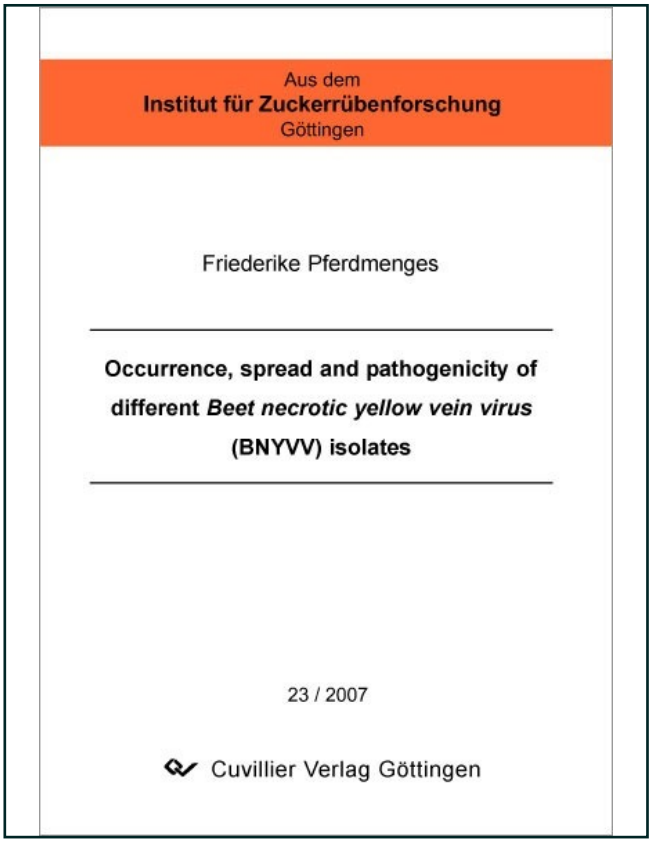
Occurrence, spread and pathogenicity of different Beet necrotic yellow vein virus (BNYVV) isolates



1. GENERAL INTRODUCTION

1.1. Summary

Rhizomania represents also in the future a risk to the world-wide sugar beet production. Although at present the disease can be successfully controlled with natural derived resistances, the virus itself possesses the potential to extremely reduce yield and sugar content. BNYVV can be divided in four major subgroups by means of sequence divergence (A, B, P, and J-type) with different geographic distribution and number of RNA-segments. The disease can be controlled by cultivating partial resistant sugar beet genotypes. The few resistance genes used in practice at present do not prevent the infection with beet necrotic yellow vein virus (BNYVV) and its vector *Polymyxa betae* (a biotrophic plasmodiophoromycete). Thus, the disease spreads to further sugar beet production areas world-wide. By far more important is the fact that inoculum concentration is increasing on several infested sites. Hence, no long-term recovery from BNYVV in the soil can be expected at those locations. A high selection pressure is exerted on BNYVV by the widespread cultivation of genetically-uniform resistant plant material, which could promote the occurrence of resistance-breaking isolates. Above all, this interaction complexity is related to the fact that the naturally occurring resistances used at present do not grant complete immunity against the virus. The resistance in sugar beet only inhibits virus spread from primary infected lateral to tap roots. Further, high viruliferous *P. betae* inoculum in soil, which is able to overcome existing resistances, has already been observed. Concerns about selection of resistance breaking BNYVV isolates are supported by repeated observations of weaknesses regarding yield and sugar content in variety tests of partial resistant Rhizomania varieties. In addition a detailed molecular characterisation of the virus led to the identification of viral pathogenicity factors (P25 and P26) responsible for the development of typical Rhizomania symptoms like small yellow leaf veins, T-shaped tap roots, brownish vascular and the development of lateral root beard growth. P25 and P26 (only occurring in P- and J-isolates) are also known to be responsible for virus movement from infected lateral roots into the tap root. Sequencing P25 genes of several different BNYVV isolates revealed a high variability depending on the geographic origin of the virus. In geographically separated BNYVV infested sites an independent emergence of resistance-breaking isolates, which exhibit a divergent composition of viral pathogenicity factors, were already detected under certain conditions. The J- and P-types that only occurs in a small region around Pithiviers in France, in Kazakhstan, in Japan and on some sites in England is able to cause severe damage on partial resistant sugar beet. Higher virus titres were detected in lateral and tap roots of sugar beet plants cultivated in P-type soil,

compared to sugar beet grown in A and B soil. At present no information on the involvement of the fungal vector, which provides pathogenicity to the virus in the infection cycle as it enables the virus to cause exponentially increasing multiple secondary infections and thus, increases viral primary infection, exists. To understand these high-complex interactions of virus, vector and host, which all have impact on virus pathogenicity, studies to estimate the impact of inoculum concentration and an establishment of artificial infection procedures are urgently required.

High concentrations of viruliferous *P. betae* can overcome resistance. In addition the vector infection is unaffected by the virus resistance. Thus, verification of occurring resistance breaks in partial resistant Rhizomania varieties due to a high variability of pathogenicity genes in different BNYVV-isolates, which developed independently, was needed. Apparently other soil-borne pathogens like *Rhizoctonia solani*, *Pythium* sp., *Heterodae schachtii* and *Fusarium* sp. also influence the severity of the disease. In order to estimate the current and future disease potential and the durability of BNYVV resistance sources used at present correctly several studies of Rhizomania pathogenicity, depending on the genetic composition of virus and parts of the vector, the vector transmission, the inoculum densities of virus and vector and the influence of other soil borne pathogens, were conducted (manuscripts **I** and **II**).

1.2. Disease history

Rhizomania represents one of the economically most important diseases affecting sugar beet production. It is caused by beet necrotic yellow vein virus (BNYVV) (TAMADA & BABA, 1973), belonging to the genus *Benyvirus* (ICTV, 1997) and transmitted by the soil-borne biotrophic plasmodiophoromycete *Polymyxa betae* Keskin (KESKIN, 1964; DESSENS & MEYER, 1996; ADAMS et al., 2001, RUSH, 2003). The original description of the disease took place in Italy in the 50's in the last century (CANOVA, 1959). Ever since the virus spread into numerous sugar beet production areas world-wide (Asia, the USA, South and Central Europe, Scandinavia) (ASHER, 1993; TAMADA, 1999; LENNEFORS et al., 2000; NIELSEN et al., 2001). It can be assumed that the propagation speed of the disease is still increasing. To date, 1.6 millions hectares were examined for the occurrence of the disease within Europe; 1990 15%, 2000 38% and for 2010 56% of the sugar beet production area were predicted to be BNYVV infected (RICHARD-MOLARD & CARIOLLE, 2001).

1.3. The vector of BNYVV: *Polymyxa betae* Keskin

Polymyxa betae Keskin is a biotrophic plasmodiophorid that is hardly influencing plant growth in the field. Greenhouse experiments displayed slight differences in virulence of various virus-free

P. betae isolates where some isolates apparently reduced tap root growth in sugar beet (GERIK & DUFFUS, 1988, BLUNT et al., 1991; KASTIRR et al., 1994).

1.3.1. Vector taxonomy

Polymyxa betae, P. graminis, and *Spongospora subterranea* were considered to be economically unimportant. A proper taxonomy of these soil-borne pathogens was long-term disregarded and led to uncertainness in the last decades when investigations proved the capability of this group to vector viruses. Molecular characterisations proved this group to be unrelated to *Ascomycetes*, *Basidiomycetes*, *Oomycetes*, or *Myxomycetes*, a placement within the *Protozoa* was favoured (BRASELTON, 1995; WARD et al., 2003). Due to characteristics similar to those of plasmodiophorids the recognition as a valid taxonomic group was justified. Typical characters are: obligate intracellular parasitism, cruciform nuclear division, zoospores with two, anterior, unequal whiplash flagella, multinucleate plasmodia, and environmentally resistant, long-living resting spores (cysts) that are often clustered together to form a sporosorus (cystosorus). Development of zoospores and long-living sporosori are also typical for plasmodiophorids (ADAMS, 1990; BARR, 1992; BRASELTON, 1995/2001; LITTELFIELD & WHALLLON, 1999; SHERWOOD & RUSH, 1999).

1.3.2. Life cycle of Polymyxa betae and host range

The life cycle of *P. betae* has been well documented (ADAMS, 1991; BARR & ASHER, 1996; CAMPBELL, 1996; LITTELFIELD & WHALLON, 1999). The protist is able to survive in thick-walled clustered resting secondary spores (sporosori) in the soil for years. There is no indication for virus multiplication in these sporosori (CAMPBELL, 1996). This implies that fields, once infested with viruliferous *P. betae,* neither recover from Rhizomania infestation through the lengthening of crop rotation nor the cultivation of nonhost crops. As soon as a host for *P. betae* is present and a soil displays near-saturated moisture conditions and the temperatures are suitable (ideal for *P. betae* propagation are temperatures around 25°C) the resting spores start to germinate and release virus carrying primary zoospores. Once the primary zoospores contact host cells they encyst immediately and inject the zoospore contents within approx. 2 hours. The primary zoospore develops into a multinucleate plasmodium. Then following two developments are possible either it develops to a sporogenic plasmodium and transforms to a zoosporangium releasing secondary zoospores or it changes into a sporogenic plasmodium converting to a sporosorus to rest in the soil. If secondary zoospores are released, they are actively swimming to new roots cells to infect them. Under favourable conditions (+25°C, high moisture soil at pH 6-8) one cycle can be completed within 60 h (reviewed in ASHER & BLUNT, 1987). The *P. betae* life cycle is schematically demonstrated in Fig. 1.

Fig. 1

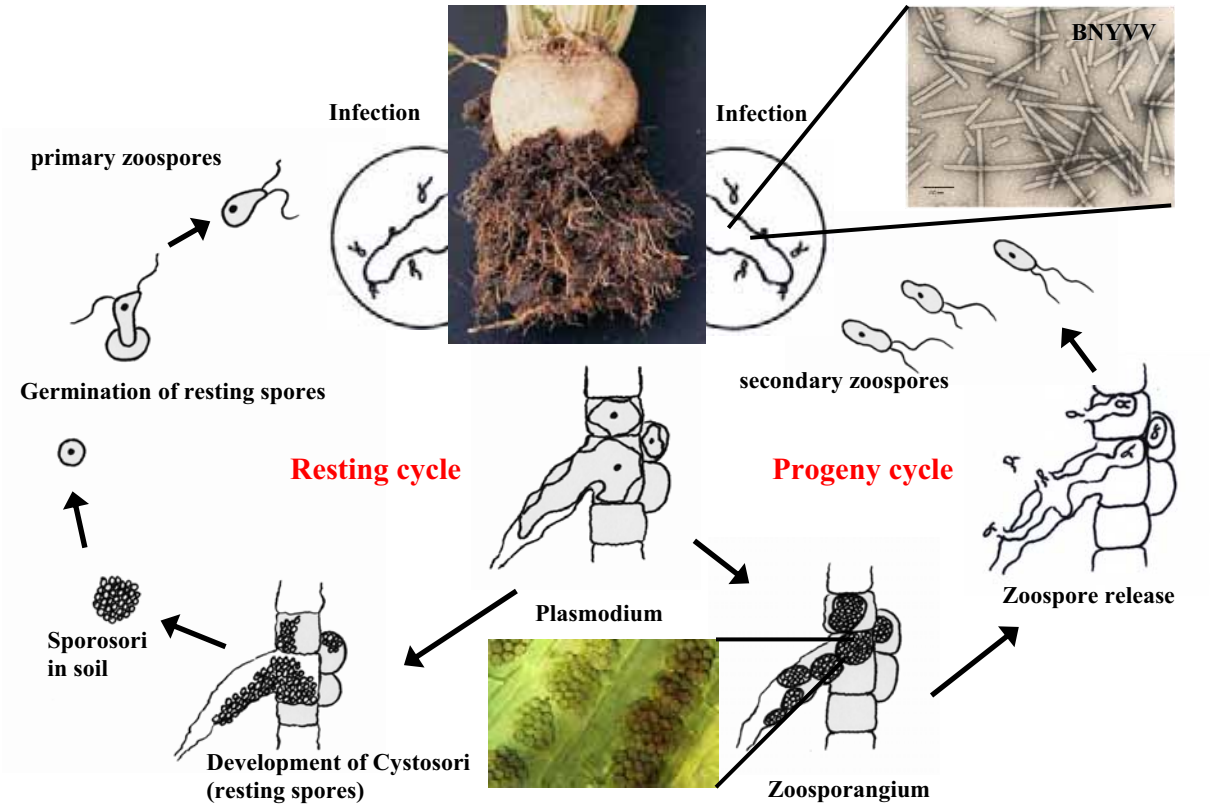


Fig. 1: Life cycle of viruliferous *Polymyxa betae* (mod. after Ruppel, unpublished)

Polymyxa betae and *P. graminis* are morphologically hardly distinguishable, thus both plasmodiophorids were separated by their host range (BARR, 1979; BARR & ASHER, 1992; BRASELTON, 1995). Today, the classification is done after molecular characterisation, strictly separating both plasmodiophorids and eliminating the hypothesis *P. betae* being a forma specialis of *P. graminis* (ADAMS & WARD, 1999; LEGRÉVE et al. 1998, 2000, 2002). *P. betae* possesses a rather small host range that is limited to species within *Chenopodiaceae*, *Amaranthaceae*, *Caryophyllaceae*, and *Portulacaceae*. *P. graminis* has a much greater host spectrum and is capable to infect both monocotyledonous as well as dicotyledonous species. Most *P. graminis* are able to infected sorghum and millet but vary in their ability to infect wheat, barley, and rye.

1.3.3. Molecular characterization of Polymyxa species

LEGRÉVE et al. (2002) conducted studies on a region of the nuclear ribosomal DNA containing the internal transcribed spacer 1 (ITS1), the 5.8S DNA and the internal transcribed spacer 2 (ITS2) for molecular characterisation of *Polymyxa betae* and *P. graminis*. In theses studies ITS sequences from isolates of *Olpidium brassicae*, *Spongospora subterranea*, *Plasmodiophora brassicae* and *Ligniera* spp. were compared to *Polymyxa graminis* and *P. betae*. However, LEGRÉVE et al. (2002 / 2003) suggested a grouping of *P. graminis* into five different distinct forma specialis: *P. graminis* f.

sp. *temperata*, *P. graminis* f. sp. *tepida*, *P. graminis* f. sp. *tropicalis, P. graminis* f. sp. *subtropicalis*, and *P. graminis* f. sp. *colombiana*. *P. betae* was also included in this sequence comparison. It was strictly separated from *P. graminis*. Further phylogenetic diversification within *P. betae* regarding its origin and the BNYVV-type carrying were not studied. Marginal sequence distinctions within *P. betae* isolates concerning the geographical origin were studied in manuscript **I**.

1.3.4. Vector detection and quantification

Polymyxa spp. are biotrophic organisms so that isolation and artificial cultivation on culture medium is impossible. GERIK (1992) described a selective growing media to grow the soil borne parasite in vitro on selective media in association with root cultures. After inoculating young sugar beet seedlings with *Agrobacterium rhizogenes*, which stimulates proliferation of fine rootlets, these cultures could be established. Because this in vitro method was not very reliable, most scientists use in practice naturally infested soil or infected sterile soil with infested dried root pieces to conduct field and greenhouse experiments (BOAG, 1986; GERIK & DUFFUS, 1988; TUITERT & HOFMEESTER 1992; TUITERT, 1993; TUITERT & BOLLEN, 1993;HARVESON et al., 1996; WISLER et al. 2003).

1.3.5. P. betae detection methods

Bait plant test: A rather simple test to prove the presence of *Polymyxa* spp. in soil by planting host into naturally infested soils. After only 8 days the sporosori can usually be observed on lateral hair roots by microscopy. For good *Polymyxa* spp. propagation the soils should be watered to near-saturation (ABE, 1987; GERIK, 1992). Soils for bait plant test should be used fresh as long-term storage influence the initiation to germinate (SHIRAKO & BRAKKE, 1983; LEGRÉVE et al., 1999).

Most probable number (MPN): Dealing with naturally infested soil the inoculum density of *Polymyxa* spp. is difficult to estimate. One solution is the determination of *P. betae* concentration in soil via MPN (CIAFARDINI, 1991; ADAMS & WELHAM, 1995, TUITERT & HOFMEESTER, 1992; TUITERT & BOLLEN, 1993). This technique has often been used to conduct ecological but also epidemiological studies. Combining the MPN with serological virus tests it is possible to estimate the percentage of viruliferous *Polymyxa* spp. in soils (TUITERT, 1990; CIAFARDINI, 1991). The MPN always delivers relative values that need to be statistically analysed to check the reliability of each test (manuscript **II**).

Serological methods: MUTASA-GOTTGENS et al. (2000) and DELFOSSE et al. (2000) developed serological methods to detect soil-borne pathogens using antiserum. Both enzyme-linked immunosorbent assays (ELISA) are suitable for qualitative and quantitative analyses of infected lateral roots, no matter which stage of fungal life development.

Molecular techniques: In general there are two different ways to detect *Polymyxa* spp. by means of nucleic acid based techniques On the one hand, the successful infestation of *Polymyxa* spp. in planta can be detected qualitatively via Polymerase chain reaction (PCR) (LEGRÉVE et al., 2003), quantitatively by real-time PCR (rtPCR) (LEES et al, 2003) or by means of DNA-probes (MUTASA et al., 1993). *Polymyxa* spp. can also be identified and quantified directly from the soil (CAMPBELL, 1996; WARD et al., 2003). Detecting Polymyxa spp. directly from the soil is difficult due to the inhomogeneous distribution of the soil-borne pathogen inhibiting the choice of a representative sample as only small amounts of soil (<10 g) for nucleic acid extraction are needed (CIAFARDINI, 1991; TUITERT & HOFMEESTER 1992). In fact often only a marginal percentage of zoospores are viruliferous (WORKNEH & RUSH, 2004). With molecular methods it is easily possible to diagnose single development stages of the soil-borne pathogen. GITTON et al. (1999) and MUMFORD et al. (2000) developed the verification and quantification by rtPCR for two viruses in parallel (soil borne wheat mosaic virus and wheat spindle streak mosaic virus as well as potato mop top virus and tobacco rattle virus, respectively) but similar methods for vectored viruses are missing for *P. betae* and BNYVV. A qualitative detection of different pathogens (BNYVV, beet soil borne virus, beet virus Q, and *P. betae*) via multiplex reverse transcriptase PCR (mRT-PCR) is published by MEUNIER et al. (2003b).

1.3.6. Virus-vector relationships

Two different ways of virus transmission via fungal vectors are known (CAMPBELL, 1996), first *in vitro* transmission and secondly *in vivo* transmission. BNYVV exhibits *in vivo* transmission (BARR, 1982; ABE & TAMADA, 1986; ADAMS, 1991; CAMPBELL, 1996). Typical *in vitro* virus transmission is demonstrated between *Olpidium brassicae* and tobacco necrosis virus (TNV), where virus transmission is much more independent from the vector. Once, *O. brassicae* transmits TNV virions, which are only absorbed to the surface of fungal membrane, the virus propagates in the host-cell and further spread is independent from the vector. If the host cell dies, fungal independent TNV virions get released into the soil (CAMPBELL, 1996). *In vivo* transmission is characterised by much more efficient virus spread than *in vitro* transmission. As soon BNYVV infected zoospores are released from resting spores and they contact a susceptible host, they start to inject the virus particles into the plant cell. The virus enters the cytoplasm to complete infestation of the cell with BNYVV and starts its replication and genome expression cycle, including virus

movement to adjacent cells. The opposite way around, if a virus-free zoospore infects a cell, which is already infected by BNYVV, the virus will be incorporated by the developed plasmodium what forms a zoosporangium, whereof secondary zoospores will be viruliferous. If the zoosporangium develops a sporogenic plasmodium it will convert to sporosori to rest in the soil, released zoospores, even after years resting in the soil, will be viruliferous. Recently, VERCHOT LUBICZ et al. (2007) proved by immunofluorescence labelling that BNYVV is accumulating in resting and zoospores of its vector *P. betae*. This would lead to the conclusion that *P. betae* is also a host for BNYVV since the virus lives and propagates inside the vector for more than one life cycle. Still most of the virus vectoring process/transfer is unknown; published data has been reviewed by DESSENS & MEYER (1996), REAVY et al. (1998), TAMADA et al. (1996), DIAO et al. (1999) and ADAMS et al. (2001). Any comparable virus-vector relationship as described for *P. betae* and BNYVV and their host the sugar beet are not puplished, yet.

1.4. BNYVV

In field BNYVV symptoms can be observed as yellow patches that are spread in the same direction of farm machinery movement. Due to ploughing, tillage, sowing and harvesting operations, infested soil is spread not only within the field but also to other sites. Thus the acreage of Rhizomania infested field is continuously increasing (RICHARD MOLARD & CARIOLLE, 2001). On weaker damaged sugar beets, pale, long and upright growing leaves can be observed. Whereas severe symptoms like rudimentary developed tap roots and extreme root beard development consisting of dark brownish lateral roots as well as systemic spread to leaves, causing yellow veins (TAMADA & BABA, 1973; TAMADA, 1975; JOHANSSON, 1985; ASHER, 1993) are nowadays rarely monitored since the cultivation of partially resistant sugar beet in most of the infested sugar beet growing areas. White sugar and root yield can be reduced up to 90% in susceptible cultivars (JOHANSSON, 1985). ASHER (1993) reported the broad possibilities of Rhizomania to spread into none or less infested field via seed potatoes and onions, farm machinery, irrigation, flooding and wind erosion.

1.4.1. Virus taxonomy

Since 1997 BNYVV is accepted by the International Committee on Taxonomy of Viruses (ICTV) as member of the genus *Benyvirus*. BNYVV consists of 4-5 rod-shaped particles, which encapsidate 4-5 genomic ss (+) strand RNAs, depending on the isolates (BOUZOUBAA et al., 1985, 1986, 1987; TAMADA et al. 1989; KIGUCHI et al., 1996; KOENIG et al. 1997).

1.4.2. Genome organisation of BNYVV

The BNYVV genome organisation and the known functions of the viral gene products expressed is displayed in Fig. 2.

Fig. 2

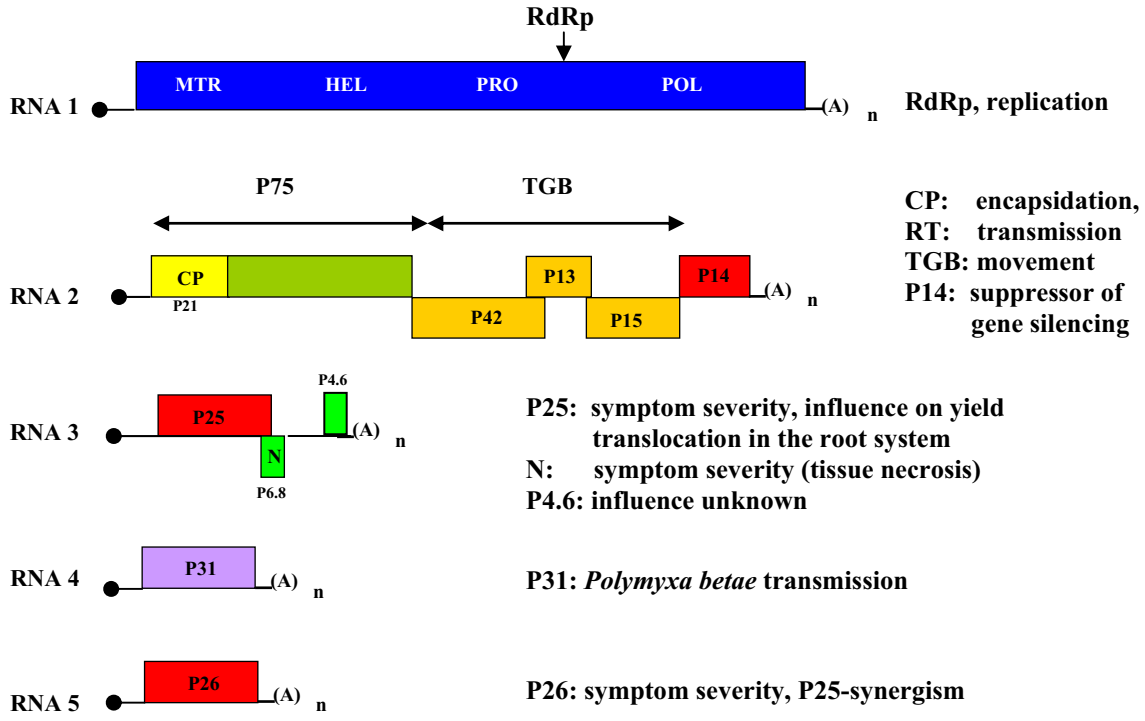


Fig. 2: Beet necrotic yellow-vein virus (BNYVV) genome expression und translation strategy, subdivided in five RNA segments, whereas only P- and J- types obtain the fifth RNA. All segments possess a cap structure at the 5′end and a poly A-tail (A) at the 3′end. Each box displays an open reading frame (ORF) in the genome, colours indicate the gene functions (blue = replication, yellow = coat protein, green = vector interaction, orange = cell-to-cell movement, red = pathogenicity, light green = connected to pathogenicity, but further functions are still unknown, lilac = cell-to-cell movement (vector transmission). RdRp = RNA dependent RNA polymerase, CP = coat protein, RT = readthrough protein, TGB = triple gene block, N = ORF inducing tissue necrosis only when sequences upstream are deleted.

RNA1

The RNA1 (in total 6746 nucleotides long, excluding the poly(A)-tail) encodes an ORF for a 237 kDa polypeptide possessing motifs for methyl transferase, helicase and RNA dependent RNA polymerase (RdRp). Thus the RNA1 is assumed to function as viral replicase protein (BOUZOUBAA et al., 1987). By a papain-like protease activity between the helicase and RNA dependent RNA polymerase (RdRp) motifs this protein is processed into a 150-kDa and 66-kDa product (the latter containing the polymerase domain) (HEHN et al., 1997).