Plants were grown in the greenhouse at 18°-23°C during the night and day periods, respectively, with 16 hours light-exposure.

Total RNA extraction and detection of viral RNAs by Northern blot

C. quinoa inoculated leaves were harvested 7 or 10 days post-inoculation (dpi). Polysome extraction (Jupin *et al*., 1990) was performed on half of each leaf for total RNA extraction, followed by phenol extraction and ethanol precipitation of the RNAs. For each RNA extract, 3 µg of total RNA was spotted on agarose gel with 8.5% formaldehyde. Diluted transcripts of BSBV RNAs 1+2+3 ($^{+}$C BS), BNYVV RNAs 1+2+3+4 ($^{+}$C BN) and BVQ RNAs 1+2+3 ($^{+}$C BQ) were also spotted as positive controls. After migration of RNAs, gels were over-night blotted on Amersham Hybond-NX membranes (GE Healthcare). Random DNA probes, synthesized with the Prime-a-Gene® Labelling System (Roche) and labeled with [α-^{32}P]dCTP (Perkin Elmer), were used to detect the viral RNAs. The PCR products used to produce probes were amplified from cDNA full-length constructs and the primers matching positions on BNYVV and BVQ genomes were: 2113-2132 and 2584-2604 (pB15), 573-596 and 805-824 (pB35), 170-189 and 464-483 (pB45), 2484-2503 and 2685-2704 (pUBQ-1-wt), 2431-2453 and 2910-2931 (pUBQ-2-wt), 1438-1457 and 2106-2125 (pUBQ-3-wt). Those used for BSBV were the same as those described by Crutzen and co-authors (2009b).

SDS-PAGE and Western blot analysis

The second half parts of the inoculated leaves were crushed directly in Laemmli buffer 2 times concentrated. Total protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 8% and 15% acrylamide-concentrated gels. After the proteins transfer from gels to nitrocellulose membranes (Biorad), viral structural proteins were detected using polyclonal antibodies directed against BSBV or BNYVV viral particles (DSMZ). Because of cross-reactivity of BSBV antibodies with BVQ, no specific antiserum was used for that last virus and structural proteins of both viruses were detected on the same membranes. Primary antibodies were 1:10 000 diluted in TBS buffer containing 0.1% Tween 20, 5% powdered skimmed milk and supplemented with total protein

extracts from healthy *C. quinoa*. Anti-rabbit alkaline phosphatase-conjugated secondary IgG (Sigma-Aldrich) was 1:10 000 diluted in TBS-Tween-milk buffer and blots were revealed with premixed BCIP-NBT solution (Sigma-Aldrich).

Expression of the BVQ and BSBV CPs in *Escherichia coli* and detection by Western blot

Coding sequences of BSBV and BVQ coat proteins were reverse transcribed and amplified using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega) and Go Taq Polymerase (Promega). Forward primers were flanked with two cytosine residues at 5' extremities of ATG start codons (Bsbv cp for-**CC**ATGGTTGATCCGCGGTATGA, Bvq cp for-**CC**ATGGTTGATCCAAGATATGA; cytosine nucleotides in bold and start codons underlined) and reverse primers included the final TAG stop codons (BSBV cp rev-CTATTCAACCCAGCGCAAAC , Bvq cp rev-CTATGAGCCGGTCCACTTCAAT; stop codons underlined). Both amplified fragments were gel-purified using QIAquick gel extraction kit (Qiagen) and cloned into the pBAD-TOPO® plasmid (Invitrogen). These constructs were *Nco*I-treated and self-ligated with T4 DNA ligase (Roche) in order to loose the N-terminal leader. Final constructs pBS-cp (BSBV CP) and pBQ-cp (BVQ-CP) were then transformed into *E. coli* One Shot® TOP10 strain (Invitrogen) and CPs expression was induced with 0.02 % and 2% L-arabinose, respectively. Bacteria were sonicated in a lysis buffer (50mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton-X-100, 10 mM Imidazole, pH 7.8) and total proteins extracts were separated by SDS-PAGE. Proteins detection by western blot was done as described previously, but primary antibodies were used 1:1000 diluted.

Alignments of 3'UTRs consensus sequences

Consensus sequences were determined for the 3' extremities of BVQ full-length cDNA clones, BSBV (accession numbers FN386612, FN386613 and FN386614) and BNYVV (accession numbers X05147, X04197, NC_003516

and DQ682453) RNAs by using Vector NTI-AlignX (Invitrogen). Consensus sequences were then aligned with the same program.

RACE of BVQ RNAs-2 and -3 by RNAs circularization

5' and 3' extremities of BVQ RNAs-2 and -3 were sequenced to check that no mutation was inserted in the cDNA full-length clones because of terminal primers. The method described below was however unsuitable for rapid amplification of cDNAs ends (RACE) of BVQ RNA-1, this last being probably two long. Protocols were adapted from Fromont-Racine and co-authors (1993) and Couttet and co-authors (1997). Original RNA extract used to construct clones of BVQ was treated with Alkaline phosphatase from calf intestine (CIP) (Promega), Tobacco acidic pyrophosphatase (TAP) (Ambion) and T4 RNA ligase (Ambion) in order to de-phosphorylate, de-cap and circularize RNAs, respectively. Four treatment combinations CIP-TAP-T4, TAP-T4, CIP-T4 and CIP-TAP were performed, the two first leading to circularization of capped RNAs. Purified by means of phenol-chloroform extraction and ethanol precipitation after each treatment, RNAs were then reverse transcribed and amplified using M-MLV RT (Promega) and Go Taq® Flexi DNA polymerase (Promega). Forward and reverse primers matching on the 3' and 5' extremities of BVQ RNAs-2 and -3 were used to amplify ligated terminal sequences of circularized RNAs. Amplicons were gel-purified using QIAquick Gel Extraction Kit (Qiagen) and nested-PCR with the same primers was performed to get enough DNA for sequencing.

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GENERAL DISCUSSION

Over the last 30 years, major efforts have been focused on the harmful BNYVV, leaving aside the other sugar beet pomoviruses. However, investigating the particular functioning of BSBV and BVQ pomoviruses by means of full-length cDNA clones is greatly facilitated because of their genomic organization. The putative replication, structural and movement proteins being encoded on separate RNAs, one or the other function may be investigated individually without interfering directly with the other ones. RNAs-2 encoded CPs RT domains, especially, could be functionally characterized throughout entire length. By contrast, studying the RT domain of BNYVV CP is limited because ORFs encoding for TGB proteins are located on the same RNA-2, just downstream the CP-RT coding sequence. Changes in the C-terminal part of BNYVV CP-RT ORF are thus expected to interfere with the expression of the p42 movement protein and affect the virus infectiousness (Gilmer *et al*., 1992). This makes therefore BSBV and BVQ good candidates for studying the particular role of each encoded protein in the multiplication dynamic of soil-borne sugar beet viruses.

BSBV and BVQ RT domains from a multifunctional point of view

Following descriptive and experimental approaches, it appeared that BSBV entire and BVQ C-terminal part of CPs RT domains were not required for the viral replication *in planta*. Indeed, the BVQ strain described by Koenig and co-authors (1998) is reported to be multiplied efficiently in *C. quinoa* leaves despite the major rearrangements in the C-terminal moiety of the RT domain described in chapter II. The same observation was made for BSBV when introducing a frameshift mutation at the beginning of RT coding sequence. Such results are consistent with the RT domain dispensability for viral replication described for closely related furoviruses *sensu lato*, but also aphid-transmitted viruses from *Luteoviridae* family. The RT domain of the CP may be involved in functions as different as encapsidation, long-distance movement, transmission or symptom severity, but has never been reported to be part of the replication mechanisms. The potential multifunctional roles of the CP RT domains of sugar beet pomoviruses may therefore be investigated without interfering with the indispensable viral replication *in planta*.

The involvement of the CP RT domain as a pathogenic determinant is in fact rather variable between RNA plant viruses. While the PMTV CP RT domain is responsible for the mosaic yellowing on *N. benthamiana* leaves (Torrance *et al*., 2009), that of BNYVV is not required for the symptom production on leaves of *B. macrocarpa*, *C. quinoa* and *T. expansa*. Nevertheless, it is reported to modulate somehow the shape and color of the lesions caused by BNYVV infection (Tamada and Kusume, 1991; Schmitt *et al*., 1992; Tamada *et al*., 1996). On the contrary, the RT domain may also act as an avirulence factor and attenuate the symptomatic reaction, like described for the SBWMV (Chen *et al*., 1994). Looking at the leaves of BSBV- or BVQ-infected *C. quinoa*, the RT domains of both viruses were not responsible for any particular symptom expression, nor did they modulate it. In light of these observations, the CP RT domains of BSBV and BVQ should not be considered as crucial pathogenic determinants, at least in *C. quinoa*.

Despite the more or less important deletions that have been described in CP RT domains of BNYVV, BSBV, PMTV and SBWMV, N-terminal parts are always preserved from the major rearrangements (Tamada and Kusume, 1991; Chen *et al*., 1995; Koenig *et al*., 1997a; Reavy *et al*., 1998; Koenig, 2000; Sandgren *et al*., 2001). The present work evidenced that BVQ is not an exception to the rule, but the reason of such characteristic deletion pattern remains unclear. Even when the N-terminal portion is known to direct the BNYVV assembly (Schmitt *et al*., 1992), such function is not attributed to the PMTV RT domain (Torrance *et al*., 2009) and has still never been reported for the other CP-RT encoding viruses. As replicative transcripts from full-length cDNA clones are from now on available for both BVQ and BSBV, the potential involvement of the CP-proximal part of the RT domain in viral encapsidation could be easily investigated by means of directed mutagenesis and checking viral particles formation under the electron microscope. Nevertheless, BSBV should be preferred for such purposes as BVQ transcripts are not self-infectious.

When the involvement of the RT domain in symptoms severity and virus encapsidation is rather proper to one or another virus species, its importance for the transmission of viruses by aphids or protists vectors is generally accepted. Clearly demonstrated for a limited number of viruses, the common role of CP RT domain in viral transmission is however also supported by indirect observations from viral genome adaptation and bioinformatic predictions. As a result from the investigations on the BVQ 76 kDa RT domain, both major rearrangements in the CP RT C-terminal part in the laboratory-maintained strain (Koenig *et al*., 1998) and the identification of predicted TM1 and TM2 argue strongly in favor of the implication of BVQ CP RT in the viral transmission by the putative vector, *P. betae*. As the BSBV RT domain is also reported to be susceptible to spontaneous deletion (Koenig *et al*., 1997a) and to contain the two predicted TMs (Adams *et al*., 2001), sugar beet pomoviruses are thus keeping with the general pattern of transmission of other soil-borne viruses. A model of TMs interaction can therefore be proposed for BSBV and BVQ (Fig. 15), following that suggested for BNYVV (Diao *et al*., 1999a; Adams *et al*., 2001). However, this model includes another TM2 for BSBV than the weak one partially structured in α-helix, with multiple charged amino acid residues and no amino acid interacting at the inner face of the plasma membrane, that is proposed but much disputed by Adams and co-authors themselves (2001). Sequencing of the full-length infectious clones of BSBV and prediction of transmembrane helices in the RT domain as described for BVQ (Crutzen *et al*., 2009a) evidenced a better candidate, TM2bis. This one was found to fold completely in α-helix, contain only one charged residue at the N-terminal extremity (a glutamic acid residue, E) and an aspartic acid (D) residue potentially interacting at the inner face of the plasma membrane with an arginine (R) residue from predicted TM1. A single amino acid change arginine(R)-alanine(A) in TM2bis amino acid sequence between the BSBV strain originally described and that from full-length cDNA clone, respectively, is responsible for this major difference of TMs prediction (alanine in black on figure 15).

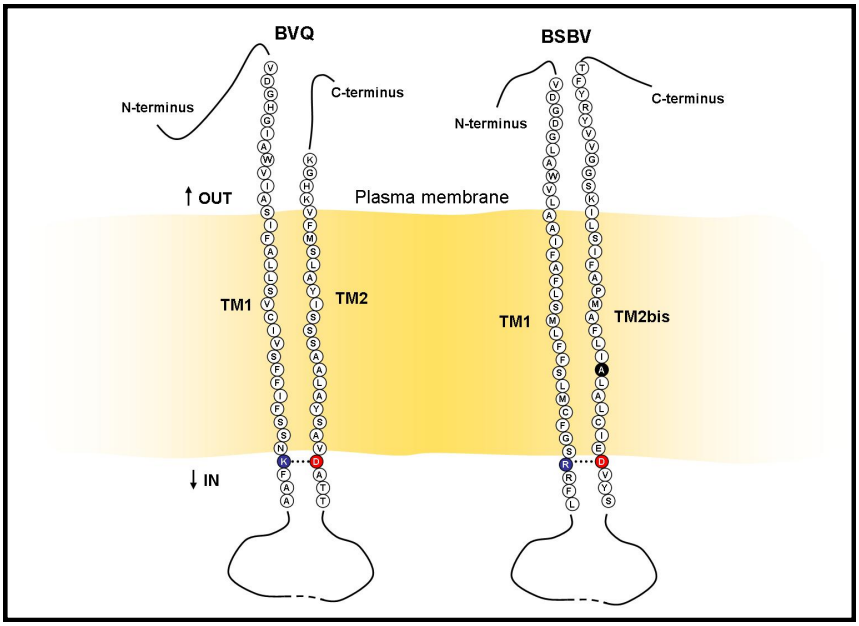

Fig. 15. Model of interaction for BVQ and BSBV predicted transmembrane motifs in CPs RT domains. Aspartic acid residues (D) interacting potentially at the inner side of the plasma membrane with lysine (K) or arginine (R) amino acids are shown. Alanine (A) residue in BSBV TM2bis differing from the arginine one previously reported is in black.

The positively charged arginine makes the TM2bis unsuitable for transmembrane purposes. Knowing that the BSBV strain originally sequenced for the CP-RT encoding RNA has been propagated on *C. quinoa* leaves like BVQ, this crucial change in the potential TM2bis might have occurred because of the serial passages. Indeed, looking at the Yu2P and Rg1 isolates of BNYVV that have been maintained in *C. quinoa* leaves for more than 15 years (Koenig, 2000), the predicted TM2 is also directly affected by deletions of 7 and 1 amino acid, respectively. It could therefore be hypothesized that, in addition to the large deletions reported for BNYVV, BSBV, BVQ, SBWMV and PMTV RT domains, the putative TM2 could also be specifically targeted by small deletions and amino acid substitutions. Nevertheless, further functional experiments should be carried out to

confirm and if so to precise the involvement of these putative transmembrane helices in the viral transmission by the plasmodiophorid vector. In this view, BSBV transcripts mutated for the predicted TM1 and/or TM2bis could be inoculated to *B. macrocarpa* or *B. vulgaris* to check for their effective transmission by *P. betae* (Tamada *et al*., 1996). However, direct inoculation of the roots (Koenig and Stein, 1990) should be preferred as BSBV is not systemic on these plants (Henry *et al*., 1986; Crutzen F., personal observations).

Potential PTMs of CP-RT for viral transmission by *P. betae*?

The first report of the *in vivo* expression of BSBV and BVQ CP-RTs evidenced a higher MM of ~10-15 kDa for both proteins. Interestingly, a shift in the migration pattern of the CP-RT of BNYVV is also reported and an estimated MM of ~85 kDa is commonly observed instead of the 75 kDa predicted one (Ziegler *et al*., 1985; Niesbach-Klösgen *et al*., 1990). Given the major role of the RT domain in viral transmission and knowing that the three viruses are supposed to share the same vector *P. betae*, it would make sense to propose that such shift in the migration pattern could be related to this particular function.

Intrinsic properties might be responsible for slowing-down the CP-RTs in SDS-PAGE conditions. As Rath and co-authors (2009) have demonstrated recently that "gel shifting" of transmembrane proteins is commonly observed as a result from differential binding of SDS, potential TMs in CPs RT domains of BNYVV, BSBV and BVQ might therefore affect their gel mobility. But, although containing the predicted TMs1 and -2, the PMTV CP-RT is reported to migrate according to the predicted MM (Reavy *et al*., 1998; Torrance *et al*., 2009). PTMs of the CP-RTs specific to the *P. betae*-transmitted viruses would then constitute another plausible explanation. When glycosylation, phosphorylation and ubiquitination are reported for structural proteins of some plant viruses, no role in viral transmission has yet been attributed to these PTMs specifically (Hazelwood and Zaitlin, 1990; Atabekov *et al*., 2001; Fernández-Fernández *et al*., 2002; Baratova *et al*., 2004; Akamatsu *et al*., 2007). An interesting comparison could however be

made when looking at animal viruses. Ubiquitine tagging of specific viral proteins has been shown to be part of the viral release for some enveloped retroviruses (Morita and Sunquist, 2004; Martin-Serrano, 2007; Isaacson and Ploegh, 2009; Raiborg and Stenmark, 2009). Thus, ubiquitination of the membrane-associated Gag protein of *Human immunodeficiency virus* (HIV) is thought to favor the interaction of this viral protein with a specific cellular factor, Tsg101 (Tumor susceptibility gene 101). Classically targeting mono-ubiquitinilated proteins in the multivesicular body (MVBs) pathway together with ESCRT (Endosomal sorting complex required for transport), Tsg101 would then recruit this last when interacting with a PS/TAP (proline, serine or threonine, alanine and proline) motif present in the late-budding domain (L-domain) of Gag protein. The Tsg101-ESCRT complex, bound to Gag protein, would finally enable the viral release by membrane budding. Interestingly, CP-RTs of BNYVV, BSBV and BVQ present some similarities with the HIV Gag protein, starting with the fact that they seem all to be membrane-associated proteins. In addition, the structural protein of these soil-borne viruses might be ubiquitinilated, as suspected by the gel shifting. This hypothesis was supported by preliminary predictions of ubiquitination sites in CP RT domains using Ubipred and Ubpred programs (Tung and Ho, 2008; Radivojac *et al*., 2010) (Table 6). And, finally, a PSAP motif is present at C-terminal extremity of BNYVV RT domain, mimicking that in Gag L-domain. When the PS/TAP or other known motifs are absent from pomoviruses CP RT domain, this doesn't rule out the possibility of a functional equivalent one like recently identified for the TBSV (Barajas and Nagy, 2010). As cellular factors homologous to those involved in the MVB pathway in animals, i.e. Tsg101 and ESCRTs, have been recently identified in *Arabidopsis thaliana* (Spitzer *et al*., 2006), an original model of viral budding might be proposed for these soil-borne viruses. Adams and co-authors (2001) have already proposed that viral TMs1 and -2 in CPs RT domains might facilitate the viral uptake by *P. betae* inside root cells. RT domains incorporated at one extremity of viral particles (Haeberlé *et al*., 1994) could therefore recruit cellular factors involved in plant MVB

Table 6. Ubiquitination site prediction with amino acid sequences of BNYVV, BSBV and BVQ CP RT domains using Ubipred (A) and Ubpred (B) programs (Tung and Ho, 2008; Radivojac *et al*., 2010). Ubiquitination scores for each lysine position indicate low (0.62 < s < 0.69), medium (0.69 < s < 0.84) or high (0.84 < s < 1.00) confidence for the prediction. Lysine residues localized between predicted TMs1 and -2 are grey shaded.

BNYVV		
Pos.	**A**	**B**
15	0.59	-
53	0.69	-
271	-	0.77
314	-	0.76
331	-	0.76
339	0.64	0.78
347	0.52	0.68
364	0.50	0.75
477	0.57	-

BSBV		
Pos.	**A**	**B**
8	0.59	-
23	0.71	-
41	0.77	0.69
79	0.77	0.69
99	0.58	-
106	0.70	-
111	0.79	-
191	0.53	-
208	0.60	-
225	0.54	0.66
226	0.56	0.64
239	0.68	-
325	0.62	-
330	0.51	-
343	0.64	-
391	0.85	0.87
404	-	0.68
406	0.63	-
564	0.70	-
576	0.70	0.78
618	0.63	0.76
624	-	0.77
649	0.57	-
675	0.80	-
678	0.67	-
680	0.70	-
689	0.53	0.62
705	0.53	0.94
711	0.54	0.84
741	-	0.70
759	-	0.82

BVQ		
Pos.	**A**	**B**
55	0.60	-
226	0.68	-
264	0.70	-
279	-	0.65
318	0.59	-
328	0.59	-
331	0.66	-
373	0.65	0.70
379	-	0.72
385	-	0.87
389	0.64	0.87
565	0.74	0.66
621	-	0.72
643	0.59	0.76
657	0.82	0.83

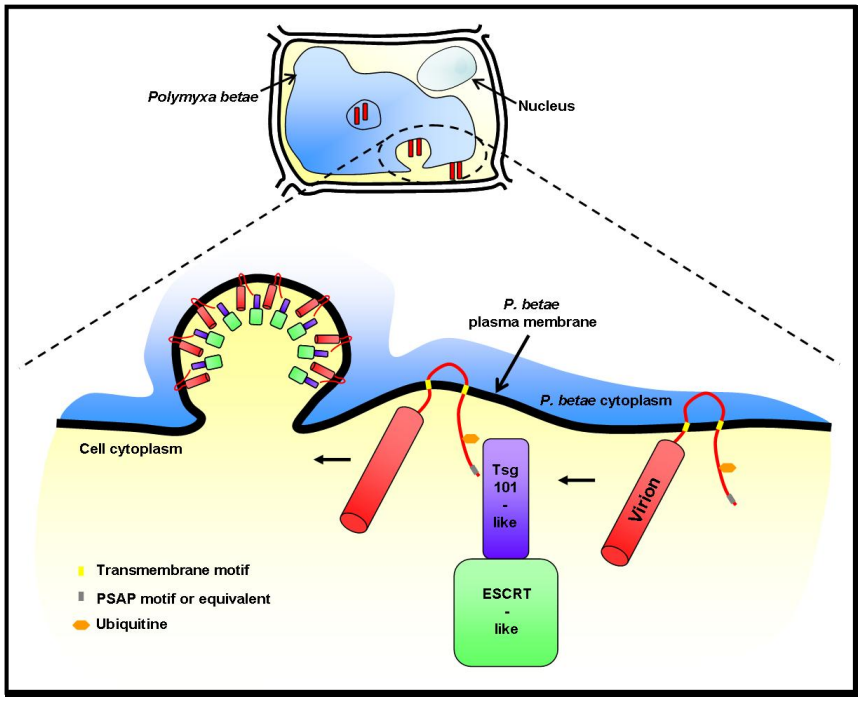

Fig. 16. Proposition of budding model for *P. betae*-transmitted viruses.

pathway in order to facilitate the internalization of the virus by the protist vector (Fig. 16). Following this model, RT domains should be ubiquitinated on the C-terminal part, the middle section between both TMs being putatively on the other side of the membrane and inaccessible for ESCRT- and Tgs101-like budding proteins.

Though such ubiquitination sites are predicted for BNYVV (but only a weak one), BSBV and BVQ, potential ubiquitinated residues are also predicted between TM1 and TM2. With the emerging role of ubiquitine-mediated cellular trafficking, it wouldn't be surprising that viruses also resort to ubiquitination for intra-cellular targeting (Acconcia *et al*., 2009; Raiborg and Stenmark, 2009).

Regarding these hypotheses, it would be of great interest to determine the underlying intrinsic properties and/or PTMs that could be responsible for the

common gel-shifting of BNYVV, BSBV and BVQ CP-RTs. Two types of approaches dealing with purified virus could be envisaged to identify potential PTMs. On one hand, viral particles might be treated with glycosydases (Seddas and Boissinot, 2006) enzymes to observe a potential shift in the migrating pattern of CP-RT compared with non-treated samples. On the other hand, anti-phosphate (Fernandez-Fernandez *et al*., 2002) or -ubiquitine (Hazelwood and Zaitlin, 1990) antibodies could be used directly in western blot. The hard task of this approach lies in obtaining particles of BVQ and BSBV, reported to be extremely difficult to purify (Koenig and Loss, 1997; Koenig *et al*., 1998; Crutzen F., personal observation). In addition, site-directed mutagenesis could be used to identify amino acids potentially linked to glucide, phosphate or ubiquitine molecules. Modifying residues in the predicted transmembrane helices might also be performed to investigate the potential role of TMs in gel shifting (Rath *et al*., 2009).

Replicase-driven switching mechanism and genome rearrangements

Although deletions in the RT domain of CPs are a well-established phenomenon described for at least five virus species, the underlying mechanisms of RNAs rearrangements have never been really investigated. Nevertheless, a replicase-driven switching mechanism has been proposed that seems to fit with such deletions events (Nagy and Simon, 1997). Following that model, viral replicase pauses during synthesis of the plus- or minus-strand RNA, probably because of hairpin structured sequences, and reinitiates elongation further downstream (Fig. 17). Such jumps when reading the minus-strand RNAs of PMTV and SBWMV are thought to be responsible for the spontaneous deletions reported in their CP RT domains (Chen *et al*., 1994; Sandgren *et al*., 2001). It might therefore be postulated that deletions in the BSBV and BVQ RT domains of CPs occurred by the same mechanism.

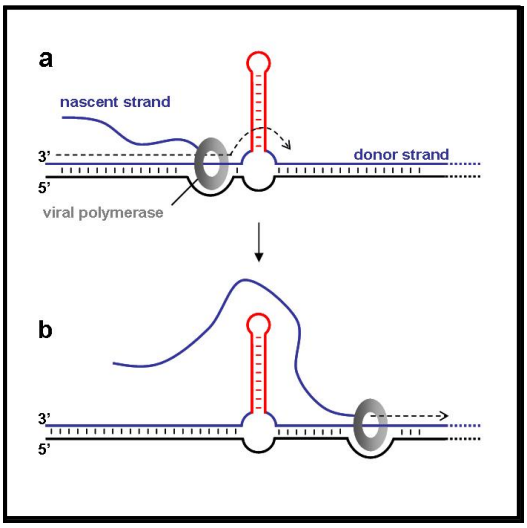

Fig. 17. Replicase-driven switching mechanism potentially leading to deletion events in sequences coding for BSBV and BVQ read-through (RT) domains of coat proteins (CP) (according to Chen *et al*., 1995; Nagy and Simon, 1997; Sandgren *et al*., 2001). (a) When replicating the viral genome, the polymerase might pause because of RNA secondary structures (in red). Such stop would favor the dissociation of the viral polymerase from the donor strand and (b) transcription reinitiation downstream of the secondary structure.

No CRP for sugar beet pomoviruses

Curiously, the BSBV and BVQ genomes are both devoid of any CRP encoding sequence, though CRPs are largely reported among plant RNA viruses (Koonin *et al*., 1991). These little proteins have been shown to act as pathogenic determinants and may be involved in suppression of PTGS (Dunoyer *et al*., 2002; Lukhovitskaya *et al*., 2005; Yelina *et al*., 2002; Bragg and Jackson, 2004; Ghazala *et al*., 2008). In this way, p8 of PMTV is a virulence factor that is dispensable for the symptom production on *N. benthamiana* and doesn't suppress PTGS (Lukhovitskaya *et al*., 2005), whereas p14 of BNYVV exhibits a PTGS suppression activity and modulates the symptom expression (Gilmer *et al*., 1992; Dunoyer *et al*., 2002). BNYVV RNAs-1 and -2 with mutated p14 produce similar number of lesions on *C. quinoa* as with wild-type RNA-2, but smaller in size and with necrotic centers. In addition, BNYVV RNAs accumulation is greatly reduced when p14 is absent (Gilmer *et al*., 1992). BSBV, although lacking a CRP, induced numerous small necrotic lesions and chlorosis on *C. quinoa* leaves and high quantities of viral RNAs were detected in both *C. quinoa*

and *B. macrocarpa* plants. Thus, BSBV accumulated in *C. quinoa* leaves to levels comparable to that of BNYVV when this last expressed p14. BSBV and BNYVV, both infecting sugar beet, behave therefore differently *in planta* when expressing homologous genes. While the lack of CRP significantly affects the BNYVV multiplication, this seems to be not restrictive for BSBV. Nevertheless, as a PTGS suppressor activity has been attributed to some viral replicases, movement or coat proteins, one of the BSBV gene products might act as such (Voinnet, 2005). It would therefore be of great interest searching for a CRP-alternative silencing suppressor among BSBV proteins. In addition, potential effects of heterologous PTGS suppressors like CRPs could also be tested on BSBV multiplication. As multiple infections frequently occur with BNYVV in sugar beet fields (Meunier *et al*., 2003; Ratti *et al*., 2005), simultaneous presence of p14 might play a role in BSBV infection.

Rescue of BVQ transcripts by BNYVV

Despite highly similar genomic organizations and both infecting commonly sugar beet, BSBV and BVQ presented different multiplication dynamics through reverse genetic strategies. On one hand, the BSBV putative replicase and movement proteins proved to be essential and sufficient for local infection of *B. macrocarpa* and *C. quinoa* leaves. On the other hand, multiplication of BVQ RNAs transcripts relied on the simultaneous presence of BNYVV RNAs-1 and -2, but not on BSBV RNAs. Although co-occurrence of BNYVV and BVQ is largely reported, BVQ is also found in sugar beet roots with BSBV but without BNYVV and has been previously shown to be self-infectious when inoculated from crude sap of infected plant (Koenig *et al*., 1998; Rubies Autonell *et al*., 2006). Depending on whether one works with transcripts from cDNA constructs or viral particles, BVQ appears as BNYVV-dependant or independent, respectively. The major difference between both approaches lying clearly in the viral inoculum, three scenarios should be considered.

The reverse genetic strategy and construction of full-length cDNA clones guarantee the single-virus infection, but may introduce mutations in the viral

sequence cloned. To avoid such lethal modification of BVQ full-length clone, an enzyme with proof-reading activity that gave good results for single-pieces cloning of BSBV RNAs was used to amplify the BVQ genome (Crutzen *et al*., 2009b). In addition, replicative potential of BVQ transcripts in presence of BNYVV together with RACE results both evidenced that the putative replication-related (Duggal *et al*., 1994) BVQ UTRs were not affected by cloning. However, internal mutations introduced in BVQ constructs cannot be excluded. A particular attention should therefore be paid to the full-length cDNA clones integrity and especially to RNA-3 encoded TGB proteins.

Assessing BVQ infectiousness by means of infected plant material may also constitute a risk. Mixed-infections of BNYVV, BSBV and/or BVQ being largely reported, working with a single virus often requires a specific selection of the one of interest. In addition, as viral interactions may repress the expression of the coat protein of one or the other virus in cross-infections (Mahmood and Rush, 1999), the virus detection by means of ELISA could miss one of the viruses. The present work combined BVQ with BNYVV and BSBV, but other viruses than BNYVV might also complement BVQ *in planta*. As no less than fifteen virus species are listed as infecting sugar beet (Table 7), multiple viral interactions could potentially happen. Interestingly, the question of potential synergies between sugar beet infecting viruses was already raised by Lindsten (1993) as well as Meunier and co-authors (2003), given the frequent mixed-infection of pomo- and benyviruses. But, when these interactions could be of major importance for the viral pathogenicity on sugar beet, they might also reflect crucial associations for viruses themselves. In this way, the frequent co-occurence of BVQ and BNYVV in fields could be the expression of the BVQ dependence on BNYVV for multiplying in plants. This situation resembles that of the satellite particles, widespread among plant RNA viruses (Roossinck *et al*., 1992). They rely on the presence of a helper virus to replicate in the host cells and differ from the so-called “defective interfering (DI)” particles in that they are not derived from the genome of the helper virus (Simon *et al*., 2004). In addition, distinction is made between satellite viruses, which encode their own coat

Table 7. Viruses infecting sugar beet according to Stevens and co-authors (2006) and the ICTV. Virus genus, genome organization and type of transmission are indicated.

Genus	Virus name	Genome	Transmission
Benyvirus	*Beet necrotic yellow vein virus*	4-5 linear ss(+)RNAs	Soil-borne
	Beet soil-borne mosaic virus	4 linear ss(+)RNAs	Soil-borne
Pomovirus	*Beet soil-borne virus*	3 linear ss(+)RNAs	Soil-borne
	Beet virus Q	3 linear ss(+)RNAs	Soil-borne
Necrovirus	*Beet black scorch virus*	1 linear ss(+)RNA	Soil-borne
Nepovirus	*Beet ringspot virus*	2 linear ss(+)RNAs	Soil-borne?
Closterovirus	*Beet yellow stunt virus*	1 linear ss(+)RNA	Aphids
	Beet yellows virus	1 linear ss(+)RNA	Aphids
Polerovirus	*Beet chlorosis virus*	1 linear ss(+)RNA	Aphids
	Beet mild yellowing virus	1 linear ss(+)RNA	Aphids
	Beet western yellows virus	1 linear ss(+)RNA	Aphids
Potyvirus	*Beet mosaic virus*	1 linear ss(+)RNA	Aphids
Alphacryptovirus	*Beet cryptic virus*	2 dsRNAs	Seed & pollen
Curtovirus	*Beet curly top virus*	1 circular ssDNA	Leafhopper
Rhabdovirus	*Beet leaf curl virus*	1 linear ss (-)RNA	Lacewing

protein, and satellite nucleic acids that rely on the CP of the helper virus for encapsidation. In that context, BVQ from full-length transcripts would be considered as a satellite virus from the BNYVV RNAs-1 and -2, relying on these unrelated helper RNAs to multiply *in planta* but encoding its own CP and being dispensable for BNYVV replication. However, whereas satellites are mostly composed of a single DNA or RNA molecule and encode very few proteins (Mayo *et al*., 2005), the BVQ genome is multipartite and encodes for seven putative structural and non-structural proteins. The synergy observed between the two viruses might therefore be based on

targeted complementation of one or several of these gene products rather than full support of the BVQ functions.
Finally, another possibility is also worth to be discussed. Viewing the results presented in this study and those reported from other works, two kinds of BVQ populations might exist in crops, one relying on the presence of the helper BNYVV and the other being self-sufficient. As the BVQ strain used to construct full-length cDNA clones derived from a mixed infection with BNYVV, we would have selected a dependent one unbeknownst to us.

Given the doubts hanging on the infectiousness of the BVQ transcripts from full-length cDNA clones, further experiments need to be performed to better characterize the molecular interactions governing the BVQ-BNYVV synergy. BVQ RNA-1 transcripts should be first inoculated to *C. quinoa* protoplasts in order to check whether the BVQ putative replicase is efficient or not. Protoplasts preparation and transfection could be performed following the method of Veidt and co-authors (1992). The functional complementation of one or the other RNA of BVQ might also be evidenced *in planta* through partial genome combinations of BVQ transcripts mixed with BNYVV RNAs-1 and -2. Knowing that replication and movement proteins are essential for the virus survival *in planta*, the dispensability of BVQ RNA-1 and/or -3 would designate the potential lacking vital function for BVQ. BSBV and BVQ RNAs sharing high similarities of sequence, they could also be exchanged following the example of BNYVV and BSBMV (Ratti *et al*., 2009). This could give further indications on the functionality of each BVQ transcript while avoiding competition between homologous RNAs. In addition, the integrity of BVQ full-length clones could be investigated by comparing them with the few other sequences available for this virus. Sequencing of the original BVQ strain used for constructing the clones could also be carried out in the same goal. Concerning the symptom changes when co-inoculating the four RNAs of BNYVV with BVQ transcripts, this could be investigated by working with GFP-labeled BNYVV (Erhardt *et al*., 2001). When the synergy modifies or not the BNYVV movement in *C. quinoa* leaves would be easily shown by such approach.

Finally, it would be of much interest mixing BVQ transcripts with other unrelated viruses such as the *Beet black scorch virus* (BBSV) or even the TMV. In addition to give further indications on the type of complementation required by BVQ transcripts, this could provide insight into the potential network of interaction that could take place in sugar beet crops.

CONCLUSIONS

The main objective of this thesis was to investigate the multiplication dynamics of BSBV and BVQ pomoviruses. Tackling with genome organization and multiplication requirements by means of new reverse genetic tools and *in silico* or *in vivo* analyses, we evidenced common but also specific characters for these sugar beet viruses.

Genome organization and *in silico* analyses

As a first step in the BVQ multiplication dynamic, the common organization of BVQ CP RT domain encoded by genomic RNA-2 was investigated. A putative 76 kDa RT domain was evidenced for ten geographically distinct strains from Europe instead of the 35 kDa one previously described (Koenig *et al*., 1998), with nucleotides and amino acids variabilities between strains as little as 0.0-0.95% and 0.0-1.29%, respectively. Three nucleotide additions of 5, 285 and 1 nt were localized in the 76 kDa RT coding sequence that were absent from the 35 kDa RT encoding RNA. Moreover, this longer RT was predicted to contain two transmembrane helices TM1 and TM2, whereas the shorter 35 kDa RT contains only TM1. As proposed for other plasmodiophorid-transmitted beny-, bymo-, furo- and pomoviruses (Diao *et al*., 1999a; Adams *et al*., 2001), BVQ TMs might be involved in the transmission by the putative vector *P. betae*. From these results, it was hypothesized that three deletion events removed a total of 291 nt potentially necessary for natural soil-borne transmission because the first sequenced strain of BVQ was multiplied through repeated mechanical inoculations on *C. quinoa* leaves. This phenomenon, already reported for other related viruses such as BNYVV, BSBV, PMTV and SBWMV, evidences however for the first time the occurrence of a triple-deletion event, as only single- or double-deletion events have been reported until now (Tamada and Kusume, 1991; Chen *et al*., 1995; Reavy *et al*., 1998; Koenig, 2000; Sandgren *et al*.,

2001). As hypothesized for SBWMV and PMTV (Chen *et al*., 1995; Sandgren *et al*., 2001), a replicase-driven switching mechanism potentially initiated because of strong RNAs secondary structures might have caused the major rearrangement of the initially described BVQ RNA-2 (Nagy and Simon, 1997).

In addition, predictions of transmembrane helices were performed in the BSBV CP RT domain from full-length cDNA clone of genomic RNA-2, following the example of BVQ. A new candidate sequence for BSBV TM2 was evidenced and that is not proposed by Adams and co-authors (2001) because of a basic arginine residue in the CP RT domain previously described for BSBV (Koenig *et al*., 1997a). Such modification in the coding sequence of the transmission-associated TM2 might have occurred because this strain of BSBV was maintained on *C. quinoa* as well.

When the KT**E**R motif is of crucial importance for the BNYVV transmission (Tamada *et al*., 1996), homologous TA**D**V and YV**D**E motifs were identified on N-terminal side of BVQ and BSBV predicted TMs2, respectively. Like the glutamic acid (E) residue of BNYVV, aspartate residues (D) might also interact with a basic lysine (K) or arginine (R) from C-terminal end of predicted TMs1.

Publication of the results on the BVQ CP RT domain contributed to a better characterization of the BVQ genome organization and added an example of viral adaptation to unnatural propagation through genomic rearrangements. Moreover, the newly available sequences for both BVQ and BSBV and the preliminary predictions of motifs putatively involved in viral transmission by *P. betae* make the way open to further functional studies of their CPs RT domains.

Multiplication requirements, *in vivo* analyses

Multiplication dynamics of BSBV and BVQ were also investigated *in vivo* by means of transcripts from full-length cDNA clones. Despite identical genome organizations and a common natural host, viral transcripts of BSBV and BVQ behaved differently *in planta*.

On one hand, the BSBV putative replicase (RNA-1) and movement proteins (RNA-3) proved to be essential and sufficient for viral multiplication in plants, like PMTV (Savenkov *et al*., 2003). RNA-2 encoded CP and CP-RT were, in turn, dispensable for virus survival and symptom expression. As reported by previous observations (Henry *et al*., 1986), BSBV transcripts induced symptoms on leaves of *C. quinoa* only, the *B. macrocarpa* infection being asymptomatic. BSBV would therefore not encode for proteins able to induce strong symptomatic reaction on *B. macrocarpa* leaves such as p25 and p31 expressed from BNYVV RNAs-3 and -4, respectively (Tamada *et al*., 1989; Rahim *et al*., 2007). Contrary to many other plant RNA viruses (Koonin *et al*., 1991), the BSBV genome comprises no CRP coding sequence. This seemed however to have no impact on its accumulation *in planta*.

On the other hand, transcripts from full-length cDNA clones of BVQ proved to rely on the presence of at least BNYVV RNAs-1 and -2 for multiplying in *C. quinoa* leaves. They did not multiply when inoculated alone or even combined with BSBV infectious transcripts. While trans-replication of BVQ transcripts by BNYVV seems rather unlikely because of sequence and structure dissimilarities, complementation of viral movement might be strongly envisaged. The vital contribution of a PTGS suppressor through BNYVV p14 should also not be excluded. BVQ being previously reported to be self-infectious when working with viral particles from sap of infected beet, further investigations need to be performed on the underlying mechanisms of BVQ transcripts rescue by BNYVV.

The western blot detection of both BSBV and BVQ CPs and CP-RTs in samples infected with transcripts from full-length cDNA clones also evidenced particular migration patterns in SDS-PAGE conditions for these proteins. While the BSBV 19 kDa CP migrated according to its predicted MM, the BVQ 19 kDa predicted CP was found to migrate faster with an estimated MM of 16.5-17 kDa. The same migration pattern was observed when both viral CPs were expressed in *E. coli* but the underlying mechanism or proper physico-chemical properties responsible for this difference of MM are unknown. Conversely, MM higher than the predicted ones of 104 and 95

kDa were estimated for BSBV and BVQ CP-RTs, with ~115 and 107-110 kDa, respectively. Intrinsic properties like transmembrane domains (Rath *et al*., 2009) or PTMs such as phosphorylation, glycosylation or ubiquitination were proposed to potentially affect the CP-RTs MM in SDS-PAGE conditions. Similar gel-shifting being also reported for the CP-RT of the other *P. betae*-transmitted BNYVV (Ziegler-Graff *et al*., 1985; Niesbach-Klösgen *et al*., 1990), it was postulated to be potentially related to one of the CP RT domain major function, i.e. viral transmission by the protist vector. In this view, potential ubiquitination of these RT domains could be involved in viral uptake by *P. betae* following the budding mechanism of some enveloped viruses (Isaacson and Ploegh, 2009). The presence of the "late-domain like" PSAP motif at C-terminal extremity of BNYVV RT domain as well as potential ubiquitination sites in CPs RT domains of BNYVV, BSBV and BVQ support this hypothesis.

The *in vivo* approach, through the use of the first full-length cDNA clones available for BSBV and BVQ, describes pioneering results in the field of beet pomoviruses multiplication requirements and proteins expression. From now on, a new powerful tool is therefore useable to further study the BSBV and BVQ particular multiplication dynamics and, at least for BSBV, the potential pathogenicity on sugar beet and involvement in the complex rhizomania syndrome.

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APPENDIX

RNA and DNA processing

RNA extractions

Working on plant RNA viruses, extracting total RNAs from fresh infected plant material is essential to deal with the viral genome. This was performed using kits or following the classical phenol-chloroform protocol.
FastRNA® Pro Green kit (Qbiogen) or SV Total RNA Isolation System (Promega) were used for RNAs extractions from roots of *B. vulgaris* used as bait plants in infected soil mixed with sterile sand, according to the manufacturers' instructions. RNAs extracts were subsequently used for targeted sequencing of viral genes or construction of full-length cDNA clones.
To extract RNA from infected *B. macrocarpa* or *C. quinoa* leaves, the Polysome extraction method was preferred (Jupin *et al*., 1990). 100-200 mg of fresh or frozen leave is crushed in a sterile mortar with 1.5 ml of Polysome buffer (TrisHCl 200mM pH 9, KCl 400 mM, saccharose 200 mM, $MgCl_2$ 35 mM, EGTA 25 mM), on ice. 500 µl of homogenate is then mixed with the same volume of saturated phenol pH 7.9 (VWR), vortexed and centrifuged for 20 min at 13000 rpm, 4°C. Supernatant (450-500 µl) is removed, mixed with one volume of acid phenol-chloroform solution pH 4.5 (Ambion) and centrifuged again. The upper aqueous phase containing the RNAs molecules is then precipitated with two volumes of 100% ethanol and sodium acetate (150 mM final concentration) at -20°C for 1 hour or over-night (O/N) period. After centrifugation during 15 min at 13000 rpm and 4°C, the pellet is washed with 70% ethanol, dried and finally resuspended in sterile demineralized water.

RNA-DNA amplification and visualization

From a limited amount of nucleic acid sequences, the polymerase chain reaction (PCR) may generate millions of DNA copies that are useable for direct visualization or further manipulations. While DNA is immediately suitable for such amplification, RNA must be reverse transcribed first.

Reverse transcription

The reverse transcription step aims to synthesize for each RNA of interest the anti-complementary DNA strand, also called cDNA. This last is then amplifyied by polymerase chain reaction (PCR). Two reverse transcriptases were used in the present study.

Moloney murine leukemia reverse transcriptase (M-MLV RT) (Promega) was used for the common production of short fragments (up to 1-2 kb). RNA samples, mixed with 1 µl reverse primer (25 µM) and DEPC-treated H_2O (diethylpyrocarbonate, inhibits RNAses) up to 10 µl final volume, is first heated during 10 min at 65°C in a MJ Mini™ Personal Thermal Cycler or iCycler™ (Biorad) to disrupt secondary structures. The next elongation step is performed at 42°C during 1 h after the addition of 4 µl of 5x-concentrated buffer (Tris-HCl 250 mM, KCl 375 mM, $MgCl_2$ 15 mM and DTT 50mM), 2 µl dNTPs (10 mM), 0.25 µl M-MLV RT (200 U/µl) and 375 µl DEPC water.

When high quantity of longer cDNA fragments (1.5-6.0 kb) needed to be synthesized, Expand Reverse Transcriptase (Roche) was preferred. This enzyme is a modified version of the M-MLV RT, which RNAse H activity, i.e. the ability to degrade RNA, is considerably reduced. The first denaturation step is identical to that with M-MLV RT but, after adding 4µl 5x-concentrated buffer (Tris-HCl 250 mM, KCl 200 mM, $MgCl_2$ 25 mM, Tween 20 2.5%, pH 8,3), 2 µl dNTPs (10 mM), 2 µl dithiothreitol (DTT, 100 mM), 1 µl Expand RT (50 U/µl) and 1 µl DEPC-treated water, elongation is performed during 60 min at 43°C.

Polymerase chain reaction

Two types of thermostable DNA polymerases synthesizing dsDNA were used in the presented experiments.

Go Taq® Flexi DNA polymerase (Promega) was used to amplify 1-2 kb fragments that didn't require high accuracy of copying, e.g. viruses detection or clones screening. 2.5 µl of cDNA from RT step is mixed with 5 µl Go Taq® Flexi buffer 5x-concentrated, 2.5 µl $MgCl_2$ (25 mM), 0.75 µl dNTPs

(10 mM), 0.5 µl of each primer forward and reverse (25 µM), 0.12 µl of Go Taq® DNA polymerase (5 U/µl) and sterile water up to 25 µl final volume.
When 1.5-6.0 kb DNA fragments were amplified for subsequent cloning or sequencing, Expand Long Template PCR System (Roche) was preferred. Because this last includes a mix of classical Taq DNA polymerase and another proof-reading Tgo DNA polymerase, the amplification accuracy is threefold higher compared to systems using only the first enzyme. The reaction mix is however somehow different, adding 2.5 µl Buffer n°1 10x-concentrated, 0.9 µl dNTPs (10 mM), 0.75 µl of each forward and reverse primer (25 µM), and 0.4 µl polymerase mix (5 U/µl)to 2.5 µl cDNA from reverse transcription with Expand RT. Volume is adjusted with sterile water to 25 µl.
Samples are then submitted to thermal cycling as shown on figure 18.

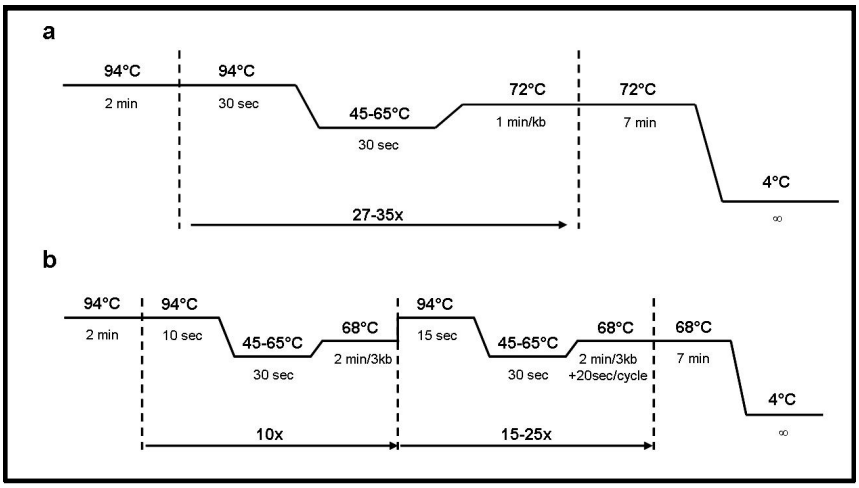

Fig. 18. (a) Go Taq Polymerase (Promega) and (b) Expand Long Template PCR System (Roche) thermal cycles for DNA amplification. 94℃ steps ensure DNA denaturation and enable primer annealing on template strands at 45°-65℃. Hybridization T° is calculated according to the specific primer nucleotide sequence. DNA synthesis is then performed at 72℃ or 68℃ depending on the polymerase used and, after completion of the elongation step, the cycle between doted lines starts again with DNA denaturation. The number of cycles is indicated above arrows and incubation times are shown underneath full lines. Elongation time is adjusted according to the length of DNA fragments to be amplified. Final incubation at 4℃ maintains DNA integrity until used.

Separation of DNA fragments on agarose gel by electrophoresis

DNA molecules, whether coming from PCR reaction or other applications (see after), may be separated according to their size. For such purpose, gels are prepared by melting Multi Purpose agarose (Roche) in 1x-concentrated Tris-Borate-EDTA buffer (TBE, Tris-borate 89 mM, boric acid 89 mM and EDTA 2 mM) to concentrations varying between 0.7 and 2.0%, depending on the fragments size. Using Biorad Power Pac 300 or Modell 1000/500 power supply (Biorad), DNA fragments are subsequently forced to migrate through the gel in TBE buffer 1x-concentrated towards the anode, as being negatively charged. Ethidium bromide (BET) (Sigma), beforehand added to the gel at 0.1 mg/ml, inserts between both strands of migrating DNA and allows their visualization under UVs. Using 1 kb or 100 bp DNA ladders (Promega), the approximate size of the observed fragments can be determined.

In vitro transcriptions

Conversely to the production of DNA from RNA by RT-PCR, RNA may also be synthesized *in vitro* from DNA sequences by transcription. This strategy is widely used to produce viral single-stranded RNAs from DNA constructions (see after). Two conditions are required to allow efficient transcription. First, a transcription promoter should be upstream the DNA sequence to be transcribed and, in the present study, the one from phage T7 (TAATACGACTCACTATAGGG) was chosen. Secondly the DNA should be linearized where transcription has to stop or the polymerase will continue transcribing. The RiboMAX^{TM} Large Scale RNA Production System – T7 (Promega) kit was used to synthesize RNAs of interest. 1-2 µg of template DNA is mixed with 4 µl transcription buffer 5x-concentrated, 1.5 µl of each of the three rATPs (100 mM), rUTPs (100 mM), rCTPs (100 mM), 0.1 µl of rGTPs (100 mM), 0.5 µl of RNAseOUT (40 U/µl) (Invitrogen), 4µl of $m^7G^{5'}ppp^{5'}$ CAP analog (New England Bioloabs) 2 µl of T7 enzyme mix and sterile water up to 19 µl final volume. After 30 minutes incubation at 37°C, 1.5 µl of rGTPs (100 mM) is added to the reaction mix that is then left for

3 h 30 min at 37°C to complete transcription. Belated addition of rGTPs in the reaction mix favors the incorporation of CAP analogs in the newly synthesized transcripts. The transcription efficiency is checked by running a 1x-TBE agarose gel with 0.5 µl of each transcription sample. However, as migration of RNAs is performed in non-denaturating conditions, the real size of RNAs molecules cannot be determined by using a DNA ladder.

Purifications of nucleic acids

DNA and RNA molecules may be purified in the view of further manipulations. Three types of purification methods were used in this work, depending on the nature of the molecules (DNA or RNA) and the aim of the purification step.

When a single-type/length of DNA had to be selected among molecules of different sizes, all fragments were separated on an agarose gel and the one of interest was selected by cutting in the gel. DNA was subsequently extracted from the piece of agarose by using affinity columns of QIAquick Gel Extraction kit (Qiagen) and eluted with sterile water.

In order to eliminate salts and chemicals present in solution with DNA from a previous reaction and make the DNA clean for further applications, QIAquick PCR Purification (Qiagen) or MSB® Spin PCRapace (Invitek) kits were used and DNA was eluted with sterile water.

If RNA needed to be purified, the phenol-chloroform method was preferred. Equal volumes of hydrophobic acid-phenol:chloroform (pH 4.5) solution (Ambion) and RNA with water are mixed and centrifuged 15 min at 13000 rpm and 4°C. The upper aqueous phase containing the RNAs molecules is then precipitated with two volumes of 100% ethanol and sodium acetate (150 mM final concentration) at -20°C during 1 h. After 15 min centrifugation at 13000 rpm and 4°C, the pellet is washed with 70% ethanol, dried and resuspended in sterile demineralized water.

RACE by RNAs circularization

While RNAs internal sequences are easily targeted by classical RT-PCR, rapid amplification of cDNAs ends (RACE) requires RNA pre-treatment. Annealing sites upstream 5' end and downstream 3' end should be provided as primers hybridization sites are necessary on both sides of the target sequence for amplification. Circularization of viral RNAs was therefore performed to determine terminal nucleotide sequences. The pomoviruses genomic RNAs being putatively 5'-capped, this last had to be remove in order to allow intramolecular ligation. Moreover, to maximize self-ligation of viral capped RNAs, preliminary dephosphorylation of non-capped RNAs was performed. The strategy is illustrated on figure 19.

4 µg of total RNA extract from roots of infected *B. vulgaris* are mixed with 1 µl of **Alkaline phosphatase from calf intestine** (CIP) (1 U/µl) (Promega), 2 µl of 10x-concentrated buffer (500 mM Tris-HCl pH 9.3, 10 mM $MgCl_2$, 1 mM ZnCl2 and 10 mM spermidine), 1 µl DTT (100 mM), 1 µl RNAseOUT (40 U/µl) (Invitrogen) and water to a final volume of 20 µl. Tubes are incubated for 30 min at 37°C. RNAs are then purified following a phenol-chloroform protocole (see Appendix I.4), resuspended in 10 µl sterile water and mixed with 1 µl **Tobacco acid pyrophosphatase** (TAP) (5 U/µl), 2.5 µl buffer 10x-concentrated (500 mM sodium acetate pH 6.0, 10 mM EDTA, 1% β-mercaptoethanol and 0.1% Triton X-100), 1 µl DTT (100mM), 1 µl RNAseOUT (40 U/µl) and water to a final volume of 25 µl. De-capping is performed at 37°C for 60 min. RNAs are purified again, resuspended in 10 µl sterile water and aliquots of 5 µl, 2 µl and 1 µl are mixed with 2 µl **T4 RNA ligase** (5 U/µl) (Epicentre), 5 µl of 10x-concentrated ligation buffer (330 mM Tris-acetate pH 7.8, 660 mM potassium acetate, 100 mM magnesium acetate and 5 mM DTT), 5 µl ATP (10 mM), 1 µl RNAseOUT (40 U/µl) and water to a final volume of 50 µl. Diluting the RNAs in a bigger reaction volume promotes intramolecular ligation rather than intermolecular linkage. Ligation reactions are left for 60 min at 37°C. After final purification, RNAs are used to amplify 5' and 3' ends of self-ligated viral RNAs.

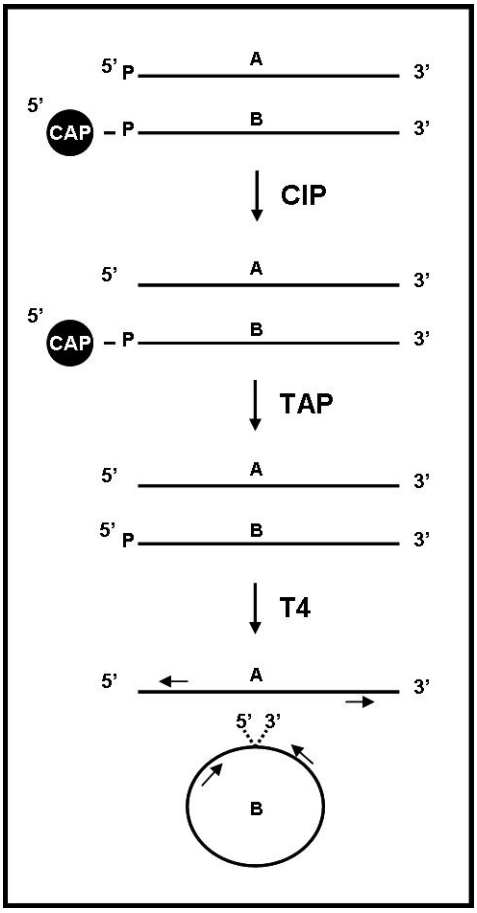

Fig. 19. Strategy of RNA circularization for amplification of cDNA ends. Successive treatments of total RNAs with alkaline phosphatase from calf intestine (CIP), tobacco acid pyrophosphatase (TAP) and T4 RNA ligase (T4) lead to the circularization of capped molecules (B). 5' phosphate groups (P), indispensable for intra-molecular ligation, is removed by CIP treatment for non-capped RNAs (A). Arrows represent primers positions used for amplification of cDNAs ends by RT-PCR.

Cloning

Bacterial plasmids

Three plasmids were used for cloning DNA fragments. **pGEM®-T** (Promega) is sold as a 3 kb linear vector with thymines overhanging at 3' extremities. These prevent the plasmid from recircularization and facilitate the insertion of PCR products with 5' adenines overhangs, added by classical polymerases with no proofreading activity. T7 and SP6 transcription promoters are also included on both sides of the insertion site that is inside a multiple cloning site (MCS) with numerous restriction sites. pGEM®-T vector is widely used for common cloning of PCR products. **pBAD-TOPO®** (Invitrogen) is also a linear plasmid of 4.1 kb, with single 3'-T overhangs linked to Topoisomerases I that facilitate the insertion of PCR products. Through a pBAD promoter upstream the insertion site, the transcription and expression of the cloned sequence are regulated by the presence L-arabinose. This vector is therefore useful for the production of proteins in bacteria. Contrary to the above-mentioned plasmids, the 2.7 kb **pUC19** should be open by means of restriction treatment (see after) in the MCS to insert the DNA sequence of interest. The simple arrangement of this vector makes it easy to work with, as it doesn't contain specific transcription promoters near the cloning site and its MCS is composed of many different restriction sites. All three types of plasmid carry an ampicilline resistance gene that makes bacteria containing the desired plasmid resistant against the antibiotic. In addition, MCS of these vectors are situated inside another gene coding for the β-galactosidase. If disrupted by insertion of a PCR product, the enzyme is not able to degrade the X-galactose anymore and bacteria appear white on Petri dishes.

Plasmid and insert processing

To obtain the desired final construct with the insert of interest in a specific vector, DNA often needed to be processed.

A-overhang

When PCR products were amplified using the Expand long template PCR system, the proof-reading activity of Tgo enzyme removed 5'-A overhangs from amplicons. For an efficient cloning of the amplified fragments in a vector with 3'-T overhang, adenines are added at 5' extremities of DNA fragments before cloning. The PCR product is therefore purified, mixed with 1 µl Go Taq polymerase (5 U/µl), 1 µl 10x-concentrated buffer, 0.5 µl dNTPs (10 mM) and 1 µl $MgCl_2$ (25 mM) in a final volume of 10 µl, and incubated at 72°C for 30 min.

Restriction

When DNA fragments were transferred from the pGEM-T vector to pUC19 or directly inserted into this last, treatment with restriction enzymes were necessary. These proteins recognize specific DNA sequences – commonly of 6 nt, but sometimes smaller or longer – and cleave the double-strand to produce two cohesive or protrusive, 3' or 5', extremities. 1 µg of template DNA to be restricted is incubated with 0.2 µl of restriction enzyme (10 U/µl) and the appropriate restriction buffer at the temperature required by the enzyme (mostly 37°C) during O/N.

Dephosphorylation

To avoid self-ligation of the restricted plasmid and favor the insertion of the DNA of interest, phosphate groups from 5'-extremities of the linearized vector may be removed. As these are necessary for the ligation of DNA strands, the fragment to be inserted brings the only phosphate groups available and insertion is the only way to get circularization. 1 µg of linearized vector to be dephosphorylated is mixed with 1 µl of alkaline phosphatase from calf intestine (20 U/µl) (Roche), 2 µl dephosphorylation buffer 10x-concentrated (0.5 M Tris-HCl, 1 mM EDTA, pH 8.5) and water to 20 µl final volume. The reaction is performed at 37°C for 60 min.

Ligation

Ligation reactions depend on the vector used. When ligating a DNA fragment into pGEM®-T or pUC19 vector, 50 ng of plasmid are mixed with 3 units of T4 DNA ligase (Promega or Roche, the appropriate ligation buffer and a length-adapted* quantity of PCR product. The reactions are incubated over-night at 4°C or at room temperature. Regarding pBAD-TOPO® cloning, PCR products ligate themselves to the vector by recovering the energy from vector-topoisomerases links. No ligase is needed for such cloning and ligation reaction is set up at room temperature during 5 to 30 min.

*To optimize cloning conditions, a one for three ratio of vector/insert as well as the length of both DNA fragments always determined the quantity of insert to add to the ligation reaction, following next formula:

$$\text{qtity of insert to clone (ng)} = \frac{\text{vector qtity (ng)} \times \text{insert length (kb)}}{\text{vector length (kb)}} \times \frac{3}{1}$$

Mutagenesis

Different kind of sequence modifications may be introduced in constructs. Among the multiple deletion, insertion and directed mutagenesis techniques, two were used in the present work.

With the view of disrupting a specific coding sequence, a shift may be introduced in the ribosomes reading frame by adding nucleotides. This technique relies on the presence of a restriction site where the mutation has to be done and that is not present elsewhere in the construct. Moreover, 5'-protruding termini should be produced after the cut as DNA elongation, and therefore gap-filling, goes always in a 5' to 3' direction (Fig. 20a). See chapter I, communication in Journal of General Virology, for the **frameshift** insertion into the pUBS-2-wt construct.

Inserting a restriction site can be performed by means of PCR and using a polymerase with proof-reading activity. Such a strategy has been used to insert new restriction sites at 5' extremities of BVQ full-length clones in pUC19 (see Chapter III, Project paper). Constructs were amplified using primers with the new restriction sequences in floating queue (Fig. 20b) and Expand Long Template PCR System. PCR products were subsequently purified and O/N treated with the enzyme cutting the newly inserted restriction site. Purified restriction products were finally self-ligated.

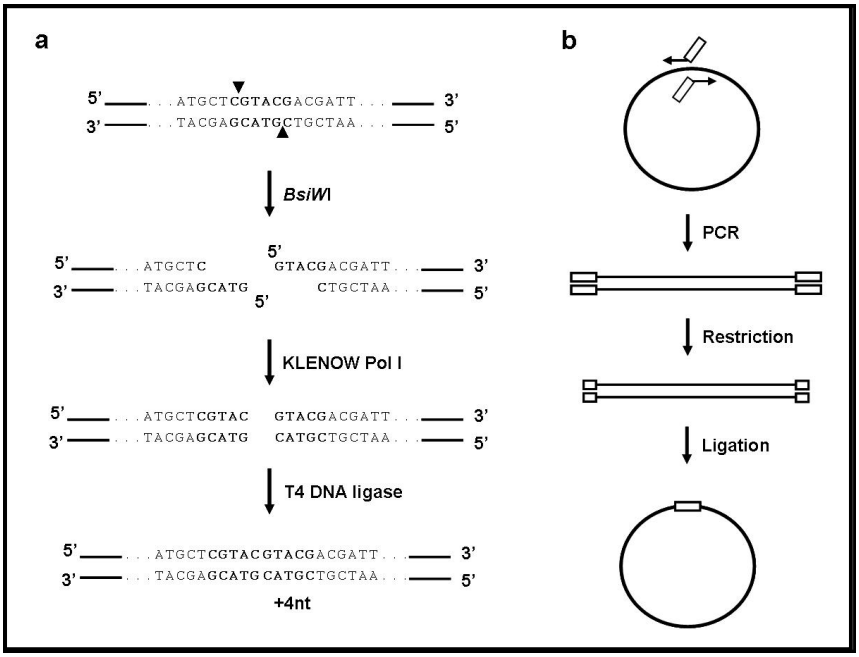

Fig. 20. Strategies for introducing (a) a *BsiW*I frameshift mutation or (b) restriction sites in constructs. (a) Restriction sequence recognized by *BsiW*I is in bold and cutting sites are indicated (▼). Gap-filling by the Klenow fragment of DNA polymerase I is followed by intra-molecular ligation using T4 DNA ligase. 4 nucleotides are added, GTAC. (b) Primers used for amplification of the construct are flanked by the restriction sequence to be inserted in floating queue (boxes). PCR products with restriction sites at both extremities are then restricted with corresponding enzyme and self-ligated with T4 DNA ligase.

Constructs multiplication in *Escherichia coli*

Processed *in vitro*, constructs have to be transferred into *in vivo* systems for both selecting the rare successful events but also multiplying them.

Escherichia coli strains

The common laboratory bacteria used multiplying constructs is the gram negative *E. coli*, an *Enterobacteriaceae* from warm-blooded animals. Two strains were used in the presented studies.
E. coli strain **DH5-α** [genotype F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1] is very popular for common cloning and was mostly used in the present work.
E. coli strain **MC1022** [genotype *ara*D139 Δ(ara leu)7697 Δ(lacZ)M15 *galU galK rpsL*] was rather used for difficult cloning, e.g. those involving toxic inserts.

Preparation and transformation of chimio-competent cells

To optimize constructs uptake by bacteria – bacterial transformation – cells may be submitted to special treatments that increase their potential of transformation, also called competence. Such prepared bacteria, when briefly chocked by means of electricity or temperature, incorporate more easily the desired constructs. Depending on the type of chock used for transformation, cells are prepared differently and distinction is made between electro- and chimio-competent cells. The *E. coli* DH5-α and MC1022 strains were prepared following a Rubidium chloride protocol (Promega), with the view of making them chimio-competent.
A fresh culture of 2.5 ml LB liquid medium inoculated with a single bacteria colony from a LB plate is first grown O/N, at 37°C and 180 rpm. Next day morning, this pre-culture is mixed with 250 ml LB liquid medium with 20 mM $MgSO_4$ in a 1 L flask. This last is placed at 37°C, 180 rpm until the absorbance of the culture at 600 nm (A_{600}) reaches 0.5 (around 150 min for *E. coli* DH5-α). The culture is then distributed in four 50 ml sterile falcons, centrifuged for 5 min at 4500 g and 4°C. Each of the four bacteria pellets is

gently resuspended in 20 ml of ice-cold TFB1 (CH_3COOK 30 mM, $CaCl_2$ 10 mM, $MnCl_2$ 50 mM, RbCl 100 mM, Glycerol solution 15%, pH 5.8, filtered through 0.2 µm acrodisc) on ice. After 5 min incubation on ice, tubes are centrifuged again in the same conditions. Pelleted bacteria are resuspended in 2 ml of ice-cold TFB2 (PIPES 10 mM, $CaCl_2$ 75 mM, RbCl 10 mM, glycerol solution 15%, pH 6.5, filtered through 0.2 µm acrodisc) on ice and the four tubes are pooled in one. After 45 min incubation on ice, the final 8 ml of prepared bacteria are distributed in 150 µl aliquots that are flash-freezed in liquid nitrogen before storage at -80°C.
For the transformation of constructs into these chimio-competent cells, up to 3 µl of the ligation reaction is gently mixed with 50 µl of chimio-competent cells thawed on ice and left for 20-30 min on ice before 45 sec incubation at 42°C. Following this heat shock, the tube is placed on ice for 2 min before adding 250 µl of SOC medium (see next point) and 1 h incubation at 37°C on a rotating wheel. Finally, bacteria are plated on LB medium with 100 µg/ml ampicillin and incubated O/N at 37°C.

Media for cultures of bacteria

Two types of media were used for growing bacteria. When plated on Petri dishes, solid **Luria-Bertani (LB)** medium (Bacto™ Trypton 10 g/l, Bacto™ Yeast Extract 5 g/l, NaCl 5 g/l, Agar 15 g/l) was used. If needed, 100 µg/ml ampicillin was added after sterilization by auto-clave. Liquid LB medium doesn't contain agar.
The enriched **SOC** medium is composed of Tryptone (20 g/l), Yeast Extract (5 g/l), NaCl (10 mM), KCl (2.5 mM), $MgCl_2$ (10 mM), $MgSO_4$ (20 mM) and Glucose (20 mM).

Sequencing

The nucleotide sequence of PCR products or cloned amplicons may be checked by sequencing. With this view, the DNA fragment to be sequenced is mixed with one primer (forward or reverse) and, following the method of Sanger, the incorporation of fluorescent chain-terminator di-deoxynucleotides during an elongation reaction by a DNA polymerase makes DNA sequences “readable”. In the presented studies, many primers were designed especially for such purposes. Tables 8 and 9 contain all primers used for sequencing of BSBV and BVQ, respectively.

Table 8. BSBV-specific sequencing primers.

BSBV f l 1.1 F	ACCAGCGATTCTTCTGAGAT
BSBV f l 1.2 F	TCCTTTGTTTGTTGTGGATG
BSBV f l 1.3 F	TAAGGAAAAGACGGTAGATG
BSBV f l 1.4 F	AGAAGCGATCCGAGGCACTA
BSBV f l 1.5 F	AAAACTGTACGCGGTTCGAC
BSBV f l 1.6 F	AGTGTACTACACACGTGCTG
BSBV lr 1.6' F	TGATAGAGAGCGTTCTGGCAC
BSBV f l 1.7 F	TGATGTTAAGCCTAAGTTGG
BSBV f l 1.8 F	TATAAGTTTGCTCCGGATCC
BSBV f l 1.1 R	ACCAACGTCCGTTCAAAGCA
BSBV lr 1.1 R	ACCAACGTCCGTTCAAAGCG
BSBV lr 1.1' R	CATCAATCACCCTACACAAAG
BSBV f l 1.2 R	CAACCGTTAACTGATTCTCC
BSBV f l 1.3 R	CGTTTATCAACACGTGCACG
BSBV f l 1.4 R	TCAGTCATCGCAGACTTATC
BSBV f l 1.5 R	GTAAACCATACCAGCATGCG
BSBV f l 1.6 R	TATCGACTCGTACTTCGACC
BSBV f l 1.7 R	ATCTCCTTCATCACAGGACC
BSBV f l 1.8 R	TGTAGTCATGATAAGAACGG
BSBV f l 2.1 F	GCTACTATACTGTCGCAGGT
BSBV f l 2.2 F	CCGAATTGACATAAAAGGCG
BSBV lr 2.2 F	CCGAATTGACATAAGAGGCG
BSBV f l 2.3 F	GTGTCGAGGAAGGTTAGCAT
BSBV lr 2.3 F	GTGTCGAGGAAAGTTAGCAA
BSBV f l 2.4 F	CCATAGAAGCTTTTGAAAGGC
BSBV lr 2.4 F	CCATAGAAGCTTTGGAAAGAC
BSBV f l 2.5 F	GATAAAGGCATCGAGTTCGG
BSBV f l 2.1 R	TTCTTATCCTGGCCTCACTC
BSBV lr 2.1 R	TTCTTATCCTGGCCTCACTT
BSBV f l 2.2 R	CCAAAACCCAGGCTAAACCA
BSBV f l 2.3 R	TATCACCTCCTCCATACTGC
BSBV f l 2.4 R	TTCGGCAAGGTGTTAGTACC
BSBV lr 2.4 R	TTCGGCAGGGTGTTGGTACC
BSBV f l 2.5 R	GTCACGTACTGTGTTATTGG
BSBV f l 3.1 F	GAAGAAGACGTTGTGGACGG
BSBV lr 3.1 F	GAGGAAGACGTTGCGGACGA
BSBV f l 3.2 F	TACGTTATTGCAGAACGGCG
BSBV lr 3.2 F	TACGTTGTTACAGAACGGTG
BSBV f l 3.3 F	GTATTGGCCAATAGTCGTGG
BSBV lr 3.3 F	GTATTGGCCAATTGTCGTAG
BSBV f l 3.4 F	AGGAACGTGCCGAGGTGAAT
BSBV lr 3.4 F	AGGAACGTGCCGAGGTGAAT
BSBV f l 3.1 R	TTGGAATCCACACCAGCTCC
BSBV f l 3.2 R	ACTGGAATCTGCAACCACTC
BSBV f l 3.3 R	TGAACACGCTTATTGCCGTC
BSBV f l 3.4 R	TAGAAACGACACTTTACCAC
BSBV lr 3.4 R	TAGGAACGACACTTAACCAC

Table 9. BVQ-specific sequencing primers.

BQ 1 s-a F	CGGAACGTATTCGAGACGAT
BQ 1 s-a(2) F	CGGCCATGAGGAAGATTGAA
BQ 1 s-a(3) F	TGACTGAAGCAACGGAAGCC
BQ 1 s-b F	TCGCAGAGATACTTCTGAGG
BQ 1 s-b(2) F	GGGTCATTTCAGTAGTGAGC
BQ 1 s-b(3) F	GATAGTGATAACGGTGGTGG
BQ 1 s-c F	GGACAGGTATGACTTCCATC
BQ 1 s-c(2) F	TTCGATGAAGCGCTAATGGC
BQ 1 s-c(3) F	GTACCTGATGTAGGTGAAGC
BQ 1 s-d F	GCTGTGTGGAACTTTGACTG
BQ 1 s-d(2) F	ACTACACATTCGTGCGGTGT
BQ 1 s-a R	ATCGTCTCGAATACGTTCCG
BQ 1 s-a(2) R	CATTGACGGTAGGTAACTCG
BQ 1 s-b R	CCTCAGAAGTATCTCTGCGA
BQ 1 s-b(2) R	CACTATCGTCTGAGTCATCG
BQ 1 s-b(3) R	TTACTTCAACGAACGCAGGG
BQ 1 s-c R	GATGGAAGTCATACCTGTCC
BQ 1 s-c(2) R	TTACACTTCTTCGGTGCCGT
BQ 1 s-c(3) R	CAACCTGGCACACCATCAAT
BQ 1 s-d R	CAGTCAAAGTTCCACACAGC
BQ 1 s-d(2) R	TTTAGTGCCTCTAGTACGCG
BQ 1 s-d(3) R	AAAGCAAACCGAATGCGCTC
BQ 2 s-a F	TGCATTGCAAAGTGTGCGAG
BQ 2 s-b F	GGTGACTTAAGTACTCGTCG
BQ 2 s-b(2) F	ATACTGACATGAAAGCCGCG
BQ 2 s-b(3) F	CGTGGTGCATACGTTAATGC
BQ 2 s-a R	CTCGCACACTTTGCAATGCA
BQ 2 s-b R	CGACGAGTACTTAAGTCACC
BQ 2 s-b(2) R	GTACATTGGGTAAGCCACAC
BQ 2 s-b(3) R	AAAGATCCATACTCCACCCC
BQ 3 s-a F	TGCGACGAATTCAGACGGAA
BQ 3 s-a(2) F	AGGTTCTGAACGGTACACAG
BQ 3 s-a(3) F	CGATCAGTTGCTTTTACCCG
BQ 3 s-a R	TTCCGTCTGAATTCGTCGCA
BQ 3 s-a(2) R	GCACAAAGAGGCGGTTGATT
BQ 3 s-b(2) F	ATCATCTCGTGGTGCATACG
BQ 3 s-b(2) R	TCAACCTCAGCACCAGACTT
BQ 3 s-b(3) R	CCAAACGGTGCTTCCTGAAA
BVQ RT rev	GCATTAACGTATGCACCACGT
BVQ RT for	CGCAGGACAAATTCGAGCTA
REV1	CGCGTCTTTAAACGCGTCAC
REV2	TTCGCGGCTTTCATGTCAGT

Detection of viral components

Three different techniques were used to detect viral RNAs or proteins, depending on what had to be evidenced.

RT-PCR

Multiplex RT-PCR, aiming to detect simultaneously *P. betae*, BNYVV, BSBV and BVQ, was developed by Meunier and co-authors in 2003 and used in the present work as a common tool to identify RNAs extracts from beet roots that contained BVQ or BSBV. However, RT-PCR proved to be too sensitive when working with full-length infectious clones and the following Northern and Western blot techniques were therefore preferred.

Northern blot

Viral RNAs were detected by Northern blot in RNAs extracts from samples infected with full-length infectious transcripts. Extractions were performed following the Polysome protocol.

Electrophoresis

Total RNAs samples are first separated according to their size on an agarose gel. Composed of TBE 1x-concentrated and 1% melted agarose, it also contains 3.8% formaldehyde that ensures denaturation of migrating RNAs. For each sample, one volume of 2-3 µg RNAs is mixed with four volumes of RNA loading buffer (HEPES 24.4 mM, EDTA 1.2 mM, KOH 20.7 mM, deionized formamide 61%, formaldehyde 7.4%, bromophenol blue 0,007%, ethidium bromide 25 µg/ml, glycerol 3.7%) before spotted in the gel's wells. Migration is performed under the hood in HEPES buffer 1x-concentrated (HEPES 20 mM, EDTA 1 mM and KOH 17 mM) at 50-80 volts depending on the gel size.

Blotting and fixation

After the gel migration, RNAs are transferred on a nylon Hybond-NX membrane (GE Healthcare) by capillarity action with a high salt SSC 20x-

concentrated solution (NaCl 3 M and Citrate triNa 0.3 M). The following day, RNAs are fixed on the membrane by exposition to 254nm-UVs, 100 000 µJoules/cm^2, in a CL-1000 UV Crosslinker (UVP). The transfer efficiency is then checked by visualization of cellular RNAs, as BET from RNA loading buffer makes them visible under UVs.

Probes synthesis and purification

Viral RNAs are detected on the nylon membrane through hybridization of specific radio-labeled probes, which sequences are anti-complementary to a limited part of the targeted RNAs. In the present work, DNA probes were used rather than RNA ones as these last proved to be much less specific. 220-750 nt long PCR products were amplified from full-length constructs of the targeted RNAs as templates for probes synthesis. 30 ng of purified PCR fragments in 20 µl sterile water are first denatured at 100°C for 2 min, put on ice and mixed with 12 µl H_2O, 10 µl Labeling buffer 5x-concentrated (Promega), 2 µl Bovine serum albumine (BSA) (10 mg/ml), 2 µl d(AGT)TPs PCR Grade (500 µM each) (Roche) and 1 µl DNA Polymerase Klenow (5 U/µl) (Promega). 25-30 µCi [α^{32}-P]dCTP (3000Ci/mmol) are added to each reaction tube for the probes radioactive labeling. Left for 60-90 min at room temperature, reactions are stopped by heating at 100°C for 2 min and then directly chilled on ice. Finally, probes are purified on Illustra MicroSpin™ G-25 Columns (GE Healthcare).

Hybridization, washing and exposition

Membranes with cross-linked RNAs are placed in tubes with PerfectHyb™ Plus Hybridization Buffer (Sigma) inside a Hybridization Oven/Shaker (Amersham). They are first incubated 1 h at 50°C for a pre-hybridization step, so that RNA-free sites of the membranes become saturated by the buffer. Once probes have been added in the tubes, these are left for O/N hybridization at 50°C. On the following day, buffer from the tubes is collected (probes may be used several times) and membranes are washed at 65°C for three periods of 15 min with first 2x SSC - 0.1% sodium dodecylsulfate (SDS) buffer and then 0.2x SSC - 0.1% SDS buffer. Dried

membranes are left with an Amersham Hyperfilm ECL (GE Healthcare) for 20 min to one week exposure periods, depending on the radioactivity emitted by radiolabeled RNAs. Films are revealed manually.

Western blot

The same blotting strategy was applied for the detection of specific viral proteins by means of Western blot.

Samples preparation

Vegetal tissue to be analyzed for the presence of viral proteins are directly crushed in sample buffer (Tris-HCl 62.5 mM pH 6.8, glycerol 25%, SDS 2%, bromophenol blue 0.01%, β-mercapatoethanol 5%). Leaves or single lesions are crushed with 2-3ml or 50 µl of sample buffer per piece, respectively. Homogenate is then centrifuged for 10-15 min at 13000 rpm and 4°C to pellet remnants of cellular structures. Part of the supernatant is mixed with an equal volume of sample buffer and boiled or heated for 3 min at 95°C to denature proteins.

SDS-PAGE and blotting

Following the example of DNAs and RNAs fragments on agarose gels, proteins are separated according to their MM on SDS-polyacrylamide gels. However, these are composed of two superimposed gels with distinct functions. The proteins separation occurs in the lower resolving gel, which polyacrylamide concentration is adapted to the MM of the proteins to be separated. Bigger proteins require low concentration of polyacrylamide whereas high-concentrated gels are used for smaller ones. The upper stacking gel is prepared with 4 or 5% polyacrylamide and is crucial for the proteins concentration before entering in the resolving gel. Gels are casted in Mini-PROTEAN® 3 Cell or Mini-PROTEAN Tetra Electrophoresis System (Biorad) and immersed under running buffer (Tris Base 25 mM, glycine 192 mM, SDS 0.1%). Samples prepared in sample buffer and Precidion Plus Protein™ Standards Dual Color (BioRad) or PageRuler™ Plus Prestained Protein ladder (Fermentas) are then spotted in the gel's wells and migration

is performed at 80 V until samples reach the resolving gel and then 150 V to the end.
After migration, the gel is placed against a nitrocellulose membrane (Biorad) and both are sandwiched between Whatman paper sheets and two foam pads. This assembly is locked in a holder cassette, which is inserted in a Mini Trans-blot Cell (Biorad) and immersed with transfer buffer (Tris-Base 25 mM, glycine 192 mM, methanol 20%). Blotting is performed at 30 V, room temperature, O/N.

Immuno-detection and revelation

On the following day, the blotted membrane is incubated 1 h in TBS-Tween-milk blocking buffer (Tris-Base 50 mM, NaCl 40 mM, 0.1% Tween 20 and 5% skimmed milk) so that protein-free sites become saturated by proteins of the milk. Membranes are then left with primary rabbit polyclonal antibody (PAb) specific to the BSBV or BNYVV (DSMZ) and anti-rabbit alkaline phosphatase-conjugated secondary IgG (Sigma-Aldrich), successively. Incubations are performed in 20 ml TBS-Tween-milk buffer with 1:10 000 diluted Ab at room temperature for 2 h shaking at 100 rpm for the first and for 2 h at 37°C for the second. Washed 3 times for 15 min with TBS-Tween buffer (Tris-Base 50 mM, NaCl 40 mM, 0.1% Tween 20) after each of these two last treatments, membrane is finally incubated with 7.5-10 ml of premixed solution of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (SIGMA). The dimerization reaction of BCIP initiated by the alkaline phosphatase and favored by NBT leads to the formation of indigo precipitate were secondary Ab is fixed on primary Ab. Revelation reaction is left for at least 30-45 min before washing with demineralized water.