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Popular Science

Sugar beet has been cultivated for sugar production since the beginning of 19th century. It is an important source of sugar for Europe and USA, since climate in these regions in general is more appropriate for beet sugar than cane sugar production. However, sugar production is limited by the pathogens associated with the crop, being viruses of particular importance. In this thesis, I focused on the study of the most important viruses infecting sugar beet: *Beet necrotic yellow vein virus* (BNYVV), *Beet soil-borne mosaic virus* (BSBMV), *Beet soil-borne virus* (BSBV) and *Beet virus Q* (BVQ). To do so, sugar beet plants were grown in soils infested with the viral vector *Polymyxa betae* from different locations: Sweden, France, Germany, the Netherlands and USA. Among the previously mentioned viruses, more attention was given to BNYVV because of its direct association with rhizomania disease, which can severely damage the cultivation of sugar beet and reduce greatly the yield. Additionally, BSBMV was also of particular interest, due to its similar genome organization with BNYVV and its exclusive presence in the USA. The results of this project verified once more that BNYVV is spread around Europe. It was possible to identify the high diversity of the virus types and that BSBMV is found only in the USA. Interestingly, novel RNA species associated with BSBMV infection were discovered in this project. We showed that these RNA species accumulate at levels similar or higher to the accumulation of BSBMV genome components. However, their biological role is still unclear.

Abstract

Beet necrotic yellow vein virus (BNYVV, *Benyvirus*) is the causal agent of rhizomania disease. BNYVV and *Beet soil-borne mosaic virus* (BSBMV, *Benyvirus*) share high similarity in their genome organisation. These viruses as well as *Beet virus Q* (BVQ, *Pomovirus*) and *Beet soil-borne virus* (BSBV, *Pomovirus*) are vectored by the plasmodiophorid *Polymyxa betae*. BNYVV P25 protein has been reported to be associated with the development of rhizomania disease, thus being a virulence factor of the virus. Recently, resistance breaking isolates of BNYVV have been reported in the Netherlands and the United Kingdom. Additionally, BSBMV has been observed in USA in the fields where BNYVV is not present. In order to understand the dynamics of the viruses associated with rhizomania in the crop, sugar beet plants were baited with soils containing *P. betae* from USA, the Netherlands, Germany, France and Sweden. BNYVV, BSBMV, BVQ and BSBV were detected by RT-PCR from total RNA isolated from root tissue of the plants. *P. betae* was detected in samples from all the soils, as well as BSBV. BNYVV was detected in all the samples except for root samples growing in soils from USA, which only showed presence of BSBMV. BVQ was only detected in samples from German, Dutch and Swedish soils. Amino acid sequences of the 'tetrads' of the BNYVV P25 protein from isolates from Germany, Sweden, the Netherlands, and France were found to have the following amino acid sequences: AYHR, AYHR, AYPR and SYHG, respectively. These differences in the tetrad sequence of P25 might be associated with the resistance breaking events by the BNYVV isolates from the Dutch soil, which need further study.

Amplifications of full-length RNA 2 of BSBMV revealed the presence of additional RNA species. These species appeared to represent chimeric RNAs characterized by the presence of complete RNA 2 ORF for the coat protein in the 5’-terminus fused to the 3'-untranslated region of either RNA 3 or RNA 4. The presence and accumulation of chimeric RNAs were confirmed by qRT-PCR. Notably, the levels of chimeric RNAs were up to 20-fold higher as compared to accumulation of BSBMV RNA 2. The influence of the chimeric RNAs on the expression of viral symptoms and accumulation of BSBMV remains to be studied.

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1. Introduction

1.1. Sugarbeet

Sugar beet (*Beta vulgaris* subsp. *vulgaris*) is considered to be a species of high economic importance within the Amarathanceace family and the order Caryophyllales. It belongs to the same subspecies *vulgaris* as many other cultivated plants such as leaf beet, garden beet or red beet and fodder beet (Monteiro *et al.*, 2013).

The sugar beet crop covers 20% of the global sucrose production (Finkenstadt, 2014), with sugar cane (*Saccarum officinalis*, Poaceae) being responsible for the other 80% (FAO, 2009). Particularly, sugar cane was the only source of sugar for over three thousand years, until the appearance of sugar derived from sugar beet around 1750 (FAO, 2009). Sugar beet is not only a source for sucrose production, but is also utilised for biofuels, animal feed, human nutrition, pharmaceutical products and plastic. The two main by-products that are derived from the sugar extraction process are pulp and molasses (Dohm *et al.*, 2014; Finkenstadt, 2014).

The sugar beet root is white and conical, while the crown is flat with numerous leaves. Sugar is concentrated in the root of the plant during the process of photosynthesis and its content can vary from 12 to 20 % depending on the variety.

In 1747 Andreas Sigismund Marggraf, a German chemist, demonstrated that extracts from sugar beet can be crystallized into the already known sugar derived from sugar cane. Almost 50 years later, Franz Karl Achard, student of Marggraf, built the first sugar beet factory (Winner, 1993; reviewed in Lennefors, 2006). Due to political reasons, the Napoleonic war and the continuous demand for sucrose worldwide, the cultivation of sugar beet increased in continental Europe and the new sugar industry was established (Cooke & Scott, 1993). In the beginning of the 20th century, sugar beet was introduced to the United States of America and since then it has been spread and cultivated around the world (Alamzan *et al.*, 1998).

The sugar beet crop is categorised as biennial; which means that the cultivation may last for two years. The first year, plants are cultivated for production of sucrose from the tap root, while the second year, they are destined for seed production after the flowering season. When the crop is cultivated only for sucrose, plants are sown around spring and harvested in autumn/ winter (FAO, 1999). The cultivation of sugar beets is preferably done in fertile soil rich in humus in a climate with temperatures between 15 to 21^{o}C and dry periods before the harvest. Harvesting is done mechanically in order to reduce the harvesting time and keep the sugar content of the beets as high as possible. Yield can vary from 30 to 70 tons/ha, depending on different factors such as

seed quality and variety, cultivation and harvesting techniques as well as weather conditions (FAO, 2009).

Some of the most damaging pathogens of sugar beets are fungi and fungi-like organisms such as *Cercospora beticola*, *Rhizoctonia solani*, *Aphanomyces cochlioides*, *Erysiphe betae*, *Ramularia beticola*, *Fusarium spp.* and *Peronospora farinosa*, nematodes as *Heterodera schacchtii* and *Meloidogyne spp.* and viruses as *Beet necrotic yellow vein virus* (BNYVV) and *Beet curly top virus* (BCTV) (Lennefors, 2006).

Breeding of the crop has been focused on increasing yield, improving sucrose production and increasing the taproot weight. During the last 200 years, there has been an increase of 10% in the sugar content (from 8% to 18%). Moreover, breeding has been also used for development of varieties resistant to viral and fungal diseases (Dohm *et al*., 2014). In the case of BNYVV, causal agent of rhizomania disease, the resistance of sugar beet cultivars is based on the *Rz1* resistance gene of the "Holly" source. Another cultivar of sugar beet partially resistant to rhizomania disease is "Rizor". These two cultivars have been the most widespread cultivars used to control rhizomania disease for the last 30 years worldwide (Biancardi *et al.*, 2002).

1.2. Viruses

Sugar beet is the host of different soil-borne viruses such as BNYVV, *Beet soil-borne mosaic virus* (BSBMV), *Beet virus Q* (BVQ) and *Beet soil-borne virus* (BSBV), which all are vectored by *Polymyxa betae* (Tamada, 1975; Ivanović *et al.*, 1983; Abe & Tamada, 1986; Wisler *et al.*, 1994; Stas *et al.*, 2001). All four viruses have rod shaped virions. BNYVV and BSBMV belong to the genus *Benyvirus* while BSBV and BVQ are members of the genus *Pomovirus* (Koenig, 2008; Torrance, 2008). The virus grouped in the species BNYVV is responsible for rhizomania, which is considered as one of the most economically devastating diseases and can damage the whole cultivation of sugar beet (Giunchedi *et al.*, 1982).

1.2.1. *Beet necrotic yellow vein virus* (BNYVV)

The causative agent of rhizomania disease, BNYVV was reported in European countries, USA, Japan and China around 1970 (Tamada & Baba, 1973). Rhizomania is considered as the most destructive sugar beet disease worldwide. The word rhizomania literally means root madness from the Greek words 'rhiza' (root) and 'mania' (madness). The disease is characterized by root proliferation and the formation of secondary non-functional roots (Koenig, 2008) (Fig. 1B). The shape of the tap root is narrowed and its growth is reduced producing stunting (Fig. 1C). There is a change in the colouration of the vascular system to brown (Fig.1B). In the leaves of susceptible varieties, it is possible to observe vein clearing. Leaves can wilt in dry conditions because of the non-functional root system (Fig.1C). As a result of these symptoms, the sugar content in the root

is significantly reduced; some susceptible varieties may end up with 50% less sugar content (Koenig, 2008). Infection of BNYVV can create patches in the field; the patches follow the movement of the farm machinery and indicate the parts of the soil that are infested (Fig.1A). The virus can also spread mechanically to other alternative hosts such as species within the families Amaranthaceae, Caryophyllaceae and some species of family Aizoaceae (Koenig, 2008). Experimentally, the model plants that are used to establish systemic infection of the virus are *Nicotiana benthamiana* and *Beta macrocarpa* (Koenig, 2008).

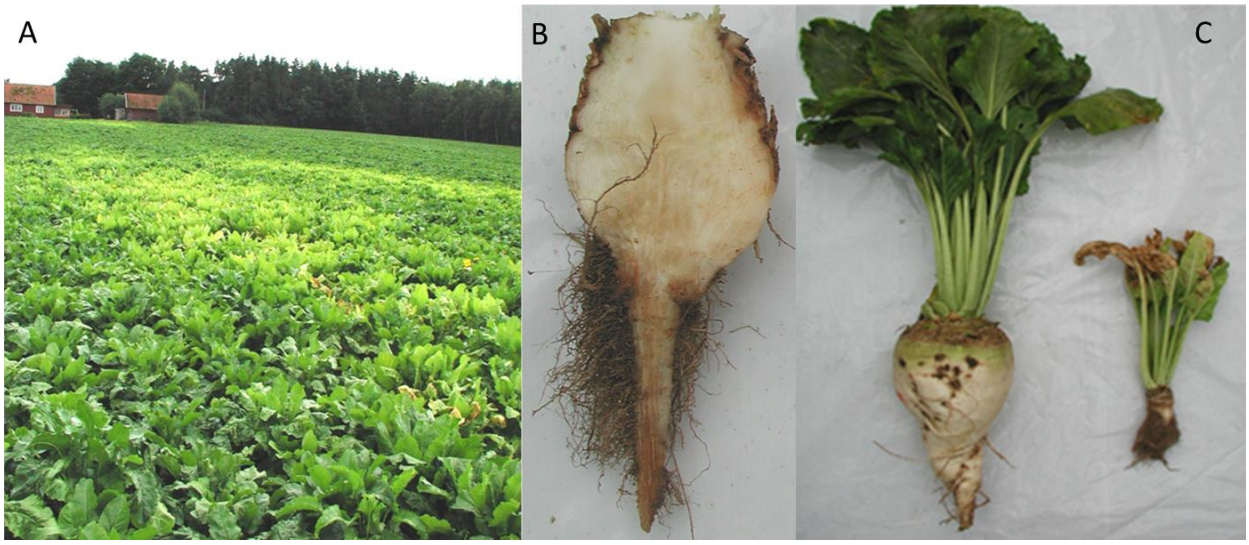

Figure 1. A. Infected sugar beet crop with yellow patches in the field. B. Infected main taproot of sugar beet with formation of secondary roots. C. Sugar beet plants cultivated in rhizomania affected field, rhizomania-resistant sugar beet (left) and susceptible variety (right) (by courtesy of Britt-Louise Lennefors).

BNYVV has a genome consisting of four to five positive sense single-stranded (ss) RNA components. They possess a cap structure at the 5’ end and a poly A-tail at the 3' terminus (Fig. 2). The viral genomic components are separately encapsidated by the same coat protein of the virus (Pferdmenges, 2007; Putz, 1977).

RNA 1 is 6746 nucleotide residues in length without counting the poly A-tail. This RNA molecule has one long open reading frame (ORF) that encodes a protein of 237 kDa (P237) with domains for methyltransferase, helicase, protease and RNA-dependent RNA polymerase (RdRp). This RNA segment encodes the proteins responsible for replication of the virus (Bouzoubaa *et al.*, 1987; Pferdmenges, 2007). The primary product of the translation of RNA 1 can be cleaved by a papain-like proteinase into two proteins, P150 and P66, with the second one carrying the polymerase domain (Hehn *et al*., 1997) (Fig. 2).

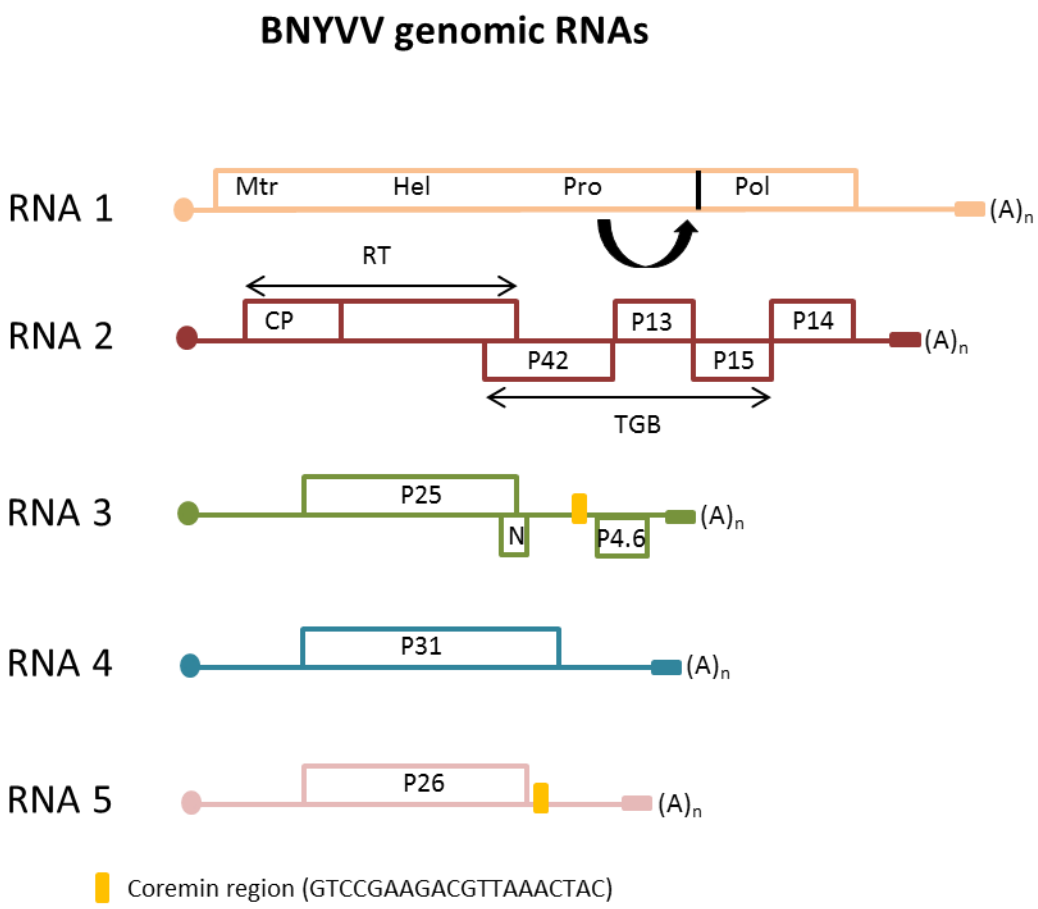

Figure 2. BNYVV genomic components. All RNAs have a cap structure in the 5' end and a poly A-tail at the 3' end. Boxes represent the ORFs of the genome. (Mtr: Methyltransferase, Hel: Helicase, Pro: Protease, Pol: Polymerase, RT: Read Through, CP: Coat Protein, TGB: Triple Gene Block)

RNA 2 is 4612 nucleotide residues long, excluding the poly A-tail and contains six ORFs (Bouzoubaa *et al*., 1986). The first ORF encodes the viral coat protein (CP) P21. The P75 is a read-through (RT) protein which is expressed by overcoming the termination codon of P21 and it is responsible for virion assembly and vector transmission (Ziegler *et al.*, 1985). The following ORFs encode triple gene block (TGB) of movement proteins (P42, P13 and P15). The TGB proteins interact with each other to facilitate the cell-to-cell movement of viral RNAs (Gilmer *et al*., 1992). More specifically, P42 can bind the viral RNAs (Bleykasten *et al.*, 1996) and with the help of the docking site that P13 and P15 create, alter the permeability of plasmodesmata in order to move the viral RNAs from cell-to-cell (Lauber *et al*., 1998). The last ORF of RNA 2 encodes P14, which is a suppressor of RNA silencing, an important host defence mechanism against viruses (Dunoyer *et al*., 2002) (Fig. 2).

RNA 3 has a length of 1775 nucleotide residues, excluding the poly A-tail and contains 3 ORFs, which encode the proteins P25, N and P4.6 (Fig. 2). P25 is involved in symptom expression on roots of sugar beet and in the leaves of experimental plants such as *Chenopodium quinoa* and *Tetragonia expansa* (Tamada *et al*., 1989; Jupin *et al*., 1992; reviewed by Pferdmenges, 2007). P25 is considered to be the most important protein associated with symptom severity.

Mutations or deletions in the P25 ORF can affect the intensity of the symptoms induced by the virus on leaves. According to Jupin *et al.* (1992), the symptoms on leaves of *C. quinoa* and *T. expansa* after mechanical inoculation of mutant viruses with mutated or deleted P25 ORF were milder compared to the wild type. The severity of the symptoms is also affected by the cellular localisation of P25 in the infected cells. Immuno-gold electron microscopy has shown that P25 can be localised both in the nucleus and in the cytoplasm of infected cells (Haeberlé & Stussi-Garaud, 1995). When the protein was able to access both the nucleus and the cytoplasm, the severity of the symptoms increased in inoculated *C. quinoa* leaves (Vetter *et al*., 2004).

P25 is found to be highly variable between amino acid residues 67-70, with residue 68 showing the highest variability. The protein is also variable at amino acid position 133. These variations affect the pathogenicity of different isolates and allow the discrimination of different "types" of isolates (Schirmer, 2005; Rush *et al*., 2006).

In RNA 3, there are two additional short ORFs. ORF N overlaps with the 3' terminus of P25 and it is translated only when a part of the nucleotide sequence is deleted, resulting in strong necrosis on the leaves of experimental plants. The last ORF in this genomic component encodes the P4.6 peptide that has not been associated with symptom expression in experimental plants (Jupin *et al*., 1991).

RNA 4 is 1431 nucleotide residues long and codes for the P31 protein (Bouzoubaa *et al.*, 1985) (Fig. 2). The protein is important for transmission of the virus by its vector *P. betae* and also acts as a suppressor of RNA silencing (Tamada & Abe, 1989; Rahim *et al*., 2007).

Three different types of BNYVV (A, B and P) have been described in Europe, and among the isolates sequenced so far, the nucleotide sequences of the viral components differ from 1 to 4 % between the types. Interestingly, the P type is characterized by having an additional RNA genomic component (RNA 5) (Koenig *et al.*, 2008). Isolates of BNYVV are sequenced for the P25 gene in order to analyse the different tetrads of the P25 protein sequence in the different types of the virus. The CP sequence is also important for differentiating or categorizing virus types. In Europe, the A type is spread in the southern countries, but also in the north-west in the Netherlands, while the B type of the virus has been found mostly in Germany and France. The P type has been identified only in Pithiviers, a region of northern France, in Kazakhstan and a few

fields in the UK (Tamada *et al*., 1989; Koenig *et al*., 1997b; Koenig & Lennefors, 2000; reviewed in Lennefors 2006).

As mentioned above, the fifth RNA of BNYVV has been found only in a few isolates from Asia, France and the UK (Tamada *et al*., 1989; Koenig *et al*., 1997b; reviewed in Lennefors 2006). RNA 5 encodes the P26 protein and it has been shown that isolates containing the fifth RNA can induce more severe symptoms in comparison to isolates without it (Koenig *et al*., 1997b) (Fig. 2).

1.2.2. *Beet soil-borne mosaic virus* (BSBMV)

BSBMV belongs to the genus *Benyvirus* and has a genome organisation similar to that of BNYVV, having four ssRNA genomic segments of positive polarity (Fig. 3). RNA 1 is 6683 nucleotide residues long and has a single ORF that encodes a protein of 239 kDa with the domain organisation similar to that of BNYVV P237. BSBMV RNA 1 and BNYVV RNA 1 share 77% nucleotide identity. BSBMV RNA 2 is 4615 nucleotide residues in total and shares 67% nucleotide identity with BNYVV RNA 2. BSBMV RNA 2 contains six ORFs similar to BNYVV RNA 2; they share 56% identity at the average amino acid level. BSMV RNA 3 is 1720 nucleotide residues in length, sharing 60% nucleotide identity with BNYVV RNA 3. The P29 protein encoded by the first ORF of RNA 3 shares 23% identity with the P25 protein of BNYVV. The last RNA component, RNA 4, has a length of 1730 nucleotides and shares 35% identity with BNYVV RNA 3 (Lee *et al*., 2001; D'alonzo *et al.*, 2012).

Symptoms induced by the virus can differ from those induced by BNYVV. Sugar beet plants infected with BSBMV may show no symptoms in the root or symptoms similar to rhizomania disease (Koenig, 2008). BSBMV symptoms on leaves can be variable from yellow stripes to pale spots. Generally, in comparison to BNYVV, BSBMV is less harmful to sugar beet plants. Experimentally, the virus can be transmitted mechanically to *C. quinoa*, *C. album* and *T. tetragonioides* resulting in a local infection. On the other hand, plants of *B. maritima* can be infected systemically (Koenig, 2008).

BSBMV has been found in the central and western regions of the USA and it has not been reported in other countries. Initially, BSBMV was hypothesized to be a milder strain of BNYVV, but even though the two viruses are morphologically similar, their coat proteins are serologically distinct (Lee *et al*., 2001). Sugar beet cultivars with tolerance or certain level of resistance to BNYVV can be infected by BSBMV. Thus, these viruses can be discriminated by different sugar beet varieties (Lee *et al*., 2001).

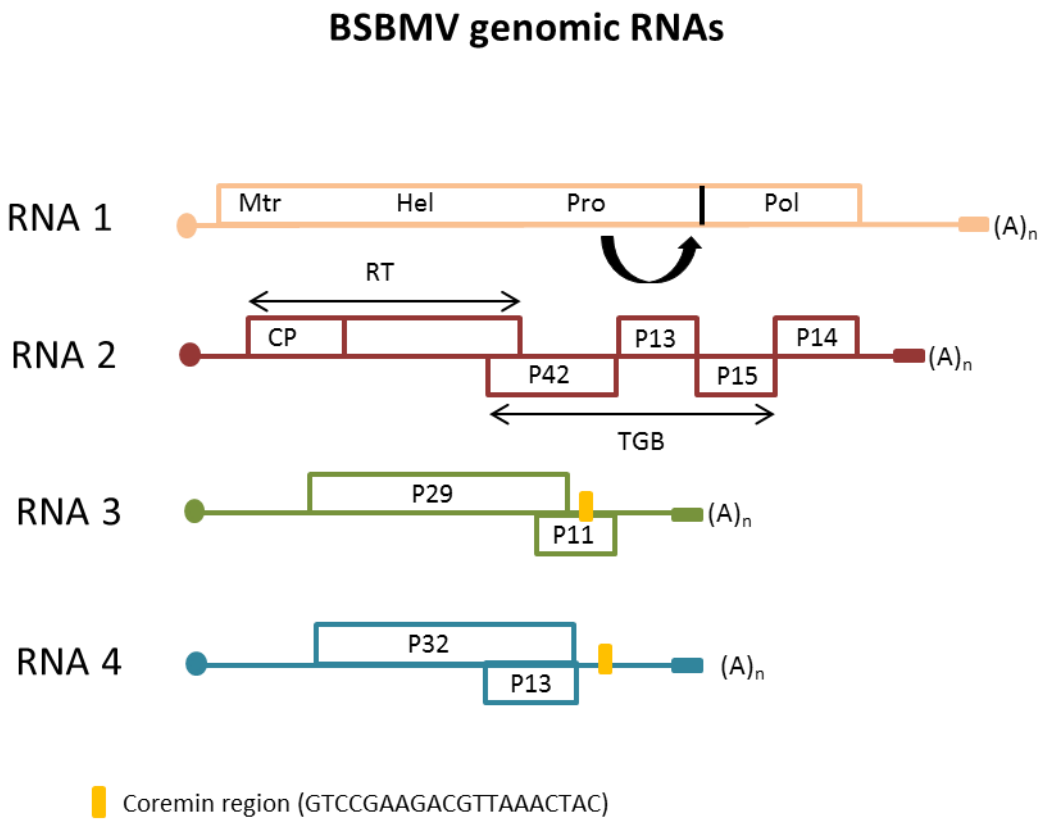

Figure 3. BSBMV genomic components. All RNAs have a cap structure in the 5' end and a poly A-tail in the 3' end. Boxes represent the ORFs. (Mtr: Methyltransferase, Hel: Helicase, Pro: Protease, Pol: Polymerase, RT: Read Through, CP: Coat Protein, TGB: Triple Gene Block)

1.2.3. *Beet soil-borne virus* (BSBV)

BSBV belongs to the genus *Pomovirus*. The virus is widespread in many sugar beet-growing countries around the world. BSBV shares many properties with BNYVV such as particle shape, hosts and vector (Koenig *et al*., 1996). The BSBV genome consists of three ssRNAs of positive polarity. The first RNA encodes a protein of 204 kDa that contains domains of methyltransferase, helicase and RdRp (Koenig and Loss, 1997). RNA 2 encodes a read-through protein of 104 kDa and the CP of the virus (Koenig *et al*., 1997a). The third RNA encodes a TGB (Koenig *et al*., 1996). BSBV has highly variable sequences among different isolates. Even isolates from the same field show a high polymorphism. Symptoms induced by the virus can lead to yield losses up to 70%, depending on the virus isolate and the sugar beet variety (Koenig *et al*., 2000).

1.2.4. *Beet virus Q* (BVQ)

BVQ is also member of the genus *Pomovirus* and it has a genome organization similar to BSBV (Koenig *et al*., 1998). It has been reported throughout Europe, in countries such as Bulgaria, Belgium, France, Germany, Hungary, Italy and the Netherlands. In all the cases, BVQ was not reported as single infections, but always together with BSBV or BNYVV (Meunier *et al*., 2003).

1.3. *Polymyxa betae*

P. betae belongs to the family of *Plasmodiophorida* and it is a soil-borne protist (Cercozoa; Cavalier-Smith & Chao, 2003). It is a parasite of the roots of plants in the Amaranthanceae family and it causes little damage to the plants when it does not carry the viruses (Ciafardini, 1991). The severity of the symptoms caused by *P. betae* will depend on the specific isolate, regardless if it is virulent or not. Some of the isolates cause reduction in the size of the sugar beet root (Gerik & Duffus, 1988; Ciafardini, 1991; Lennefors, 2006).

The life cycle of *P. betae* starts when cystosori, thick-walled resting spores, are released into the soil after the deterioration of infected plant roots. The cystosori can survive for many years and germinate under specific conditions of high soil moisture and in the presence of a susceptible plant (Fig. 4A). Cystosori release primary zoospores that come in contact with the epidermal cells of rootlets and penetrate them. Within two hours, zoospores have released their content into plant host cells, resulting in infection. At this stage, multiplication of the zoospores takes place inside the cell of the host. After cell penetration, zoospores develop a multinuclear plasmodium. This plasmodium will later form a zoosporangium, which will give origin to secondary zoospores or, it can instead give origin to a sporogenic plasmodium, which will be involved in the formation of new cystosori (Rush, 2003; Lennefors, 2006) (Fig. 4B).

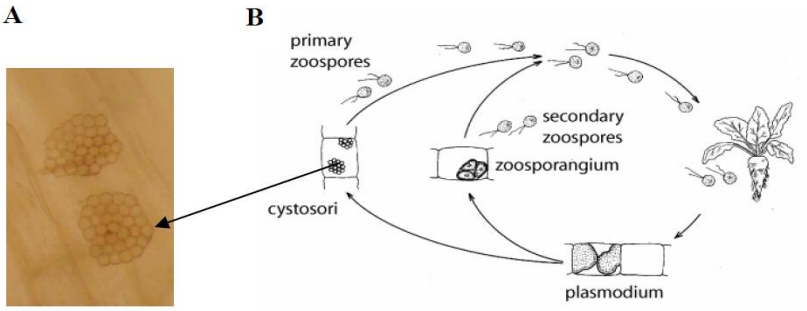

Figure 4. A. Resting spores of *P. betae* in sugar beet roots. **B.** Life cycle of *P. betae* (by courtesy of Britt-Louise Lennefors).

2. Aim and hypothesis of this study

The objective of this study was to enhance our knowledge about BNYVV and BSBMV.
More specifically:

- To determine BNYVV P25 sequences from isolates of different geographic origin.
- To develop a protocol for transient expression of viral virulence factors in leaves of sugar beet.
- To characterize a new isolate of BSBMV at the molecular level.

We hypothesize that:

- The BNYVV amino acid sequence of the P25 protein differs between isolates from various geographic locations.
- Enhanced virulence of new US strain of BSBMV might be associated with differences in the genome organization/content of BSBMV or/and presence of additional virulence factors/RNA species.

3. Materials and methods

3.1. Plant material

Sugar beet (*Beta vulgaris*) plants were grown in a greenhouse at the department of Plant Biology, Swedish University of Agricultural Sciences (SLU), Ultuna for overexpression studies. The growing conditions were 22^{o}C day temperature and 20^{o}C during night with 16 hours daylight, the humidity was fluctuating between 40 to 60%. Seeds of sugar beet were supplied by Syngenta Seeds AB, and sown in inert substrate of light peat, black peat, perlite, sand and lime. One week old plantlets were transferred to pots of 0.5 L, having one plant per pot.

3.2. Virus sources

Total RNA was isolated from baited plants growing in soils from sugar beet growing regions infested with *P. betae* carrying BNYVV, BSBMV, BSBV and BVQ. The soils were taken from Sweden, the Netherlands, Germany, France (Pithiviers) and United States of America (USA). There were also control samples used as a negative indicator. The cultivation of sugar beet plants and the isolation of RNA took place in the facilities of Syngenta Company in Landskrona, Sweden.

3.3. RNA extraction and Reverse transcription-Polymerase chain reaction (RT-PCR)

RNA extraction was performed using the Spectrum Plant Total RNA Kit (Sigma Aldrich), according to the manufacturer's instructions.

Depending on the purpose, RT-PCRs were performed differently. For virus detection, RT-PCR was carried out in two steps. RT was done using 1x reaction buffer RT, 2.5 μM of reverse primer, 0.5 mM dNTPs, 1 U RiboLock RNase Inhibitor and 5 U of RevertAid Reverse Transcriptase (Thermo Scientific) to a total volume of 20 μL. The reaction was primed using a specific reverse primer for each virus in the case of pomoviruses and oligo (dT) for benyviruses. PCR was done in a total volume of 50 μL, using 1x of buffer DreamTaq Green buffer including 2 mM of $MgCl_2$, 0.6 μM of each primer, 0.2 mM of dNTPs and 0.05 U of DreamTaq DNA polymerase (Thermo Scientific). Forward and reverse primers for the RT and PCR are listed in Supplementary Table 1. The concentrations given are the final.

For cloning genes of interest, and/or viral genomic components, RT-PCR was performed also in two steps. For the RT reaction, SuperScript III Reverse Transcriptase (Invitrogen) was used at final concentration of 10 U, supplemented with 1x First Strand Buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1 mM DTT), 2.5 μM of specific reverse primer and 0.5 mM dNTPs to a final volume of 20 μL. PCR was done using 0.02 U Phusion High-Fidelity DNA

Polymerase (Thermo Scientific), 1x Phusion HF Buffer (1.5 mM $MgCl_2$, 0.2 mM of dNTPs) and 0.6 µM of each primer to a final volume of 50 µL. Forward and reverse primers used for cloning are listed in Supplementary Tables 2, 3 and 5. In case of cloning RNAs of BSBMV the reverse primer used was oligo (dT). The concentrations given are the final.

In cases of cloning long amplicons, such as RNA 1 of BSBMV, the Q5 High-Fidelity DNA Polymerase (New England BioLabs Inc.) was used. The reaction was performed in a final volume of 50 µL with 0.02 U of the polymerase, 1x Q5 Reaction Buffer, 0.2 mM of dNTPs and 0.6 µM of each primer (Supplementary Table 3). The concentrations given are the final.

For every PCR, a blank (reaction without template) was included to verify the absence of contamination.

All RT-PCRs were verified in 1% agarose gels stained with ethidium bromide and visualized in a UV Transilluminator (Syngene).

When needed, PCR products were purified from gel using the GeneJet Gel Extraction Kit (Thermo Scientific) following manufacturer's instructions.

3.4. Real time PCR (qPCR)

Reverse transcription for qPCR was performed using 1 µg of total RNA from each sample. IScript cDNA synthesis kit (BIO-RAD) was used according to manufacturer's instructions. DyNAmo Flash SYBR Green qPCR kit (Thermo Scientific) was used to perform qPCR, according to manufacturers' instructions. Primer sequences for this experiment are available in Supplementary Table 4. Relative qPCR was used to compare the accumulation of viral RNA between the different RNAs in the samples, while absolute qPCR was used to estimate the accumulation of viral RNA in the samples. The values of relative qPCR were normalized based on RNA 1 of BSBMV using the ΔCt method. The RNA 1 was selected because its accumulation verifies the existence of BSBMV in the sample. For absolute qPCR, a standard curve for each target was prepared using ten-fold serial dilutions of plasmids, from 100 pg/µl to 1 fg/µl. The number of copies of total RNA was estimated by the following formula: number of copies= (ng * 6.0221 * 10^{23})/ (length * 660 * 1* 10^{9}) (Olmos *et al.*, 2005). In all the cases, there were three technical replications and the statistical analysis of the data was done in Excel Microsoft Office.

3.5. Cloning

PCR fragments for RNA 1, RNA 2, RNA 3 and RNA 4 of BSBMV were cloned using CloneJet PCR Cloning Kit (Thermo Scientific) using the Blunt-End Cloning Protocol, according to manufacturer's instructions.

Gateway cloning was performed to produce the constructs for *A. tumefaciens* transformation and infiltration in *B. vulgaris* plants. BNYVV P25 gene from different isolates (Germany, the Netherlands, France and Sweden) were cloned into the binary vectors pGWB17 and pGWB18 in order to have detectable proteins with Myc-tag for further analysis. Gateway BP Clonase II Enzyme mix and Gateway LR Clonase II Enzyme mix (Invitrogen) were used according to manufacturer's instructions. The Gateway pDONR/Zeo Vector (Invitrogen) was utilized as donor vector for the BP reaction.

Chimeric RNAs were cloned into pCB vector (Supplementary Table 6) using the In-Fusion HD Cloning Kit (Clontech), according to manufacturer's instructions. This was done in order to use the plasmids of chimeric RNAs as a standard for the absolute qPCR.

3.6. Transformation of *Escherichia coli* strains

In order to obtain plasmids with desired insert, transformation of *Escherichia coli* competent cells with the ligation reactions was performed. *E. coli* strain used was NEB 10-beta (New England BioLabs Inc.). NEB 10 was used to clone fragments destined for sequencing and Gateway cloning. The procedure of transformation was performed according to manufacturer instructions for the competent cells. After transformation the cells were plated on LB agar with the appropriate antibiotics for each vector (Supplementary Table 6).

3.7. *Agrobacterium* transformation and infiltration

In order to study the transient expression of the virulence factor of BNYVV, *P25* gene, in plants, plasmids carrying the construct of interest were transferred into *Agrobacterium tumefaciens* cells (Supplementary Table 7). After the transformation, *Agrobacterium* cells were plated on LB plates with antibiotics according to the resistance of plasmid and specific *Agrobacterium* strain that had been used and incubated for 48 hours at 28^{o}C.

Agrobacterium infiltration was performed in *B. vulgaris*. The infiltration mixture was prepared in two steps. First, an *Agrobacterium* culture containing 10 mM MES, 20 μM acetosyringone, the appropriate antibiotics for each strain and LB liquid was set overnight at 28^{o}C. Second, the bacterial culture was precipitated by centrifugation for 10 minutes at 3500 rpm and re-suspended with induction buffer (10 mM $MgCl_2$, 10 mM MES and 300 μM acetosyringone). O.D. of the culture was adjusted to 0.5, followed by infiltration using a syringe without a needle into the backside of the leaf area of *B. vulgaris*. *Agrobacterium* constructs expressing green fluorescence protein (GFP) were used as a control of transformation.

3.8. Sequencing

Sequencing was carried out at Macrogen, the Netherlands, using either universal sequencing primers or specific primers depending on the target sample. Capillary sequencing was requested in all the cases. The results from the sequencing were analyzed by DNAstar Lasergene 12 Core Suite program or CLC sequence viewer 7 (Qiagen Aarhus A/S).

4. Results

4.1. Virus detection in soil samples

After RNA extraction and reverse transcription, PCR was performed to detect the presence of BNYVV, BSBMV, BSBV and BVQ as well as their vector *P. betae*. The PCRs were performed on the different RNA samples, which were extracted from roots of susceptible sugar beet varieties growing in soil collected from infested fields in Sweden, the Netherlands, Germany, France (Pithiviers) and USA.

PCR detection of BNYVV revealed that the virus was present in samples from the Netherlands, Sweden, France (Pithiviers) and Germany, but not in the USA. On the other hand, BSBMV was present only in soil samples from the USA (Fig. 5), but not in other samples analyzed. PCR detection of BSBV showed that the virus was present in every soil sample and also in the control sample that was supposed to be virus free. This indicated that even the negative control soil (sand sample) was contaminated with *P. betae*. PCR for the detection of *P. betae* showed also the presence of vector in every soil sample. BVQ was present in soils from the Netherlands, Sweden and Germany (Fig. 5).

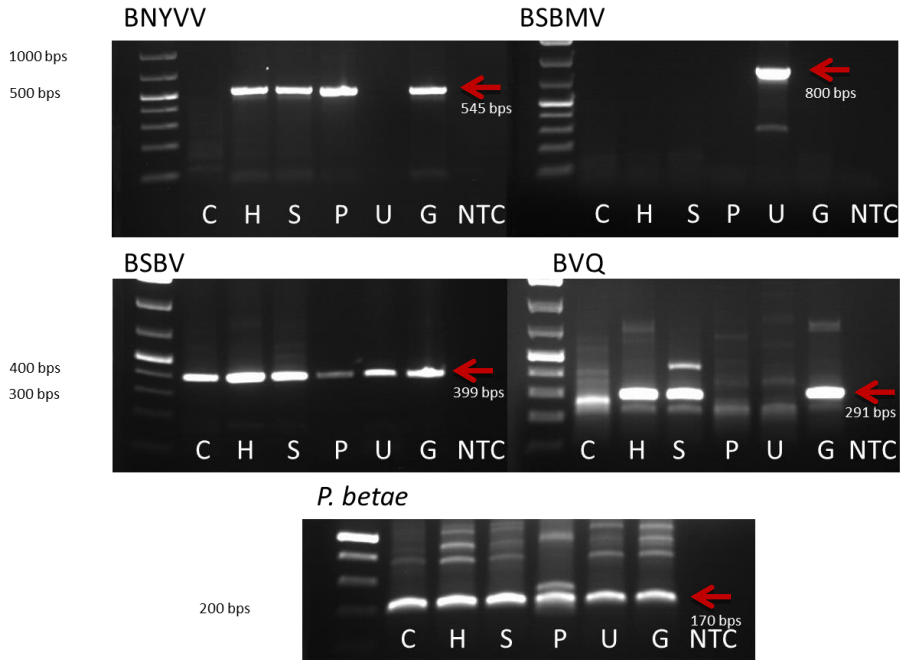

Figure 5. Gel electrophoresis (1% agarose) for virus detection in different countries, C: negative control, H: the Netherlands, S: Sweden, P: France (Pithiviers), U: USA, G: Germany and NTC: no template control. Name of the test is indicated at the upper left corner of each picture. Red arrows indicate fragments of the expected size.

4.2. Sequencing of BNYVV gene encoding the P25 protein

Samples containing BNYVV were selected for cloning and sequencing in order to determine the amino acid sequence of the P25 protein, the virulence factor of the virus. More specifically, the *P25* gene from isolates from the Netherlands, Sweden, France (Pithiviers) and Germany was sequenced to determine the amino acid variability of the encoded protein. The results showed that P25 sequences at position 67- 70 varied between the different isolates. In the Dutch isolate the specific tetrad of the P25 protein was "AYPR", in the French isolate it was "SYHG", while in the Swedish and German isolates the amino acid tetrad was "AYHR" (Fig. 6).

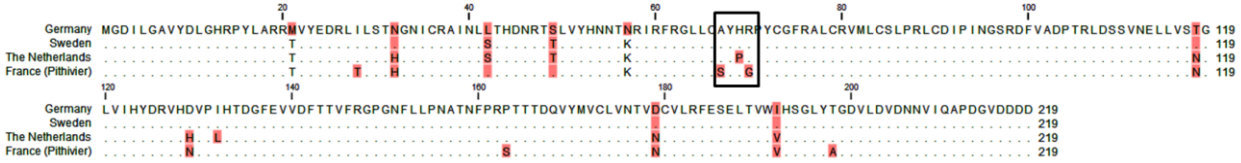

Figure 6. Amino acid sequence of the P25 protein of BNYVV for four European isolates from Germany, Sweden, the Netherlands and France (Pithiviers) that were used in the present project. The box depicts position 67-70 of the amino acid sequence. The differences in the amino acid sequence between the different isolates are labelled in red.

4.3. Transient expression of BNYVV P25 protein in sugar beet

The *P25* binary constructs (in plasmids pGWB17 and pGWB18) were infiltrated into the leaves of sugar beets to test transient expression of the P25 protein. However, *Agrobacterium*-mediated transformation of sugar beet has been reported to be of very low efficiency, making it difficult to estimate protein expression in the tissue. GFP was used as a reporter for transient expression, infiltrated in a sugar beet plant selected as control. Even though the leaves could take up the infiltration media, no expression of the fluorescence protein was detected when exposed to UV light (Fig. 7). As a result the transformation was considered unsuccessful and there was no expression and no symptoms of the P25 of BNYVV. In order to improve the technique, three different strains of *Agrobacterium* were used (Supplementary Table 7) and the concentration of the acetosyringone were doubled, but, again, the result was negative – no GFP fluorescence observed.

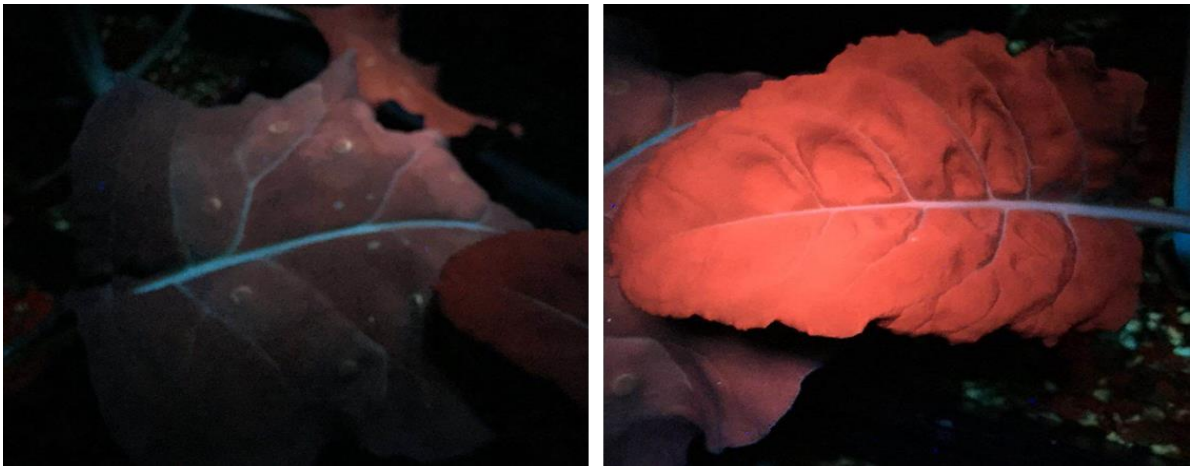

Figure 7. Agrobacterium-mediated transient expression in sugar beet leaves. Left, leaf of sugar beet infiltrated with GFP construct, but without any visible expression of GFP fluorescence. Right, un-infiltrated leaf of sugar beet for comparison.

4.4. Molecular characterization of BSBMV

Amplification of the complete genome components of BSBMV was the main purpose of this project. Thus, RNA 1, RNA 2, RNA 3 and RNA 4 were amplified to engineer infectious clones of the different viral components for downstream experiments.

The amplification of RNA 2 resulted in the expected product of 4.6 kb. Additionally, two extra bands of lower molecular weight were also observed in all the cases. The bigger one has a length of 2 kb and the smaller of 1.4 kb (Fig. 8). These two amplification products were cloned and sequenced. Analysis of the sequences showed that these two additional amplification products appeared to be chimeric RNA species.

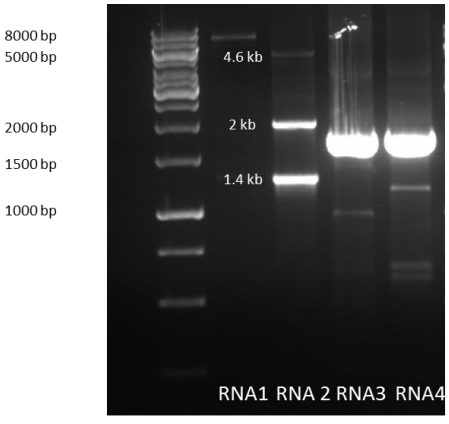

Figure 8. Gel electrophoresis (1% agarose). Amplification of RNAs of BSBMV from a USA sample. RNA 2 as well as the two additional amplification products are shown along with their molecular weight.

More specifically, the chR2-R3 (1.2 kb) represented 5' untranslated region and the CP coding region of RNA 2 fused with the 3' untranslated region of RNA 3 (Fig. 9). The sequence of chR2-R4 (1.2 kb) had the same part of RNA 2 as chR2-R3 but it was fused with the 3' untranslated region of RNA 4. Ch5R2-R4 (2 kb) was a chimera consisting of the 5' untranslated region of RNA 2 and a small fragment of the CP coding region merged with the complete RNA 4. All of the above described chimeric RNA species contained a 'coremin' sequence, which was characterized by the sequence GTCCGAAGACGTTAAACTAC (Fig. 9).

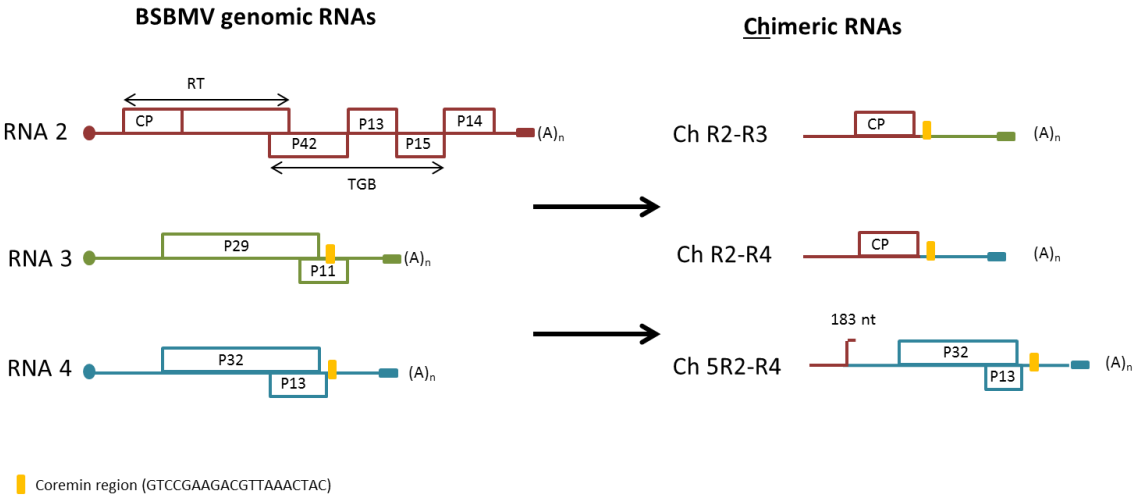

Figure 9. BSBMV chimeric RNAs. On the left side, genomic components of BSBMV (RNA 2, RNA 3 and RNA 4) are shown. On the right side, chimeric RNAs are shown in a graphical representation. All RNAs have a poly A-tail in the 3' end. Boxes represent the ORFs of the genome. (RT: Read Through, CP: Coat Protein, TGB: Triple Gene Block)

4.5. Accumulation of the chimeric RNAs of BSBMV

To confirm the natural existence of the chimeric RNAs, relative and quantitative real time PCRs were conducted to estimate the levels of chimeric RNA and compare them to the accumulation of BSBMV genome components. Experiments of relative qPCR were conducted to test the accumulation of the chimeric RNAs as compared to the RNA 2 accumulation. Six different root samples baited with soil from USA containing the virus were selected and qPCR was performed. All of the samples, but one showed greater accumulation of the chimeric RNAs as compared to RNA 2 (Fig. 10). From the graph it can be observed that chimerics R2-R3 and R2-R4 had higher accumulation in the samples U4, U5, U6, U7 and U8. In sample U3, however, there was a lower accumulation of chimeric RNAs in comparison with RNA 2 (Fig. 10).

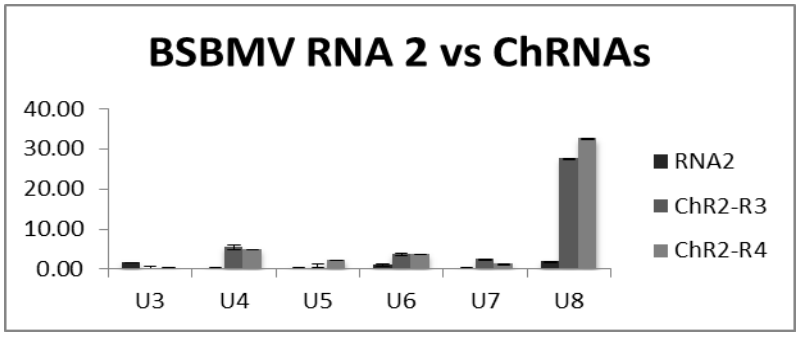

Figure 10. Relative levels of accumulation of chimeric R2-R3 and R2-R4 in comparison with RNA 2 of BSBMV in six root samples. Bars represent standard deviation of technical replications.

The results of the absolute qPCR showed that the estimated copy number of the chimeric RNAs as compared to the viral components was higher. More specifically, in case of sample U1, the accumulation of two types of chimeric RNAs, chR2-R3 and chR2-R4, were 2- and 10-fold higher than of RNA 2, respectively (Fig. 11). When compared to RNA 4, chR2-R4 was found to be accumulated to a level of 20-fold higher. In the case of ch5R2-R4, the genomic components of the virus RNA 2 and RNA 4 resulted in higher accumulation (11 and 5 times, respectively, compared to ch5R2-R4). Accumulation of chR2-R3 was found to be 4-fold higher than BSBMV RNA 3 (Fig. 11).

In case of sample U2, the accumulation of the viral components of BSBMV was in most cases higher than the chimeric RNAs. The accumulation of RNA 2 was 28 times; 4 times and 3 times higher compared to chR2-R4, chR2-R3 and ch5R2-R4, respectively (Fig. 11). The level of RNA 4 was compared with those of chR2-R4 and ch5R2-R4 and RNA 4 resulted in 10 and 3 times, respectively, higher accumulation compared to the chimeric RNAs. In comparison to the level of RNA 3, chR2-R3 had 2 times higher accumulation (Fig. 11). Despite the greater number of copies of the viral components in comparison with the chimeric RNAs in the specific sample, their accumulation remained high for a product of natural recombination.

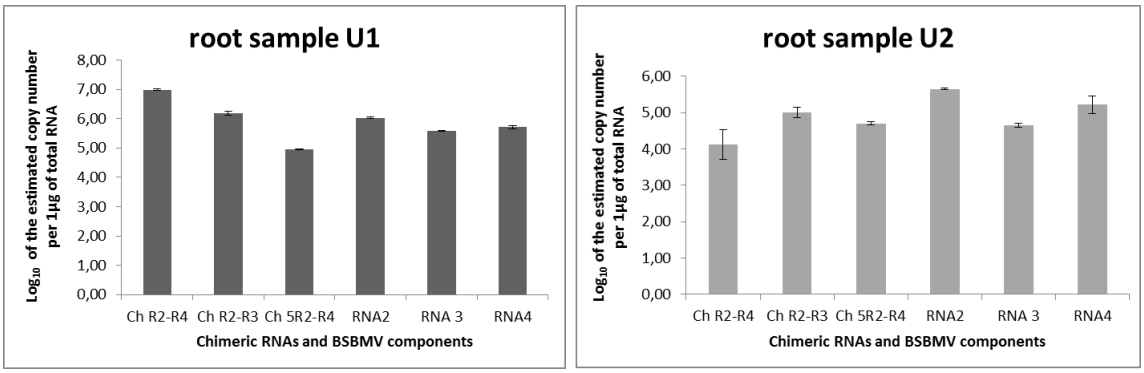

Figure 11. Estimated copy number per 1 μg of total RNA expressed in $\log_{10}$ of BSBMV chimeric RNAs and the genomic components of BSBMV. Bars represent standard deviation of technical replications.

4.6. Defective RNAs of BSBMV

In addition to the chimeric RNAs, amplification of RNA 2 resulted also in the identification of a defective RNA from RNA 2. The defective RNA was composed of the 5' untranslated region of RNA 2 and the coding region of the CP gene as well as a coding part of the P75 protein gene. The 3' end of the defective RNA consisted of a part of the coding region of the last ORF of the TGB, the whole ORF for the P14 protein and the 3' untranslated region of RNA 2 (Fig. 12).

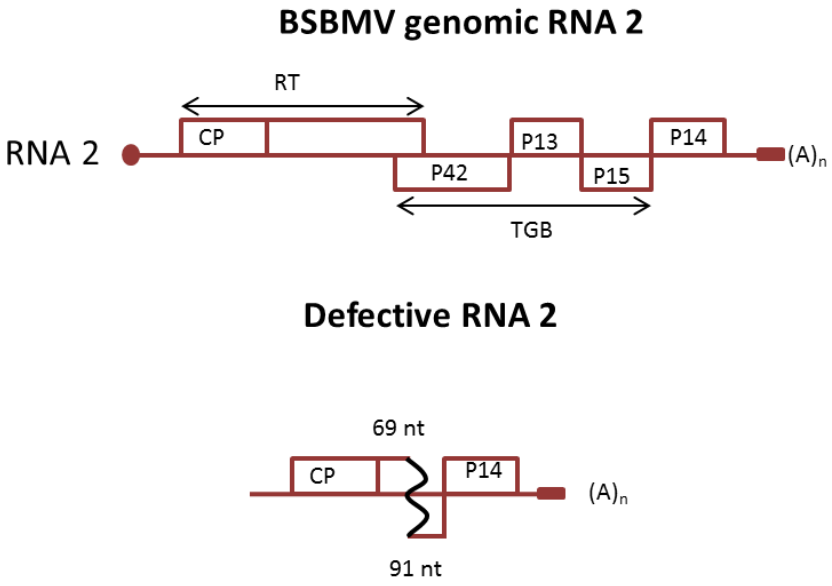

Figure 12. Defective RNA 2 of BSBMV. Organization of the complete RNA 2 (upper) is depicted in comparison with that of the defective RNA 2 (below). All RNAs have a poly A-tail in the 3' end. Boxes represent the ORFs of the genome. (RT: Read Through, CP: Coat Protein, TGB: Triple Gene Block)

5. Discussion

The present study focused initially on the detection of BNYVV, BSBMV, BSBV, BVQ and their vector *P. betae* in the root samples of susceptible sugar beets varieties grown in soils from different countries. The results showed that BNYVV was present in the soils from the Netherlands, Sweden, France (Pithiviers) and Germany, but not in soil from USA, whereas BSBMV was detected in the US soil. These results are consistent with previous reports, which suggest that the presence of BSBMV is restricted to the USA (D'Alonzo *et al.*, 2012). BSBMV and BNYVV appear to have a complex relationship; previous studies have shown that RNA 3 of BSBMV can be amplified and encapsidated by RNA 1 and 2 of BNYVV, but, the presence of BNYVV RNA3 weakens the replication of BSBMV RNA 3 due to competition (Ratti *et al.*, 2009). Additionally, D'Alonzo *et al.* (2012) reported the ability of BSBMV RNA 4 to be replicated by RNA 1 and 2 of BNYVV, to induce symptoms in *C. quinoa* leaves and to replace BNYVV RNA 4 in cases of virus transmission by the vector to sugar beet plants.

When the two pomoviruses BSBV and BVQ were detected in the roots of susceptible sugar beet grown in soils infested with the vector *P. betae*, it was found that BSBV was present in every sample from Europe and the United States, while BVQ was detected in samples from the Netherlands, Sweden and Germany. Meunier *et al.* (2003) stated that BVQ had never been detected alone in a sample; it is always being accompanied by BSBV or BNYVV. This is consistent with what is shown in this report. The presence of BSBV in negative control samples indicates that the soil had been contaminated with *P. betae*, as was proven by the detection of the vector in all the samples. However, there was no amplification of the benyviruses or BVQ from these samples. Reliability of the PCRs was confirmed based on the no template control, which showed no amplification.

Analysis of the BNYVV P25 protein at amino acid positions 67-70 showed that isolates from the Netherlands contained the "AYPR" tetrad of the A type of BNYVV that is associated with breaking down resistance based on the *Rz1* gene (Bornemann *et al.*, 2015). In the French isolate from Pithiviers the tetrad of P25 was identified as "SYHG" (P type), which is considered to be associated with the most aggressive type of the virus. The high virulence of this type derives from the additional RNA 5 and the P26 protein encoded by this genomic component (Koenig *et al.*, 1997b). Swedish and German isolates had the same tetrad of P25: "AYHR". Koenig *et al.* (2008) characterized this tetrad in German isolates as a 'standard' P25 B type detected also in many countries around Germany, such as Austria, France, Czech Republic and Switzerland. According to Lennefors (2006) and Lennefors *et al.* (2000), in Sweden both A and B types of BNYVV are present, and the virus has been introduced twice from other countries, probably from plant material contaminated with the vector *P. betae*. The Swedish isolate with the ''AYHR'' tetrad identified in this project belonged to the A type (Britt-Louise Lennefors,

personal communication). The fact that resistance breaking strains of BNYVV have been observed lately creates the need for more resistant varieties of sugar beet. Lennefors *et al.* (2006) described a transgenic approach to develop sugar beet plants resistant to BNYVV, based on the use of an inverted repeat of the BNYVV replicase gene.

Inoculation of sugar beet plants with gene constructs using *A. tumefaciens* resulted in no expression of the GFP reporter gene, and there was no expression of BNYVV P25 either. Transformation of sugar beet leaves with *A. tumefaciens* is of low efficiency. In the present work, different strains of *A. tumefaciens* and two concentration of acetosyringone were tested, but there was no GFP fluorescence achieved indicating lack of transformation. Pferdmerges (2007) reported successful transformation of sugar beet using *A. tumefaciens.* Although we followed the same protocol in this study, we failed to transform sugar beet leaves by agroinfiltration.

Recombination of viral RNA takes place when the RdRp changes its template during the process of RNA replication. The result of this mistake can lead to homologous, aberrant homologous or non-homologous recombination (Lai, 1992). The new recombinants can help the virus to overcome resistance of its host and sometimes even to create a new strain or new viruses (Cheng *et al.*, 2006). The existence of chimeric RNAs of BSBMV also has been described by D'Alonzo *et al.* (2012). They described BSBMV chimera of RNA 3 fused with RNA 4 after 21 passages in experimental plants. It was also stated as the first natural recombinant of the virus ever reported. In the present project, three new chimeric RNAs were identified directly from infected roots of sugar beet. They can be considered as another example of natural recombination of the viral components, since their existence was verified and confirmed by qPCR. However, more experiments should be done in sugar beet plants and experimental plants to gain more knowledge about the role of these novel RNA species in virus infection.

Every type of chimeric RNA that had been detected in the present work contained a specific sequence of nucleotides known as a coremin sequence. This sequence has been associated with long distance movement of BNYVV in *B. macrocarpa* (Lauber *et al.*, 1999). However, D'Alonzo *et al.* (2012) mentioned that the coremin sequence of their chimeric RNA 3-4 had no effect in long distance movement. In experiments in *B. macrocarpa*, the chimeric RNA 3-4 and RNA 4 could not complement the absence of RNA 3 for long distance movement of the virus.

Along with the detection of chimeric RNAs, the existence of BSBMV defective RNA was also confirmed in this study. According to Simon *et al.* (2004), defective RNAs can minimize the symptoms induced by a helper virus or in some cases as *Turnip crinkle virus* (TCV), they can enhance the aggressiveness of the virus. The defective RNAs can also help the plant to suppress virus accumulation, as in the case of *Potato mop-top virus* (PMTV) (Lukhovitskaya *et al.*, 2013). However, since the biological role of the defective RNAs is not fully understood, more

experiments are required to enhance our knowledge about them and specifically, about the one found in this project.

6. Conclusion

In this project, the presence of the viruses BNYVV, BSBMV, BSBV and BVQ was verified in soils from five countries containing resting spores of their vector *P. betae*. BNYVV was detected in the European soils while BSBMV was detected only in soils from USA. BSBV was present in all the soil samples and BVQ was detected in soils from the Netherlands, Sweden and Germany. The P25 amino acid sequence of BNYVV was analyzed and the results indicated that the Dutch isolate has a resistance-breaking genotype, P type from France (Pithiviers), the common B type of the German isolate and the A type from Sweden. Molecular characterization of BSBMV resulted in identification of chimeric RNAs of the virus and a defective RNA. The chimeric RNAs are the result of natural recombination as was verified by qPCR, but their role in the pathogenicity of the virus is still under investigation. The hypotheses raised in this project were addressed. The conducted analysis confirmed that the amino acid sequence of P25 was different depending on the virus isolate; novel RNA species (chimeric RNAs) were shown to be associated with BSBMV infection.

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9. Supplementary Material

Supplementary Table 1. Sequences of forward (Fw) and reverse (Rv) primers for the detection of sugar beet viruses and their vector *P. betae* (Meunier *et al.*, 2003).

Primers for virus and vector detection	
BNYVV Fw	ACA TTT CTA TCC TCC TCC AC
BNYVV Rv	ACC CCA ACA AAC TCT CTA AC
BSBMV Fw	TTT CTG TTG TCG TTG GTA TTT AAC GTC
BSBMV Rv	TAG ATG TTT AAC GTC AAG AGA CGA AAA ATT
BSBV Fw	CTT ACG CTG TTC ACT TTT ATG CC
BSBV Rv	GTC CGC ACT CTT TTC AAC TGT TC
BVQ Fw	GCT GGA GTA TAT CAC CGA TGA C
BVQ Rv	AAA ATC TCG GAT AGC ATC CAA C
PB Fw	CAA ACG CCT GAA ATC ATC TAA C
PB Rv	GAT GGC CCA ATT CCT TAC AC

Supplementary Table 2. Sequences of forward (Fw) and reverse (Rv) primers for Gateway cloning of BNYVV P25 protein gene.

Primers for P25 Gateway cloning	
BNYVV P25 Fw	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT GAC CAT GGG TGA TAT ATT AGG CGC A
BNYVV P25 Rv no stop codon	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA ATC ATC ATC ATC AAC ACC GTC A
BNYVV P25 Rv	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CTA ATC ATC ATC ATC AAC ACC GTC

Supplementary Table 3. Sequences of forward (Fw) primers for amplification of BSBMV genomic components for cloning purposes.

Primers for BSBMV RNAs	
BSBMV RNA 1 Fw	TTT TTA ATT AAT ACG ACT CAC TAT AGG AAA TTC GAT CTT TCC CAC CCA
BSBMV RNA 2 Fw	TTT TTA ATT AAT ACG ACT CAC TAT AGG AAA TTC TAA TTA TTA TCT CCA TTG AAT AGA
BSBMV RNA 3 Fw	TTT TTA ATT AAT ACG ACT CAC TAT AGG AAA TTT AAA TCT ATC ACC ACA TTA
BSBMV RNA 4 Fw	TTT TTA ATT AAT ACG ACT CAC TAT AGG AAA TTC AAA ACT CAA AAA TAT AAT T

Supplementary Table 4. Sequences of forward (Fw) and reverse (Rv) primers for qPCR reactions for the specific detection of chimeric RNAs and BSBMV genomic components.

Primers for qPCR	
BSBMV RNA 1 Fw	CCC CTT GAC CGG AAT CAC AA
BSBMV RNA 1 Rv	CCA TGA GTA CTG TTC CGG GG
BSBMV RNA 2 Fw	GAG AGC TTG AGG TTG AGC GT
BSBMV RNA 2 Rev	AGC TAA AGC ACC AGC GAG TT
BSBMV RNA 3 Fw	TGC TTG TGT GAC CGA TTG GA
BSBMV RNA 3 Rv	CGA GGA CCG TCG TCT AAA CC
BSBMV RNA 4 Fw	GTG TTT CGC GTG TTG GTC AG
BSBMV RNA 4 Rv	ACA TCC AGG GAT CAC TTG CC
BSBMV ChR2-R3 Fw	TAG GGG TGT TAC TCC TGC CG
BSBMV ChR2-R3 Rv	TTT ACG TCT TCG GAC CCA CA
BSBMV ChR2-R4 Fw	CGT TCC ACG CGC CAA TTA
BSBMV ChR2-R4 Rv	TGC GAG GAA CAC ACC AAT CG
BSBMV Ch5R2-R4 Fw	TGC TTA CAA TAT GTC TGA TGA AGG T
BSBMV Ch5R2-R4 Rv	TCA GAC TCG AGT TGT TAA TCG GA

Supplementary Table 5. Sequences of forward (Fw) and reverse (Rv) primers for In-fusion cloning of chimeric RNAs.

Primers for In- fusion cloning	
BSBMV Ch R2-R3/R2-R4 Fw	TTT CAT TTG GAG AGG GAA ATT CTA ATT ATT ATC TCC ATT GAA TAG AAT TTC AC
BSBMV Ch R2-R3 Rv	ATG CCA TGC CGA CCC CTT CAA TAT ACT GAA GGT ACA CCC TAC AAG
BSBMV Ch R2-R4 Rv	ATG CCA TGC CGA CCC CAA TAA ACT GAA AAT AAA CCC TAC AAG GAC
BSBMV Ch 5R2-R4 Fw	TGC TTA CAA TAT GTC TGA TGA AGG T
BSBMV Ch 5R2-R4 Rv	TCA GAC TCG AGT TGT TAA TCG GA

Supplementary Table 6. Information about vectors used for cloning and cloning purpose.

Cloning vectors		
Vector	**Cloning Purpose**	**Resistance**
pJET	Genomic components BSBMV	Ampicillin
pUC 19	Genomic components BSBMV and Chimerics	Ampicillin
pCB or pDIVA	Genomic components BSBMV and Chimerics	Kanamycin
pDONR/Zeo	P25 protein BNYVV	Zeocin
pGWB 17/18	P25 protein BNYVV	Kanamycin

Supplementary Table 7. *Agrobacterium tumefaciens* strains used for infiltration of P25 of BNYVV.

***Agrobacterium tumefaciens* strains**	
Strain	**Resistance**
EHA 105	Rifampicin
C58C1	Rifampicin, Carbenicillin
GV3101	Rifampicin, Gentamycin

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