PROCCEDINGS OF THE EIGHTH SYMPOSIUM OF THE INTERNATIONAL WORKING GROUP ON PLANT VIRUSES WITH FUNGAL VECTORS

Editor: U. Merz

**Université Catholique de Louvain
Earth and Life Institute
Louvain-La-Neuve, Belgium**

July 6-8, 2011

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PREFACE

The International Working Group on Plant Viruses with Fungal Vectors (IWGPVFV) was formed in 1988 at Kyoto, Japan, with Dr. Chuji Hiruki as chairperson. Drs. Renate Koenig, John Sherwood, Gail Wisler, and most recently Ueli Merz also have served as Chairperson. There are approximately 65 members, representing 16 countries. The goal of the working group is to provide a forum to facilitate international collaboration and multidisciplinary research on all aspects of plant viruses with fungal vectors. Members of the group meet every three years to present research on a wide variety of topics including virus taxonomy and characterization, vector biology and ecology, virus-vector interactions, and disease epidemiology and management. The high quality of information presented in an informal setting to an international group with common interests always makes for an enjoyable, professionally rewarding meeting.

Symposia of the working group have been held at the Biologische Bundesanstalt (BBA), Braunschweig, Germany (1990), McGill University, Montreal, Canada (1993), West Park Conference Centre, University of Dundee, Dundee, Scotland (1996), Asilomar Conference Center, Monterey, California (1999), Plant Pathology, Institute of Plant Sciences, ETH Zürich, Zürich, Switzerland (2002), Alma Mater Studiorum, Universita Di Bologna, Bologna, Italy (2005), Julius Kühn-Institute, Federal Research Centre for Cultivated Plants, Quedlinburg, Germany (2008) and Université Catholique de Louvain, Louvain-la-Neuve, Belgium (2011). This volume serves as a record of material presented at this most recent meeting. It is hoped that the information in this Proceedings will be useful to members of the IWGPVFV and all those interested in viruses with fungal vectors.

The IWGPVFV is a totally volunteer group and success of its meetings is in large part a result of the hard work and contributions of the local organizing committee and sponsors. The 2011 meeting in Louvain-la-Neuve was no exception, and the hospitality and good times will be remembered fondly. Those most responsible for the success of the recent meeting are listed on the next page. The names and e-mail addresses of the current program committee are also listed. Please

contact a member of the program committee if you wish to be included in any future mailings of the IWGPVFV.

A main agenda item of the General Assembly at the end of the last meeting was a basic discussion about the future of the working group. This discussion was initiated by Claude Bragard, the local organizer, as the number of participants was lowest compared to all other meetings. The discussion resulted in the resolution to continue the activities of the group, especially as participants from Japan volunteered to organize the next meeting. The committee decided that the next symposium is scheduled to be held in 2013 in Obihiro, Hokkaido, Japan.

Ueli Merz

Proceedings editor

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BEET NECROTIC YELLOW VEIN VIRUS, PAST, PRESENT, WHAT'S NEXT?

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Summary

Reported half a century ago, the rhizomania viral disease never stopped spreading among sugar beet growing places. Its particular mode of transmission requires an obligatory parasite vector that rests in soils for decades. This makes the disease the major viral beet threat. Yield losses were stabilized mainly by the use of tolerant crops but some viral isolates tend to overcome genetic traits; such new threat cannot be stopped even if some genetically modified organisms are efficient against the virus but not allowed. Therefore, major attempts are made to identify the *Beet necrotic yellow vein virus* (BNYVV) virulence factors and their mode of action. BNYVV biology will be described with the last known properties of the RNA-3 encoded p25 pathogenicity factor and its role in the 'omics' balance. A revisiting role of the RNA-3 and its link with other viral components will be presented.

The disease

Rhizomania has been related the *Beet necrotic yellow vein virus* infection and its transmission by *Polymyxa betae* vector (Canova, 1959; Tamada, 1999; Tamada, Baba, and Aba, 1971). The viral disease provokes taproot stunting, necrosis and the proliferation of bared roots, which decrease the sugar yields up to 70% (Richard-Molard, 1985).

Such soil borne disease is able to rest for decades as *P. betae* life cycle includes a resting spores strategy that keep viral loads in the soil. In favorable conditions, such viral load is delivered to plant rootlets and later from plant to plant by viruliferous swimming zoospores (Rush, 2003).

The countermeasures

To prevent the disease progression, methyl bromide fumigation was an alternative to control the vector but is not allowed since the 1987 Montreal treaty (UNEP, 1987). Beside chemical aspects, breeders selected rhizomania tolerant varieties (*Rz1* –Holly-, *Rz2* carrying sugar beets) that are today extensively used worldwide (Scholten & Lange, 2000). Other approaches have been

developed, either based on pathogen derived resistance such as viral coat protein or mutated viral movement proteins expression, viral genome targeting by post transcriptional gene silencing, viral cycle interference by the expression of single chain variable fragment (scFv) towards CP or non structural proteins (Fecker, Koenig, & Obermeier, 1997; Lauber *et al.*, 1998a; Lennefors *et al.*, 2006; Lennefors *et al.*, 2008; Peltier *et al.*, 2008).

The virus

BNYVV belongs to the *Benyvirus* genus that includes as well *Beet soil-borne mosaic virus* (BSBMV) with the tentative members *Rice stripe necrosis virus* and *Burdock mosaic virus* (Gilmer & Ratti, in press). Full-length infectious clones of BNYVV and now BSBMV allowed many reverse genetic studies, which permitted to identify the functions of the viral RNA encoded proteins.

Interestingly, Benyviruses possess a unique multipartite RNA genome that completely mimic cellular messenger RNAs, since vRNAs possess m^{7}Gppp (cap) structure and a polyA tail. However, BNYVV uses extensive expression strategies to express multicistronic RNAs. Minor coat protein is expressed by a read-through of the major coat protein stop codon and the TGB3 movement protein is produced by internal translation initiation from subgenomic RNAs. Beside such features, BNYVV express as well a polyprotein with replicase signatures that appear closer to *Hepatitis E virus* than to other described phytoviruses. The four components of the virus, five in some isolates, are essential to carry out biological cycle in the field. However, only RNA1 and RNA2 appear essential when the virus is passage onto local host plants by the mean of mechanical inoculation. Such property allowed deriving cDNA infectious clones of small viral RNAs as viral expression vectors (Ratti *et al.*, 2009; Schmidlin *et al.*, 2005).

Viral pathogenicity determinant is encoded by RNA3

In 1992, Jupin et al demonstrated that the presence of BNYVV RNA3 and the expression of the p25 protein were affecting leaves symptoms (Jupin *et al.*, 1992). Tamada et al evidence the role of the p25 protein by the use of natural RNA3 deleted isolates that were unable to provoke rhizomania symptoms (Tamada *et al.*, 1999). The p25 protein possesses a nuclear localization signal as well as a nuclear export sequence that allows the protein to shuttle between the cytoplasm and the nucleus of the cells (Vetter *et al.*, 2004). If p25 sequence is conserved between BNYVV isolates, a four amino acids domain, known as Tetrad (Schirmer *et al.*, 2005), appears highly variable and linked to resistance breaking (Acosta-Leal *et al.*, 2010; Chiba *et al.*, 2008; Koenig *et al.*, 2009; Liu, Sears, and Lewellen, 2005) as well as influencing p25 protein known properties (Klein *et al.*, 2007).

Ectopic expression of the p25 protein in sugar beet leaves induces a yellowing of the infiltrated area (Peltier *et al.*, 2011) whereas its constitutive expression in sugar beet has never been obtained, suggesting its lethal effect. However, transgenic *Arabidopsis thaliana* Columbia have been successfully obtained and display a dominant root branching phenotype that has been linked to an auxin hormone accumulation and a decrease of jasmonic acid as well as ACC ethylene precursor (Peltier *et al.*, 2011). Since no drastic phenotypic modification occurred in transgenic plants expressing the p25 protein, a homeostatic balance has been proposed to explain the huge transcriptomic changes identified by chip analyses. Within the identified deregulated messengers, some gene products were as well described in other transcriptomic and proteomic studies (Larson *et al.*, 2008; Schmidlin *et al.*, 2008; Thiel & Varrelmann, 2009). Interestingly, one p25 protein partner identified by the group of M. Varrelmann is an F-box protein. Together with M. Varrelmann's team, we determined that the F-box p25 partner of p25 acts through a Skp2 mediated function which seems inhibited by the p25 protein in a yeast two-hybrid system using a bridge vector. Using a similar approach, other p25 partners are under characterization.

Another pathogenicity determinant linked to BNYVV RNA3 was pointed out by the work of E. Lauber *et al.* (1998b). This study identified the RNA core sequence responsible for the long distance movement of BNYVV and later the work from C. Ratti *et al.* predicted that a 20 nts long sequence, known as "coremin" sequence, was involved in such viral spread (Ratti *et al.*, 2009). Such "coremin" sequence is also present on BSBMV RNA3 and 4, BNYVV RNA5 as well as CMV group II RNAs (Peltier *et al.*, submitted).

Using transgenic approaches as well as mutagenesis, we demonstrated the importance of the "coremin" sequence in both the long distance movement of the virus and the stabilization of the RNA3-sub species that appear to be a non coding RNA issued from a cleavage and a degradation of genomic RNA3 (Peltier *et al.*, submitted). Comparative studies between BNYVV and BSBMV have been initiated and will help to understand mechanisms involved in benyviruses biology.

System biology of healthy and infected sugar beet is required to draw deregulation pathways that could explain the rhizomania syndrome which involve 'omics' deregulations affecting the disease progression. Parallel approaches may focus as well on the soil effect, particularly the microbiome, which may interfere positively or negatively on *P. betae* biology, which still constitute our black-box.

Concluding remarks

The rhizomania disease is linked to the RNA3 encoded p25 protein, which plays a major role in the transcriptome, proteome and hormonal balance of the

plant. Beside this protein activity, RNA3 itself is a pathogenicity determinant that produces ncRNA acting in favor of viral propagation within sugar beet.

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AGROINFECTION: A NEW TOOL FOR *BEET NECROTIC YELLOW VEIN VIRUS* STUDY AND FOR RHIZOMANIA-RESISTANCE ASSESSMENT

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Summary

Agroinfection consists in a tissue infiltration with a suspension of *Agrobacterium tumefaciens* cells carrying binary plasmids harboring full-length cDNA copy of a viral genome component. It is a quick and easy way for plant infection so we decided to clone *Beet necrotic yellow vein virus* RNAs and replicons expressing viral and fluorescent proteins in the pJL89 binary vector under the control of the *Cauliflower mosaic virus* 35S promoter. BNYVV infection of *Nicotiana benthamiana* and *Beta macrocarpa* plants have been successfully performed by leaf agroinfiltration and the capability of ours clones to reproduce a complete viral cycle, from replication to transmission by the vector, has also been verified.

Moreover, a new protocol for agroinfection of *B. vulgaris* roots has been evaluated, in order to test resistance to Rhizomania, the sugar beet disease caused by BNYVV.

Introduction

The *Beet necrotic yellow vein virus* (Tamada & Baba, 1973), the most important infectious agent that affects sugar beet, is a member of *Benyvirus* genus and is transmitted through the soil from the plasmodiophorid *Polymyxa betae.* BNYVV is the causal agent of Rhizomania disease (Canova, 1959), leading to an abnormal rootlet proliferation that inhibits sugar accumulation.

BNYVV genome consists in four plus-sense 5' capped and 3' polyadenylated RNAs: RNA-1 and -2 are essential for infection and replication while RNA-3 and -4 play important roles in plant and vector interactions, respectively. According to nucleotide sequences analysis the existence of three types was revealed: A, B and P. The last one, characterized by the presence of RNA-5, is typically more aggressive.

Up to now several and different studies have been performed, using a reverse genetic approach (Quillet *et al.*, 1989; Ratti *et al.*, 2009) or field isolates

(Koenig *et al.*, 1991; Bournemann & Varrelmann, 2011), to investigate or assess Benyviruses-plant-vector interaction.

A useful alternative to these methods is agroinfection (Grimsley, 1986), that appears to be a less expensive and more reproducible strategy to infect plants with the virus of interest. Agroinfection consists in tissue infiltration with a suspension of *Agrobacterium tumefaciens* cells carrying binary plasmids harboring full-length cDNA copy of a viral genome component. A plant-functional promoter and the cDNA of a RNA viral genome are transferred as T-DNA from *A. tumefaciens* into plant cells and, after transcription, they can generate biologically active viral RNAs able to initiate the infection.

Here we describe the production of BNYVV agroinfectious clones. Moreover a new protocol for massive inoculation of sugar beet plants has been evaluated for Rhizomania-resistance assessment.

Materials and Methods

Full-length cDNA clones of BNYVV RNA-1 to -5 as well as replicon based on RNA-3 and -5 that express BSBMV and BNYVV viral proteins or the green fluorescent protein (GFP) have been introduced in the pJL89 binary vector (Fig. 1), previously digested with *Stu*I/*Sma*I enzymes, downstream of the *Cauliflower mosaic virus* 35S promoter. The binary vector constructs were then transferred by electroporation into *A. tumefaciens* cells (strain C58C1).

Several steps have been necessary to clone cDNA of BNYVV RNA-1 into the binary vector. First, *Sph*I restriction site has been removed from pJL89 then cDNA of

Figure 1. pJL89 binary vector.

RNA-1, without fragments *Sph*I/*Sph*I (1995-3742 nt) and *Mlu*I/*Mlu*I (4125-5396 nt), has been introduced in the new pJL89-SphI plasmid. The two missing fragments have been then added in two different steps. All intermediate plasmids were cloned in *E. coli* cells (strain M1022). The complete cDNA of RNA-1 in pJL89 was sequentially introduced into *A. tumefaciens* cells.

Cultures of transformed *A. tumefaciens* cells were grown O/N at 28°C in 5ml LB supplemented with 100µg/ml kanamycin and 50µg/ml rifampicin. The bacteria were collected by centrifugation and resuspended in MA buffer (10mM MgCl$_2$ and 200µM Acetosyringone) in order to have an OD600 value of 0.6 for
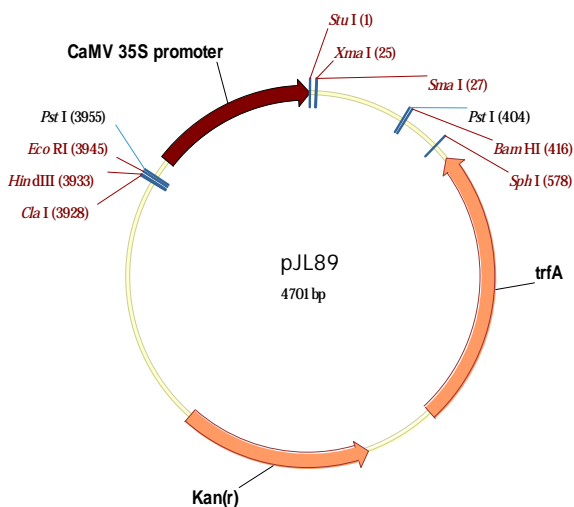
Nicotiana benthamiana and 0.3 for *Beta macrocarpa* agroinfiltration. Cells were mixed in a ratio 1:1 and left at room temperature for 3-4 hours before to be agroinfiltrated in 3-weeks old plants.

Results

    A. tumefasciens cells, carrying clones able to reconstruct a viral genome, were mixed and infiltrated onto host plant leaves. Local and systemic symptoms were produced in both *N. benthamiana* and *B. macrocarpa* plants (Fig. 2). Local symptoms appeared as chlorotic spots whereas systemic symptoms showed leaves distortions giving evidences of the efficient viral movement into the plant. In all samples BNYVV RNAs were visualized by nothern blot and the coat protein detected by western blot. Replicon-mediated GFP expression was observed in *N. benthamiana* leaves and characteristic rod-shape particles seen in all tissues including roots.

    The capability of our clones to reproduce a complete viral cycle, from replication to the transmission through the vector, has been tested. *B. macrocarpa* plants were agroinfected by BNYVV RNA-1 to -4 and infested with aviruliferous *P. betae* zoospores. After three weeks BNYVV particles were detected in the roots using Transmission Electron Microscope (TEM). Formation of cystosori into the roots was also observed using light microscope. Infected roots were then collected, air-dried and crumbled into a pot carrying *B. vulgaris* seeds. After 2 weeks BNYVV particles were observed by TEM on the new seedling roots demonstrating the viral transmission, through the vector, to new host plants.

    Finally, *B. vulgaris* plants have been directly agro-infected. Roots of 10-days old sugar beet seedlings have been vortexed in a suspension of *A. tumefaciens* cells containing

Figure 2. Systemic infection from BNYVV agroinfectious clones infiltration in *B. macrocarpa*. Progressive evolution of the symptoms from the left (healthy plant) to the right (infected plant with leaves completely distorted).

carborundum. Three weeks after infection ELISA test confirmed viral infection and BNYVV particles and GFP expression have been visualized in the roots, respectively, by TEM and confocal microscope.

Discussion

Our data demonstrated that BNYVV agroinfectious clones are perfectly functional as they generate biologically active viral RNAs able to complete a viral cycle, from the replication to the transmission. Induced symptoms and behavior of viral progeny, derived from RNA produced by our clones, are actually identical to those of BNYVV wild type isolate.

Agroinfection is therefore a quick and economic way to infect plants, useful to perform different experiments *in planta*, such as interaction test between different viruses, protein expression and virus-vector interaction. In particular we are now working to produce BSBMV agroinfective clones in order to verify synergistic or antagonistic effects, as well as cross protection, between BNYVV and BSBMV.

Agroclones of replicons Rep3, Rep5 or RepIII (Bleykasten-Grosshans *et al.*, 1997; Schmidlin *et al.*, 2005; Ratti *et al.*, 2009) expressing different viral or foreign proteins can be also produced with the purpose to test their expression and/or their effects on the plant. Moreover, using our clones, transmission test through the vector can be performed easily infecting plants by agroinfection and therefore skipping the use of infectious transcripts or field isolates.

Finally direct agroinfection of sugar beet roots was successful and could represent a new tool, useful to perform Rhizomania-resistance test in different sugar beet cultivars, characterized by high homogeny of viral infection pressure ensured by precise quantification of *A. tumefaciens* cells.

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DEVELOPMENT OF NEW TOOLS TO STUDY *BEET VIRUS Q*

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Summary

Beet virus Q (BVQ), often detected in sugar beet roots infected by *Beet necrotic yellow vein virus* (BNYVV), the causal agent of the rhizomania syndrome, and in co-infection with *Beet soil-borne virus* (BSBV), is still poorly characterized. In order to investigate the functioning and potential pathogenicity of BVQ *in planta*, full-length cDNA clones were developed for the three genomic RNAs. Transcripts from BVQ constructs were shown to be infectious and able to produce clear symptoms on both *Chenopodium quinoa* and *Beta macrocarpa*.

Introduction

Beet virus Q (BVQ), first identified in 1998 as a distinct species from the closely related *Beet soil-borne virus* (BSBV) (Koenig *et al.*, 1998), is widely reported among beet crops. Both viruses belong to *Pomovirus* and have a multipartite genome composed of three single-stranded plus-sense RNAs. RNA-1 (6.0 kb) encodes for the putative viral replicase. 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). Three open reading frames (ORFs) coding for putative Triple Gene Block (TGB) proteins are identified on BVQ RNA-3 (2.5 kb). TGB proteins are known to be required for viral movement from cell to cell (Morozov & Solovyev, 2003).

Although BVQ is commonly detected with BSBV and *Beet necrotic yellow vein virus* (BNYVV) (Meunier *et al.*, 2003), a benyvirus responsible for the rhizomania disease of sugar beet (Tamada & Baba, 1973), the functioning and pathogenicity of this pomovirus remain unclear. Moreover, the co-occurrence of these three beet viruses transmitted by the soil-borne protist *Polymyxa betae* raises questions about the potential viral interactions that could take place in crops.

In order to tackle with the functioning and pathogenicity of BNYVV and BSBV under controlled conditions, full-length cDNA clones have been constructed for both viruses (Ziegler-Graff *et al.*, 1988; Quillet *et al.*, 1989; Crutzen *et al.*, 2009b). Such a tool was however still lacking for BVQ. To

investigate the behavior of BVQ *in planta* and the possible interactions between the three soil-borne beet viruses, full-length cDNA clones were therefore developed for the three BVQ genomic RNAs. The infectiousness of BVQ transcripts from full-length cDNA clones was first tested in *Chenopodium quinoa* and *Beta macrocarpa* leaves.

Materials and Methods

The BVQ full-length cDNA clones were developed following the example of Crutzen and co-authors (2009b) with minor changes to obtain the plasmid constructs pUBQ-1-wt (RNA-1), pUBQ-2-wt (RNA-2) and pUBQ-3-wt (RNA-3). The plasmids containing BVQ full-length cDNA clones were linearized and transcripts were subsequently produced using the RiboMAX™ large-scale RNA production system – T7 (Promega). BVQ transcripts were mechanically inoculated on *C. quinoa* and *B. macrocarpa* leaves and viral structural proteins were searched by western blot as described by Crutzen and co-authors (2009b). For each host, four plants were inoculated with BVQ transcripts and two others were mock-inoculated. Plants were kept in the greenhouse with 16h of photoperiod, at 13-20°C for night and day periods, respectively.

Results

As shown on Figure 1, BVQ transcripts from full-length cDNA clones proved to be able to produce clear symptoms on the inoculated leaves of both C. quinoa and B. macrocarpa. Tiny chlorotic and necrotic lesions were induced as soon as 3-4 days post-inoculation (dpi) on C. quinoa leaves whereas B.
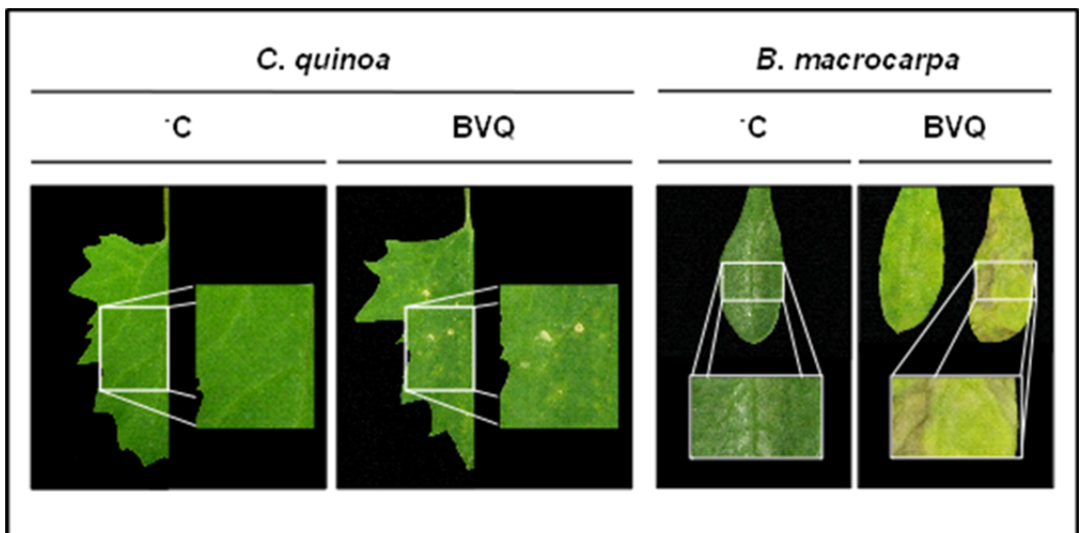

Figure 1. Symptoms produced after the rub inoculation of *in vitro* transcripts produced from BVQ from full-length cDNA clones on *Chenopodium quinoa* and *Beta macrocarpa* leaves compared with mock-inoculated samples (˙C), 10 days post-inoculation.

macrocarpa inoculated leaves developed strong overall chlorosis and necrosis 7 dpi. Such symptoms were observed on all plant leaves inoculated with BVQ transcripts.
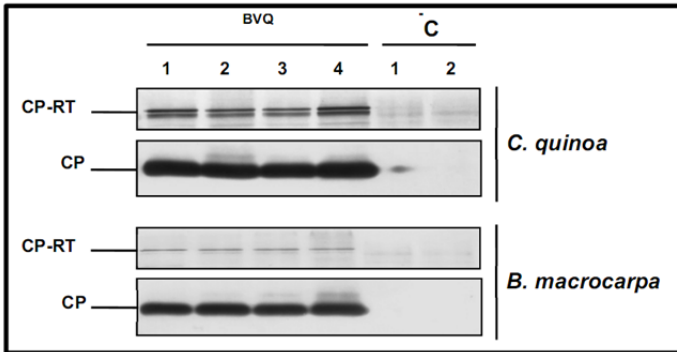

Figure 2. Western blot detection of BVQ structural CP and CP-RT in leaves of *C. quinoa* and *B. macrocarpa* inoculated with BVQ transcripts or mock-inoculated (-C).

The infectiousness of BVQ transcripts was confirmed by the immunodetection of the viral structural proteins using a polyclonal antibody directed against BSBV particles (DSMZ). Cross-reactivity of the serum with BVQ allowed both the CP (19kDa) and CP-RT (95 kDa) to be detected in all leaves of *C. quinoa* and *B. macrocarpa* inoculated with BVQ transcripts (Fig. 2).

Interestingly, multiple bands were specifically immunodetected in the infected samples and not in the control experiments when searching for the BVQ CP-RT. If this detection of proteins isoforms clearly occurs in *C. quinoa*, it appears less obvious on *B. macrocarpa* inoculated leaves (Fig. 2). The origin of CP-RT multiple bands needs to be further investigated.

Discussion

Viewing the results presented herein, the transcripts from full-length cDNA clones of BVQ proved to be able to produce symptoms on both *C. quinoa* and *B. macrocarpa* leaves and their infectiousness was confirmed by detecting BVQ structural proteins. When compared with the closely related BSBV, it is worth noting that this last has been shown in previous studies to be unable to induce clear symptoms on beet species, using sap from infected plants or transcripts from full-length cDNA clones (Henry *et al.*, 1986; Crutzen *et al.*, 2009b). Though the genomes of both pomoviruses are highly similar in structure and sequence (Koenig *et al.*, 1998), BSBV and BVQ exhibit different behaviours depending on the infected host. The gene(s) responsible for such specificities should nevertheless still be identified.

The Western blot results also evidenced that multiple forms of the BVQ CP-RT, with different molecular masses (MM), might be detected in the infected leaves. As suggested for BSBV, which CP-RT exhibits a higher MM in SDS-

PAGE conditions than the predicted one (Crutzen *et al.*, 2009b), post-translational modifications could be responsible for such a migration pattern of BVQ CP-RT. This remains however to be determined experimentally.

This study aimed to develop new tools to further investigate the functioning and pathogenicity of BVQ. From now on, full-length cDNA clones are available for beny- and pomoviruses infecting beet. The role of potential interactions between one and the other of these viruses in the rhizomania syndrome could therefore be studied under controlled conditions.

Acknowledgements

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PREPARATION AND USE OF INFECTIOUS IN VITRO TRANSCRIPTS FOR FOUR FUROVIRUSES AND ONE BYMOVIRUS FOR THE STUDY OF VIRUS-HOST INTERACTIONS

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Summary

Infectious *in vitro* transcripts derived from full-length cDNA clones are required for reverse genetics studies on positive-sense RNA viruses. We have been attempting to construct full-length infectious cDNA clones for viruses in the genera *Furovirus* and *Bymovirus*, which include agriculturally important virus species that infect cereal crops. To date, we have obtained full-length infectious cDNA clones for a US isolate of *Soil-borne wheat mosaic virus* (SBWMV) from Nebraska, a Japanese isolate of *Japanese soil-borne wheat mosaic virus* (JSBWMV) from Tochigi, a British isolate of *Soil-borne cereal mosaic virus* (SBCMV) from Wiltshire, a Japanese isolate of *Chinese wheat mosaic virus* (CWMV) from Iwate and a Japanese isolate of *Barley yellow mosaic virus* (BaYMV) Pathotype II from Kurashiki. The preparation and propagation of these cDNA clones required different plasmid vectors and *Escherichia coli* strains, and some cDNA clones could only be kept as isolated plasmid DNAs in TE and not in bacterial hosts in the form of frozen glycerol stocks. These infectious cDNA clones are useful for studying the biology of furoviruses and bymoviruses at the molecular level, and these constructs are available upon request.

Introduction

Reverse genetics systems have already been established for the majority of plant RNA viruses. These systems allow the study of the functions of viral RNA elements and the encoded proteins and the analysis of the interactions between viruses and both their host plants and transmission vectors. However, reverse genetics systems for viruses in the genera *Furovirus* and *Bymovirus* have not been develped except for systems for one virus species in each genus, JSBWMV in the genus *Furovirus* and BaYMV in the genus *Bymovirus*. One challenge in the development of reverse genetics systems is that viral cDNA fragments are foreign to bacterial hosts and that portions of the cDNA fragments or the encoded proteins could be toxic to the bacterial hosts, resulting in the failure of stable cloning and of the maintenance of a full-length cDNA insert in a plasmid.

In this study, we investigated the use of two *Escherichia coli* plasmid vectors with different copy numbers and of six *E. coli* K12 strains for the stable cloning of full-length cDNA inserts derived from four furoviruses and one bymovirus.

Materials and Methods

Infectious cDNA clones for JSBWMV RNAs 1 and 2 (pJS1 and pJS2, respectively), for SBWMV RNA2 (pNE2) and for BaYMV RNAs 1 and 2 (pBY1 and pBY2, respectively) were described previously (Yamamiya & Shirako, 2000; Miyanishi *et al.*, 2002; You & Shirako, 2010). SBWMV RNA1 cDNA clones prepared for nucleotide sequencing were used for the constructing of a full-length cDNA clone (Shirako & Wilson, 1993). The CWMV Iwate isolate was obtained in a field in Iwate Prefecture, Japan in the spring of 2006. The SBCMV Wiltshire isolate was kindly provided by Gerard Clover.

Full-length cDNA inserts for SBWMV RNA1, CWMV RNAs 1 and 2, and SBCMV RNAs 1 and 2 were constructed such that there was a T7 promoter immediately upstream of the 5' end and a unique restriction enzyme site next to the 3' end of the viral genomic RNA. Two plasmid vectors were used in the cloning of each full-length cDNA insert: one was derived from pBR322 by removing the tetracycline-resistance gene (Rice *et al.*, 1987) and the other was derived from pACYC177 (New England Biolabs) after removal of the kanamycin-resistance gene. For plasmid amplification, *E. coli* K12 strains MC1061 (Casadaban & Cohen, 1980), JM109 (Promega), DH5-α (Invitrogen), XL1-blue (Stratagene), SURE (Stratagene) and Stbl4 (Invitrogen) were used as hosts. Plasmids were extracted from *E. coli* cells with a modified boiling method and were purified using the PureLink plasmid purification kit (Invitrogen). Plasmids were linearized using the restriction enzyme site inserted immediately downstream of the 3' end of the cDNA insert and were transcribed *in vitro* using SP6 RNA polymerase for pJS1 and pJS2 or T7 RNA polymerase for all others. The *in vitro* transcripts were diluted in RNA inoculation buffer (50 mM glycine, 50 mM K_2HPO_4, pH 9.2) and rubbed on the leaf surfaces of *Chenopodium quinoa*, wheat (cv. Fukuhokomugi) or barley (cv. Ryofu) plants at the 2-3 leaf stage after dusting leaves with carborundum (600 mesh). The plants inoculated with the four furoviruses were grown at 17 °C, and the plants inoculated with BaYMV were grown at 15 °C.

Results

A summary of the preparation of the cDNA clones for the four furoviruses and the manipulations of these constructs is listed in Table 1.

(1) SBWMV RNA1 cDNA clone, pNE1AC. Previously, the 5'-terminal and 3'-terminal cDNA fragments were individually cloned into pBR322-derivative plasmids in MC1061 cells, and the two overlapping cDNA inserts were combined *in vitro* by PCR for the preparation of a full-length template (Miyanishi

et al., 2002). However, the cloning of a full-length cDNA was not possible, with only a limited number of colonies being formed on ampicillin-containing LB agar plates. The plasmids from these colonies contained aberrant cDNA inserts with a large deletion or an IS insertion. When a low-copy-number plasmid derived from pACYC177 was used for the cloning of a full-length cDNA insert, MC1061 cells could be transformed, and a number of minute colonies formed along with a few medium-sized colonies on an LB-ampicillin plate. Plasmid DNA containing the full-length cDNA could be isolated from minute colonies, whereas the medium-sized colonies harbored plasmids with aberrant cDNA inserts. Liquid cultures started from the minute colonies were incubated at 37 °C for 20 hours. The yield of the isolated plasmid DNA was approximately 200 ng per 6-ml culture. Full-length cDNA clones could not be obtained using other *E. coli* strains. For unknown reasons, a secondary liquid culture derived from the primary culture did not yield detectable amounts of plasmid DNA using the same isolation method as used for the primary culture. Therefore, pNE1AC DNA could be saved as a suspension in TE at 4 °C but not in the bacterial host as a glycerol stock at -70 °C.

(2) CMWV RNA1 cDNA clone, pJN1AC. The full-length cDNA for CWMV RNA1 was successfully cloned into a pACYC177-derived plasmid using MC1061 cells as the host, but cloning was not successful when using pBR322-derivative plasmids and MC1061, SURE or JM109 cells. The yield of the plasmid DNA was approximately 200 ng from a 6-ml culture after growth at 37 °C for 20 hours. pJN1AC DNA could be stored at 4 °C but not as a glycerol stock of the transformed *E. coli* cells in a freezer, as in the case of pNE1AC.

(3) SBCMV RNA1 cDNA clone, pUK1. The full-length cDNA was cloned into a pBR322-derived plasmid and propagated in SURE and JM109 cells. The plasmid yields after the growth of recombinant bacterial cells at 37 °C for 16 hours were approximately 500 ng and 200 ng, respectively, from 6-ml cultures. MC1061, DH5-α, Stbl4 and XL1-blue cells could not be used for the cloning of the full-length cDNA due to the lack of the plasmid DNA from a primary culture or due to the generation of recombinant cDNA inserts. As in the cases of pNE1AC and pJN1AC, pUK1 DNA could not be maintained in transformed bacterial cells but could be kept only as a DNA suspension in TE at 4 °C.

(4) CWMV and SBCMV RNA2 cDNA clones. Full-length cDNA inserts for CWMV RNA2 and SBCMV RNA2 were cloned into a pBR322-derived plasmid and propagated in MC1061 cells. The yields were approximately 2 µg from 6 ml cultures.

Using the 4 sets of infectious *in vitro* transcripts, we examined the compatibility of the RNA1 and RNA2 transcripts of different furovirus species in infectivity using *C. quinoa* as a local-lesion host and wheat (cv Fukuhokomugi) as a systemic host. As shown in Fig. 1, only the combination of SBWMV RNA1 and JSBWMV RNA2 did not cause systemic infection in wheat plants. This combination resulted in minute lesions on *C. quinoa* leaves, but CP could not be detected from these lesions by Western blot analysis. Thus the four furoviruses are biologically quite similar even though they are classified as separate species within the genus.

Table 1. Colony formation, bacterial growth and plasmid yields

Virus	RNA	Clone name	Plasmid	DH5-α	JM109	MC1061	Stbl4	SURE	XL1-blue
JSBWMV	1	pJS1	pBR322	X[1]	◇	○[1]	X[2]	◇	X[2]
	2	pJS2	pBR322	n.t.	n.t.	○[1]	n.t.	n.t.	n.t.
SBWMV	1	pNE1AC	pACYC177	X[2]	X[2]	○[2] + X[3]	X[1]	X[2]	X[3]
	2	pNE2	pGEM3Z	n.t.	n.t.	○[1]	n.t.	n.t.	n.t.
CWMV	1	pJN1AC	pACYC177	n.t.	◇	○[2]	n.t.	X[2]	n.t.
	2	pJN2	pBR322	n.t.	n.t.	○[1]	n.t.	n.t.	n.t.
SBCMV	1	pUK1	pACYC177	X[2]	◇	X[3]	X[2]	○[2]	X[2]
	2	pUK2	pBR322	n.t.	n.t.	○[1]	n.t.	n.t.	n.t.

pBR322: Tetracycline resistance gene was deleted (Rice *et al.*, 1987).
pACYC177: Kanamycin resistance gene was deleted.
○[1]: Plasmids with a full-length cDNA insert were recovered and stored as a glycerol stock at -70 °C.
○[2]: Plasmids with a full-length cDNA insert were recovered but only from a primary culture.
◇: Plasmids with a full-length cDNA insert were recovered but in a smaller amount than for ○[1].
X[1]: Colonies were not formed on agar plates.
X[2]: Colonies were formed on agar plates but plasmids were not recovered from liquid cultures.
X[3]: Plasmids were recovered from liquid cultures but the cDNA inserts harbored aberrant recombinant sequences.
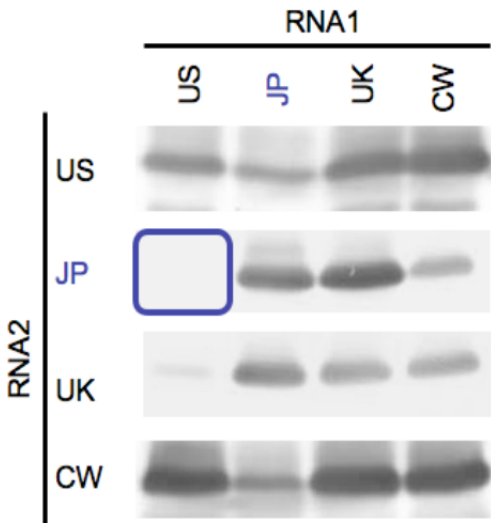n.t.: Not tested.

Figure 1 Reassortment infectivity assay in wheat plants inoculated with homologous and heterologous combinations of the RNA1 and RNA2 transcripts of SBWMV (US), JSBWMV (JP), SBCMV (UK) and CWMV (CW). CP was detected from upper uninoculated leaves by western blotting.

Discussion

Since the first report by Ahlquist *et al.* in 1984, infectious cDNA clones have been established for the majority of plant RNA virus species for use in structural and functional studies of the virus genome. However, infectious cDNA clones had not been developed for viruses in the genera *Furovirus* and *Bymovirus* until recently. Viruses in these two genera present common challenges in molecular biological studies because of the lack of infectious *in vitro* transcripts. Although these viruses are transmissible by mechanical inoculation using virus-containing sap or viral RNA, the infectivity is very low and it takes several weeks for inoculated plants to develop symptoms. The host range of these viruses are narrow, and bymoviruses do not infect dicot plants. In addition, the optimal temperatures for infectivity are 12°-17 °C, and therefore, reliable temperature-controlled growth cabinets are required.

Infectious transcripts that are generated *in vitro* from full-length cDNA clones overcome the limitations associated with the virus and viral RNA in infectivity assays at the whole-plant level and at the cellular level using isolated protoplasts (You & Shirako, 2012). Although cDNA clones for RNA1 of SBWMV, SBCMV and CWMV still present challenges with respect to handling, the availability of sets of infectious cDNA clones allows molecular biological studies of furoviruses and bymoviruses to be conducted. We hope that these materials will be used widely by researchers in this field to answer important biological questions and to develop effective measures for controlling diseases caused by furoviruses and bymoviruses.

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DEFECTIVE RNA OF THE *POTATO MOP-TOP VIRUS* AND ITS ROLE IN THE VIRUS INFECTION

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The genome of PMTV consist of three species of single stranded positive sense RNA. RNA1 encodes the replicase. RNA2 contains ORFs encoding the capsid protein and a larger protein produced by translational read through of the CP termination codone. RNA3 contains four overlapping ORFs three of which encode triple gene block movement proteins and a small cysteine-rich protein p8.

In addition to three genomic RNAs and two subgenomic RNAs *Potato mop-top virus* produces a redundant defective RNA (DI). DI RNA encompass the 5' 1-480 and 3' 2591-2964 nucleotides of RNA-TGB. This spontaneous deletion in RNA3 was identified by RT PCR and northern blot. Sequence deletion is a well known phenomenon among positive stranded RNA viruses If the D molecules interfere with the replication and reduce symptom production, these are termed DI.

D-RNA production have been attributed to errors in RNA replication such as a copy choice mechanism where the RNA polymerase dissociates and primes else-where on the template during RNA synthesis. A model can be proposed where the deletion occurs during the synthesis of plus strand RNA3 from minus strand RNA templates. The end and the beginning of the minus strand can base-pair to form a steam. This would bring nucleotides 480 and 2592 into direct proximity at the base of the steam and the deletion would be produced by template switching.

Previously we studied the role of the p8 CRP in the PMTV infection and we were able to produce a few mutants of the PMTV RNA3 that do not encode p8. Some of them, like PMTV 25KO also do not produce D-RNA. The accumulation level of all mutant viruses was slightly lower compared to the wild type. Furthermore, those that still produce D-RNA were less accumulated than those that didn't. To separate the effects of CRP and DI abolishing we made an additional mutant with point-non-sense mutations in the beginning of the TGB1 that prevent DI formation. The titer of this mutant virus was higher than that of the wild type. And we clearly saw that DI decreased virus accumulation. When we compared the efficiency of infection of different mutants that do not produce DI or CRP or both we could see that mutants that do not produce neither DI nor CRP had the same titer as the wild type but those that produce DI but not CRP had a lower titer and those that produce CRP but not DI had a higher titer. From these data we can conclude that DI-RNA and CRP act antagonistically in virus infection and that defective RNA of PMTV which interferes with PMTV accumulation means that it is bona-fide DI-RNA.

The next question was: does DI-RNA move long distances. We made a full length cDNA copy of DI-RNA and co-inoculated it with PMTV delta DI mutant. DI-RNA accumulates in inoculated but not in upper non-inoculated leaves. Therefore DI-RNA efficiently replicates and moves cell-to-cell, but does not move systemically. Thus, PMTV DI-RNA is generated *de novo* during virus replication cycles.

DI-RNA encodes an in-frame fusion protein consisting of the N terminus of TGB1 and the C terminus of CRP (DI-Protein). To confirm DIP existence we were made an N tagged mycTGB1 into viral copy of RNA3. Western blot in addition to mycTGB1 dimers and monomers revealed a band corresponding by size to mycDIP. To investigate the role of DIP in virus infection we compared the efficiency of infection of the four component viruses delta DI mutant with DI and delta DI mutants with DI without AUG that was abolished to produce DI protein. Knockout of the DIP ORF resulted in higher virus accumulation in the inoculated leaves. Than the CRP gene of PMTV was inserted into engineered cDNAs of PVX under the control of duplicated subgenomic CP promoter. Wild type PVX caused no visible symptoms on the inoculated leaves and displayed mild mosaic symptoms in the systemically infected leaves. Chimeric virus PVX-DIP showed a different phenotype: necrotic lesions on inoculated leaves and necrotic mosaic on systemic leaves. This phenotype is very similar to those induced by PVX-PMTV CRP

Localized necrotic lesions about 2 mm in diameter first appeared on inoculated leaves at 4 d.p.i. and expanded later to cover a substantial part of the leaf followed by the development of severe necrosis and wilting of the whole upper non-inoculated leaves 9–10 days p.i. DIP was spliced onto TGB1 and CRP derived parts and these parts were inserted into PVX genome and inoculated on *N. benthamiana* in parallel with PVX DIP and PVX wild type, PVX Np1 induces the same symptoms as a wild type PVX, and PVX Cp8 symptoms were very similar to PVX DIP symptoms. Thus CRP derived part of DIP is responsible for the HR induction. CRP is an integral membrane protein associated with ER-derived membranes. Expression of p8 in epidermal cells induced dramatic rearrangements of the ER structure. Localization of DIP is different: DIP localizes in small motile vesicles in cytoplasm. This difference probably explains an opposite role of DIP and p8 on PMTV infection instead of similar influence on the PVX infection.

POWDERY SCAB RESISTANCE: AN ASSESSMENT OF THE HOST GENOTYPE X LOCAL *SPONGOSPORA SUBTERRANEA* POPULATION RELATIONSHIP

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Summary

Despite many years of research, no efficient and economically sound control method is available for powdery scab. A disease management strategy which integrates several components is the best long term approach to powdery scab control and host resistance is an essential component. There is little information about the possibility of host genotype x local pathogen interaction. To test this hypothesis, ten reference cultivars thought to have a range of powdery scab and *Potato mop-top virus* susceptibilities according to previous tests were cropped over four years in 4-8 locations across Europe and disease levels on roots and tubers were assessed using standardised scoring scales. Soil contamination was tested using two different methods (real-time PCR, ELISA). The cultivars performed as expected according to previous characterization, with one exception. No relationship was found between tuber and root susceptibility. Assessment of powdery scab symptoms one month before harvest gave results comparable to those assessed two months after harvest. Neither real-time PCR nor ELISA soil test results were closely related to disease index data. The field trial results indicate that environmental conditions and/or soil inoculum level play a minor role and that there is either no difference between the pathogen populations in each location or that the resistance of most of the cultivars is polygenic.

Introduction

Powdery scab, a potato tuber disease caused by the zoosporic plasmodiophorid pathogen *Spongospora subterranea* f.sp. *subterranea* (*Sss*) is

in some potato production regions one of the most important problems (Merz & Falloon, 2009). *Sss*, as a soil-borne organism, is difficult to control. Efficient chemicals are not available. The pathogen survives in the form of resting spores, or sporosori, which are highly resistant to environmental stresses. Contaminated soils stay infectious for many years. To circumvent these difficulties, an integrated disease management strategy is necessary with host resistance in the focus (de Boer, 2000). Many of the commercially grown cultivars are susceptible to the disease, none has been found to be immune so far (Schwärzel, 2002). Some cultivars show only susceptibility on the tubers, others on the tubers and roots. It appears that resistance of roots and tubers may be under independent genetic control (Merz *et al.*, 2004). The nature of the resistance mechanisms is not known. It seems that powdery scab resistance is under polygenic control as field trials for assessing disease resistance with contaminated soils have shown that there are gradual differences in susceptibility to disease development between the most resistant and most susceptible cultivars and lines (Schwärzel, 2002; Merz & Falloon, 2009).

At the 1st European Powdery Scab Workshop (Gans, 2000), country-specific standard keys to assess powdery scab severity on potato tubers, symptom misidentification (common scab vs powdery scab) and the possibility of the existence of pathotypes of *Sss* (Merz *et al.*, 2004) were identified as a factor causing the discrepancies observed in resistance ratings of cultivars between countries. Missing of disease scoring standards and the existence of obvious resistance rating discrepancies were once again a topic of the Powdery Scab Scoring Workshop, 2002, in La Frètaz, Switzerland (http://www.spongospora.ethz.ch/LaFretaz/index.htm). To identify the main factors causing such discrepancies, a set of ten reference cultivars thought to have a range of susceptibility to powdery scab and mop-top (caused by *Potato mop-top virus*, vectored by *Sss*) according to previous tests (Genet *et al.*, 1995; Falloon *et al.*, 1997; Bus, 2000; Schwärzel, 2002) was selected by the participants. They decided to crop these cultivars at least for three years at four locations and assess levels of tuber and root disease using standard methods.

Materials and Methods

Trials: The 10 selected cultivars (Table 1) were, with some exceptions, cropped in four to six locations across Europe (CH: Wal + (LaF), Scot, Den, Fra and Ger; all known to be contaminated) over four years (2003 – 2006). The basic trial layout was 25 plants/row and five replicated rows/cultivar (total 125 plants per cultivar), applying standard agricultural practices in each country, including irrigation.

Assessment of root disease Root galling was assessed at 3 sites (Wal, LaF, Scot) in all 4 years and in Ger in 2005 and 2006 by digging 5-10 plants per row one month before harvest and estimating galling per plant according to a standard scale (www. spongospora.ethz.ch/LaFretaz/scoringtablegalls.htm): 0 =

no galls; 1 = 1-2 galls; 2 = 3-10 galls; 3 =>10 galls, but most in clusters; 4 = many galls, regularly distributed over the roots. A disease index (Houser & Davidson, 2010) by multiplying disease incidence (mean % roots infected) with mean disease severity (0-4 scale) was calculated per row and cultivar at each location.

Cultivar	Putative resistance level
Agria	Susceptible to powdery scab
Estima	Susceptible to powdery scab
Kennebec	Susceptible to powdery scab
Desiree	Intermediate to powdery scab
Santé	Resistant to powdery scab
Russet Burbank	Resistant to powdery scab
Gladiator	Resistant to powdery scab
Nicola	Intermediate to powdery scab, susceptible to mop-top
Ratte	Susceptible to powdery scab, tolerant to mop-top
Saturna	Susceptible to powdery scab, susceptible to mop-top

Table 1. Resistance categories of 10 potato cultivars to powdery scab on tubers (and mop-top).

Powdery scab assessment Tubers from the remaining plants were all harvested separately per row (five replications), stored and disease assessment was made approximately two months later with washed tubers and individual disease severity scoring with a visual scoring scale (0 to 6; www.spongospora.ethz.ch/LaFretaz/scoringtable.htm). An index similar to that used for root infection was calculated per row and cultivar at each location by multiplying disease incidence (mean % tubers infected) with mean disease severity (0-6 scale) for all cultivars.

Results

Gladiator showed the smallest root disease index, even under high disease pressure situations e.g. in Scotland and ranked as the most resistant cultivar to root galling over all years and locations, closely followed by Santé (Fig. 1). When tuber disease index data were averaged and rank was compared, the powdery scab susceptible (Agria, Estima, Kennebec) and resistant (Gladiator, Santé) cultivars performed as would be expected over all years and locations, with Gladiator being again the most disease resistant (Fig. 1). Only Russet Burbank was an outlier. Most of the cultivars didn't show much relationship between tuber and root disease index, except Gladiator and Santé and Agria.

Discussion

A range of selected potato cultivars have been tested for the first time at different European locations over several years. Although the ten cultivars were cropped in different environments they performed similarly and thus allowed an estimation of their environmental stability. The results suggest the absence of a genotype x location interaction

Fig .1 Potato cultivars ranked according to post-harvest tuber disease indices for all years (2003-2006) and at all locations (Wal, LaF, Scot, Ger, Fra, Den) and compared to ranked mean root gall indices at locations Wal. LaF, Scot and Ger (bars show standard deviation).

due to low genetic variation in the pathogen population. Another explanation could be that the resistance is based on several genes which act as environmental stress buffers. A similar conclusion was made by Genet *et al.* (2005) who found a consistent continuum of susceptibility to powdery scab from very resistant to very susceptible when more than 100 cultivars were tested over several years. Russet Burbank may represent a different genotype that displays an interaction between disease and inoculum level.

The use of a standard scoring scale is strongly recommended allowing the reliable assessment of relative resistance of cultivars (root and tubers) and generating disease resistance scores which are reliably useful for growers in other countries. The consistent performance of Gladiator, introduced in New Zealand in 1995 and known to be highly resistant (Genet *et al.*, 1995). suggests that breeding in one location can produce powdery scab resistant cultivars useful for other countries. Research on the population genetics of *Sss* will show definitively whether there is pathogen variation and whether this relates to phenotypic traits.
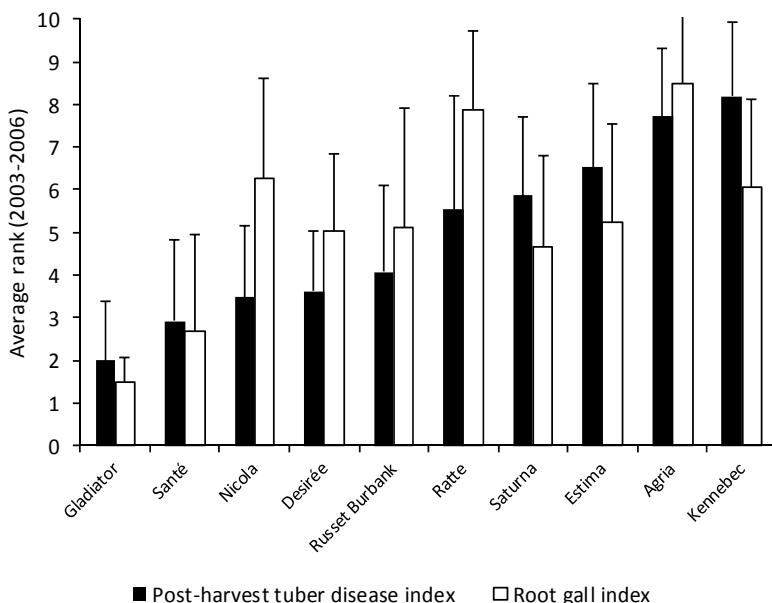
Acknowledgements

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RHIZOMANIA - WHAT IS TO BE LEARNED FROM THE FIELD?

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Summary

Since the widespread use of rhizomania partially resistant cultivars, bearing the Rz1 gene along with high yield capacities, multiple Rz1 resistance-breaking events have been reported both in the USA and in Europe. A single mutation from alanine to valine in the p25 hypervariable amino acid tetrad was linked with such resistance breaking (Koenig *et al.*, 2009). Although there are difficulties for determining the effect of virus variation along with the role of other soil-borne pathogens, inoculum densities as well as questions regarding a possible genetic erosion of the resistance enhancer genes, understanding the dynamic of the emergence of resistance breaking isolates as well as evaluating their fitness and ability to spread is of the uttermost importance.

Between the 2007 and 2010 sugar beet growing season, rhizomania affected fields have been surveyed in France, in the Pithiviers region. More than 600 samples were collected both in disease and non-disease expressing areas. Samples were tested for the presence of BNYVV by RT-PCR targeting mostly the p14, p25 and p26 genes. The multiplex RT-PCR (Meunier *et al.*, 2003) was also used to detect the rhizomania-associated pomoviruses *Beet soil-borne virus* and *Beet virus Q*, together with their vector *Polymyxa betae*. The presence of the *Beet black scorch virus* was also checked in selected samples.

In the frame of a long-term study of rhizomania, field trials were set up in 2009 and 2010 to follow up the evolution of the disease within a single sugar beet growing seasons.

The results confirmed the large presence of BNYVV type B and P in the surveyed areas, with mixed infections in single beets. Conversely to reports from other areas, the canonical p25 A-V mutation was almost not found while the AYHR and SYHG tetrads were frequently detected. The results stressed also the need to set up convergent survey methods to facilitate the exchange of data on viral resistance-breaking isolates.

Introduction

The *Beet necrotic yellow vein virus* (BNYVV) is the causal agent of the rhizomania syndrome and two soil-borne *Pomovirus*, the *Beet soil-borne virus* (BSBV) and the *Beet virus Q* (BVQ), are often associated with (Lindsten, 1993; Prillwitz & Schlösser, 1992; Koenig *et al.*, 1998). These viruses are transmitted by the protist *Polymyxa betae* (Ivanoviç *et al.*, 1983; Tamada & Kusume, 1991; Tamada *et al.*, 1996). *Beet black scorch virus* (BBSV-*Necrovirus*) transmitted by *Olpidium brassicae* appears to exacerbate symptoms produced by BNYVV (Cui *et al.*, 1991).

BNYVV p25 (RNA-3) and p26 (RNA-5) are known to play a major role in the pathogenicity (Koenig *et al.*, 1997). Moreover, BNYVV p14 (RNA-2) seems to be a suppressor of PTGS (post-transcriptional gene silencing) (Dunoyer *et al.*, 2002). Therefore, these three proteins seem to be in high and direct relation with the plant. The use of different types (Rz1-Holly or Rz2-Maritima) and level of plant resistance may induce a selection pressure (McGrann *et al.*, 2009; Lewellen *et al.*, 1987; Lewellen, 1997). The diversity of virus association is reported here in varietal field assays from 10 different localities around the Pithiviers region in France.

Materials and Methods

Total RNA was extracted from homogenized plant material using RNeasy plant mini-kit (Qiagen, Germany). The p25, p26 and p14 coding sequences of BNYVV were amplified by RT-PCR using M-MLV reverse transcriptase and *Gotaq*® polymerase (Promega, USA). Amplicons were purified using MSB® Spin PCRapace Kit (Invitek GmbH, Berlin, Germany) and subsequently sequenced with three pairs of primers, forward and/or reverse, in an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA). Consensus sequences were obtained with ContigExpress and Vector NTI version 7.1 software (Invitrogen, Carlsbad, USA). p25 tetrads from RNA-3 were identified. RNAs of BNYVV, BSBV, BVQ and *P. betae* were also detected as described by Meunier *et al.* (2003). BBSV was detected as described by Weiland *et al.* (2006).

Results

In France, the partial sequencing of BNYVV RNA-3 evidenced different mutation positions between the A-type, B-type and P-type in the p25 amino acid sequence (position amino acid: 21, 23, 28, 30, 32, 42, 49, 67, 68, 69, 70, 118, 129, 132, 163, 179, 192, 198, 213 and 219). Up to 71% of the 600 analyzed samples carried the AYHR tetrad, usually related to the B-type, whereas 25% contained the SYHG tetrad. For the last 4% samples, tetrads were usually

related to the A-type. Only 0.3% of the French samples had the tetrad VCHG, which is often found in the USA.

In the field where the SYHG tetrad was found, the presence of the fifth RNA was demonstrated. We recorded occasionally the P-type outside the Pithiviers area. Concerning all the samples analyzed, 78% where positive for BNYVV, 76% for BSBV and 88% for BVQ. In most of the cases, we also found BSBV and BVQ associated with BNYVV. In several samples we identified also mixed infection between the three different viruses of the rhizomania complex (BNYVV, BSBV and BVQ).The presence of *P. betae* was also identified in all samples. However, we never found BBSV.

According to the different varieties used in the field-assay (Ludwinia, Julietta, Sophia, Python, Bison, Bering, Annouchka, Adriana and Fiorenza), we found a lot of different tetrads (SYHG, AYHR, TYHR, VCHG, AYPR, AFHR, TFPR, AYHG, SYHR, AFPR and AFHG) related either to the A-, B- or P-type. The variety with the highest tetrad diversity was Ludwinia. We also identified type mixed infection in some single sugarbeet roots. More than 11 different tetrads were evidenced within the samples analyzed (Fig. 1).

The variety Ludwinia with the double dominant tolerant gene (Rz1rz1Rz2rz2) showed also a resistance against the BSBV, but not BVQ.

Discussion

It seems clear that the situation between the USA. and France is completely different. Several resistant breaking events have been reported in the USA (Acosta-Leal *et al.,* 2007). Koenig and co-authors (2009) have also identified a single U/C nucleotide substitution changing an alanine to valine in the p25 tetrad domain and allowing increased virus accumulation in roots as well as the emergence of aggressive resistance-breaking strains, hence indicating that the switch from the amino acid tetrad ACHG to VCHG was linked to the resistance breaking. According to the data from our surveys in France, eight to ten different mutations were recorded on the whole p25 sequence between the different strains. Nevertheless, only two tetrads were found in the majority of the fields: AYHR usually linked to the B-type and SYHG usually linked to the P-type. The tetrad VCHG was never encountered, except for a peculiar and single red beet sample. Such situation is raising the question whether other single mutations along the BNYVV genome or virus types association might be responsible for the rhizomania problems observed in the South of Paris, in France. The occurence of BNYVV P-type in fields with strong rhizomania symptoms is higher than the one of B-type. Besides these two types, the presence of BNYVV A-type was also recorded, but to a lesser extend (only 4% of the samples).

The discovery of the BNYVV P-type outside the Pithiviers area is also raising the question of the fitness of such pathotype: is it able to spread easily, knowing that it has been recorded since more than 20 years in France ?
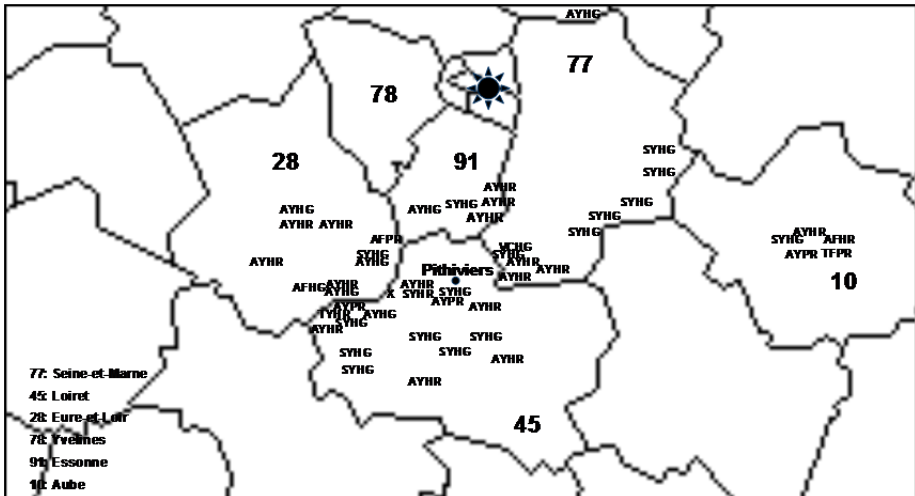

Figure 1: Tetrad diversity around the region of Pithiviers (Galein Y., 2011)

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SBCMV-RESISTANCE IN DURUM WHEAT: RESULTS OF A SIX-PARENT DIALLEL CROSS.

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Summary

The F_1s resulting from a six-parent durum wheat diallel cross were grown along with their parents in a naturally SBCMV-infected field near Bologna, and evaluated for SBCMV-resistance on the basis of symptom severity and DAS-ELISA value. SBCMV-resistance was inherited as an incompletely recessive trait. Results, moreover, suggested that the three resistant parental cvs. carry a fully recessive major SBCMV-resistance gene or gene-block, and that the six parental cvs carry prevalently dominant SBCMV-resistance modifiers.

Introduction

In 2005, following the results of sequence and alignments' analyses (Diao *et al.*, 1999; Yang *et al.*, 2001; Koenig & Huth, 2003), the *Soil-borne mosaic virus* isolates prevalent in North America, Europe and Far East Asia were subdivided into three distinct species within the *Furovirus* genus (Torrance & Koenig, 2005) denominated, respectively, *Soil-borne wheat mosaic virus* (SBWMV), *Soil-borne cereal mosaic virus* (SBCMV) and *Chinese wheat mosaic virus* (CWMV).

In common wheat (*T. aestivum* L) resistance to SBWMV and to CWMV have been reported to be governed either by 1-2 dominant genes (Miyake, 1938; Dubey *et al.*, 1970; Modawi *et al.*, 1982; Merkle & Smith, 1983; Barbosa *et al.*, 2001), by an incompletely dominant gene plus a modifier (Shaalan *et al.*, 1966), or by two recessive genes plus an inhibor with epistatic effects on the first two (Nakagawa *et al.*, 1958 and 1959). In durum wheat (*Triticum durum* Desf.) SBCMV-resistance was found to be controlled by major genes and by a plethora of genes with small effects (Ratti *et al.*, 2006; Vallega *et al.*,2006; Rubies *et al.*, 2008; Maccaferri *et al.*, 2011; Russo *et al.*, 2009 and 2011)**.** Interestingly, pedigree analysis revealed that a high proportion of the cvs. found

resistant to SBCMV in Italy derive from "Edmore" (Ratti *et al.*, 2006), an SBCMV-resistant cultivar bred in North Dakota (USA).

Two major Quantitative Trait Loci (QTLs) for response to *Soil-borne mosaic viruses* have been identified in common wheat: *Sbm1*, effective against both SBWMV in the U.S. (Narasimhamoorthy *et al.,* 2006; Hall *et al.,* 2009) and SBCMV in Europe (Bass *et al.,* 2006; Perovic *et al.,*2009), and *Sbm2* (located in the distal telomeric region of chromosome 2BS), effective against SBCMV in Europe (Bayles *et al.,* 2007). Major QTLs for response to SBCMV seemingly homologous to the *Sbm2* QTL detected in common wheat have been identified in durum wheat "Meridiano" (Maccaferri *et al.*, 2011) as well as in "Neodur" (Russo *et al.*, 2011), a cv. derived from "Edmore".

Casual observations by the senior author on durum wheat F_1 plants grown near Rome for general breeding purposes suggested that some durum wheat cvs. carry either recessive or co-dominant SBCMV-resistance genes. In particular, F_1 plants from crosses between SBCMV-resistant derivatives of cv. Edmore and susceptible cvs. showed severe symptoms. A six-parent diallel cross without reciprocals was set up to verify these observations.

Materials and Methods

The parents included cvs. Ionio (resistant = R), Neodur (R), Duilio (moderately resistant = MR), Cirillo (susceptible = S), Valnova (S) and Simeto (moderately susceptible = MS). Cultivars Neodur and Ionio, both derived from cv. Edmore, are known to carry a major SBCMV-resistance QTL on 2BS (Russo *et al.*, 2009 and 2011). Cultivar Duilio too carries a major QTL on 2BS (Maccaferri *et al.*, 2011), possibly the same as cvs. Neodur and Ionio. Based on their response in varietal trials (Rubies-Autonell *et al.,* 2008) and on the results of genetic and molecular marker studies, all the cvs. intercrossed carry minor resistance genes. The six parental cvs. were grown during 2008-2009 in a field free of SBCMV near Foggia, and intercrossed in all combinations excluding reciprocals. In the following season the resulting 15 F_1s and their parents were seeded October 29 in a field with SBCMV near Bologna in plots consisting of single 1.5m long rows, distributed according to a randomized-block design with three replicates. Twenty seeds were sown in each row. Symptom-severity (SS) was rated April 7 on a 0-4 scale (Vallega & Rubies-Autonell, 1985). DAS-ELISA was performed as reported previously (Ratti *et al.,* 2006) on extracts from a bulk of the basal portions of the two youngest fully expanded leaves collected April 9 from ten plants per plot.

Results and Discussion

SBCMV-pressure was severe, as testified by the high mean SS score recorded for the susceptible parents (Table 1). ELISA values and SS scores were significantly correlated (r = 0.887; P 0.001). The nine F_1s derived from

crosses between resistant and susceptible parents manifested a clear susceptible reaction in terms of SS (mean = 3.0; range = 2.5 - 3.4), in all cases significantly higher than that recorded for any of the three resistant parents (mean = 0.8; range = 0.6 – 1.0). The noticeable difference (2.16) between the mean SS recorded for the RxS F_1s and for the three resistant parents closely corresponds to the effect estimated for the major SBCMV-resistance QTLs identified in studies performed on RILs derived from the durum wheat crosses "Meridiano x Claudio" and "Neodur x Cirillo" (Maccaferri *et al.*, 2011; Russo *et al.*, 2009 and 2011). ELISA values for the nine RxS F_1s (mean ELISA = 0.87; range = 0.70 - 1.12) too were much closer to those recorded for the susceptible parents (mean ELISA = 1.09; range = 1.06 - 1.15) than for the resistant ones (mean ELISA = 0.18; range = 0.03 - 0.47). On the other hand, the nine RxS F_1s showed a somewhat greater degree of SBCMV-resistance than the susceptible parents both in terms of SS and ELISA value.

Table 1. Mean SBCMV symptom score (on a 0-4 scale) and mean ELISA value for parents and F_1 hybrids of a six-parent diallel cross between durum wheat cultivars.

Genotypic groups	Symptom score (April 7)		DAS-ELISA value (April 9)	
Resistant parents (3)	0.79	c*	0.178	b
RxR F1s (3)	0.48	c	0.344	b
RxS F1s (9)	2.95	b	0.872	a
SxS F1s (3)	3.58	a	1.011	a
Susceptible parents (3)	3.66	a	1.094	a

* Within columns, means followed by the same letters are not significantly different (P= 0.05) according to Duncan's multiple range test.

Based on the above results we concluded that: a) in the cvs. examined SBCMV-resistance was inherited as an incompletely recessive trait; b) the parental durum wheat cvs. Ionio, Neodur and Duilio carry a fully recessive major SBCMV-resistance gene or gene-block; and c) the SBCMV-resistance modifiers contributed by the parental cvs. were prevalently dominant. Given the close affinity between durum and common wheat as well as between SBWMV, SBCMV and CWMV, the results we obtained for SBCMV in durum wheat are difficult to reconcile with the dominance generally reported for SBWMV and CWMV in common wheat. In this respect, however, it should be noted that all such reports, based solely on symptomatology, were exceedingly vague in regard to the phenotyping criteria adopted and to the disease pressure encountered. Moreover, a number of them contain gross errors: one research group, for instance, concluded that SBWMV-resistance was inherited as a dominant character (Dubey *et al.,* 1971) despite reporting 1R:1S ratios for all

backcross families from (RxS) x R crosses and 0R:1S ratios for all backcross families from (RxS) x S crosses.

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IRANIAN DIVERSITY OF *BEET BLACK SCORCH VIRUS* AND SATELLITE

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Summary

Over 200 BNYVV infested iranian soils have been investigated for the presence of *Beet black scorch virus* and its satellite, using soil bioassays and RT-PCR. Such survey allowed to evidence up to 61 different Iranian BBSB sources, a large number of which were associated with the satellite. To our knowledge, this is the first large survey for this soil-borne necrovirus species. Such approach allowed to perceive the diversity of BBSV over a large territory like Iran, as well as allowing to find new virus & satellite sources. Full length clones of BBSV were generated by simply amplifying the viral genome before cloning into the pDrive vector (Qiagen) before generating infectious RNA transcripts. Six different BBSV and four BBSV satellite sources have been fully sequenced and compared with respectively, the four virus genomes and three satellites already available.

Introduction

Beet black scorch virus (BBSV) is a *Necrovirus* of the *Tombusviridae* family (Van Regenmortel *et al.*, 2000), described as causing severe, systemic disease symptoms of black scorched leaves and severe stunting in sugar beet (*Beta vulgaris* L.) in China (Yuan *et al.*, 2006; Weiland *et al.*, 2007). The virus is transmitted efficiently through the soil in a non-persistent manner by zoospores of the chytrid *Olpidium brassicae* (Jiang, 1999). It was first identified in Inner Mongolia, China, in the late 1980s (Cui, 1988) and was recently reported from the USA (Weiland *et al.*, 2007), Iran (Koenig & Valizadeh, 2008) and Europe (Gonzàles-Vàzquez, 2009) where it did not show black scorching on the leaves, but bore exacerbated symptoms of rhizomania, such as rootlet proliferation (Weiland *et al.*, 2007; Gonzàles-Vàzquez, 2009). Symptomless infections have also been observed in sugar beet (Weiland *et al.*, 2007). The virus can be detected in the host plants by ELISA, RT-PCR and by retro-inoculation to *Chenopodium quinoa* (Yuan *et al.*, 2006).

BBSV shares the highest sequence identity (61%) with *Tobacco necrosis virus* D (TNV-D) (Coutts *et al.,* 1991). Complete sequences of one Chinese (BBSV-N) and one US (BBSV-CO) BBSV isolate comprise seven and eight open reading frames (ORFs), respectively (Yuan *et al.,* 2006; Weiland *et al.,* 2007). The *P23* and *P82* ORFs encode for the putative viral RNA polymerase. The functions of the small *P5*, *P7a* and *P7b* ORFs located in the central region of the BBSV RNA genome were identified by Yuan *et al.* (2006): they are required for localized movement, accumulation of viral RNAs and formation of local lesions on the leaves of *C. amaranticolor*. An ORF encoding for a 24 KDa protein is predicted to encode the coat protein.

A small single-stranded RNA (ssRNA), similar to the satellite RNAs (sat-RNA) of other necroviruses, was found associated with Chinese isolates (Guo *et al.,* 2005). The sat-RNA of BBSV belongs to the small linear satellite RNA subgroup, which contains several subviral RNAs associated with helper viruses in the *Tombusviridae* family. This small RNA multiplied in test plants only when associated with BBSV genomic RNA, either from the same viral isolate or from other sources of BBSV (Guo *et al.,* 2005).

The objective of this work was to assess the presence of BBSV and its satellite over a large area such as Iran, close to where BBSV was first identified by Cui *et al.* (1988) in China, in order to gain a better understanding of the virus diversity and its potential risk in areas where sugar beet is cultivated extensively. It was also to verify the feasibility of full length clones using Iranian virus sources, as already done for the Chinese and American BBSV sources (Weiland *et al.*, 2007).

Materials and Methods

For trapping the virus, the bait plant technique used by Meunier *et al.* (2003) was used. The soil was filtered to discard all stones and visible prior grinding. Uninfected seedlings were grown under the same conditions in sand-sterile soil mixture as a control.

Plants rootlets were washed and ground in 0.01 M sodium phosphate buffer, pH 7.0. The extract inoculated in upper side of *C. quinoa*, *B. macrocarpa* and sugar beet leaves mechanically. Test of host range performed by transfer of virus from infected *C. quinoa* to the leaves of healthy test plants using mechanical inoculation as aforementioned. Presence of virus in test plants was confirmed by back inoculation to *C. quinoa*. Rootlets from infected sugar beet and single chlorotic lesion of *C. quinoa* leaves were used for RNA extraction using a SV Total RNA Extraction Kit (Promega).

For the detection of BBSV, primers BBSV1F and BBSV1R, which amplify a fragment of 315 bp within the 3' UTR of BBSV RNA were used (Weiland *et al.*, 2007). Sequences were analyzed by the Clustal W program of the Genetic Computer Group.

Total RNA was extracted from healthy and infected *C. quinoa* and sugar beet roots. Infectivity of the RNA extracted from virus preparation and of transcript RNA was tested by suspending 2 to 5 μg of RNA in GKP buffer (50 Mm glycine, 30 mM K2HPO4, 1% bentonite, and celite, pH 9.2) and mechanically inoculated on leaves of *C. quinoa*, *Nicotiana benthamiana*, *Beta macrocarpa* and sugar beet (*Beta vulgaris*) (Weiland & Edwards, 1994).

The Expand Reverse Transcriptase kit (Roche), which includes a high-fidelity, thermo-stable polymerase, was used to generate a complete cDNA fragments from the virion RNA. Primer designated BBT75fwd and BBSV3rev (Weiland *et al.*, 2007) were used for the amplification of complete BBSV genome-length amplicon using the Expand Long Template PCR System kit (Roche). The amplicon was cloned into pDrive vector (Qiagen, Germany). EcoR1 digestion of clones liberated the clone inserted from the plasmid vector, providing a template from which transcription would initiate one G nucleotide before the viral sequence.

Plasmid pBBSV-Ha1, pBBSV-Ha2, pBBSV-Msh1, pBBSV-AzGh1, pBBSV-Ksh1 and pBBSV-Sh1 were sequenced using primers positioned at an average of 400-bp intervals and the sequences assembled using vector NTI Advance 10 program (Invitrogen) & the ORF finder program (NCBI). Sequence alignments were performed using Clustal W (Thompson *et al.*, 1994) and Geneious (Drumond *et al.*, 2010)

Transcription reaction with T7 RNA polymerase for generating uncapped RNA was used to produce genome-length RNA for inoculation to plant leaves. Released construct was transcribed by transcription T7 kit (Promega). *In vitro* transcripts were inoculated on *C. quinoa* and *N. benthamiana* leaves.

Results and Discussion

A total of 62 BBSV different sources from all the sugar beet growing areas were collected, altogether with 19 BBSV satellites (Mehrvar & Bragard, 2008). From these sources, six where chosen based on their different geographical origins as well as on the divergence of their 3'UTR sequence, for full length sequencing. The sequence obtained were compared to already available genomes, the Iranian BBSV sequenced by Koenig & Valizadeh (2008), two Chinese BBSV as well a US one. The general genome organization is in general similar to that reported for the previous Iranian isolate, with a small putative ORF for a *ca* 5 KDa protein located downstream the coat protein gene, except for the three BBSV sources from Hamadan and Kermanshah. In a general manner, the differences were marked between BBSV from distant geographical origins. In Figure 1, Chinese BBSV appear clustered together, not far from the US strain. Interestingly, the Iranian strains clustered in two separate groups corresponding to their different genome organisation. No clear link between the geographical origin in Iran and the different clusters obtained by

genome comparison was possible, nor with the association with the virus satellite, found in almost all Iranian regions.
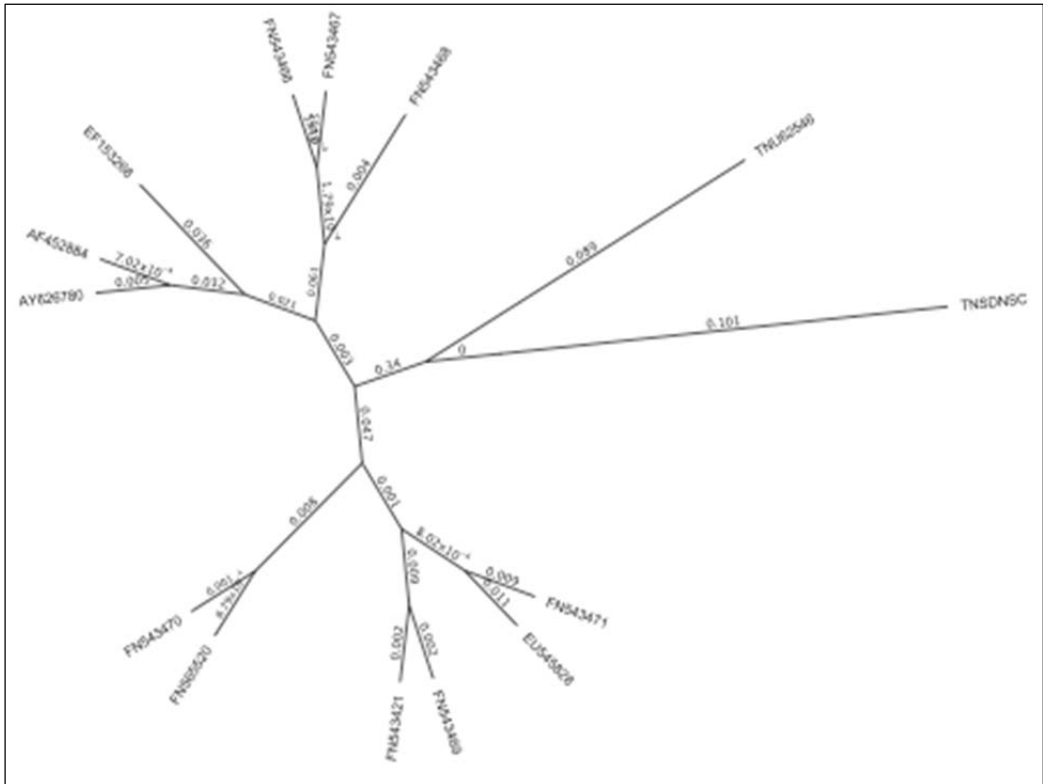

Figure 1: Distance tree generated by aligning the BBSV genomes with the algorithm Muscle, (Geneious - Drumond *et al.*, 2011), tree building, Juke-Kantor genetic distance, the neighbour joining and *Tobacco necrosis virus D* (TNSDNSC) as outgroup. Note the separate clusters formed by 1) the Chinese BBSV (AY626780 – AF452884) along with the US Colorado BBSV (EF153268), and two separate clusters of Iranian BBSV sources: a first group from Hamadan and Kermanshah (west Iran) (FN543466 to 68), and two sub-groups, one from Hamadan & Mashad (west and north east) (FN543470 – FN565520), and another from Azarbaeijan Gharbi, Kermanshah and Khorasan Shomali (west) (FN543421-FN543469-EU545828 - FN543471).

It was possible to generate infectious full length clones of Iranian BBSV, able to reproduce typical symptoms on *Nicotiana benthamiana* or *Chenopodium quinoa*.

The Iranian BBSV satellite, if they were detected in approximately one sample over five, did not show high level of variation (Fig. 2). Instead, they appeared quite different from their Chinese counterpart, with more than 100 different mutations along the satellite genome, comprising between 615 and 617 nucleotides. Interestingly, the presence of a 50 bp nucleotide stretch lying within the discovered satellites, homolog to an Argonaute region of *Pisum sativum,* was discovered. By sequencing similar areas of AGOI from *Beta vulgaris* or *Chenopodium quinoa*, it was possible to show that such sequences are even

more related to the satellite than the previously used one. Though it is tempting to speculate about a role of such a sequence for suppressing the gene silencing by targeting the core of the system itself, such a hypothesis still needs to be verified.
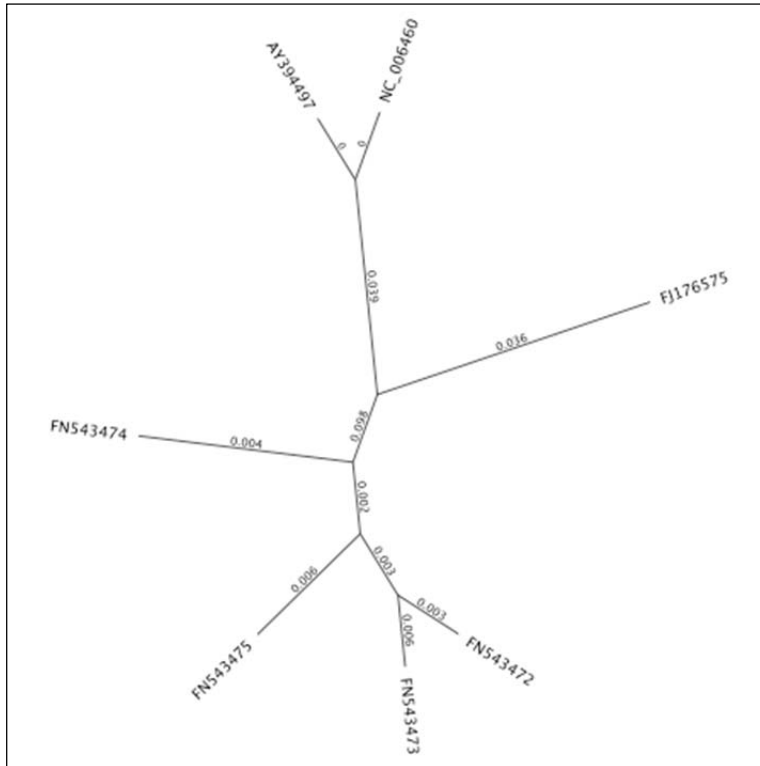

Figure 2: Distance tree generated by aligning the BBSV satellite genomes with the algorithm Muscle, (Geneious - Drumond *et al.*, 2011), tree building, Juke-Kantor genetic distance, no outgroup. Chinese BBSV satellites NC_06460, FJ176575, AY394497, Khorasan Shomali (FN543475), Khorasan Razavi (FN543474), Mashad (FN543473), Hamadan (FN543472).

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FUTURE CHALLENGES IN VIRUS TRANSMISSION BY *POLYMYXA*

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Summary

The incidence of phytoviruses transmitted by *Polymyxa graminis* and *P. betae* remains economically important in major crops such as sugar beet and cereals. Over the past 20 years, the use of varieties with resistance to *Beet necrotic yellow vein virus* or to cereal mosaic viruses such as *Barley yellow mosaic virus* or *Soil-borne cereal mosaic virus* has been the only way to achieve profitable yields on highly rhizomania- or mosaic-infested soils, respectively. The effectiveness of this strategy is limited, however, by the occurrence of resistance-breaking viral isolates or the absence of resistance in some plant species. In the context of integrated pest management, the development of control strategies against the vector would be useful, but this requires better knowledge of the soil ecology and an understanding of the molecular interaction between the vector *Polymyxa* and the host plant, as well as between *Polymyxa* and the virus it transmits. Recent studies have sought to develop new models that overcome the difficulties associated with the specific parasitism of *Polymyxa* spp. We describe here how the recent identification of a compatible *Arabidopsis thaliana*-*P. betae* interaction and the development of a dual *in vitro* culture system of *P. betae* on sugar hairy roots could help to improve knowledge in this area. The new approaches for investigating the virus acquisition and transmission processes by *Polymyxa* under controlled conditions, and for evaluating the specificity of the interaction between *Polymyxa* spp. and f.spp. isolates and the viruses vectored by them, are presented from the perspective of a risk assessment of the increased prevalence of viruses and vectors associated with distinct pathosystems.

Introduction

The transmission of viruses by *Polymyxa* confers particular epidemiological characteristics on the diseases caused by these viruses, such as dissemination through soil and very long survival in infested soils. Until now, genetic resistance against viral particles has been the only efficient way to control the diseases caused by these viruses. A strategy targeting the vector would limit the inoculum potential in the soil. Genetic resistance against the vector has been analyzed (Legrève *et al.*, 2000; McGrann *et al.*, 2007 and 2009), but the difficulty of growing the vector and the differences in host ranges between *Polymyxa*

populations has complicated these studies, and this strategy has therefore not been fully developed. The development of new strategies to control viral diseases transmitted by *Polymyxa* spp. requires a better understanding of the molecular interaction between the main actors in the pathosystems – i.e., the plant, the virus and the vector. Since *Polymyxa* is an obligate root endoparasite, studies are complicated by the fact that the genome of the vectors has been only partially described and by the difficulty of growing it in an axenic medium. In addition, the complete genome of major host plants (e.g., sugar beet, peanut, wheat and barley) is not yet known. New models have been developed to overcome these constraints, two of which are described here: a compatible interaction between *P. betae* and *A. thaliana*; and a dual culture system for *P. betae* in sugar beet hairy roots. The first model was developed in order to provide a useful tool for understanding the molecular interaction between *P. betae* and the host plant. Knowledge of the complete genome of *A. thaliana* enables the mechanisms involved in the interaction to be understood and contributes to knowledge about the *Polymyxa* genome. The second model was developed to enable *Polymyxa* in roots to be multiplied in an *in vitro* system without any other organism or root contaminant.

Another important issue regarding the epidemiology of viral diseases transmitted by *Polymyxa* relates to the specificity of virus transmission by *Polymyxa* spp. More than 17 viruses belonging to bymy-, furo-, peclu- and pomoviruses are transmitted by *Polymyxa* species. Several of them can occur in a same plant (e.g., *Beet necrotic yellow vein virus* [BNYVV], *Beet soil-borne virus* and *Beet virus Q*), and some soils are infested by species involved in several pathosystems (e.g., P. *graminis* f.sp. *temperata*, *P. graminis* f.sp. *tepida*, *Barley yellow mosaic virus* [BaYMV] and *Barley mild mosaic virus* [Vaianopoulos *et al*., 2007]*, and P. graminis* f.sp. *temperata* and *P. betae* associated or not with BaYMV or BNYVV, respectively). Given the host specificity of some of these species, some virus-vector interactions are unlikely. For example, the interaction between *Soil borne cereal mosaic virus* and *P. betae* is unlikely because the first infects cereals and the second, mainly, *Chenopodiaceae*. The recovery of the host ranges of other species, however, allows the question of the specificity of viral transmission by vector populations to be considered. The acquisition and transmission of *Peanut clump virus* (PCV) by different *formae speciales* of *P. graminis* on cereals has been studied under controlled conditions, using sugar cane as the common host for virus and vector (Dieryck *et al*., 2011). This experiment demonstrated the role of *P. graminis* f.sp. *tropicalis* and *subtropicalis* zoospores in PCV transmission and clarified the issue of the specificity between virus, *Polymyxa* and plant species.

Materials and Methods

Detailed information on the materials and methods used in the studies described here is provided by Desoignies and Legrève (2011), Desoignies *et al.*

(2011) and Dieryck *et al.* (2011).

Results and Discussion

Identification of a compatible *Arabidopsis thaliana*-*P. betae* interaction:
    The potential of infection of 14 *A. thaliana* accessions by *P. betae* was evaluated by testing the capacity of the zoospores released from sugar beet to infect and parasitise the roots (Desoignies *et al.*, 2011). *Polymyxa betae* infection occurred in all but one accession (Ita-0 from Morocco), but there were differences in the level of infection, depending on the accession. The greatest infection was observed in the ecotype Cvi-0 (N1096) from Cape Verde Island. In this accession, some plasmodia of the protist were detected in the roots 21 days after inoculation; 12 and 24 days later the same structures, and sporosori, were visible in the roots. In the accessions Cvi-0 (N902) from Cape Verde Islands, Bla-3 from Spain, Bs-1 from Switzerland, Kas-1 from India, Gr-1 from Austria and Tu-1 from Italia, *P. betae* plasmodia and sporosori were also detected, but the infection level was lower than in Cvi-0 (N902). In the other tested accessions (Bd-0 from Germany, Co-2 from Portugal, Col-0 from USA, Kil-0 from UK, Mh-0 from Poland and Van-0 from Canada), the infection was slight and sporadic. Although *P. betae* infection was observed in all but one *A. thaliana* tested accession, the infection level was lower than in the *P. betae-Beta vulgaris* interaction, and the sporogenic phase of the life cycle was favored over the sporangial phase. In addition, the phenotype of *P. betae* in *A. thaliana* differed from the one in *B. vulgaris*; the dimensions of the plasmodia were smaller and the resting spores were not grouped, or only a few spores were grouped. This phenotype could be related to the host cell. The compatible interaction between *P. betae* and *A. thaliana* has also recently been reported in a study by Smith *et al.* (2011). These authors described the infection of *A. thaliana* by *Polymyxa* spp. after growth on *Polymyxa*-infested soils. The compatible interactions highlighted in two independent studies offers new possibilities for studies on plant-parasite interactions.

Dual *in vitro* culture system of *P. betae* on sugar beet hairy roots:
    A dual *in vitro* culture system of *P. betae* on sugar beet hairy roots was developed by inoculating fragments of the roots transformed by *Agrobacterium rhizogenes* with *P. betae* zoospores and transplanting the inoculated fragments on Gamborg B5 liquid medium with sucrose (Desoignies & Legrève, 2011). The zoosporangia-infected roots, produced in the automatic immersion device classically used to culture *P. betae* (Legrève *et al.,* 1998), were disinfected in Ca(ClO)$_2$ solution and used to inoculate sugar beet hairy roots. After 24 h incubation in the presence of inoculum, the roots were transplanted into the liquid medium and incubated in the dark at 18-23°C under sterile conditions. Ten weeks after incubation, *P. betae* was detected by microscopy and specific PCR in half of the inoculated roots. Typical *P. betae* plasmodia and sporosori

were observed and the severity of infection in parts of roots after inoculation revealed the occurrence of a secondary infection in the system. This new *in vitro* culture system will be useful for multiplying and conserving *P. betae* strains/isolates. The production of an axenic inoculum of *P. betae* will also be useful for the molecular characterization of the species.

Study on the specificity of the transmission of viruses by *Polymyxa* spp.:

A new way of studying PCV transmission by the protist vector *P. graminis* on cereal under controlled conditions was developed. Using sugar cane as common host for this virus and distinct *formae speciales* of the vector, viruliferous zoospores of *P. graminis* f.sp. *temperata, tropicalis* and *subtropicalis* were produced in systemically PCV-infected plants grown in automatic immersion devices. The same numbers of viruliferous zoospores of each special form were then inoculated in distinct cereal species. Effective PCV transmission by *P. graminis* f.sp. *tropicalis* occurred in pearl millet (44%), barley (13%), sorghum (4%) and wheat (6%), and by *P. graminis* f.sp. *subtropicalis* in barley (6%) and wheat (10%), but not in pearl millet and sorghum. No PCV transmission by *P. graminis* f.sp. *temperata* was observed, although PCV had been detected by RT-PCR in the zoospore suspensions released from PCV-infected sugar cane and used to inoculate cereal plants. The experiment therefore demonstrated the role of *P. graminis* f.sp. *tropicalis* and *subtropicalis* zoospores in PCV transmission. It also demonstrated differences in transmission efficiency depending on the *P. graminis formae speciales*, highlighting the specificity of the interaction between the virus and its vector. Although molecular interaction between PCV and *P. graminis* has not yet been identified, a co-evolution mechanism has been hypothesized to explain the specificity of the PCV-*P. graminis* interaction based on the origin of viral and vector isolates used in this study. The absence of PCV transmission by *P. graminis* f.sp. *temperata*, despite the virus being detected in the zoospores suspension, suggests that the mechanism involved in the acquisition of the virus by the vector could differ from that involved in the release of the virus by the vector after the infection. The molecular characterization of such interaction will clarify such mechanisms.

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ESSENTIAL ROLE OF BSBMV RNA4'S P32 ON VIRUS TRANSMISSION BY *POLYMYXA BETAE* IN *BETA VULGARIS* PLANTS.

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Summary

The *Benyvirus* genus ascribes both *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV), vectored by the plasmodiophorid *Polymyxa betae*. We recently reported the identification of new BSBMV RNA4 form. In this study, we demonstrate that BSBMV p32 expression is essential for virus transmission by *P. betae* in *Beta vulgaris* plants. This protein has been molecularly and functionally characterized.

Introduction

Beet soil-borne mosaic virus (BSBMV) is a member of *Benyvirus* genus together with *Beet necrotic yellow vein virus* (BNYVV), both vectored by *Polymyxa betae* (Gilmer & Ratti, in press). BSBMV was first identified in Texas as a sugar beet virus similar to BNYVV but serologically distinct (Heidel *et al.*, 1997) and, up to date, is only widely distributed in the United States. Subsequent sequence analysis of BSBMV RNAs showed similar genomic organization to that of BNYVV but sufficient molecular differences to distinct BSBMV and BNYVV into different species (Lee *et al.*, 2001). Recent data evidence the ability BNYVV helper strain (RNA-1 and -2) to replicate and encapsidate BSBMV RNA-3 and RNA-4 (Ratti *et al.*, 2009; D'Alonzo *et al.*, 2008) confirming a strong correlation between this two virus and suggesting a common and conserved viral RNA selection mechanism for both species.

Benyvirus field isolates usually contain four genomic RNAs but some BNYVV isolates contain a fifth genomic RNA. The described BSBMV genome consists of four RNAs capped at the 5' end and polyadenylated at the 3' end. RNA-1 and -2 are, respectively, 6,683 and 4,615 nucleotides in length and contain putative ORFs similar to which identified on BNYVV RNA-1 and 2. The 1,720 nt BSBMV RNA-3 has a 29 kDa ORF that shares 23% amino acid sequence identity with the 25 kDa ORF of BNYVV RNA-3. The role of BSBMV

RNA-3 on symptoms determination on *Chenopodium quinoa* plants has been recently described (Ratti *et al.*, 2009). Lee and co-authors (2001) described a single putative ORF on the 1,203 nts BSBMV RNA-4 that encodes a putative protein with a predicted mass (13 kDa) considerably smaller than the BNYVV RNA-4 31 kDa product. We recently identify and described a new form of BSBMV RNA-4, length 1,730 (GenBank accession number: FJ424610) with two putative ORF: 32 kDa (nucleotides 383 to 1,234) and 13 kDa (nucleotides 885 to 1,244) (D'Alonzo *et al.*, 2008). BNYVV RNA-4 is essential for efficient transmission of the virus by the plasmodiophorid vector (Tamada & Abe, 1989), moreover encodes p31 protein that is required for efficient vector transmission and is also involved in enhanced symptom expression in host-specific manner on *Nicotiana benthamiana* (Rahim *et al.*, 2007). We already demonstrated that full length BSBMV RNA-4 form promote BNYVV RNA-1, 2 and 3 transmission through the vector *P. betae* in *B. vulgaris* plants (D'Alonzo *et al.*, 2008), here we report our recent investigations on the role of the BSBMV RNA-4 encoded protein in plant-virus-vector interaction.

Materials and Methods

Full-length cDNA infectious clone of BSBMV RNA-4 (pUC47, FJ424610) constituted the base from which many clones have been produced to investigate putative ORF encoding 32 kDa protein (p32) expressed fused to Flag epitope tag (DYKDDDDK) at N-terminal. Different PCR reactions were performed using specific primer pairs carrying *Nco*I and *Bam*HI restriction sites added on forward or reverse primers, respectively, to facilitate cloning procedure of BSBMV RNA-4 p32 or BNYVV RNA-4 p31 sequences into Rep3 or Rep5 viral vector (Schmidlin *et al.*, 2005) to obtain Rep3-FlagP32, Rep3-FlagP31 or Rep5-P32 and Rep5-P31.

Deleted forms of the p32 coding sequence have been produced as shown in Fig. 1. All the sequence have been tagged with the Flag epitope at their N-terminal extremity. The lengths of the six deletion mutants were 588 bp (P32 Δ1; 22 kDa), 381 bp (P32 Δ2; 14 kDa), 192 bp (P32 Δ3; 7 kDa), 639 bp (P32 Δ4; 25 kDa), 405 bp (P32 Δ5; 16 kDa), 378 bp (P32 Δ6; 15 kDa). Series of mutants were generated by PCR mutagenesis to obtain the clone Rep3-FSp3 in which the p32 ORF is disrupted by frame shift mutations. Moreover, PCR site-directed mutagenesis were performed to mutate predicted post-translational modification sites [18]NVTG[21] or [69]NVSV[72] (glycosylation) and [39]GVLCNI[44] (myristoylation) on the P32 protein sequence. PCR products, carrying FLAG sequences at N-terminal part, with single or double mutation were digested and cloned into Rep3 viral vector. The clones are presented in Table 1.

Full length and deleted forms of p32 sequence described in Fig. 1 were cloned into pGAD424 and pGBT9 (Clontech) plasmids to tested transcription activation and self-interaction by using the matchmaker yeast two-hybrid system as previously described (Link *et al.*, 2005).
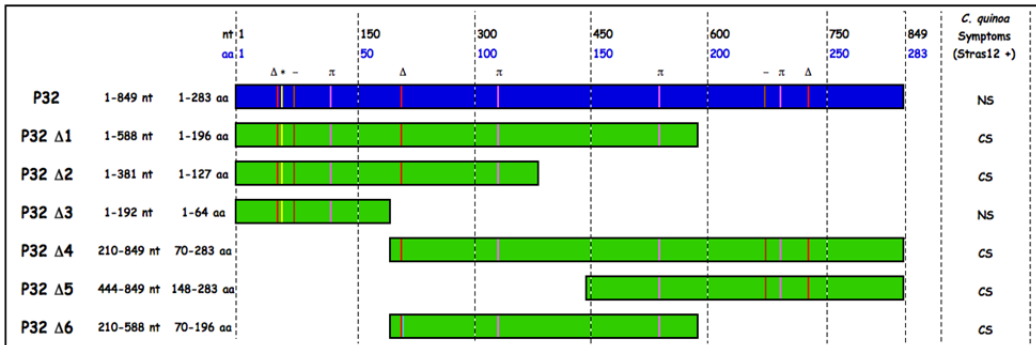
Figure 1. Deleted forms of P32 protein and symptoms observed on *C. quinoa* leaves. Blue bar represents full length, wild type P32; Green bars represent deleted forms tagged with Flag at N-terminal. NS necrotic spots; CS chlorotic spots. Prosite motifs are shown: Δ N-glycosylation site (red line); * Casein kinase phosphorylation site (yellow line); - Protein kinase C phosphorylation (brown line); π Myristoylation site (purple line).

Clone	Construction	Mutation
MD150	Rep3-p32 N18G	$N^{18}G \Rightarrow M1$
MD151	Rep3-p32 N68G	$N^{69}G \Rightarrow M3$
MD157	Rep3-p32 N18G N69G	$N^{18}G, N^{69}G \Rightarrow M1, M3$
MD168	Rep3-p32 G37V-G39V	$G^{37}V, G^{39}V \Rightarrow M2$
MD169	Rep3-p32 N18G-G37V-G39V	$N^{18}G, G^{37}V, G^{39}V \Rightarrow M1, M2$

Table 1. P32 mutated sequences expressed in Rep3

Transient expression, based on pBIN61 plasmid (Voinnet *et al*., 2000); (Dunoyer *et al.*, 2002), were produced to drive the expression of full-length or mutated forms of p32 protein fused to GFP at N- or C-terminals. Plasmids were transformed into *Agrobacterium tumefaciens* and subsequently co-agroinfiltrated with the p19 gene silencing suppressor from *Tomato bushy stunt virus* (TBSV) into *N. benthamiana* leaves.

Results and Discussion

Sequence alignment of BSBMV p32 and BNYVV p31 proteins evidenced amino acids identity of 49.8% and similarity of 68.4% demonstrating that p32 is much closer to p31 than to BSBM p13 described by Lee *et al.* (2001). Moreover, using Prosite motif search (htt://expasy.org/prosite) we identified possible post-translational modification sites on BSBMV p32 that include various

N_glycosylation, N-myristoylation and some phosphorylation sites as indicated on Fig. 1.

Rep5 viral vector clones, carrying BSBMV p32 or BSBMV p31 nucleotides sequences, were employed, using protocol as previously described (D'Alonzo *et al.*, 2008), to successfully verify the capability of these two proteins to promote BNVV RNA-1, -2 and -3 transmission through the vector *P. betae* in *B. vulgaris* plants. Our results demonstrated, for the first time, that the correct expression of BSBMV p32 is essential for an efficient transmission of the virus.

BSBMV RNA-4 cDNA (pUC47) and Rep3-FlagP32, Rep5-P32, Rep3-FlagP31, Rep5-p31 and Rep5-FS-P32 clones were linearized, *in vitro* transcribed and rub-inoculated together with BNYVV RNA-1 and -2 (namely Stras12) helper strain transcripts (Quillet *et al.*, 1989) on *C. quinoa* plant leaves, as described by Klein *et al.* (2007). Typical chlorotic spots appeared 7 days post inoculation (dpi) on leaves of plant inoculated with Stras12 helper strain. When Rep3-FlagP32 or Rep5-P32 transcripts were supplemented to Stras12, local necrotic spots appeared. Such symptoms were very similar to those induced by full-length BSBMV RNA-4 transcripts. On the opposite, chlorotic spots appeared when a non functional protein was expressed (Rep5-FS-P32). Similarly to p32, the use of replicons expressing the wild type BNYVV protein (Rep3-FlagP31 or Rep5-P31) induced the development of local necrotic ring spots on *C. quinoa* leaves. Northern blot analyses were performed, as previous described (Link *et al.*, 2005) and confirmed correct replication of all RNAs.

Similarly, Flag-tagged p32 deletion mutants were also analysed for their ability to induce necrotic local lesions *in planta*. When inoculated into *C. quinoa* leaves, together with Stras12, local lesions appeared 5-7 d.p.i. Stras12-like chlorotic spots were obtained with all deleted form but necrotic spots were observed when Rep3-P32 Δ3 mutant was used. Lesions obtained in the presence of Rep3-P32, Rep3-FSP32 and deletion mutants were analysed by western blot as previously described (Link *et al.*, 2005).

Immunodetection of the flag-tagged proteins was performed using an anti-FLAG antibody conjugated to peroxidase. A specific signal of the expected size (15 kDa) was detected for mutant p32 Δ6 carrying a deletion of both N- and C-terminal part of the protein (Fig. 1 and Fig. 2). A protein of 25kDa was detected in sample expressing P32 Δ3 but no protein of the 7kDa expected size was found. Taken together, our results suggest that the first 64 amino acids of the P32 protein are responsible for the induction of the necrotic spots on *C. quinoa* leaves. Moreover the P32 protein could be glycosylated or myristoylated on N-terminal part in the predicted site respectively, [18]NVTG[21] (M1) or [69]NVSV[72] (M2) and [39]GVLCNI[44] (M3), may explain the modified electrophoretic mobility observed for protein expressed by P32 Δ3 clone.

Transcripts corresponding to single and double mutants reported on Table 1, were then mechanically inoculated in the presence of Stras12 isolate. Results evidenced that double mutations of two glycosylation predicted sites (M1 + M2) or glycosylation and myristoylation (M1 + M3) predicted sites as well as single

mutation on myristoylation predicted site (M3) prevented the induction of necrotic spots on *C. quinoa* leaves. Moreover western blot analysis specifically revealed a protein of about 32 kDa in all mutated p32 forms, as well as wild type p32.
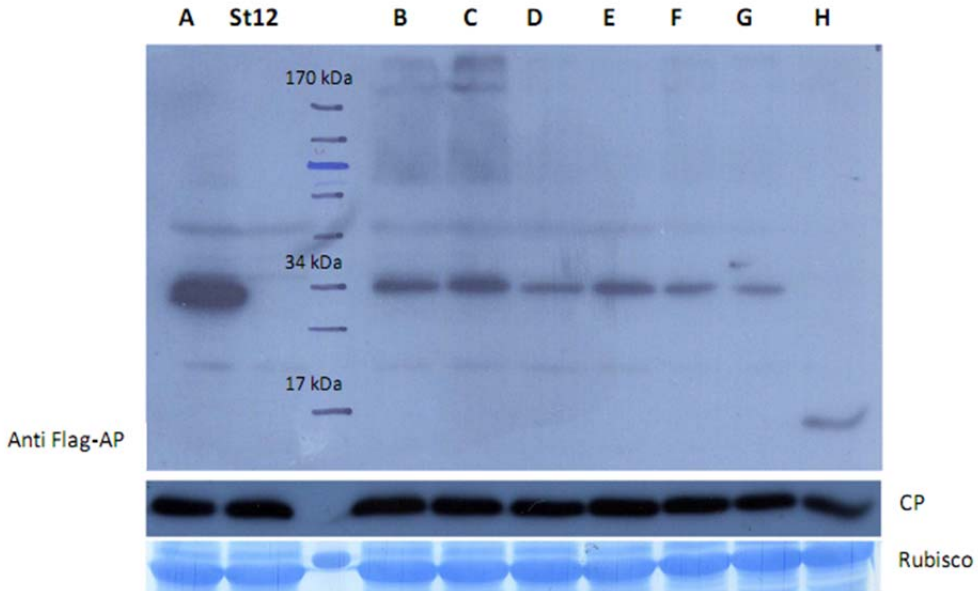

Figure 2. Western blot analysis of *C. quinoa* leaves inoculated with different P32 mutated forms. Order of samples: A= Rep3-FlagP29; St12= Stras12; B= Rep3-FlagP32; C= FlagP32 M1; D= FlagP32 M3; E= FlagP32 M1-M3; F= FlagP32 M2; G= FlagP32 M1-M2 and H= FlagP32 Δ6. Anti Flag conjugate with alkaline phosphatase and anti BNYVV CP conjugate with peroxidase were use at dilution of 1:7500 and 1:60,000, respectively. Coomassie brilliant blue stain was used to reveal Rubisco protein.

Subcellular localization analysis, performed on *N. benthamiana* leaves, evidenced that the GFP-p32 or p32-GFP proteins localized preferentially in the nucleus after 72 h. The observation of such nuclear localization suggests the absence of myristoylation modification that is supposed to enhance protein-membrane or protein-protein interactions (Podell & Gribskov, 2004). Moreover, after 96 h GFP-p32 or p32-GFP proteins re-localized into the cytoplasm, forming fluorescent aggregates close to the membrane that were observed before cells death. According to our observation and bibliographic studies, we formulated the hypothesis that p32 myristoylation occurs, post-translationally, when internal glycine residues become exposed by cleavage during apoptosis associated to necrotic spots (Zha *et al.*, 2000). According to this hypothesis we cannot exclude that, in the presence of *P. betae* vector, p32 protein follows a different metabolic pathway where interaction with protozoa protein prevents its

myristoylation. This aspect will need a better investigation when specific p32 antibody will be available.

Yeast two-hybrid system analysis resulted in the absence of growth in histidine-depleted media letting us to conclude that BSBMV p32 protein is not able to self-activate the transcription nor interact with itself in such systems. Moreover using the same approach, we excluded any interaction within BSBMV p32 protein and viral proteins encoded by RNA-2, -3 and -5 of BNYVV.

In conclusion, our results demonstrated that correct expression of BSBMV p32 protein can support efficient transmission of BNYVV RNA-1, 2 and -3 thought *P. betae* on *B. vulgaris* plants.

More experiments are needed to complete molecular characterization of BSBMV p32. In particular the use of p32 deleted forms and p32 protein carrying point mutations will be tested for transmission thought the vector in order to establish a possible correlation between the p32 domain(s) related to transmission and necrosis induction. More experiments will also be developed with the intent to detect host protein(s) involved on transmission and then may opening a new strategy to develop sugar beet plants resistant to Rhizomania disease.

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CONTROL OF *POLYMYXA BETAE* BY *BACILLUS AMYLOLIQUEFACIENS* LIPOPEPTIDES

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Summary

Rhizomania is one of the most important diseases of sugar beet. It is caused by *Beet necrotic yellow vein virus* (BNYVV), which is transmitted by the protist *Polymyxa betae*. Until now, only a few resistance genes against BNYVV have been used in the control of the disease. Within the context of integrated pest management, the effect of molecules stimulating the host defence mechanism in *P. betae* infection was explored. Systemic acquired resistance (SAR) was induced by spraying the leaves with a synthetic analog of salicylic acid three times before inoculation with *P. betae* zoospores. Also before inoculation, induced systemic resistance (ISR) was elicited twice, with a 1-week interval, by watering the sugar beet roots with a *Bacillus amyloliquefaciens* lipopeptides solution. One week after inoculation, the plants were harvested and *P. betae* was quantified in the roots using a quantitative PCR assay. The ISR was controlled by assessing the expression of the *NPR1* gene and three PR genes. Both systemic resistances were induced in corresponding treated plants (SAR-salicylic acid analog; ISR-lipopeptides). Although no reduction in *P. betae* infection was observed in SAR-induced plants, *P. betae* infection decreased significantly for the two concentrations of zoospores from 10 times (5000 zoospores) to 16 times (500 zoospores) when the plants were treated with the bacterial lipopeptides. These results accord with the fact that *P. betae* induced the SAR. It therefore seems to be insensitive to the defences induced by SAR but not to those by ISR. This decrease in infection, due to ISR, might affect viral transmission and hence rhizomania pressure in fields. From an integrated pest management perspective, the decrease in disease pressure due to bacterial lipopeptides could help to preserve genetic resistance to the virus longer and provide a new way to manage *P. betae* inoculum potential in soils.

Introduction

The control of rhizomania in the field is still based mainly on sugar beet tolerance genes against *Beet necrotic yellow vein virus.* In the context of integrated pest management, the possibility of using partial genetic resistance

from wild *Beta* species against the vector *Polymyxa betae*, as suggested by Barr *et al.* (1995) and Mc Grann *et al.* (2009), is an attractive proposition. The control of this vector would help to reduce both virus and vector titers in sugar beet roots and hence in soils. Taking a similar approach, we tested the possibility of controlling *P. betae* infection by inducing the systemic resistance in the plants. Plants have two "immune systemic reaction" strategies (Van Loon *et al.*, 1998). The first occurs when a pathogen attacks the plant. Recognition of the pathogen leads to a response in the tissues surrounding the pathogen, which can spread throughout the plant by means of a signal molecule, salicylic acid. This kind of response is called 'systemic acquired resistance' (SAR). The second type of response, quite similar, is induced by non-pathogenic organisms such as plant growth-promoting rhizobacteria. This resistance is mediated by jasmonate and ethylene, and is called 'induced systemic resistance' (ISR).

The events leading to these types of resistance can be mimicked by synthesis molecules, such as the analogues of salicylic acid. Recently, cyclic lipopeptides of rhizobacteria, such as non-pathogenic *Pseudomonas* sp. and *Bacillus* sp., have been shown to confer resistance against plant pathogens by inducing ISR (Tran *et al.*, 2007; Ongena *et al.*, 2007). In this study, the efficiency of the each type of immune response was assessed.

Materials and Methods

Two-week-old sugar beet plants were treated twice, at an interval of 1 week, with Acybenzolar-S-methyl (ASM), a functional analogue of salicylic acid. Other 3-week-old sugar beet plants were treated with cyclic lipopeptides from *Bacillus* sp. (30 mg/L), or with water as the control. The plants were then inoculated with two concentrations of *P. betae* zoospores (500 and 5000 zoospores) 1 week after the last treatment. At inoculation time, the roots and leaves of some treated and control plants were harvested. The expression of two genes, markers of the establishment of the immune response, was tested by RT-PCR in both the root and leaf samples in order to assess the induction of the systemic response.

One week after inoculation, the *P. betae* infection in the treated and control plants was quantified by a qPCR assay targeting the ITS region. This quantification was normalized with the quantification of sugar beet DNA, and compared using ANOVA-1.

In order to control the absence of direct action by the lipopeptides on the zoospores, the zoospores were put in contact with lipopeptide concentrations from 0 mg/l (control) to 120mg/l. for 3 hours and their viability was evaluated by quantitation of the ATP using a luminescent assay.

Results and Discussion

The assessment of gene expression implicated in the systemic resistance responses showed that the genes were expressed or overexpressed in treated plants, whereas in the controls they were expressed as negatives in RT-PCR or poorly positive. Each treatment (ASM or lipopeptides) induced a systemic response in the whole plant.

No significant difference in *P. betae* infection between the ASM-treated and untreated plants was detected for either of the tested concentrations of zoospores. In contrast, there was a significant decrease in *P. betae* infection in the lipopeptide-treated roots: this decrease reached 93% or 88%, depending on the level of zoospore concentration (Fig. 1). No difference in ATP quantitation between *P. betae* zoospores in lipopeptide solution or Hoagland solution was observed. The likelihood of direct action by the lipopeptides on the zoospores membranes can be discounted. These results indicate that the systemic response induced by the bacterial-produced lipopeptides greatly reduced the infection rates of sugar beet by *P. betae.*
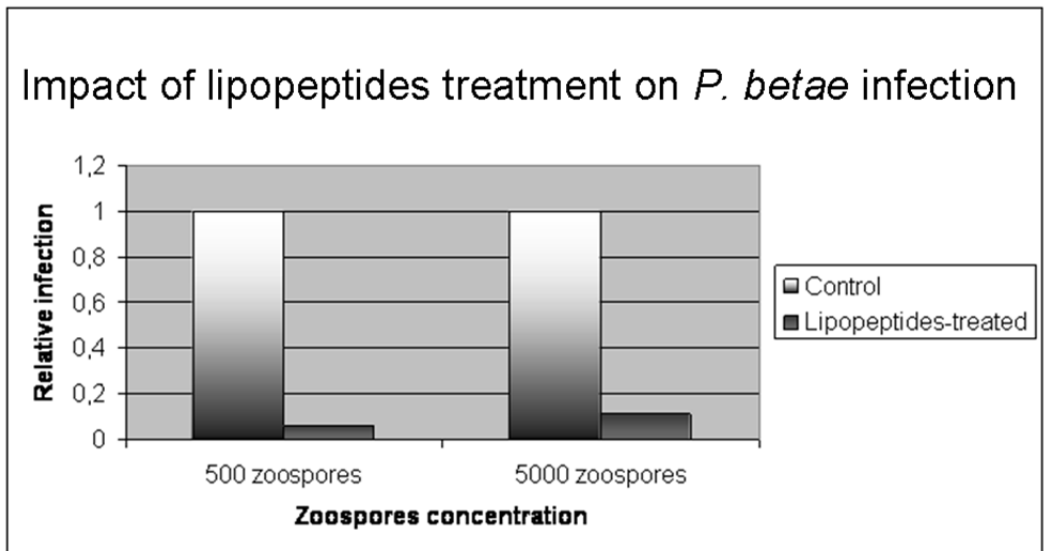

Figure 1. Comparison of *P. betae* infection in untreated plants (control) and lipopeptide-treated plants, 1 week after inoculation with two concentrations of zoospores

From these results it can be said that the systemic resistance induced by lipopeptides is active against *P. betae* under controlled conditions. In the context of integrated pest management, these results offer possible new ways of controlling rhizomania disease based on action against the virus vector, followed by a decrease in inoculum potential of the disease.

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FIRST DETECTION AND PARTIAL CHARACTERIZATION OF A *FUROVIRUS* ISOLATED FROM WINTER BARLEY IN GERMANY

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Summary

The yellow mosaic virus complex of winter barley which is caused by the two bymoviruses *Barley mild mosaic virus* (BaMMV) and *Barley yellow mosaic virus* (BaYMV), is one of the most severe threats to the crop growing of winter barley in Germany. Other soil-borne cereal viruses like *Soil-borne cereal mosaic virus* (SBCMV) or *Soil-borne wheat mosaic virus* (SBWMV) also transmitted by *Polymyxa graminis* and infecting wheat, rye and triticale have never been observed up to now on winter barley under field conditions in Germany. However field examinations of yellow mosaic virus resistant barley genotypes has shown that plants carrying mosaic symptoms contained rod-shaped virus particles. Several isolates were obtained by mechanical inoculation of test plants and one isolate originating from variety 'Jorinde' (harbouring the rym5 resistance gene) was designated as Jorinde-1 and was further characterized. The biological, serological and molecular data obtained so far indicate that the German isolate Jorinde-1 belongs to the genus *Furovirus* and is closely related to Japanese SBWMV (JT) and the French *Soil-borne barley mosaic virus* (SBBMV, later renamed as SBWMV-Mar). Phylogenetic analysis by means of sequencing data for RNA2 coding for cistrons of coat protein, read through protein and cysteine rich protein revealed that Jorinde-1, SBBMV and SBWMV (JT) form a separate cluster and may represent a new species in the genus *Furovirus* within the family *Virgaviridae*.

Introduction

Besides virus diseases transmitted by insects, the soil-borne viruses of cereals represent the greatest danger potential for the production of cereals in Germany. In recent years, yield losses in winter barley caused by the barley yellow mosaic virus complex have been reduced by growing new varieties carrying different resistance genes. In order to assess further barley accessions for virus resistance, annual field trials were carried out at different locations in cooperation with the Federal Plant Variety Office (Bundessortenamt). The

resistant varieties 'Jorinde' and 'Nerz' e.g. first released in 2005 and 2008, respectively remained virus free in all preceding examinations (Rabenstein *et al.*, 2008).

However, during field inspections in spring 2010 unexpected yellowing and mosaic symptoms were observed in both control varieties 'Jorinde' and 'Nerz' harbouring resistance allel rym5 that confers resistance to bymovirus isolates including to the pathotype *Barley yellow mosaic virus* 2 (BaYMV-2) (Hofinger *et al.*, 2011; Kanyuka *et al.*, 2005; Stein *et al.*, 2005). Therefore, it was initially assumed that a new resistance-breaking pathotype had emerged. However, the plants contained, instead of bymoviruses, rod-shaped furovirus-like particles which could be transmitted mechanically. A discrete isolate originating from the variety 'Jorinde', designated as Jorinde-1, was further investigated and characterized.

Materials and Methods

The field trials were carried out at one site near Braunschweig (JKI) where the soil was contaminated with both BaYMV-1 and BaMMV and at two further locations (Bornum and Schladen) additionally infested with the resistance breaking pathotype BaYMV-2. All field trials were inspected for virus symptoms at three different times in the early spring. Plants from varieties with or without visible symptoms were tested serologically using polyclonal antisera to BaMMV and BaYMV in DAS-ELISA. Serologically negative tested plants were further assayed by RT-PCR using virus specific primer combinations (Steyer *et al.*, 2005; Götz & Huth, 2005). Additionally, plants with suspect symptoms were tested by TAS-ELISA using specific monoclonal antibodies (Rabenstein *et al.*, 2005) for the presence of other cereal infecting viruses like *Soil-borne wheat mosaic virus* (SBCMV) and *Soil-borne cereal mosaic virus* (SBWMV). IC-RT-PCR and electron microscopy including immunogold labelling were performed as described by Rabenstein *et al.* (2011). Phylogenetic analysis was performed as described by Shirako *et al.* (2000).

Figure 1: Symptoms on barley variety 'Kathleen' after inoculation with isolate Jorinde-1.

Results and Discussion

Preliminary host range studies showed that the isolate Jorinde-1 was mechanically transmissible to all tested barley varieties. All recently listed new varieties as 'Kathleen' (Fig.1), 'Yokohama' and 'Otto' possessing resistance to viruses of the barley yellow mosaic disease complex could be infected and large amounts of virus particles arranged in bundles were observed in ultrathin sections as shown as an example in Figure 2 for the barley variety 'Kathleen'.
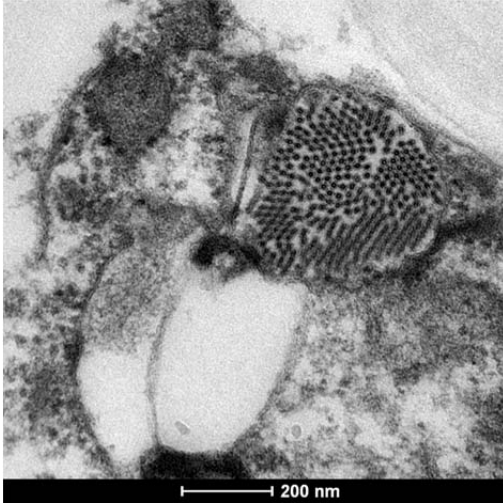

Figure 2: Virus particles arranged in bundles in the cytoplasm in ultrathin sections of barley variety 'Kathleen' after inoculation with isolate Jorinde-1, bar 200 nm.

In addition the isolate Jorinde-1 infected a few wheat varieties but could never be mechanically transmitted to rye. On inoculated leaves of *Nicotiana tabacum* varieties 'Samsun - NN' or 'Samsun - nn' local lesions appeared as early as 4 dpi. In contrast to a German isolate of SBWMV originally obtained from naturally infested wheat plants in the south if Germany (Heddesheim) isolate Jorinde-1 also infected *N. benthamiana* systemically.

Initial ELISA and Western blotting experiments using rabbit antisera and specific monoclonal antibodies (MABs) to SBCMV or SBWMV (Rabenstein *et al.*, 2005) were confirmed by electron microscopic decoration tests and immunogold labeling. By using a SBCMV specific MAB (4G11) all tested SBCMV isolates and Jorinde-1 were labeled similarly as shown in Figure 3. In contrast MAB 4G4 specific for SBWMV gave no labeling and reacted only with the type strain of SBWMV (ATCC) and the German SBWMV isolate Heddesheim (data not shown). The new furovirus isolated from barley variety 'Jorinde' was in this way serologically more closely related to SBCMV than to SBWMV, which occurs so far only in wheat, rye and triticale in Germany.

Using movement protein-specific primers for RNA1 of SBCMV or SBWMV in RT-PCR only amplification products for the homologous furovirus isolates from wheat, rye and triticale were obtained, but not for the Jorinde-1 isolate, indicating differences in the movement protein gene sequence. So far, for the *German barley furovirus* the complete genome sequence was obtained only for RNA2. Blast analysis revealed close homology to the isolate Marne (F), first described as '*French barley mosaic virus'* or '*Soil-borne barley mosaic virus*' (SBBMV)' in France in 2001 (Hariri, 2004), and to a virus designed as SBWMV-JT, which was originally isolated from barley in Japan (Shirako & Ehara, 1986). On the nucleotide level, the Jorinde-1 sequence showed 99% identity to the
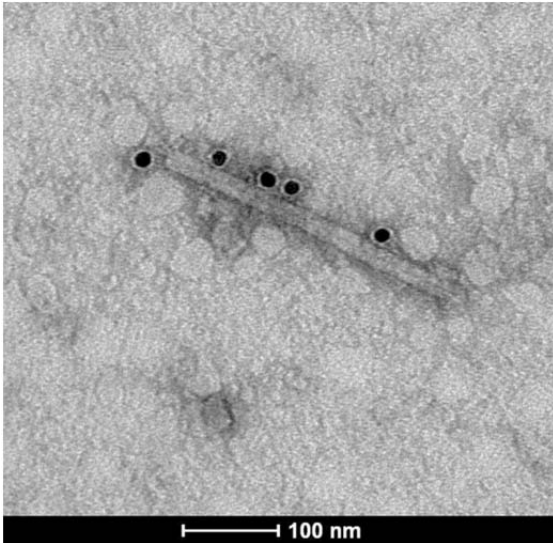
Figure 3: Immunogold labeling of virus particles found in barley variety 'Jorinde' with SBCMV specific monoclonal antibody 4G11, bar 100 nm.

incomplete sequence of the French SBBMV and 94% identity to the complete RNA2 sequence of SBWMV-JT. The amino acid sequence of the CP of isolate Jorinde-1 was nearly identical to that of SBBMV with only phenylalanine at position 63 replaced by leucine.

The CP and the sequences for cystein rich protein of SBWMV-JT and SBBMV formed a single clade with Jorinde-1 in phylogenetic analyses that was well supported by bootstrapping as shown in Figure 4A using the example of CP amino acid sequences.

A slightly divergent cluster was formed using the read through protein amino acid sequences of several members of the genus *Furovirus* (Fig. 4B). Isolate Jorinde-1 formed together with SBWMV-JT, SBBMV and SBCMV isolate G a single well-supported clade. SBCMV-G isolated from rye was the most divergent from other sources of SBCMV found in Germany (Koenig *et al.,* 1999).

In conclusion, the biological, serological and phylogenetic data obtained so far indicate that the German furovirus from barley is closely related to Japanese SBWMV (JT) and the French SBBMV, later renamed as SBWMV-Mar (Hariri & Meyer, 2007). Future investigations will focus on economic relevance and the distribution of this virus in German fields.
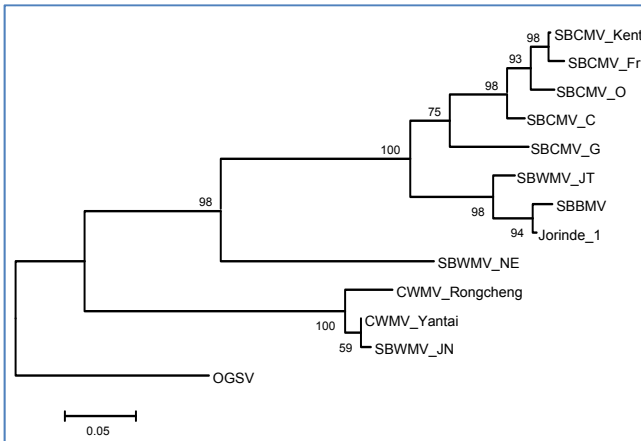
Acknowledgements

Figure 4A: Phylogenetic (neighbor-joining) tree of the coat protein amino acid sequences of members of the genus *Furovirus* (SBCM *Soil-borne cereal mosaic virus* isolates from Europe, SBWMV_NE *Soil-borne wheat mosaic virus* type strain Nebraska, CWMV *Chinese wheat mosaic virus* isolates and SBWMV_JN strain "Japanese Northern, OGS *Oat golden stripe virus*".
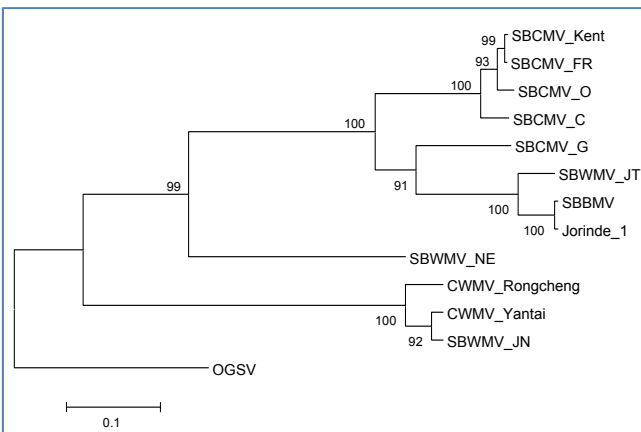
Figure 4B: Phylogenetic (neighbor-joining) tree of the read-through protein amino acid sequences of members of the genus *Furovirus*

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THE ASSUMPTION ON THE SPREAD OF *POTATO MOP-TOP VIRUS* IN JAPAN BASED ON FIELD SURVEY

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Summary

In Japan, potato spraing caused by *Potato mop-top virus* (PMTV) was first found in 1980 in Hiroshima, western Japan (Imoto *et al.,* 1986). The disease has not been recorded until the recent outbreak in 2005 in Hokkaido, northern Japan (Nakayama *et al.,* 2010). A survey was subsequently conducted in 2006 - 2010 to elucidate the geographic distribution of the virus. A total of 847 soil samples were collected from northern (Hokkaido), central (Nagano, Shizuoka, etc.), and southern (Nagasaki, Kagoshima, etc.) parts of Japan. They were then examined for the virus, using the soil diagnostic method that consisted of a bioassay using tomato plants as baits to trap the vector of PMTV, *Spongospora subterranea*, the causal agent of potato powdery scab and reverse transcription-polymerase chain reaction-microplate hybridization (RT-PCR-MPH) to detect the virus from roots of bait plants (Nakayama *et al.,* 2010). PMTV was detected from 195 of 847 samples, indicating that infested soils were widely distributed throughout Japan. Four soil samples collected from the fields planted with landrace potatoes in central Japan were revealed to be positive. These fields were geographically isolated from each other, and modern potato varieties have never been grown there so far. The history of potato cultivation in Japan led us to assume that PMTV was introduced and distributed with the spread of landrace potatoes throughout Japan before the dissemination of modern potato varieties.

Introduction

Spraing of potato (*Solanum tuberosum*) is characterized by brownish arcs and rings on the surface and inside the flesh of tubers. The disease is caused by *Potato mop-top virus* (PMTV), the type species of the genus *Pomovirus,* family *Vigraviridae.* Spraing was first recorded by Calvert and Harrison (1966) in Scotland and northern Ireland and then reported from various parts of European countries, South America, China, Canada and recently in the USA. In Japan, the first report of spraing was made by Imoto *et al.* (1986) on cv. Norin No.1 for autumn crop in Hiroshima in 1980, but no further incidence was recorded for 25 years until a second outbreak in Tokachi, the main potato-producing area of Japan, Hokkaido, northern Japan in 2005 (Nakayama *et al.*, 2010). PMTV is known to be transmitted by a Plasmodiophorid, *Spongospora subterranea* f.sp. *subterranea*, the causal agent of potato powdery scab. To prevent the expansion of infested areas, we surveyed accurate distribution of PMTV in Hokkaido using a bait plant bioassay combined with reverse transcription–polymerase chain reaction-microplate hybridization (RT-PCR-MPH); 137 of 224 soil samples (61.2%) from fields adjacent to the infested field were found positive (Nakayama *et al.*, 2010). Here, we report results from a subsequent, extensive field survey conducted throughout Japan to present an assumption on the introduction and spread of PMTV.

Materials and Methods

Soil samples were collected from 847 fields in Japan (Table 1) by the method of Nakayama *et al.* (2010) in 2006 - 2010 and examined on the presence of PMTV by the soil diagnosis method (Nakayama *et al.,* 2010). The samples included those from the fields adjacent to that of second outbreak (Table 1, area 1, published by Nakayama *et al.,* 2010). Surveyed fields were located in 21 areas of Hokkaido (northern), Honsyu (central) and Kyusyu and Okinawa (southern) islands of Japan.

Results and Discussion

PMTV was detected from the soil in 195 out of 847 fields (Table 1); the virus was detected from 10 out of 11 areas of Hokkaido at various ratios from 0 to 60%, however, spraing potato was not found except one field where PMTV was rediscovered in 2005 (data not shown). PMTV was detected from 4 out of 7 and 2 out of 3 areas in central and southern regions, respectively (Table 1), and no spraing potato was found from these fields so far (data not shown). Soil diagnosis for PMTV revealed that 23% of the fields were infested although only a single field produced potatoes with spraing symptoms. From these results, we concluded that PMTV was widely present throughout Japan but that soil infestation with PMTV did not always result in spraing occurrence.

Interestingly, PMTV was detected from four fields where landrace potatoes were cultivated (Table 1, area 12). These fields were located in mountains and geographically isolated with no history of modern potato cultivation. Since the first introduction of potato from the Netherlands to Nagasaki in 1600, potatoes spread throughout Japan and have been grown as landraces for 400 years in Japan. Modern potatoes such as Irish Cobbler (named as Danshakuimo) were first introduced and released in 1908. Certified seed potatoes have been produced for commercial cultivation under the plant quarantine law in 1947. Results from our survey, as well as historical aspects, led us to assume that PMTV was introduced and distributed with the spread of landrace potatoes.

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Table 1. Summary of field survey of *Potato mop-top virus* in Japan in 2006 – 2010

Area	Region	No. of fields	PMTV	
			Positive	Negative
1	Northern	224	137	87
2	Northern	10	4	6
3	Northern	24	1	23
4	Northern	84	4	80
5	Northern	220	1	219
6	Northern	2	0	2
7	Northern	57	3	54
8	Northern	13	1	12
9	Northern	57	13	44
10	Northern	40	13	27
11	Northern	46	4	42
12	Central	13	4	9
13	Central	2	0	2
14	Central	12	0	12
15	Central	1	0	1
16	Central	3	1	2
17	Central	8	2	6
18	Central	2	2	0
19	Southern	5	3	2
20	Southern	17	2	15
21	Southern	7	0	7
total		847	195	652