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

Editor: U. Merz

Obihiro
Hokkaido, Japan

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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 normally 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), Université Catholique de Louvain, Louvain-la-Neuve, Belgium (2011) and Hokkaido Hotel, Obihiro. Hokkaido, Japan (2013). 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 2013 meeting in Obihiro 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. Unfortunately not many group members from overseas participated. Our Japanese friends volunteered as local organisers to enable a budget saving combination with the ICPP congress in Beijing but the editor was the only who took this opportunity.

A main agenda item of the General Assembly (minutes: http://www.iwgpvfv.ethz.ch/) during the Obihiro meeting was the usual discussion about the next venue in combination with the question about the future of the working group. Although our group is constantly shrinking, at least at the symposia, it was agreed that we still should continue. One suggestion was to hold the next meeting (2016) in France but this needs clarification. Another suggestion was made by Ch. Hiruki to ask a Chinese scientist who he thinks would be willing to organize. The Cairman accepted to contact him in Beijing

The names and e-mail addresses of the current program committee are listed below. Please contact a member of the program committee if you wish to be included in any future mailings of the IWGPVFV.

Ueli Merz

Proceedings editor

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EVIDENCE FOR *BEET NECROTIC YELLOW VEIN VIRUS* BNYVV REASSORTMENT AND DIVERSITY OF THE P25 AVIRULENCE GENE IN FRANCE

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Summary

Since the beginning of 1990, in the region of Pithiviers, the sugar beet crop is under a strong pressure of rhizomania disease. The causal agent, *Beet necrotic yellow vein virus* (BNYVV) has evolved through the selection pressure linked with the progressive introduction of Rz1 tolerant sugar beets. Field-based research was carried out to analyze both the virus and the vector (*Polymyxa betae*).

A very high diversity of the BNYVV RNA-3 tetrad, indicative of resistance-breaking was evidenced in the Pithiviers region. BNYVV was detected in 835 of the 1058 samples collected between 2008 and 2012. Twenty-one different variants of the highly variable amino acid tetrad at positions 67–70 of p25 were identified, i.e. AYHR, TYHR, SYHG, SYHR, SYHN, SFHG, SCHG, AYPR, AYHG, AFHG, AFHR, AFLG, AFPR, VCHG, VYHG, VYHR, TFPR, AYHT, AYHS and ASHR. The first variant, AYHR, was found most commonly followed by SYHG. In a single sugar beet root, presumptive indications of BNYVV reassortment were found in ca 20 % of the samples. Several different tetrads associated with the A- or B-type were found with the fifth RNA.

Moreover, one of the variety Rz1rz1Rz2rz2 showed lower titer with *Beet soil-borne virus* (BSBV), a *Pomovirus* associated with rhizomania. This variety showed also lower root symptom and lower virus inoculum potential at the end of the beet season compared to the others. Results will be discussed in comparison with those provided by Schirmer *et al*. (2005) from the same area.

Keywords : BNYVV, soil-borne virus, *Polymyxa betae*, resistance-breaking, molecular epidemiology, resistance durability

Introduction

Multiple Rz1 resistance-breaking events have been reported throughout the world, both in Europe and in the U.S.A. where partially resistant sugar beet cultivars to rhizomania have been used widely to counter to the disease progress. In the U.S.A., such resistance breaking was explained by the emergence of a single mutation from Alanine to Valine in the BNYVV type A, p25 hypervariable amino acid tetrad (from ACHG to VCHG) was linked (Koenig *et al*., 2009). Such mutation was also proposed to explain the field problems observed in Spain (Pferdmenges *et al*., 2009). Nevertheless, the exact role of the p25 in the disease syndrome is still a matter of debate, despite progress made in its cellular localization, its ability to activate transcription and its confirmed role as an avirulence factor (Peltier *et al*., 2008 ; Chiba *et al*., 2011). Furthermore, it should be noted that several places where strong disease symptoms have been noted under a Rz1rz1 resistance context have not been associated with this VCHG tetrad. Similarly, Koenig *et al.* (2009) also evidenced a possible reassortment of the virus to explain the resistance-breaking problem. Also, a BNYVV strain a RNA5 close to asian strains has also been evidenced in Germany.

Following the surveys conducted by Schirmer *et al.* (2005) in the area of Pithiviers, fields were surveyed in France. More than 1000 samples were collected both in disease and non-disease expressing areas. Samples where tested for the detection of BNYVV by RT-PCR targeting mostly the p14, p25 and p26 genes. The multiplex RT-PCR proposed by Meunier *et al.* (2003) was also used to detect the RT-CP on RNA-2, the rhizomania associated pomoviruses *Beet soil-borne virus* and *Beet virus Q*, together with their vector *Polymyxa betae*. In the frame of a long-term study of rhizomania, field trials set up in 2009 and 2010 to follow up the evolution of the disease within a single sugar beet growing seasons.

The results confirm the large presence of BNYVV type B and P in the surveyed areas, with mix infections in single beet. 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 stress also the need to set up convergent survey methods to facilitate the exchange of data on viral resistance-breaking isolates.

Materials and Methods

Sampling. Samples were collected by the French Technical Sugar Beet Institute, based on reports made by farmers. Additionally, ten varietal field assays have been set up each year since 2009. Two French departments have been mainly studied, the department “Loiret” and the department “Seine-et-Marne” but other departments were also investigated as “Aisne”, “Eure-et-Loire”, “Aube”, “Oise”, “Yvelines” and “Essonne” (Figure 1).

Total RNA extraction. Each rootlet was lyophilised and homogeneized for uniformity. The total RNA was extracted from each 100 mg of homogenized root powder sample using the RNeasy extraction kit (Qiagen, Hilden, Germany).

RT-PCR detection of the viruses and vector. A duplex RT-PCR assay was used for the detection of RNA-3 and RNA-5 of BNYVV. For the RNA-3, the primer pair CAGTTTATGATTTAGGGCACA / ATCATCATCAACACCGTCAG was used to amplify a p25-gene portion of BNYVV RNA-3 from homogenized root samples of field-grown sugar beets. For the RNA-5, the primer pair ATGTTTGTTGGTCCCCCGCT / CGAGCCCGTAAACACCGCAT was used to amplify a p26-gene-containing portion.

Sequencing. Before sequencing, the PCR products were purified by the MSB® Spin PCRapace Kit (Invitek GmbH, Berlin, Germany). The nucleotide sequences of the PCR products from the p25 were obtained using an ABI377 Sequencer-Genetic Analyser and the “Big Dye Terminator Cycle Sequencing Kit” (Applied Biosystems).

Results and Discussion

In an area of 150 km^2 corresponding to the severe rhizomania disease foci, targeted BNYVV genes have been analyzed by RT-PCR and sequencing. The p25 amino acid tetrads detected between 2008 and 2012, and their location in the Pithiviers area are given in Figure 1.

The total number of positive samples represents 835 BNYVV isolates out of 1058 samples analyzed. Amongst these isolates, 482 isolates were found as single infection in a single sugar beet root while 353 isolates were found as mixed infection in a single beet. In comparison with the situation before 2005 (Schirmer *et al*., 2005), a much higher diversity was observed, raising the question whether the generalization of increased level of resistance had triggered a diversification within the BNYVV population around Pithiviers.

Before 2005, three types and five tetrads known in the area (based on 40 isolates) were as follow: the BNYVV B-type associated AYHR (54%), the BNYVV P-type associated SYHG (32%) and finally the BNYVV A-type associated AFHR (3%), AHHG (3%) and ALHG (6%). In comparison, amongst the 835 BNYVV positive detections, only a single mutation alanine-valine in the tetrads (tetrad VCHG) was detected in France, from a red beet. Single infections were observed in more or less 80 % and mixed infection were observed in 20 % of the samples. In this study, the most abundant tetrad was the BNYVV B-type associated AYHR (frequency occurrence: 56.49 %) followed by the BNYVV P-type associated SYHG (32.42 %). The BNYVV A-type tetrads AHHG and ALHG have not been found anymore in roots samples collected between 2008 and 2011. Conversely, tetrads that have been linked to resistance-breaking events previously have been found (SYHG, AYHR, AYPR, AFHG, AFHR, VCHG, and VLHG).

Our results stress i) the very high diversity recorded in the area, conversely from the situation described by Schirmer *et al.* (2005) but similarly to other world areas (Mehrvar *et al*., 2009), ii) the presence, sometimes simultaneous within a single sugar beet, of the different BNYYV type A, B and P and iii) indications, trough RT-PCR results, of the co-occurrence of BNYVV genome segments from different BNYVV types within a single sugar beet. These results raise the question whether the virus is able, beyond mutating within the p25 tetrad, to re-assort or possibly recombine between BNYVV types

(Koenig *et al*., 2009, Meunier *et al*., 2005). They are also indicative of a spread of the BNYVV outside the historical epidemic foci around the Pithiviers locality.

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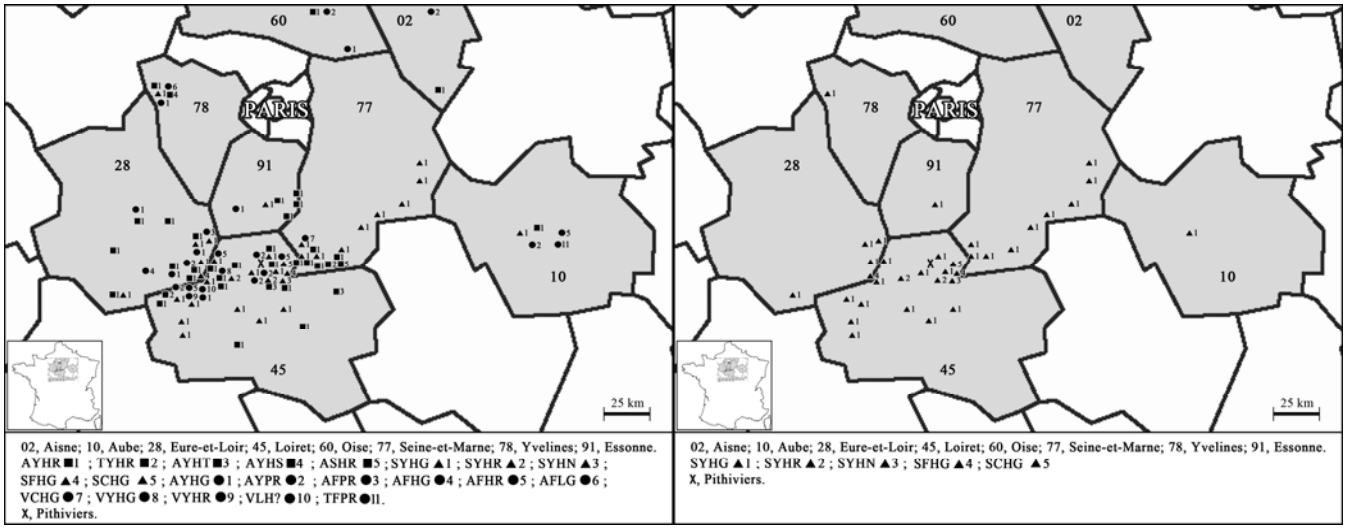

Figure 1 Distribution of BNYVV and the associated tetrads in the Pithiviers area. The figure in right shows all the tetrad diversity of BNYVV and the figure in left shows the P-type associated tetrad dispersion in the Pithiviers area. The most abundant tetrad is the BNYVV B-type associated AYHR (frequency occurrence based on 835 positively infected samples: 56.49 %) followed by the BNYVV P-type associated SYHG (32.42 %), mostly located South-East of Pithiviers (X).

PRODUCTION AND INVESTIGATION OF BIOLOGICAL PROPERTIES OF *BEET NECROTIC YELLOW VEIN VIRUS* AND *BEET SOIL-BORNE MOSAIC VIRUS* CHIMERAS

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Summary

The genus *Benyvirus* (*Benyvidiae*) includes the most important and widespread sugar beet viruses. In particular, *Beet necrotic yellow vein virus* (BNYVV) is worldwide distributed and causes an abnormal rootlet proliferation known as rhizomania, whereas *Beet soil-borne mosaic virus* (BSBMV) is present only in the United States of America. These two viruses have the same genomic organization, infect the same host range and are both transmitted by *Polymyxa betae*. However, no chimeric forms have been described in nature so far. Thus, we investigate the behavior of artificial viral chimeras.

Introduction

Recombination and reassortment occur in many families of RNA viruses and may have a large impact on their evolution, emergence and epidemiology. These processes correspond to the formation of chimeric molecules from parental genomes of mixed origin and require that two or more viruses infect the same host cell (Simon-Loriere and Holmes, 2011).

As many positive sense and single stranded RNA viruses, *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV) have segmented genomes that are encapsidated into separated particles. BNYVV and BSBMV belong to the genus *Benyvirus,* infect the same host and are both transmitted by *Polymyxa betae*. The genome of these viruses is composed by four to five RNAs: RNAs-1 and -2 carry housekeeping genes, RNAs-3 are necessary for long-distance movement, RNAs-4 are involved in the transmission through the vector and a fifth RNA is present only in some field isolates of BNYVV.

In the United States, BNYVV and BSBMV are sometimes present in the same fields and frequently co-infect a common host leading to mixed infections. However, no chimeric benyviruses have been described in the nature so far.

Previous laboratory experiments performed with *in vitro* infectious transcripts revealed that BSBMV RNA-3 and -4 can be replicated and encapsidated by BNYVV RNA-1 and -2 allowing viral long distance movement and transmission through the vector (Ratti *et al*., 2009; D'Alonzo *et al*., 2012). Thus, we evaluated the viability and the biological properties of chimeras with BNYVV/BSBMV RNA-1 and -2 exchanges and the possibility that they may arise in nature.

Materials and Methods

Full-length cDNA copies of BNYVV and BSBMV RNAs-1 and -2 have been previously cloned under the T7 promoter giving rise to pB15, pB214, EUB11, EUB22 (Quillet *et al*., 1989; D'Alonzo, 2011). The RiboMAX Large Scale RNA Production System–T7 kit (Promega, Madison, CA) was used to synthesize RNAs of interest, following manufacturer's protocol.

Leaves of *C. quinoa* were mechanical rub-inoculated with different combinations of transcribed RNAs named: Stras12 (BNYVV RNA-1 + -2), Bo12 (BSBMV RNA-1 + -2), BoStras12 (BSBMV RNA-1 + BNYVV RNA-2) and StrasBo12 (BNYVV RNA-1 + BSBMV RNA-2). Each leaf was dusted with Celite to promote mechanical lesions and then gently rubbed with the inoculum solution composed by 10 µg of each RNAs transcripts, 10 µl of potassium phosphate buffer (0,5 M KH_2PO_4 pH 7.5), 8 µl of macaloid 0.5% and sterile water up to 100 µl. After 7 days lesions from inoculated leaves were recovered and used for total RNAs and proteins extraction.

Cultures of *A. tumefaciens* cells carrying BNYVV and BSBMV RNAs-1 and -2 agroclones (Delbianco *et al*., 2013; Delbianco, 2013) 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. Cells were mixed in a ratio 1:1 and left at room temperature for 3-4 hours before agroinfiltration in leaves of 3-weeks old *N. benthamiana* plants.

Results

In vitro transcripts of chimeral combinations BoStras12 and StrasBo12 have been mechanically inoculated onto C. quinoa leaves and compared to the wild type Stras12 and Bo12. Chlorotic local lesions appeared 7 d.p.i. on leaves of plants inoculated with StrasBo12 combination showing a phenotype comparable to those of Stras12 and Bo12. On the contrary, the BoStras12 combination induced large necrotic lesions with chlorotic borders (Fig.1).

Northern and western blot analysis revealed the efficient amplification of viral RNAs and expression of CPs and p14s in all the inoculated leaves.

The presence of viral RNAs and proteins suggests that a classical viral cycle, including suppression of post-transcriptional gene silencing was efficient in both *wild type* and chimerical combinations. Necrotic lesions are obtained in the absence of viral silencing suppressor (VSR). The clone BNYVV RNA-2Δp14, carrying a frame-shift mutation in p14 gene (Hleibieh, 2010), has been used to assay efficiency of post-transcriptional gene silencing suppression of BoStras12 chimera. Such clone is not able to express the BNYVV VSR and induces small necrotic local lesions when co-inoculated together with RNA-1 (Chiba *et al.*, 2013).

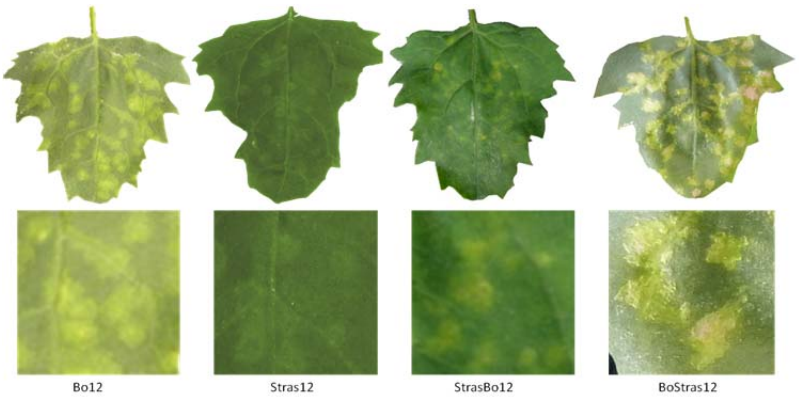

Figure 1 *C. quinoa* inoculated leaves and detail of the local lesions observed 7 d.p.i.

BSBMV RNA-1 and BNYVV RNA-2Δp14 *in vitro* transcripts have been mechanically inoculated onto *C. quinoa* leaves originating the combination BoStras12Δp14. Symptoms expression has been evaluated 7 d.p.i. and compared to those induced by BoStras12 chimera. Small necrotic lesions, morphologically similar to phenotype obtained using Stras12Δp14 and different from lesions obtained with BoStras12, appeared on inoculated leaves. Northern and western blot analysis confirmed the *quasi* absence of viral RNAs and protein expression for both Stras12Δp14 and BoStras12Δp14 combinations when compared to construct containing *wild type* RNAs. These results suggest that suppression of post-transcriptional gene silencing is fully efficient in BoStras12 chimera, as both viral RNAs and proteins can be detected in the large necrotic lesions.

The appearance of necrosis in the lesions produced with BoStras12 chimera let us evaluate the role of the p14 protein. We investigated the role of p14s within BoStras12 combination using complementation experiments with BSBMV and BNYVV p14s through *in vitro* transcripts of viral replicons Rep3-BNYVVp14 or Rep3-BSBMVp14 and Rep5-BSBMVp14.

When *C. quinoa* leaves were inoculated with BoStras12 supplemented with Rep5-p14 BSBMV, chlorotic local lesions appeared 7 d.p.i., whereas necrotic local lesions similar to those obtained with BoStras12 were observed in the presence of the empty replicon Rep5. To extend our study we tested the following combinations of *in vitro* transcripts inoculation on *C. quinoa* leaves:

- Stras12Δp14 + Rep3 p14 BSBMV
- Stras12Δp14 + Rep3 p14 BNYVV
- BoStras12Δp14 + Rep3 p14 BSBMV
- BoStras12Δp14 + Rep3 p14 BNYVV

Seven d.p.i. chlorotic lesions appeared on leaves inoculated with both helper strains supplemented with Rep3-p14BSBMV and Stras12Δp14 supplemented with Rep3-p14BNYVV. Large necrotic lesions with chlorotic borders appeared on leaves inoculated by BoStras12Δp14 + Rep3 p14 BNYVV, restoring the phenotype of BoStras12 combination. Northern and western blot analyses performed on the local lesions evidenced the replication and expression of the viral products in all the combinations tested.

Taken together, the results obtained suggest that BSBMV p14 is able to complement the functions of BNYVV p14. On the contrary, BNYVV p14 is unable to fully complement BSBMV p14 in the presence of BSBMV RNA-1, suggesting the existence of a link between the BSBMV p14 protein and its cognate RNA-1. These results also give some insights that may explain the absence of reassortment in the nature.

Figure 2 Upper leaves of *N. benthamiana* plants agroinfected agroinfected with Stras12 (left) and BoStras12 (right).

BNYVV and BSBMV RNAs agroclones (Delbianco *et al.*, 2013) have been employed to investigate long distance movement of Benyviruses chimeras in *N. benthamiana* test plants. Plants agroinfiltrated with BoStras12 showed large necrotic areas with chlorotic borders in both infiltrated and not infiltrated leaves (Fig.2). Leaves samples were analyzed through western and northern blot demonstrating proteins expression and RNAs replication and the viral long distance movement.

Discussion

Similarities and complementation between BNYVV and BSBMV were already demonstrated by the ability of BNYVV machinery (RNA-1 and -2) to replicate and encapsidate BSBMV RNA-3 and -4 (Ratti *et al*., 2009; D'Alonzo *et al.*, 2012). As BNYVV and BSBMV are frequently present in the same cultivated field in the United States and often infect the same plant, it can be supposed that trans-replication also occurs in natural condition. However, no chimeras between the two viruses have been described.

Results obtained in this work show that artificial Benyvirus chimeras are able to replicate, move cell-to-cell and suppress the post-transcriptional gene silencing. Conversely, large necrotic lesions induced by BoStras12 suggest higher aggressiveness of this combination, when compared with the wild types, may inducing hypersensitive response of the host that could explain why this chimera has not been identified in nature. To demonstrate this hypothesis the hypersensitive defense response could be further investigate quantifying the expression of pathogenesis related proteins which increase during HR. The same experiments need to be conducted on *B. macrocarpa* and *B. vulgaris* plants to confirm our results and further analyze the behavior of BNYVV/BSBMV chimeras in natural benyviruses hosts and in transmission conditions.

Hence, further experiments will be addressed to analyze the possible interaction between RNA-1 and p14. Immunoprecipitation, two- or three-hybrid tests in yeast may allow to precise the p14 interaction with RNA-1 sequences and/or its encoded proteins.

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GENOMIC DIVERSITY OF POTATO MOP-TOP VIRUS IN JAPAN

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The second outbreak of potato spraing took place in Hokkaido, northern Japan, in 2005. Surveys using the soil diagnosis method (Nakayama *et al.,* 2010; Maoka *et al*., 2011) revealed the presence of Potato mop-top virus (PMTV), the causal agent of spraing, not only in fields adjacent to the field in question but also in many potato fields throughout Japan. Latvala-Kilby *et al*. (2009) found low genomic diversity in amino acid sequence among European isolates of PMTV; RT-CP region of RNA2 distinguished types I and II, and 8K protein region of RNA3 types A and B. Consequently, there are four types based on the permutations of sequence differences in RNAs 2 and 3. We examined a total of 41 isolates from soils, tubers, leaves, and resting spores of the vector, *Spongospora subterranea* to find that three genomic types, i.e., IA, IB, and IIB, were present in Japan and that type IIA was not found. The three types coexisted in a same soil sample, but this was not the case with other samples. Type IA was most prevalent. RNA2 II and RNA3 A seemed mutually exclusive. There was no correlation between PMTV types and samples or localities of samples.

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PATTERN OF SYSTEMIC MOVEMENT OF SOIL-BORNE PLANT VIRUSES: EVIDENCE OBTAINED FROM GFP-TAGGED *BEET NECROTIC YELLOW VEIN VIRUS*

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Summary

Beet necrotic yellow vein virus (BNYVV) that is transmitted by *Polymyxa betae* causes rhizomania of sugar beet, in which the virus is usually restricted in roots, but very rarely moves to the aerial parts of plants. Here, we examined the characteristic of systemic movement of BNYVV in an RNA3-dependent systemic host *Beta vulgaris* subsp*. maritima* M8 using GFP-tagged virus (BNYVV-GFP). When BNYVV-GFP was rub-inoculated to leaves, BNYVV-GFP moves first into shoots, in which the virus spread is due to cell-to-cell movement between cortical and epidermal cells rather than to long-distance movement through vascular systems. In hypocotyls and primary roots, BNYVV-GFP was restricted into vascular tissues, whereas, in lateral roots, it was observed in cortical cells and vascular tissues. In contrast to foliar-rub inoculation, when BNYVV was inoculated with *P. betae*, BNYVV was not detected in shoots until 30 days after inoculation. At this time point, BNYVV was detected by ELISA at lower levels in hypocotyls than in primary roots, suggesting that the virus may move through cortical cells rather than vascular tissues. These comparative results suggest that there are several barriers that block upward virus movement from lateral roots via taproots to aerial parts of plants. Such a restrictive pattern of systemic movement is thought to be a common characteristic for soil-borne root-infecting viruses.

Introduction

Many plant viruses infect the aerial parts of plants by invertebrate vectors and spread systemically through vascular tissue to young shoots and roots of plants, in most cases of which the virus is thought to move in parallel with photo assimilate transport (Hull, 2002). On the other hand, soil-borne viruses are directly transmitted to roots of plants by soil-inhabiting organisms, and thus the virus accumulates first in the roots, moves through the vascular systems and then spreads into the shoots. However, some viruses can complete their life cycle in the root of plants (Tamada and Kondo, 2013). A good example is *Beet necrotic yellow vein virus* (BNYVV) that is transmitted to sugar beet roots by the plasmodiophorid *Polymyxa betae*. BNYVV is usually restricted in sugar beet roots and very rarely moves to shoot of plants showing yellow vein symptoms (Tamada, 1999). BNYVV also causes rarely systemic infection in sugar beet plants by foliar-rub inoculation, but it is frequently systemically infected in *Beta macrocarpa*, *B. vulgaris* subsp. *maritima* M8, and *Nicotiana benthamiana* plants (Rahim *et al*., 2007; Tamada, 2007; Tamada *et al*., 1989). Although BNYVV RNA1 and RNA2 encode "house-keeping" genes involved in replication, assembly, and cell-to-cell movement, an additional RNA3 molecule is essential for systemic movement in *Beta* species (Lauber *et al*., 1988; Rahim *et al*., 2007). Here, we examined the patterns of systemic movement of the virus in *B. vulgaris* subsp. *maritima* M8 using a GFP-expressing virus and compared the movement patterns of virus by foliar-rub inoculation and root-vector inoculation.

Materials and Methods

Plants, virus inoculation, and growth conditions

B. vulgaris subsp. *maritima* M8 and *B. macrocarpa* plants were used for systemic hosts (Tamada, 2007). The seedlings were grown in special test tubes filled with quartz, sand in a growth cabinet at 24 C with a 16-h light (Rahim *et al*., 2007). Foliar-rub inoculation and root-vector inoculation were conducted as described previously (Rahim *et al*., 2007).

GFP-expressing virus

A constructs, pB2-RT-GFP3 (RT3), expressing the green fluorescent protein (GFP), were used (Erhardt *et al*., 2001; Valentin *et al*., 2005). It was derived from pB2-14, which contains the wild-type BNYVV RNA2 cDNA clones into pBluescribe (Clontech). The GFP sequence was amplified by the PCR using primers designated to introduce *Acc*I sites at the both ends of the GFP sequence. The GFP sequence fragment was inserted in frame into the *Acc*I1415 site of pB2-14. Capped RNA transcripts produced by *in vitro* transcription of the constructs were mixed with the wild-type BNYVV RNA1 transcript. The mixture was inoculated to leaves of *Tetragonia expansa*, and one week later, cutting yellow lesions were stored and used as an inoculum source.

Preparation of tissue samples for GFP detection

Leaf, petioles, stems and roots of plants inoculated with BNYVV-GFP were sampled. Transverse sections of the plant material were prepared by hand with a razor blade. GFP fluorescence was observed with a fluorescence microscope (Zeiss Axioskop) equipped with a 10 filter (450 to 490 nm excitation filter, 510 nm dichroic mirror, 515 to 565 nm emission filter).

Detection of BNYVV

BNYVV detection from plants inoculated by *P. betae* was conducted by ELISA as described previously (Rahim *et al*., 2007).

Results

Systemic movement of BNYVV by foliar-rub inoculation

We examined the process of systemic movement of BNYVV in *B. vulgaris* subsp*. maritima* M8 using GFP-expressing BNYVV (BNYVV-GFP). In BNYVV-GFP-inoculated leaves, visible faint chlorotic lesions appeared at 5 to 6 days post inoculation (dpi), followed by yellow lesions. First systemic symptoms appeared at 9 to 12 dpi, which differed in individual plants, and we monitored GFP expression in various parts of plants (shoots, leaves, petioles, hypocotyls, primary roots and lateral roots) at different dpi. GFP fluorescence first appeared to be in the basal part of shoot apex at 8 dpi (Fig. 1A), followed by extensive and rapid spreading GFP fluorescence in the vascular tissue and the surrounding cortical tissue (Fig. 1B). Subsequently, small GFP signals were detected in vascular tissues of hypocotyls and primary roots (Fig. 1C) at 9 dpi, but not yet in lateral roots. At the same time, in an emerging leaf, GFP fluorescence foci were found in major class I and II veins, followed by spreading in class II and III veins, and visible yellowish symptoms begun to appear in those leaf veins. In the petiole of systemically infected leaf, GFP

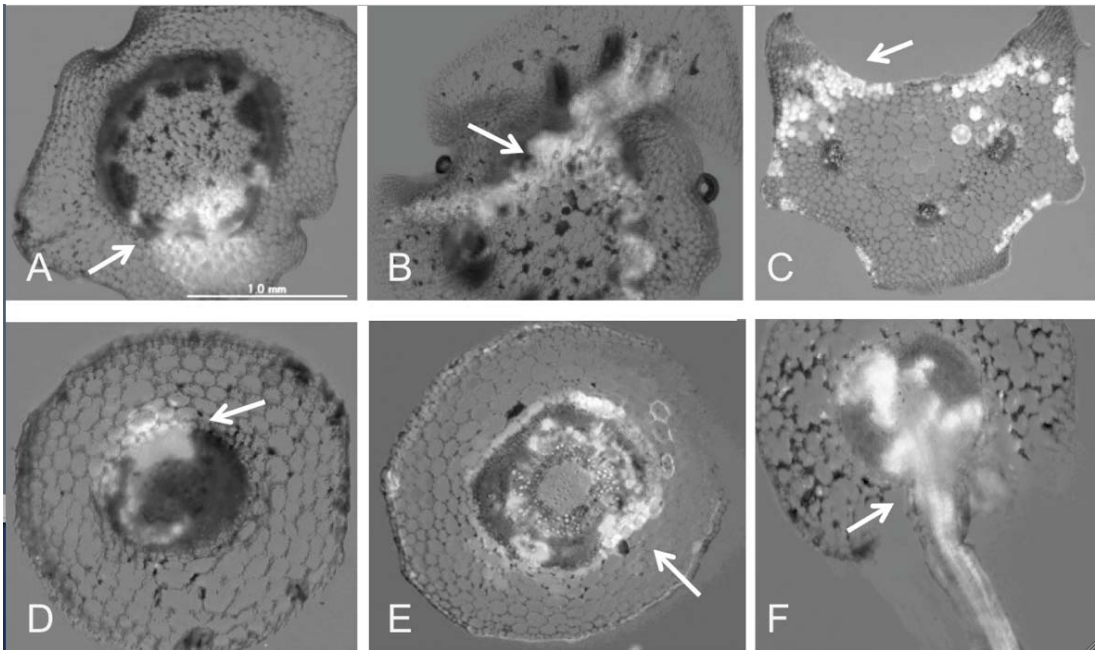

Figure 1 Distribution and accumulation of BNYVV-GFP in various parts of *B. vulgaris* subsp. *maritima* M8 plants. (A) Basal part of shoot at 8 dpi. (B) Basal part of shoot at 9 dpi. (C) Petiole of a systemically infected leaf at 15 dpi. (D) Primary root at 9 dpi. (E) Hypocotyl at 15 dpi. (F) Primary root with a branched lateral root at 15 dpi. Arrows indicate GFP fluorescent signals.

fluorescence had spread to epidermal and cortex cells, whereas only a small part of vascular tissue showed GFP signal (Fig. 1C). On the other hand, BNYVV-GFP moved from the basal shoot via vascular tissue to root apex. GFP fluorescence was detected in limited regions of vascular bundles, but not in the cortical tissue. As the symptoms progress, GFP fluorescence was observed to spread via cell to cell within the vascular tissue, and further move to inward tissues such as cambium layer cells and xylem element, but less to move to outward cortical cells (Fig. 1E). A restriction of GFP in vascular system was observed in a primary root connected with a lateral root (Fig. 1F). However, BNYVV-GFP was easily detected in both the cortical and vascular tissues in lateral roots. These results indicated that there is a boundary barrier between vascular and nonvascular tissues in virus movement within hypocotyls and primary roots.

Systemic movement of BNYVV by root-vector inoculation

In our preliminary experiments, BNYVV-GFP could not be transmitted to roots of test plants (sugar beet, *B. vulgaris* subsp*. maritima* M8 and *B. macrocarpa*) by *P. betae*. In our experimental conditions, systemic symptoms have been observed in only a few *B. vulgaris* subsp*. maritima* M8 or *B. macrocarpa* plants by 2 months after inoculation. In this study, we examined the process of systemic movement by ELISA detection in different plant parts (lateral roots, lower and upper half parts of hypocotyl, and shoot) of *B. vulgaris* subsp*. maritima* M8 at different days after inoculation with wild-type BNYVV-carrying *P. betae*. At 7 dpi, BNYVV was detected in lateral roots in which virus content was considerably high, but not in the lower part of hypocotyl. At 14 dpi, however, BNYVV was detected in the lower hypocotyl part with low virus content. At 30 dpi, BNYVV was detected in the upper hypocotyl part, but did not in shoot. Thus, for root-vector inoculation, systemic movement of BNYVV from root to shoot is very slow, which contrasts to the case of foliar-rub inoculation.

Discussion

In this study, we showed the pattern of systemic infection of BNYVV in a systemic host using a GFP-expressing virus and the difference of systemic movement of BNYVV by foliar-rub inoculation and root-vector inoculation. For foliar-rub inoculation, BNYVV first accumulates in the basal part of shoot, and then moves in two directions. Toward shoot, the virus moves mostly to cell-to-cell between cortical and epidermal cells rather than to long distance through vascular systems. Toward root, the virus moves through vascular tissues, but the virus may not enter into the sieve element. In hypocotyls and primary roots, the virus confines in vascular tissues, whereas in lateral roots, the virus moves easily from vascular tissues to nonvascular tissues.

In contrast to systemic movement by foliar-rub inoculation, for root-vector inoculation, BNYVV was suggested to move to cell-to-cell mainly through cortical cells of primary roots and hypocotyls. From these comparative results, we suggest the following barriers in virus movement from lateral roots to shoots: first barrier is between cortical and vascular tissues within lateral roots, because *P. betae* can infect epidermal cells and cortex, but not the endodermis or stele, second is between lateral roots and primary roots, in which the cortex of the primary root are separated by epidermis of the lateral root, third is between nonvascular (cortex) and vascular tissues within primary roots (include hypocotyl), and forth is between primary roots and shoot (crown). Such a restrictive pattern of systemic movement is thought to be a common characteristic for soil-borne root-infecting viruses.

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MUTATIONAL ANALYSIS OF BNYVV P25 PROTEIN FOR SYMPTOM INDUCTION IN SYSTEMIC HOST *BETA MACROCARPA*

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Summary

The p25 protein encoded by beet necrotic yellow vein virus (BNYVV) RNA-3 is known as the symptom determinant of rhizomania in sugar beet. In BNYVV-susceptible assay plant, *Beta macrocarpa* (a wild beet), the protein induces bright yellow lesions on mechanically inoculated leaves and systemic yellowing mosaic with severe stunting. Molecular mechanisms underlying these symptom expressions are not well understood. In the current study, we created a series of p25 mutants (20 variants) which have site-directed substitutions at conserved cysteine and ion-charged amino acid residues. Inoculation of the p25-mutant viruses revealed an interesting variation of systemic phenotypes in *B. macrocarpa*. A disease index was created based on the systemic symptoms and the virulence level of the mutants was scored. Obtained results would provide useful information for investigating p25-host factor interactions involved in symptom expressions.

Introduction

One of the most destructive pathogen of sugar beet is a fungal (plasmodiophorid)-born benyvirus, *Beet necrotic yellow vein virus* (BNYVV). The virus is transmitted by *Polymyxa betae* and infects rootlets of the plants, eventually causing rhizomania disease which involves root necrosis, massive proliferation of rootlets, nutrition deficiency and significant loss of sugar yield. BNYVV has 4 or 5 plus-strand RNA genomes (RNA-1 ~ -5) with capped 5'-ends and poly-A tailed 3'-ends. The RNA-1 and -2 encode house-keeping genes, while the others are appeared satellite-like RNAs mainly playing roles in symptom inductions (RNA-3 and -5) and in fungal transmission (RNA-4) (Peltier *et al*., 2008). Of these, RNA-3 is responsible for rhizomania, and the single protein p25 contributes to disease development. The protein also induces bright yellow local lesions called yellow spot (YS) on mechanically inoculated leaves of sugar beet and experimental plants, *Tetragonia expansa*, *Chenopodium quinoa*, and some *Beta* plants. In the absence of p25, BNYVV induces faint chlorotic spot (CS) at infection foci. Furthermore, the p25 induces sever symptoms in systemic host, *Beta macrocarpa*, represented by sever stunting, yellowish mosaic and massive shoot proliferation. Interestingly, p25 acts as the key molecule for triggering resistant reaction (elicitation) in some BNYVV-resistant beet cultivars and wild beet accessions in which necrotic spot (NS) or macroscopically invisible lesions are induced when mechanically inoculated (Chiba *et al*., 2008; 2011). So the situation is very similar to bacterial effector proteins (specifically *Avr* proteins).

The BNYVV p25 has been subjected to several mutagenic studies to elucidate its pathogenicity, elicitor activity, subcellular localization, oligomerization and transcription activation ability (Jupin *et al*., 1995; Vetter *et al*., 2004; Klein *et al*., 2007; Chiba *et al*., 2008). However, the molecular mechanism underlying symptom inductions is as yet to be unveiled. To construct a bridgehead addressing this question, we further conducted mutagenesis analyses on p25 by targeting conserved amino acids (aa) of cysteine and positively or negatively ion-charged residues.

Materials and Methods

Mutations in *p25* gene was introduced by PCR-based mutagenesis on the RNA-3 replicon vector plasmid, pTS3cs, which has basal sequence body of BNYVV S-isolate RNA-3 and restriction enzyme recognition sites for *Cla* I and *Sal* I at immediate upstream and downstream of p25 ORF, respectively. The 5'-end sequence of RNA-3 was directly connected to the T7 RNA polymerase promoter sequence. The wild-type p25 gene was replaced by mutant p25 cDNA fragments. The 5'-capped transcripts of recombinant RNA3 were synthesized *in vitro* using RiboMax kit (Promega). Each mutant RNA-3 was

foliar rub-inoculated on *T. expansa* leaves in the presence of total RNA preparations of *T. expansa* leaves infected with a laboratory isolate O11-4 (containing BNYVV RNA-1, -2 and -4). Phenotypes were observed at 1 week after inoculation. Subsequently, those inoculated leaves were homogenized in phosphate buffer and further inoculated to leaves of *B. macrocarpa* planted in special test tube with quartz sand which maintained in growth chamber (22 C˚, 15 h luminescence). Plants were incubated for 1 month and observed resultant systemic phenotypes. A disease index (0-4; non-pathogenic to virulent) was established based on above observation and represented in Table 1. Virus infections were confirmed by Northern blotting or RT-PCR following the procedure of Chiba *et al*. (2008) (data not shown).

Disease index	systemic infection	phenotypes
non-pathogenic		
0	-	healthy: no systemic symptom appeared
1	+	almost healthy: slight increase of shoot proliferation
2	+	mild: mild mosaic, slight stunting, increased shoot proliferation
3	+	severe: mosaic + chlorotic spot, stunting, increased shoot proliferation
4	+	very severe: yellow mosaic, severe stunting, massive shoot proliferation
virulent		

Table 1 Disease index established for *p25*-mediated symptom induction in *B. macrocarpa*

Results and Discussion

Based on aa sequence alignment of representative p25 from distinct group of BNYVV isolates proposed by Chiba *et al*. (2011), we have selected target aa residues for the mutagenesis study. That is, eight cysteine residues, and twelve positions of positively or negatively charged aa residues residing adjacent to each other. For the mutant construction, cysteine (C) residues were replaced by glycine (G), while positive- (R, K, H) and negative-charged (E, D) residues were substituted by alanine (A). Resultant RNA-3 mutants (cysteine-glycine mutants: CG-1 to -8 & ion-charged aa mutants: IC-1 to -12) are listed in Table 2. We inoculated wild-type or 20 individual mutants of RNA-3 with BNYVV RNA-1, -2 and -4 onto *T. expansa* leaves. After 1 week, most of mutants induced YS formation indistinguishable from or slightly milder than those induced by wild-type p25, whereas some mutants such as IC-1, -2, -5, -10 and -11 exhibited CS formations similar to that observed in the RNA-3-free virus inoculation (data not shown).

These BNYVV-infected *T. expansa* leaves were used as inoculums for further inoculation to *B. macrocarpa*. We observed local and systemic phenotypes after 1 week and 1 month of inoculation, respectively. As a result, local phenotypes were somewhat similar to those observed in *T. expansa*, but systemic phenotypes were varied in terms of stunting severity, yellowing and chlorosis levels of systemic mosaic, and magnitude of shoot proliferation. From those observations, a disease index 0-4 (non-pathogenic to virulent) was established as shown in Table 1. Overall, only the RNA-3-free virus belongs to index 0, two mutants (IC-2 and -10) are scored as index 1, three mutants (IC-1, -5 and -11) are marked as index 2, many of mutants (CG-1, -4, -5, -6, -7 and -8; IC-3, -4, -7, -8 and -9) are categorized in index 3, and four mutants (CG-2 and -3; IC-6 and -12) together with wild-type p25 are classified into index 4 (Table 2). These results indicate a number of aa residues are involved in symptom induction in *B. macrocarpa,* while some residues such as C^{66}, C^{73}, R^{97}, D^{98}, D^{216}, D^{217} and D^{219} are dispensable. Interestingly, symptom severities on inoculated leaves and whole plants are co-related. We have also observed root phenotypes at the same time point, however, all were unexceptionally rampant in the culture tubes (correspond to the pots), thus were unable to evaluate.

Mutations of aa residing N- and C-terminal regions tended to attenuate symptom severity (Table 2). This may indicate the importance of those residues in molecular basis of p25-host interactions involved in symptom expression, as terminal peptides are often exposed to cellular environments. Indeed, deletion analyses using the truncated forms of p25 readily resulted in failure of YS formation in *T. expansa* (data no shown), supporting above notion and thus suggested the requirement of the whole protein sequence for characteristic symptom expressions.

Klein *et al*. (2007) demonstrated that mutations in the tetrad motif (aa position 67-70) greatly affected p25-pathogenicities and protein stabilities in *T. expansa* leaves, oligomerization and transcriptional activation abilities in yeast system (two- and one-hybrid assays), hence the mutations introduced in this study may have similar impact on such biochemical trait of p25.

Recent advances in the survey of host factors presumably involved in p25-mediated symptom inductions revealed a blueprint of molecular mechanism which including disruption of hormone balance (Peltier *et al*., 2011). However, the key virus-host interaction controlling symptoms remains unknown. In this regard, useful mutants exhibiting much milder virulence (disease index 1~2) in our collection may serve as a powerful tool; the use of such mutants would identify the precise interactions between p25 and host factor(s) which controlling symptom expression(s). Moreover, such approach may answer whether the molecular mechanism underlying p25-mediated symptom inductions in inoculated leaves, on whole plants, and in root system are co-related each other or independent.

Cys-Gly (CG) mutants				Ion-charged aa (IC) mutants			
RNA-3	mutation	Ionc.[a]	systemic[b]	RNA-3	mutation	Ionc.[a]	systemic[b]
wild-type	—	YS	4	IC-1	H13A, R14A	CS	1
				IC-2	R19A, R20A	CS	2
RNA-3 free	deletion	CS	0	IC-3	E24A, D25A, R26A	mYS	3
				IC-4	H43A, D44A, R46A	mYS	3
CG1	C36G	mYS	3 (2?)	IC-5	K57A, R58A, R60A	CS	2
CG2	C66G	YS	4	IC-6	R97A, D98A	YS	4
CG3	C73G	YS	4	IC-7	R106A, D108A	mYS	3
CG4	C79G	mYS	3	IC-8	H123A, D125A, R126A	mYS	3
CG5	C84G	mYS	3	IC-9	H133A, D135A	mYS	3
CG6	C90G	mYS	3	IC-10	R183A, E185A, E187A	CS	1
CG7	C173G	mYS	3 (2?)	IC-11	D203A, D205A	CS	2
CG8	C180G	mYS	3 (2?)	IC-12	D216A, D217A, D219A	YS	4

a. Local symptoms on inoculated leaves: YS, yellow spot; mYS, mild yellow spot; CS, chlorotic spot.
b. Systemic symptoms scored based on disease index (0~4).

Table 2 Mutation introduced into *p25* and their effect on symptom induction in *B. macrocarpa*

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THE GENETIC DIVERSITY OF *WHEAT YELLOW MOSAIC VIRUS* (GENUS *BYMOVIRUS*) THAT ASSOCIATES WITH DIFFERENCE OF HOST REACTIONS

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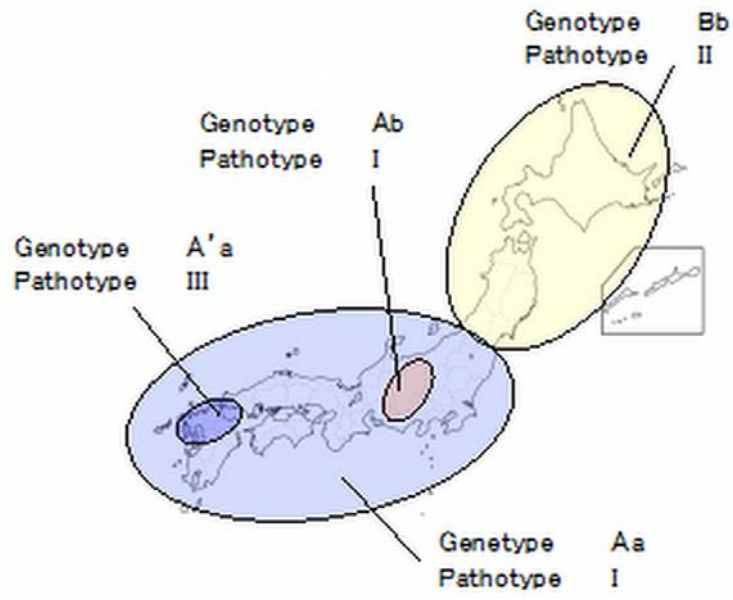
The biological and genetic diversity were compared among isolates of *Wheat yellow mosaic virus* (WYMV) in Japan. On the basis of wheat cultivar reactions, 14 isolates of WYMV from various regions of Japan were classified into pathotypes I, II or III, geographically distributed respectively in central, northern, and southern areas of Japan. In amino acid sequence comparisons of the WYMV isolates, the isolates comprised three genotypes (A, A′ and B) based on amino acid differences in RNA1 and two genotypes (a and b) based on differences in RNA2. Each isolate with the RNA1-based genotype was consistent with the pathotype, suggesting that the viral factor(s) associated with pathotypes exists in the WYMV RNA1. The RT-PCR-RFLP analysis showed that the genotype Aa and A′a distributed mainly from the central to southern regions of Japan, and genotype Bb was in northern parts of Japan. Interestingly, Chinese isolates, YA and YZ also agreed well with these genotypes; YZ and YA isolates were close to genotypes Bb and Aa, respectively. Wheat has been introduced from China to Japan in the 4th and 5th centuries, and two genotypes of WYMV might also have come from China and adapted to local wheat cultivars in Japan.



The distribution in Japan of genotypes and pathotypes of *Wheat yellow mosaic virus*

FUNGAL TRANSMISSION OF A COLOUR-BREAKING VIRUS INFECTING 'TAMA-NO-URA' CAMELLIA IN JAPAN

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Summary

A potted plant of young camellia (*Camellia japonica* L. 'Tama-no-ura') with colour-breaking was found in Goto, Japan in 2004. The colour-breaking was associated consistently with occurrence of a virus which is transmitted by an obligatory fungus, *Olpidium* sp. in the soil. The resting spores of the fungus was found to carry a virus causing colour-breaking and green spots or mottle when camellia seedlings were infected during and shortly after germination. These symptoms persisted at least 6 years in the greenhouse. A preliminary PCR test using primers for lettuce big-vein and tobacco stunt viruses failed to establish a possible relationship with the virus.

Introduction

The occurrence of Varicosa virus in camellia plants showing colour-breaking was reported previously (Hiruki, 1985). In the past, colour-breaking patterns in camellia have often considered beneficial as they increase the aesthetic value of a given specimen and used as a basis for assigning a new cultivar name in camellias worldwide. In 2004, hitherto unknown colour-breaking of 'Tama-no-ura' (*Camellia japonica* L.) was found in the Goto Archipelago in Japan. Unlike other colour-breakings, the disease is associated with the devastating reduction of commercial value of Tama-no-ura camellia by disturbing the development of white marginal picotee in the red petal tissues (Hiruki, 2012). This paper reports, for the first time, transmission by *Olpidium* of a virus that induces colour-breaking in camellia.

Materials and Methods

Plants

A camellia seedling suspected of being infected with a virus was obtained in 2004. While healthy 'Tama-no ura' plants showed an attractive regular white edge in deep red background of petals, virus-infected 'Tama-no-ura' exhibited irregular wavy pattern in the same area. The soil sample from the pot of the infected plant was used as inoculums in this investigation.

Growth conditions

Unless otherwise stated, camellia seedlings were grown in 17-cm diameter plastic pots containing a commercial premixed soil (Registered name: Sunshine mix LA4, peat/perlite/sand) from Sun Gro Horticulture Canada Ltd., Seba Beach, AB T0B 2B0, Canada. Greenhouse temperature was maintained at 21±1°C during day and 18°C Light period was 16 hrs with a light intensity of 400 mv provided with C5F54T5/835/color HO Highoutact band lamps. Fertilizer (15/30/15) was provided biweekly at a concentration of 200 ppm.

Molecular analysis

Molecular analysis of viruses was performed according to the procedures described previously (Sasaya *et al*., 2005).

Scanning electron microscopy

Methods employed were the same as published previously (Hiruki and Alderson, 1976).

Results

Detection and isolation of viruliferous *Olpidium* sp. from infested camellia soil

In preliminary tests including several species of plants, tobacco (*Nicotiana tobacum* L. 'Bright Yellow') was found to be most susceptible to *Olpidium* (Hiruki, 1999). A few week-old tobacco seedlings

were used as bait plants for detection and isolation of *Olpidium*. Zoospores and zoosporangia were found in the root tissues. Resting spores typical for *Olpidium* were found in root tissues at late stages of infection by light microscopy as well as scanning electron microscopy.

Inoculation of camellia seedlings with viruliferous *Olpidium*

After establishing a culture of viruliferous *Olpidium* in tobacco, *Olpidium*-infected roots were air-dried and pulverized with a rubber stopper in a mortar. About one gram of *Olpidium*-containing root powder was mixed with 9 gram of autoclaved soil. The mixture was spread thinly over pot soil in each pot and camellia seeds were placed in the mixture. About one month after the seeding, tobacco seeds were sowed in the same pot for *Olpidium* detection, as it was found easier to find *Olpidium* in young tobacco roots, although *Olpidium* was found in camellia roots as well.

Detection of *Olpidium* from virus-infected camellia plants

Observation of green mottle symptom development and the presence of *Olpidium* in the camellia roots and pot soil were continued for 6 years, using 5 camellia seedlings after inoculation of germinating camellia seeds in 2006 and the symptom and the presence of *Olpidium* were confirmed.

Symptomatology

'Tama-no-ura' is known for its regular white marginal picotee in deep red background of petals (Hiruki, 2008). However, once virus-infected, flowers subsequently produce misshapen, ugly patterns. Frequently wavy patterns appear along the edge of petals, but the number and size of white patches were not consistent. Leaf symptoms as green spots or mottle were developed in 3 to 5 months after inoculation. The typical green spots and mottle were developed each year as new leaves emerged. In 2009, first floral buds were formed. Typical colour-breaking of picotee in Tama-no-ura was observed in the camellia plants artificially inoculated by means of *Olpidium* in this study.

Discovery of naturally infected 'Tama-no-ura' camellia showing colour-breaking in the field

In 2006, a 'Tama-no-ura' camellia plant of about 10-year old growing in the field was found showing colour-breaking. Leaf symptoms in the infected plant were not very distinct as it was exposed to sun in the field, but flowers displayed severe colour-breaking of various degrees.

Molecular analysis

Independent PCR analyses were performed by Dr. S. Kuwata and Dr. T. Sasaya, using primers for lettuce big-vein and tobacco stunt viruses, failed to establish a possible relationship with the viruses. Both artificially inoculated plants and a naturally infected field plant of camellia were used in these analyses.

Discussion

First case of fungal transmission of a camellia virus

Sap transmission of virus is very common in many virus infections in plants. However, camellia variegation (=*Yellow mottle*) virus could not be transmitted by sap (Hiruki, 1985). In this paper, fungal transmission of the virus in 'Tama-no-ura' has been established for the first time, although the result from a previous electron microscopy study (Hiruki, 1985) suggested that camellia variegation virus morphologically belongs to *Varicosa* virus that is transmitted by *Olpidium* (Hiruki, 1996).

First case in definitive reduction of the aesthetic value of a camellia cultivar

There are numerous cases of colour-breaking in camellias and some of them are prize winners in camellia flower shows like 'Donckelaeri' (='Masayoshi'). In some cases, the introduction of virus by means of grafting was reported previously (Plakidas, 1948, 1952, 1954; Tourje, 1950) and it was suggested that grafting was a useful means of producing attractive new cultivars (Plakidas, 1948). However, in the case of 'Tama-no-ura', virus infection is definitely detrimental to its aesthetic value and results in the drastic reduction in the saleability of the seedlings in the market. Thus, virus infection of 'Tama-no-ura' should be avoided by all means.

Perspectives in the future studies

There are many cases of colour-breaking in camellia cultivars that are suspected to be caused by a virus or viruses (Milbrath *et al*., 1946). However, there are no data to relate each case to establish relationships among presumptive viruses, although a suggestion was made for the presence of strains of a camellia virus (Plakidas, 1962). Detailed molecular studies are expected to shed light to the classification of camellia viruses in the near future.

Since camellias are adapted to subtropical environment and relatively tolerant to high ambient temperatures, heat incubation of diseased plants offers a possible treatment to reduce the harmful

effects of virus infection. Such heat treatments are in progress in an attempt to cure the disease in the valuable cultivars of commercial camellias.

Acknowledgements

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TRANSMISSION OF *LETTUCE BIG-VEIN ASSOCIATED VIRUS* AND *MIRAFIORI LETTUCE BIG-VEIN VIRUS* BY *OLPIDIUM BRASSICAE*

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Summary

In order to make clear if *Olpidium brassicae* transmits *Lettuce big-vein associated virus* (LBVaV) and *Mirafiori lettuce big-vein virus* (MiLBVV), transmission experiments were conducted using the virus-infected cabbage plants. LBVaV- or MiLBVV-single infected lettuce cuttings were approach grafted on cabbage seedlings. Two months after the grafting, LBVaV or MiLBVV was detected in the grafted cabbage leaves by RT-PCR and the transmission of each virus from the virus-infected lettuce plants to cabbage plants was confirmed by RT-PCR. A virus-free strain of *O. brassicae* was inoculated into the virus-infected grafted cabbage plants and acquired each virus. The *O. brassicae* was inoculated into healthy cabbage seedlings and the transmission of each virus was checked. The cabbage seedlings that were inoculated with the LBVaV-carrying fungus induced mild mosaic symptoms on their leaves after one month and the virus infection was confirmed by RT-PCR. On the other hand, the cabbage seedlings that were inoculated with the MiLBVV-carrying one were symptomless, however the virus was detected in the cabbage seedlings by RT-PCR two months after inoculation.

Introduction

Lettuce big-vein disease is soilborne viral disease, and the causal agent is *Mirafiori lettuce big-vein virus* (MiLBVV). *Lettuce big-vein associated virus* (LBVaV), which is non-pathogenic for lettuce, is also detected from the diseased plants. These two viruses are transmitted by the soil-borne chytrid, *Olpidium virulentus* (Sasaya *et al*., 2008). *Olpidium brassicae* had been previously reported as a vector of LBVaV and MiLBVV. However, the molecular analysis of the rDNA-ITS regions of crucifer and non-crucifer strains of *O. brassicae* revealed that the non-crucifer strain is a distinct species rather than a strain of *O. brassicae*, and the non-crucifer strain was named as *Olpidium virulentus* (Sasaya and Koganezawa, 2006). *Olpidium virulentus* can infect lettuce plants and transmit LBVaV and MiLBVV to lettuce plants, while *O. brassicae* cannot infect lettuce plants and it is not shown whether it could be a vector of these viruses (Sasaya and Koganezawa, 2006).

In this research, we prepared the virus-infected cabbage plants by approach grafting onto LBVaV- or MiLBVV-single infected lettuce cuttings, and examined the virus transmissibility of *O. brassicae* by using the test plants.

Materials and Methods

Analysis of physiological differences between *O. brassicae* and *O. virulentus*

Olpidium brassicae was obtained from a cabbage field soil at Kagawa Prefecture of Japan, and *O. virulentus* was obtained from a lettuce field soil at Kagawa Pref. Each *Olpidium* was inoculated into cabbage, Chinese cabbage, lettuce, cowpea, and oriental melon seedlings. The extent of *Olpidium*-infection in roots was observed by a microscope. To extract DNA, resting spores of these *Olpidium* species were beads-beated by a multi beads shocker (Yasui Kikai, Japan). Their rDNA-ITS region was PCR-amplified by universal primers (White *et al*., 1990), and analyzed electrophoretically.

Preparation of virus-infected cabbage plants by approach grafting

LBVaV- or MiLBVV-single infected lettuce was cut off at its stem above the ground with a razor and put in water. After the cutting took root at the cut end, epidermis of a virus-infected lettuce cutting and a

healthy cabbage seedling were thinly peeled off with a razor. And then, two plants were approach grafted by making cut end surfaces stick together and transplanted to a pot. Two months after the grafting, total RNA was extracted from young leaf and roots of each grafted plants and viral infection was checked by RT-PCR.

Viral transmission assay of *Olpidium brassicae* using grafted cabbage plants

Virus-free *O. brassicae* was inoculated into the virus-infected grafted cabbage plant and propagated in the cabbage roots. A pot of *Olpidium*-inoculated grafted plants was embedded in a slightly larger plastic case. Then, healthy cabbage seedlings were transplanted around the pot in the case. The pot had a drainage hole though which zoospores of *O. brassicae* released from the grafted cabbage roots could pass and infect cabbage seedlings' roots. Total RNA was extracted from young leaf of cabbage seedlings and viral infection was checked by RT-PCR.

Results

Physiological differences between *O. brassicae* and *O. virulentus*

Olpidium brassicae infected cabbage and Chinese cabbage roots and severely multiplied (Table 1). However, it could scarcely infect non-cruciferous plants including lettuce, cowpea, and oriental melon. In contrast, *O. virulentus* could not infect the cruciferous plants, but proliferate heavily in the other plants (Table 1). In *O. brassicae*, the size of rDNA-ITS region was 600 bp and was smaller by about 30 bp than that of *O. virulentus* (Fig. 1).

	cabbage	Chinese cabbage	lettuce	cowpea	oriental melon
Olpidium brassicae	+++	+++	-	+	-
Olpidium virulentus	-	-	+++	+++	+++

extent of infection: +++,severe, ++, moderate, +, slight, -, not infected

Table 1. Host specificity of *O. brassicae* and *O. virulentus*

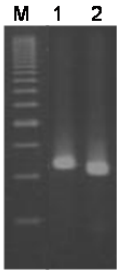

Figure 1
Electrophoretic profile of rDNA-ITS region of two *Olpidium* species.
1: *O. virulentus*, 2: *O. brassicae*, M: 200 bp DNA ladder marker

Transmission of LBVaV and MiLBVV from lettuce to cabbage by approach grafting

There was no problem with the growth of approach-grafted plants. In both cases of LBVaV and MiLBVV, the size of RT-PCR products derived from donor lettuce and recipient cabbage was the same (Fig.2).

Transmission of LBVaV and MiLBVV by *Olpidium brassicae*

One month after inoculation of *O. brassicae* released from LBVaV-infected grafted cabbage, cabbage seedlings showed mild mosaic symptoms on their true leaves (Fig. 3a)

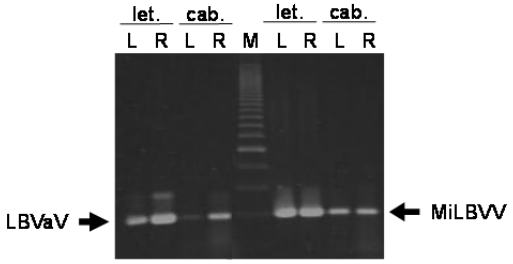

Figure 2 Viral detection from approach-grafted plants by RT-PCR. L: leaf, R: roots, M: 200 bp DNA marker

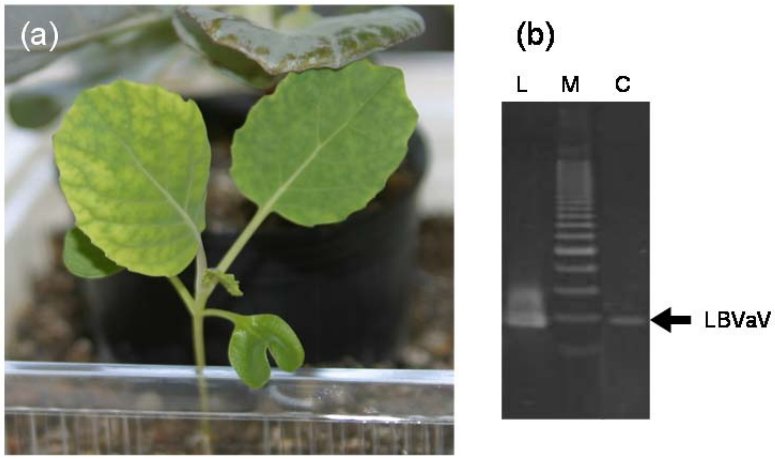

Fig. 3 LBVaV-infected cabbage seedling transmit-ted by *O. brassicae*.
(a) Mild mosaic symptom of LBVaV-infected cabbage seedling.
(b) LBVaV detection from the seedling by RT-PCR. L: lettuce, C : cabbage, M: 200 bp DNA marker

and LBVaV infection was confirmed by RT-PCR (Fig. 3b). In the case of MiLBVV transmission assay, the cabbage seedlings were symptomless. However, MiLBVV was detected in the cabbage seedlings by RT-PCR two months after inoculation (data not shown).

Discussion

Olpidium brassicae, used in this research, had almost the same characteristics of host specificity and the size of rDNA-ITS region as previously reported data (Sasaya and Koganezawa, 2006). Also, its zoosporangium morphology and sexuality were the same as shown by Koganezawa *et al*. in 2004 (data not shown). In addition, anti-*O. virulentus* resting spores IgG did not react to its resting spores (Nomiyama *et al*., 2013).

Although LBVaV and MiLBVV are supposed not to be inoculated into plants by mechanical transmission, it is interesting that these viruses can infect cruciferous cabbage from composite lettuce by approach grafting. As a result, it was elucidated that *O. brassicae* was a vector of LBVaV and MiLBVV, which could not be clarified in the previous report (Sasaya and Koganezawa, 2006). Our results contribute to the further understanding of the mechanism of *in vivo* viral transmission by *Olpidium* species.

The cabbage seedlings that were inoculated with the LBVaV-carrying *O. brassicae* induced mild mosaic symptoms on their leaves. Because LBVaV is non-pathogenic for lettuce, further experiments are required to figure out what is attributed to the symptomatic difference between them.

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OCCURRENCE CONDITIONS AND CONTROL MEASURES OF RHIZOMANIA IN SUGAR BEET IN OKHOTSK DISTRICT OF HOKKAIDO

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Summary

In the last two decades, lower yields and sugar content of sugar beet that are due to yellowing symptoms have become serious problems in the Okhotsk District (including Kitami, Enmon, and Shamou areas). Therefore, we investigated the causal factors of yellowing fields throughout this district. In 2002 to 2005, yellowing symptoms were observed in 77.9 % fields in Kitami and Enmon areas, whereas they were observed in 46.7 % fields in Shamou area. About 75 % of yellowing fields were caused by rhizomania, and 24 % of fields were caused by bad soil conditions such as wet or drought injury. Some fields were damaged by infestation of mites. Thus, it was found that most of the yellowing fields were due to rhizomania, and in addition weather and soil conditions affected the disease occurrence. Comparisons of susceptible and resistant cultivars to rhizomania in several fields showed that the use of resistant cultivars is most effective to control the disease. From these results, we recommend the introduction of rhizomania-resistant cultivars in the Okhotsk District and the improvement of subsurface soil in fields of faulty soil environment.

Introduction

The Okhotsk District (including Kitami, Enmon, and Shamou areas) as well as the Tokachi District is a major area of sugar beet cultivation in Hokkaido. In the last two decades, lower yields and sugar content of sugar beet that are due to yellowing have become serious problems especially in Kitami and Enmon areas in the Okhotsk District. In general, yellowing symptoms in sugar beet fields are thought to be due to several factors such as rhizomania, insect-borne yellowing virus diseases, nematode and mite damages, nutrition deficiency, and physical disorders (e.g. wet damage). Among these factors, rhizomania that caused by Beet necrotic yellow vein virus (BNYVV) is most common and causes severe damage in sugar beet. Yield losses depend greatly on the inoculum level in the soil, the weather conditions and the time of infection. Severe infection leads to reduction in yield of 50% or more, especially the sugar content is reduced from 16-18% to less than 10% (Asher, 1993; Tamada, 1999). BNYVV can survive in the soil for many years without any decrease in infectivity. The disease has been widely distributed in Hokkaido; BNYVV was detected by ELISA-rootlet diagnosis method from about 23% fields in 1982 (Abe, 1987). Nevertheless, until the early 2000s, resistant cultivars to rhizomania have been grown in only a few areas. Here, we further surveyed the occurrence of yellowing fields in Okhotsk District, and found that the major factor is rhizomania and confirmed the effectiveness of resistant cultivars to control the disease.

Materials and Methods

The occurrence of yellowing sugar beet fields was surveyed in total 1834 fields in September from 2002 to 2005 in Kitami, Enmon and Shamou areas in the Okhotsk District. Out of them, 158 fields in Kitami and Enmon areas were selected and investigated in detail factors affecting low sugar yield caused by yellowing. Rhizomania, soil environment and spider mites were targeted as factors. Rhizomania was analyzed by detection of BNYVV by DAS-ELISA (Tamada and Hagita, 1982). Soil environment was assessed by thickness of top soil, hardness, water permeability, moisture and pH according to fixed methods. Spider mites were counted. As for poor soil environment, the improvement

of soil condition (i.e. water and air permeability of soil) by improved pan-breaking was conducted. To control rhizomania, effectiveness of resistant cultivars was assessed. Rhizomania-resistant cultivars “Molino”, “Flöden R” and “Kitasayaka” and susceptible cultivars “Abend”, ”Skåne” and “Yukihinode” were used.

Results

Survey of yellowing sugar beet fields and their causal factors

Sugar beet fields showing yellowing symptoms (yellowing fields) were surveyed in different areas of the Okhotsk District for 4 year periods of 2002 to 2005. Out of 869 fields surveyed, yellowing symptoms were found in 77.9 % fields were in Kitami and Enmon areas, whereas they were found in 46.7 % fields in Shamou area. Table 1 shows the results of detailed analyses in 158 yellowing fields selected from Kitami and Enmon areas. Rhizomania is a major causal agent of the yellowing fields, but in some fields, other factors, mite injury and poor soil environment, were concerned doubly. Totally, BNYVV was detected from sugar beet rootlets in 118 fields (74.7 %). Poor soil environment was found in 38 fields (24.1%) and spider mites were observed in 29 fields (18.4%); however, yellowing by unknown factors was seen in 22 fields (13.9 %).

Rhizomania	Factors	Number of fields				
		Year				
		2002	2003	2004	2005	total
Positive	Rhizomania only	20	14	25	14	73
	+mites	5	9	1	0	15
	+ soil environment	9	4	8	7	28
	+ mites and soil environment	1	1	0	0	2
Negative	Mites	1	6	2	1	10
	Soil environment	4	1	0	1	6
	Mites and soil environment	1	1	0	0	2
	Unknown	8	9	3	2	22
Total		49	45	39	25	158

Table 1 Occurrence of rhizomania and other yellowing symptoms in sugar beet in the Okhotsk District

Subsoil improvement

Some yellowing fields were due to poor soil environmental conditions (e. g. wet damage), and therefore we conducted subsoil improvement by the improved pan-breaking. Eight fields with different soil conditions were selected and subsoiled at 55 to 70 cm intervals. Effects of subsoil improvement were different in different fields (Fig. 2). In fields where plowed soil layers (worked soil layer) were about 30 cm or below, root weights of sugar beet decreased in treated fields, but in fields where plowed soil layers were much thicker, the subsoil improvement was effective. Notably, the effect in rhizomania-infested fields was great. The reason for decreasing sugar yields observed in fields with thin plowed soil layers was thought to be due the mixing of a large amount of subsoil with poor nutrient content to the top soil. White and black circles show non-treated and treated fields, respectively. Black arrows show the contrast effect. A dotted line indicates rhizomania-infested fields. The worked soil layer over 45 cm may contain surface humus layer.

Effect of resistant cultivars

It is well known that use of resistant cultivars in rhizomania-infested fields is effective to control this disease (Asher, 1993; Tamada, 1999). In this study, we assessed effects of rhizomania-resistant cultivars in different levels of infestation. Five fields were heavily infested, in which BNYVV had been detected from all of the rootlets tested. Eleven fields were moderately (or less heavily) infested, in which BNYVV had been detected from less than 80% of rootlets. In heavily infested fields, resistant cultivars were much more effective than susceptible cultivars (Fig. 2A). In moderately infested fields, the

difference of the sugar yield between tolerant cultivars and susceptible cultivars varied. In fields of number 7, 9, 14, and 16, the sugar yield of resistant cultivars was less than that of susceptible cultivars and in the other fields, the sugar yield of resistant cultivars exceeded that of susceptible cultivars (Fig. 2B).

Discussion

In our survey in 2002 to 2005, yellowing fields of sugar beet occurred much more in Kitami and Enmon areas than in Shamou area. About 75 % of yellowing fields were found to be caused by rhizomania, and 24 % of yellowing fields were due to bad soil conditions such as wet or drought injury. The latter case was thought to be influenced by the weather condition. In our trial, soil condition could be improved by improved pan-breaking, except for a few fields where worked soil layers were thin. In the Enmon area, there are many fields with poor soil condition, in which improvement of water and air permeability of soil should be recommended.

This survey also confirmed the importance of rhizomania in the Okhotsk district. Especially, in Kitami and Enmon areas, the occurrence of rhizomania-infested fields was more than half of fields. Abe (1987) reported that rhizomania in Abashiri (=Okhotsk) occurred in 191 fields (about 2%) in 1970, and these infections were introduced and spread by paper pot transplantation using infested soils. Furthermore, an ELISA-rootlet detection survey conducted in 1982 showed that BNYVV was detected in 37% fields in this district, whereas it was detected in 23% out of 550 fields throughout Hokkaido. Thus, it is clear that the occurrence of rhizomania increased rapidly or gradually. The reason for a difference in yellowing fields between Kitami and Enmon area and Shamou area is not clear, but generally, soil pH strongly affects the severity of rhizomania incidence (Abe, 1987). The vector *Polymyxa betae* infects most rapidly and actively with neutral and alkaline soils (Abe, 1987).

The cultivation of resistant or tolerant cultivars is the most promising way to control the rhizomania (Asher, 1993; Tamada, 1999). Therefore, we assessed the effect of resistant cultivars in different levels of infestation and confirmed that sugar yield of resistant cultivars was higher than in susceptible cultivars in any infested field, which may depend on inoculum potentials (infestation levels). In 2002, the cultivation acreage of resistant cultivars in the Okhotsk district was only 7.2 %, but in 2012, it increased of almost 100%.

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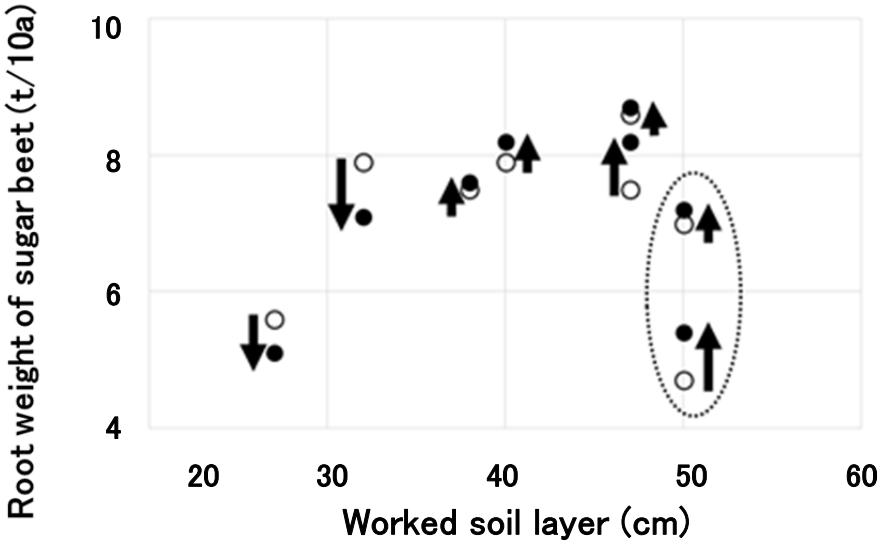

Figure 1 Effect of subsoil improvement by the improved pan-breaking on root weights of sugar beet. White and black circles show non-treated and treated fields, respectively. Black arrows show the contrast effect. A dotted line indicates rhizomania-infested fields. The worked soil layer over 45 cm may contain surface humus layer.

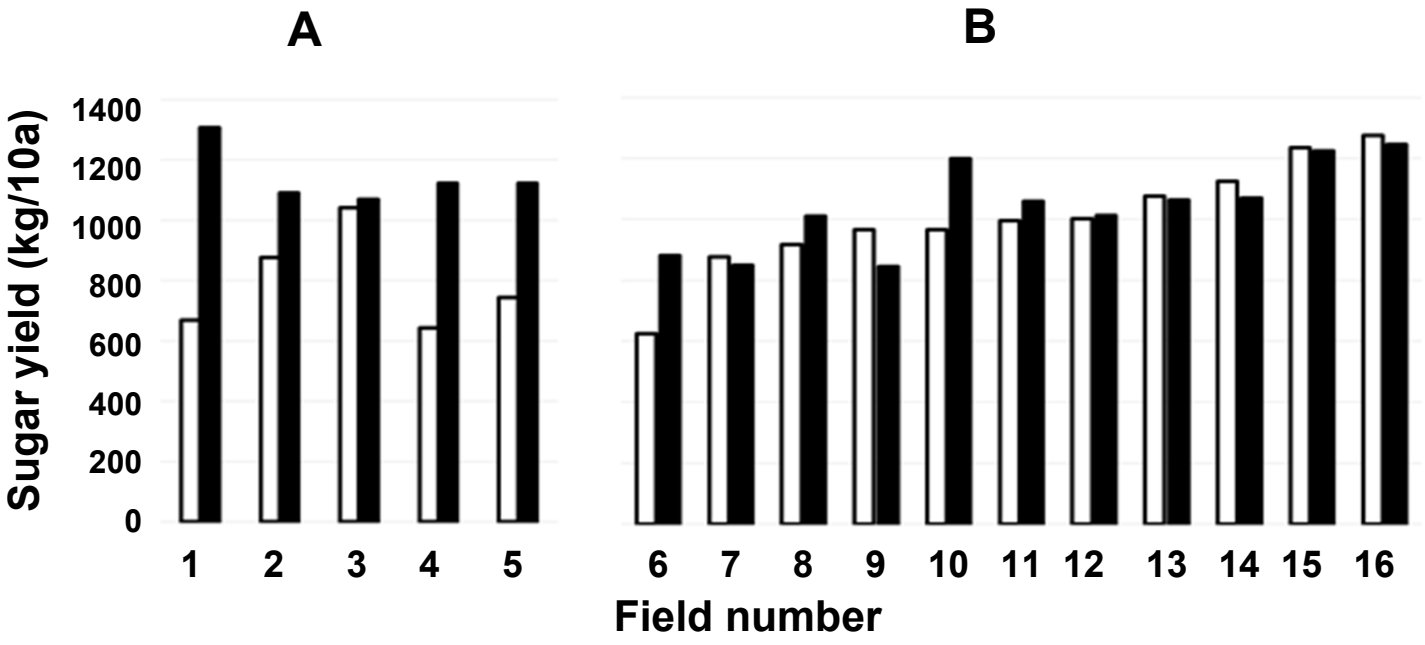

Figure 2 Comparison of sugar yield of susceptible and resistant cultivars in heavily rhizomania infested fields (A) and in moderately to less heavily rhizomania infested fields (B). White and black bars show rhizomania-susceptible and resistant cultivars, respectively.

VALIDATION OF RESISTANCE GENE FOR WHEAT YELLOW MOSAIC VIRUS IN JAPANESE WHEAT CULTIVAT 'YUMECHIKARA'

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Summary

Wheat yellow mosaic, caused by *Wheat yellow mosaic virus* (WYMV), is one of the most serious soil-borne diseases of winter wheat (*Triticum aestivum* L.). In this study, 247 doubled-haploid lines (DH) developed from 'Yumechikara' (resistant) / 'Kitahonami' (susceptible) were genotyped using the microsatellite markers closely linked to *YmIb* and tested in field trials at a nursery located in Utsunomiya in central Japan in where WYMV Type I is predominant. The result of WYMV detection by ELISA test was coincident with marker genotypes in 235 lines (95.1 %) and 238 lines (96.4 %) in 2010-2011 and 2011-2012, respectively. Results suggested the *YmIb* is also effective in Kanto region in where WYMV type I is distributed.

Introduction

Wheat yellow mosaic (WYM) is one of the most devastating soil-borne diseases of winter wheat caused by *Wheat yellow mosaic virus* (WYMV). WYMV is vectored by a soil-borne pathogen fungus-like organism *Polymyxa graminis* (Inouye, 1969), and the disease is characterized by yellow-striped leaves and stunted spring growth, which results in severe yield loss. WYMV was first reported on the Japanese mainland in the 1920s, and spread to Hokkaido, the northernmost grain production area of Japan in the 1990s. In Japan, WYMV pathogenicities are classified into three types based on the infectivity on the differential cultivars (Ohto, 2006): type I is mainly distributed in west and central part of Japan; type II is mainly distributed in northern part of Japan; and type III is distributed in a part of south west of Japan.

It has been reported that some of WYMV resistant cultivars including 'Yumechikara' which has WYMV resistance in Hokkaido (Type II) contained common resistant haplotype with 'Ibis' around the resistance gene *YmIb* region on the chromosome 2D (Nishio *et al*., 2010). The objective of this study was to identify the resistance genes of 'Yumechikara' in Kanto region (Type I).

Materials and Methods

Plant materials

A population of 247 F_1-derived doubled-haploid (DH) lines was developed from 'Yumechikara' (resistant) / 'Kitahonami' (susceptible). DH lines were developed in 2003-2004 from F_1 plants using the maize pollination procedure. Seeds from individual DH plants were multiplied in 2005-2006 for field trials.

Field experiments for wheat yellow mosaic resistance

The DH and their parents were tested in field trial at a nursery located in Utsunomiya, Kanto during 2010-2011 and 2011-2012, in where WYMV type I is distributed. Each experimental unit consisted of 10 plants in a single 0.5 m row and spaced 0.3 m apart. The DH and their parents were sown in early November and were grown under normal field condition with standard cultivation practice.

Phenotypic evaluation of WYMV response

WYMV severities were scored in March and April of both 2010-2011 and 2011-2012. A 0-5 scale was used to represent different disease severities, where 0 = no visible symptoms, 1 = slightly purple leaves stained with anthocyanin, 2 = slightly yellowish leaves with mild streak mosaic, 3 = yellowish leaves with streak mosaic, 4 = distinct yellow and streak mosaic area covering almost all of the leaves, 5 = yellowish brown leaves and more than half of the plants dead.

ELISA test and DNA marker genotyping

For ELISA test, leaf samples were collected on April 7th in year 2011 and on March 13th in year 2012. The ten bulked leaf samples per each line were used for the ELISA test. Four microsatellite markers *wmc181*, *cfd16*, *wmc41* and *cfd168* that are closely linked to *YmIb* (Nishio *et al*., 2010) were tested on the DH lines and their parents.

Results

Genotyping of DH lines by microsatellite markers

Out of the 247 DH lines, 116 lines showed the same haplotype with 'Yumechikara' (resistant parent), while 131 lines showed the same haplotype with 'Kitahonami' (susceptible parent) for the four microsatellite markers (*wmc181*, *cfd16*, *wmc41* and *cfd168*) closely linked to *YmIb*. Although these markers were mapped on the both side at distance of 2.0 to 12.4 cM from *YmIb* in previous study (Nishio *et al*. 2010), the four markers were co-segregated within the all DH lines.

Evaluation of WYMV response

In the two years field trails, 'Yumechikara' showed stable WYM resistance (disease index 1.0) while 'Kitahonami' showed severe symptoms (disease index 4.0). Distribution of disease index of the DH lines is shown in Fig. 1. The disease indices of the DH lines with resistant haplotype were distributed in lower range than that of the DH lines inherited susceptible haplotype. The average disease index of 116 DH lines inherited resistant haplotype was 1.27 and 1.66 in 2010-2011 and 2011-2012, respectively. Out of the 116 DH lines, WYMV was detected from only 5 and 9 DH lines by ELISA in 2010-2011 and 2011-2012, respectively. On the other hand, the average disease index of 131 DH lines inherited susceptible haplotype from 'Kitahonami' was 2.82 and 3.66 in 2010-2011 and 2011-2012, respectively. Out of the 131 DH lines, WYMV was detected from 124 and all 131 DH lines by ELISA in 2010-2011 and 2011-2012, respectively.

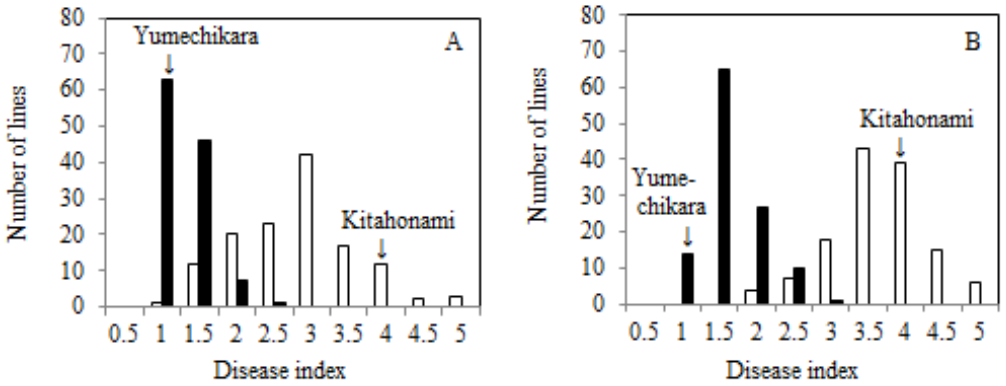

Figure 1 Distribution of wheat yellow mosaic indices for 247 doubled-haploid (DH) lines derived from 'Yumechikara'/'Kitahonami' in 2010-2011 (A) and in 2011-2012 (B). Solid bars and open bars indicate the lines which showed the same haplotype as in 'Yumechikara' and 'Kitahonami' for the microsatellite markers wmc181, wmc41, cfd168, and cfd16, respectively.

Discussion

There were significant differences between the mean disease index of the lines inherited resistant haplotype and the lines inherited susceptible haplotype in both two years. Here we identified that the resistant gene on the chromosome 2D of 'Yumechikara' is also effective for WYMV type I, suggesting the MAS using these markers would be effective. According to the meteorological data provided by Automated Meteorological Data Acquisition System (AMeDAS), average temperature from November through February in 2011-2012 was lower than that in 2010-2011. It was reported that the favorable temperature for the virus propagation in wheat plants is lower than 10°C (Ohto, 2005). Higher disease index in 2011-2012 than in 2010-2011 would be attributed to the favorable condition for WYMV in 2011-

2012. Within the two lines infected for both years even though possessing resistant haplotype, further investigation is needed to confirm the phenotype of these lines.

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QUANTITATIVE DETECTION OF MAJOR THREE *OLPIDIUM* SPECIES USING COMBINATION OF FUNGAL PROPAGATION IN ROOT AND PCR

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Summary

Few Olpidium species are recognized as plant viral vector. For control of those viral diseases, it is important to know how much amount of such fungi survives in the field soil. We developed the quantitative detection system of major three *Olpidium* species, *O. bornovanus*, *O. brassicae*, and *O. viulentus* using combination of fungal propagation in host plant root and PCR (multiplex PCR and nested multiplex PCR).

At first, 2 g of sample soil are inoculated to a pot individually reared seedlings of *Cucumis melo* or *C. melo* var. *makuwa* for 1 or 2 weeks then preparing DNA from roots of host plant inoculated. As a second step, nucleotide sequences of internal transcribed spacers of *Olpidium* species are detected in DNA isolated from host plant root using different detectable sensibility PCR, multiplex PCR and nested multiplex PCR. Following this procedure, amount of fungi in soil could be stepwise evaluated by combination of two step PCR systems.

In field trials of this detection system, sampled some soils from fields which cultivate cabbage, melon, or tulip, were put into the system to quantitatively evaluate presence of *Olpidium* species. The results showed that *Olpidium* species were detected in several field soils which were not fully correspondent to incidence of viral disease occurred in the past.

Introduction

Our group is trying to evaluate the risk of virus disease in field, especially melon necrotic disease, lettuce big vain disease, and tulip streak disease. *Olpidium bornovanus*, *O. brassicae* and *O. virulentus* which were classified into *Chytridiomycota*, are obligate plant parasite and act play as these virulent viral vectors (Sasaya and Koganezawa, 2006). Already, we tried to detect virulent virus and measure the amount of virus in field for evaluation of the risk of these virus diseases. But it is so difficult by simply and practical method. Because, detection was affected by soil components, and the amount of virus in the field are unsufficient to detect the virus. Then, we attempt to measure the amount of fungi which carried virulent virus in the field for evaluation of the risk of these virus diseases. Amount of fungi was measured by two strategies, direct detection and indirect detection. In direct detection, template DNA was isolated from soil and fungi were detected from DNA by PCR. And in indirect detection, template DNA was isolated from bite plant root and fungi were detected from DNA by PCR.

Materials and Methods

Olpidium species isolated from Japan using previously reported method (Lin *et al*., 1970) and were maintained by reported method (Mochizuki *et al*., 2012). Template DNA was isolated from soil using ISOIL for Beads Beating (NIPPON GENE) following manuscript in direct detection. Template DNA of *Olpidium* species including host plant DNA was isolated from inoculated plant (*Cucumis melo* var. makuwa) root in indirect detection. Plant was seeded on 50 ml tube filled with autoclaved vermiculite and was incubated for 1 week in green chamber at 27°C with 16 hr photoperiod. Then, 2 gram of sample soil was inoculated to this seedling and was incubated for 1 week or two weeks in green chamber at 27°C with 16 hr photoperiod. DNA was isolated from inoculated roots using DNeasy Plant mini kit (QIAGEN)

or simple DNA isolation method. To summarize of simple DNA isolation method, 0.1 g of root, 250 ul of TE, and 100 ul of PCI (50 % phenol, 48% chloroform and 2% isoamyl alcohol) put into 2 ml tube and homogenized by beads shocker (Yasui kikai). The supernatant was collected by centrifugation (20,000 x g, 10 min), diluted 10-fold with water and used as template for PCR. Primers used in this study are listed in Table 1. Each PCR carried out with KOD plus (TOYOBO), or MyTaq (Bioline), and condition followed each manuscript.

Results and Discussion

At first, we tried to construct quantitative detection of *O. bornovanus* which carried *melon necrotic spot virus*. Two methods, direct detection and indirect detection method of *O. bornovanus*, in filed were constructed and compared. In direct detection, template DNA was isolated from soil and *O. bornovanus* specific band was amplified by normal PCR and nested PCR. PCRs carried out using KOD plus in *O. bornovanus* detection. Normal PCR performed with F1-R1 primers, and primary reaction of nested PCR performed with O2-bor2 primers and secondary reaction of nested PCR performed with F1-R1 primers. Detectable level of normal PCR was lower than nested PCR. We assumed if specific band was detected in normal PCR, high amount of *O. bornovanus* were in soil, and if specific band was detected in nested PCR, middle amount of *O. bornovanus* were in soil. In indirect detection, template DNA was isolated from inoculated plant root by kit. The different amounts of *O. bornovanus* were propagated in root for different period, and these different amounts of fungi in root might affect the detectable level of PCR. We assumed, if specific band was detected in normal PCR from root DNA at 1 week after inoculation, high amount of *O. bornovanus* were in soil, if specific band was detected in nested PCR from root DNA at 1 week after inoculation and in normal PCR from DNA at 2 weeks after inoculation, middle amount of *O. bornovanus* were in soil, and if specific band was detected in nested PCR from DNA at 2 weeks after inoculation, low amount of *O. bornovanus* were in soil.

We tried to detect *O. bornovanus* in soil from melon cultivated field by direct and indirect detection. Melon necrotic spot disease was found in this filed. This soli sample was diluted 3, 9, 27 fold with sterilized sand (Table 2). In direct detection, no band was detected by normal PCR and *O. bornovanus* specific band was amplified from no diluted sample by nested PCR (Table 2). In the other hand, *O. bornovanus* specific band was amplified from 27 fold diluted soil by indirect detection and detectable level became lower when polluted soils were diluted to lower (Table 2). These results suggested that indirect detection could quantitatively detect the fungi in soil, and detectable sensitivity of indirect detection is higher than direct detection. We thought that indirect detection is practical and useful for quantitatively detection.

In this way, we developed detection method of *O. bornovanus*. But it was required that detection of other *Olpidium* species which were carried virulent virus. Then, we constructed quantitative detection method of major three *Olpidium* species, *O. bonovanus*, *O. virulentus*, and *O. brassicae*. Detection method of *Olpidium* species was constructed by improving indirect detection of *O. bornovanus*. Template DNA was isolated from inoculated plant root with simple DNA isolation method and species specific bands were detected from root DNA by multiplex PCR and nested multiplex PCR using MyTaq. Primary reaction of nested multiplex PCR performed with OLPF1 and OLPR, multiplex PCR and secondary reaction of nested multiplex PCR performed with OLPborF, OLPbra, OLPvir and OLPR2. It was confirmed that species specific bands, *O. bornovanus* specific band is about 1 kbp, *O. brassicae* specific band is about 200 bp, and *O. virulentus* specific band is about 500 bp, were amplified from infected field soil by multiplex and nested multiplex PCR.

Therefore, this method behave similar to *O. bonovanus* indirect detection, might be able to be quantitatively detected the fungi. Then, we assumed, if specific band was detected in multiplex PCR from root DNA at 1 week after inoculation, high amount of fungi were in soil, if specific band was detected in nested multiplex PCR from DNA at 1 week after inoculation and in multiplex PCR from DNA at 2 weeks after inoculation, middle amount of fungi were in soil, and if specific band was detected in nested multiplex PCR from root DNA at 2 week after inoculation, low amount of fungi were in soil.

We tried to detect *Olpidium* species in soil from melon cultivated field by O. *bornovanus* indirect detection and *Olpidium* species detection method (Table 3). Samples were collected from melon cultivated field. No disease was found in field 1 and field 2, and melon necrotic spot disease was found in field 3 to 5. Results of two methods showed middle amount of fungi are in filed 3 to 5. It suggested two methods showed same detectable level. Therefore, no one detected by *O. bonovanus* detection, but *O.*

virulentus was detected by *Olpidium* species detection in the field 2. This result suggested the possibility of existence of *Olpidium* species which were carried some virulent virus in no disease field. We thought that this *Olpidium* species detection method will be applied to evaluate the risk management in field when other crops will be cultivated, for example, crop rotation.

Actually, we are collecting soil from cultivated field (melon, lettuce, and tulip) in Japan, and are detecting *Olpdium* species by *Olpidium* species detection. These results suggested that this method could decide the existence of different amount fungi in each field and the show the tendency for higher amount of fungi found from field with high degree of disease incidence. Although, some results suggested that degree of disease incidence is not always defined by amount of *Olpidium* species, and further research is necessary to reveal the relationships among the amount of fungi and degree of disease incidence in the field.

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A GLOBAL POPULATION GENETIC STUDY OF *SPONGOSPORA SUBTERRANEA* F.SP. *SUBTERRANEA* OPENS THE POSSIBILITY FOR AN EFFETIVE CONTROL OF POWDERY SCAB OF POTATO AND SHOWS THE NEED FOR STRICT QUARANTINE MEASURES

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Summary

The protozoan organism *Spongospora subterranea* f.sp. *subterranea* (*Sss*) causes the diseases powdery scab - lesions on tubers - and root galls. Both structures contain a powdery mass of sporosori, resting structures which are highly resistant to environmental stresses. No effective control measures are available. Host resistance breeding will be a key component thus knowledge of the genetic diversity of the pathogen is needed. A combination of microsatellite and DNA sequence data derived from worldwide populations of *Sss* showed that South American populations were consistently more diverse compared to all other regions. Estimates of past and recent gene flow suggested that *Sss* was likely introduced from South America into Europe and from there, as a "bridgehead", further globally disseminated. The low global genetic diversity of *Sss* allows potato breeders to select for resistance which is likely to be durable. This was confirmed in a series of field trials in Europe over four years and at different locations with ten cultivars. New introductions of *Sss* genotypes, particularly from South America, increase the potential of more aggressive inoculum, e.g. due to recombination. Thus strict quarantine measures for potato import need to be established, or must continue with enforcement.

Introduction

Invasive plant pathogens often successfully establish in new regions and spread over large areas (Vizzini *et al.*, 2009). Despite the importance of these invasions, relatively little is known about the modes, timing or frequency with which they have occurred. Knowledge of these factors is important for prediction, prevention, and response to additional introductions (Desprez-Loustau *et al.*, 2007). Molecular genetic data can be useful to elucidate sources and routes for invasions, to identify the patterns of dispersal and the genetic composition of founding populations, and thus evaluate the reasons for invasion success.

A concept from invasion theory states that some invasive species are able to establish in a new territory after only one or a few introduction events and are then cut off from the source population. Further spread is exclusively outgoing from this introduced population to other regions. This phenomenon - called the "bridgehead effect" by Lombaert *et al*. (2010) - suggests that genetic diversity is not essential for invasion success, and that rapid adaptive evolution is possible despite strong bottlenecks and single introduction events. Although such invasive pathogens may each have a clonal genetic structure, different phenotypes can be observed under varying climatic or environmental conditions, as observed by Fry and Goodwin (1997; for *P. infestans*).

Spongospora subterranea f. sp. *subterranea* (*Cercozoa*, *Plasmodiophoridae*; hereafter abbreviated as "*Sss*"), is the causal agent of powdery scab, an economically important disease complex of potato. Powdery scab usually refers to the scabby lesions caused by the pathogen on potato tuber surfaces, but also galls on potato roots are a symptom of infection of these organs by *Sss*. The main host species of *Sss* is potato, *Solanum tuberosum* ssp. *tuberosum*. Other important hosts are *Solanum tuberosum* ssp. *andigena* and *Solanum phureja*, both of which are potatoes cropped in South America, the native region of potato, in the higher altitude areas of the Andes. In Colombia it is well recognized that root galls are commonly formed on *Sss*-infected potato plants, and that tuber lesions caused by *Sss* are less frequently observed (Gilchrist *et al.*, 2011). In most of the regions, where potato was introduced, both symptoms occur. The mechanisms behind the differences in susceptibility to the two forms of disease caused by *Sss* remain to be elucidated.

The life cycle of this obligate soilborne biotroph prevents natural long distance dispersal. However, there is a considerable global trade in potatoes, and movement of *Spongospora*-infected seed potatoes

is therefore likely to be responsible for successful short and long distance dispersal of the pathogen (Merz and Falloon, 2009). Powdery scab is difficult to manage because contaminated soils remain infectious for many years due to the formation of numerous, highly resistant resting spores. Breeding of resistant potato cultivars will play an important role in controlling the disease (Merz and Falloon, 2009). Until now, plant breeders screening cultivars and lines for susceptibility to powdery scab have been doing so without knowledge of genetic variability in *Sss*, and little is known about the role of sexual recombination in its lifecycle.

One goal of the present study was to provide the first broad scale population genetic study of an important plasmodiophorid pathogen, based on newly-developed microsatellite markers and sequences of the actin gene and ITS region. Data were obtained from sporosorus samples originating from six continents and many potato producing regions. Our second goal was to test the hypotheses that the pathogen was introduced from South America to Europe, and was subsequently dispersed with Europe acting as a bridgehead, through colonial and/or contemporary seed potato trade to the other introduced regions.

Material and Methods

Samples and DNA extraction

Global populations of *Sss* were sampled in 19 countries, representing all continents. To simplify further naming in this paper, South American samples are specified as 'native' and all others samples as 'introduced'. Total DNA from dried lesion scrapings from potato tubers or potato root galls was extracted either using a commercial extraction kit (QUIAGEN) or the CTAB method.

Nucleotide sequences and haplotype relationship

A subset of 303 *Sss* samples representing all regions was analyzed for nucleotide sequence variation at the ITS region (389 bp) and the partial actin gene (615 bp). Products were sequenced with an ABI 3730 xl sequencer (Applied Biosystems). Sequences were edited using SEQUENCHER (Gene Codes Corporation). We constructed a parsimony-based haplotype network using TCS (Clement *et al.*, 2000) and the haploNet function of R (http://www.r-project.org/), to better visualize haplotype relationship and frequencies across regions.

Microsatellite analyses and migration history

Six polymorphic microsatellite loci yielding unambiguous PCR products were selected to genotype 693 *Sss* samples. We inferred migration rates between regions on two temporal scales to test the two main hypothesis of *Sss* distribution. The first hypothesis is the historic introduction of *Sss* from South America to Europe through colonial trade (CIP, 2008). These past migration rates were estimated using the maximum-likelihood approach implemented in MIGRATE (Beerli and Palczewski, 2010). The second hypothesis to be tested was that Europe acted as a bridgehead for subsequent dispersal of *Sss* to the other introduced regions. Estimates of these recent migration rates were performed using the software BayesAss v. 3 (Rannala and Yang, 2003).

Results

All results of genotypic diversity and genetic diversity showed significantly lower values for the introduced regions compared to the native regions (Table 1). A total of 131 different multilocus genotypes (MLG) were detected among the 693 samples analyzed in the six regions. South America possessed most of the detected genotypes. Out of 127 genotyped samples, 82 MLGs were detected, resulting in the smallest clonal fraction of cf = 0.346 (Table 1). Within South America, the root gall (cf = 0.226) and tuber lesion samples (cf = 0.382) had similar diversities. In contrast, all introduced regions had significantly greater clonal fractions, ranging from cf = 0.906 (Australasia) to cf = 0.632 (Africa). Most striking was the low number of site-specific genotypes ranging from one to 13 among the introduced regions compared to 81 detected in South America.

We found strong asymmetrical gene flow. All estimates for South America tuber lesions and South America galls into Europe or into the pooled introduced regions had confidence ranges >1 and ranged from M = 2.14 to M = 7.23. In contrast, migration estimates for the opposite directions indicated no significant historical gene flow from Europe or the pooled introduced regions into South America with estimates ranging from 0.00 to 0.34.

The most striking finding of recent migration rates estimation was the high proportion of European migrants in all other introduced populations, ranging from 17% in North America to 29% in Australasia. In sharp contrast, the proportions of migrants originating from other regions than Europe were all non-significant from zero based on confidence intervals. In contrast to our estimates of past migration rates, we did not detect any significant recent gene flow between the South American regions and the introduced regions.

Region	N[a]	cnum[b]	ssg[c]	cf[d]	G[e]	H[f]	I_A[g]
Europe	215	26	11	0.879	0.885	0.225	0.0339***
Africa	57	21	13	0.632	0.917	0.234	0.0144^{ns}
Asia	98	16	7	0.837	0.835	0.249	0.0673***
Australasia	170	16	2	0.906	0.878	0.21	0.0131***
North America	26	3	1	0.885	0.151	0.013	na
Total Introduced	566	49	34	0.913	0.91	0.235	0.0205**
South America Root galls	39	29	26	0.226	0.983	0.391	0.0630**
South America Tuber lesions	88	55	54	0.382	0.972	0.314	0.0269*
Total Native	127	82	81	0.346	0.985	0.461	0.1138***

[a]N = Sample size
[b]cnum = Number of multilocus genotypes
[c]ssg = Site specific genotypes; clones specific to a region and not shared with other regions
[d]cf = Clonal fraction; proportion of individual samples originating from asexual reproduction
[e]G = Nei's corrected diversity (genotypic diversity)
[f]H = Nei's Gene Diversity
[g]I_A = Index of association to tests the null hypothesis of linkage equilibrium for multilocus data. Significance of deviation from equilibrium expectations are indicated by asterisks. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$; ns = non-significant; na = not enough diversity for estimation

Table 1 Estimates of clonal and genetic diversity parameters based on six microsatellite markers of *Spongospora subterranea* f.sp. *subterranea* sorted by sampled regions.

Discussion

To our knowledge, the present study is the first extensive population genetic characterization of a plasmodiophorid plant pathogen. Our microsatellite and sequencing data showed that South American populations were consistently more diverse compared to all other regions. Estimates of migration rates further suggested a historic gene flow from South America to Europe and recent gene flow from Europe to the other introduced regions. Consequently, we conclude that *Sss* is very likely to have been introduced from South America to Europe on contaminated potato specimens. This could possibly be through the exploration and migratory activities associated with the conquistador era in the second half of the sixteenth century. Supporting this is the first documented report of powdery scab published in Germany in 1842, describing the disease as a well-known problem for farmers (Wallroth, 1842). From the bridgehead in Europe, *Sss* was spread subsequently due to the lack of plant quarantine or control measures to the North American and European colonies in Africa, Asia and Australasia, with no or a very limited number of new introductions from the native region. According to the recently published potato trade map (Rabobank, 2009), Europe, mainly the Netherlands, is by far the greatest exporter of seed potatoes worldwide. In accordance with our results, this strongly suggests that Europe is the contemporary global distributor of potatoes potentially infected with *Sss* to other introduced regions of the world.

The present study confirms that development of potato breeding lines and cultivars resistant to powdery scab is likely to be an efficient and sustainable way to manage the disease. Given the great clonality of *Sss* in the introduced regions, resistance screening during breeding is not likely to be faced with variable virulence in pathogen populations. However, the similarities between *Sss* and other potato pathogens, i.e. *P. infestans*, must be considered, however. New introductions of *Sss* genotypes, particularly from South America, increase the potential of more aggressive inoculum, e.g. due to recombination. This could lead to multiple pathotypes and additional challenges for resistance breeding.

In order to prevent such introductions, strict quarantine measures for potato import need to be established, or where they exist, strictly enforced.

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ATTEMPTS FOR QUANTITATIVE EVALUATION OF *POTATO MOP-TOP VIRUS* IN SOIL AND SANITIZATION OF POTATO PULP CONTAMINATED WITH THE VECTOR *SPONGOSPORA*

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Summary

By combining the bioassay with the dilution series of soil suspension using tomato bait plants and the examination of the infection of *Potato mop-top virus* (PMTV) on bait roots at each level of dilution by ELISA, we successfully evaluated the relative infestation level of PMTV in soil as the most probable number (MPN). We have also investigated the lethal conditions for *Spongospora subterraea* (*Ss*), the vector of PMTV, especially contaminated in potato pulp. It was revealed that spore ball of *Ss* was highly heat tolerant though it became extinct within 5 minutes at 70 ºC under acidic condition, i.e. at pH2 (sulfuric acid). *Ss* was not detected from the bait root incubated with the potato pulp that was artificially inoculated with *Ss* and fermented for 6 months in greenhouse where the cumulative temperature was 2,689 ºC.

Spraing of potato is caused by *Potato mop-top virus* (PMTV) and is characterized by brownish arcs and rings on the surface and the tuber flesh. In Japan, the first report of spraing was made in Hiroshima in 1980 (Imoto *et al.*., 1986), but no incidence was recorded for 25 years until its rediscovery on cv.

Sayaka in Tokachi, Hokkaido, northern Japan in 2005 (Maoka *et al*., 2006). PMTV is transmitted by a member of *Plasmodiophoraceae*, *Spongospora subterranea* (*Ss*; (Jones and Harrison, 1969)), the causal agent of potato powdery scab (Harrison *et al*., 1997). After the second outbreak of spraing in

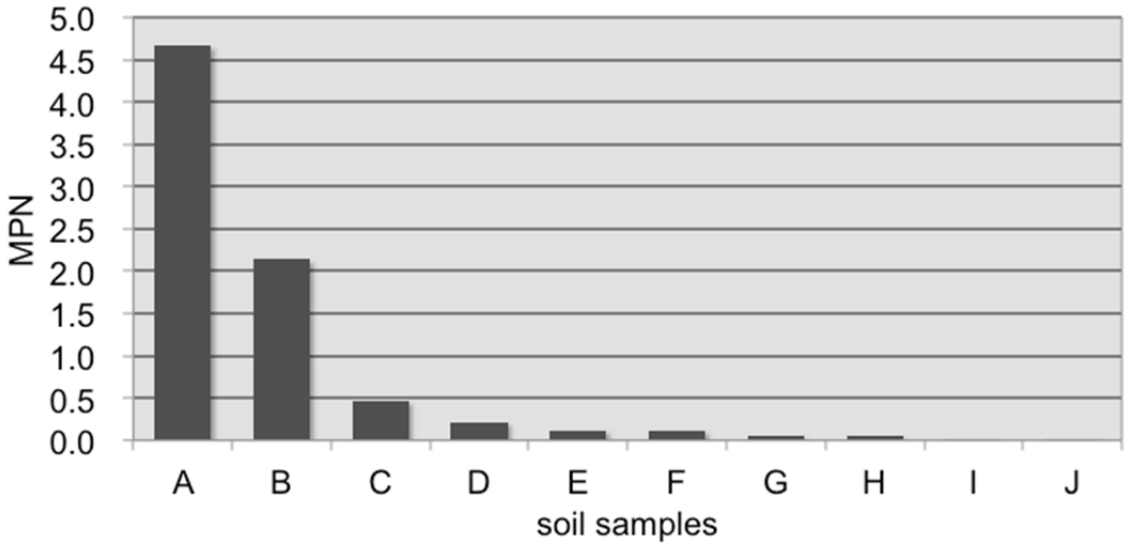

Figure 1 The relative amount of *Potato mop-top virus* represented as the most probable number (MPN) in 10 potato field soils. Soil A was collected at the spraing-affected field in Tokachi, Hokkaido. The other soil samples were from the fields that had no record of spraing so far.

Hokkaido, we have revealed that PMTV has already been distributed not only in the area close to the sprang-affected field in Hokkaido but also in a number of potato fields throughout Japan employing the soil diagnosis method (Maoka *et al*., 2011; Nakayama *et al*., 2010). Although the method we developed is highly sensitive and specific enough to reveal the infestation of PMTV in soil, we could not get quantitative information concerning PMTV contamination in soil that was considered to be necessary for risk estimation of spraing by using our diagnosis method. Besides that, in recent years, social demand

for utilization of potato pulp as animal feed has been raised in Japan, however, concern with spreading of PMTV through its fungal vector *Ss* contaminated in animal wastes fed with potato pulp is an obstacle

pH	ºC	duration (min)					
		5	7	10	15	30	60
4	80		+	+	NT	NT	NT
	70			+	+	+	+
3	75		+	-			
	80		-	-			
2	70	-	-				
DW	80						+

+: detected (survive), -: not detected (extinct), DW: suspended in distilled water (pH not adjusted), NT: not tested

Table 1 Effect of heat treatment on survival of *Spongospora subterranea* under acidic condition adjusted with sulfuric acid

to its promotion. In this study, we tried to 1) quantify the infestation level of PMTV in soil samples, and 2) investigate the lethal conditions for *Ss*, especially contaminated in potato pulp.

The dilution series (10^0 to 10^{-4}) of suspension of test soil samples were prepared by 10-fold dilution of the original soil suspension (50g of soil in 200ml of 2.5-fold nutrient solution ; Merz, 1989). The tomato seedlings (cv. Regina) grown for 3 weeks on commercial pot soil were transplanted (3 plants per

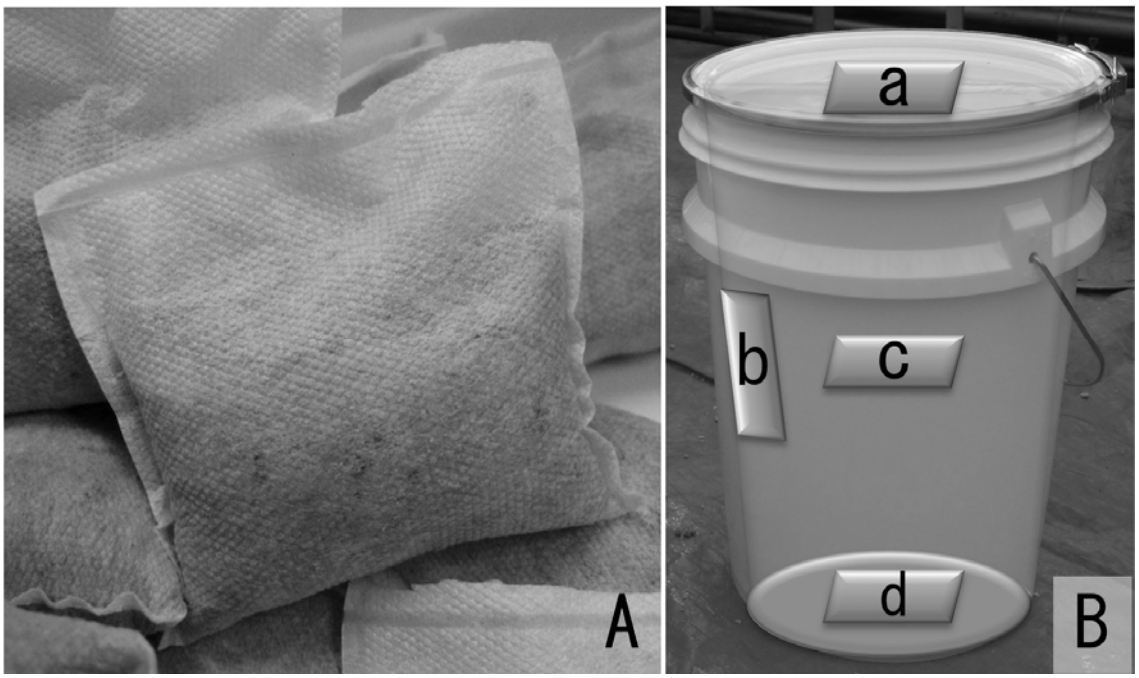

Figure 2 The nonwoven bag packed with potato pulp inoculated with *Spongospora subterranea* (A) and the plastic pail used in the experiment with the schematic position of embedded bags (B: a: top, b: side, c: center, d: bottom)

container) and pre-cultivated in the nutrient solution for 1 week. Then the tomatoes were cultivated immersing their roots in the suspension as prepared above for 3 weeks at 18 ºC (3 containers for each dilution level). The roots were sampled and extracted with phosphate buffered saline (PBS; containing 0.05% Tween 20). The infection of PMTV on the bait roots at each level of dilution was examined by DAS-ELISA (Bioreba, Switzerland) and the number of containers with tomato plants diagnosed as positive was scored. The relative infestation level of PMTV in soil was represented as the most probable number (MPN) by employing the calculation program MPN ver.2 (Wilrich, 2010). The MPN of the

spraing-affected field soil in Tokachi, Hokkaido (sample A in Fig. 1) was revealed to be the highest among other PMTV-positive fields that were diagnosed by the soil diagnosis method (Fig. 1). It was suggested that this method might be useful to estimate potential risk of spraing, however, further investigation will be required to clarify the relationship between the occasion of spraing and the MPN of soil.

Spore ball suspension of *Ss* was exposed to various thermal conditions by soaking in water bath. Then the suspension was inoculated to the nutrient solution of hydroponically cultured bait tomatoes (cv. Momotaro). The thermal death condition was determined by detection of *Ss* from the bait roots by PCR basically as described in the literature (Nakayama *et al*., 2010). Although spore ball of *Ss* was revealed to be highly heat tolerant comparing to other plant pathogens (Pulluman *et al*., 1981), *Ss* was considered extinct within 5 min at 70 ºC under acidic circumstance, i.e. under pH2 adjusted with sulfuric acid (Table 1). Heat tolerance of spore ball could be derived from its characteristic “sponge-like” structure (Lahert and Kavanagh, 1985).

storage place	fermentation period (months)	temperature (℃) average (range) [cumulative]	pulp position	detection of Ss[b)]	pH	organic acid (% fr. wt.)[c)]		
						lactic acid	acetic acid	propionic acid
A	3	5,2 (-1.1~15.3) [497.6]	top	+++	3.43	0.44	0.09	
			side	++	3.43	0.43	0.08	
			center	−	3.42	0.43	0.08	
			bottom	++	3.42	0.42	0.08	
	6	6.7 (-1.1~18.2) [1233.4]	top	++	3.39	0.45	0.11	
			side	+++	3.39	0.45	0.10	
			center	++	3.37	0.44	0.11	
			bottom	++	3.39	0.42	0.10	
B	3	13.8 (9.2~24.5) [1317.4]	top	+++	3.19	0.95	0.20	
			side	−	3.19	0.99	0.21	
			center	+	3.18	1.02	0.24	
			bottom	+++	3.16	1.06	0.22	
	6	14.6 (9.2~32.1) [2688.7]	top	−	3.13	1.02	0.40	
			side	−	3.13	1.08	0.41	0.01
			center	−	3.12	1.11	0.45	0.00
			bottom	−	3.13	1.15	0.42	0.01

Three pales were prepared for each treatment (3 replicates). Potato pulp was collected at potato-starch-producing factory in Shiribeshi, Hokkaido in 2009 and commercial lactobacilli formulation (acremo conc., Snow Brand Seed Co., Ltd.; 17mg/kg) was mixed before filling into pales in order to promote initial fermentation. a) No. of pales that Ss was detected from the pulp sample embedded at the same position. +++ : detected from all pales, ++ : detected 2 of 3 pales, + : detected 1 of 3 pales, - : no Ss was detected from all pales. b) average of 3 pale samples

Table 2 Effect of silage fermentation of potato pulp on survival of *Spongospora subterranea* contaminated and pH and organic acid accumulation in the fermented pulp.

Plastic pails (29 cm i.d. x 37 cm h) were filled up with potato pulp collected at potato-starch-producing factory in Hokkaido, sealed strictly, and then they were kept in the places with different ambient temperature conditions to let the pulp fermented anaerobically. During the filling process, the pulp inoculated with spore balls was packed in nonwoven bag (10.5 cm x 11 cm) and embedded at the bottom, side, center and top of pail (Fig. 2). The fermented potato pulp was sampled after 3 and 6 months and inoculated to hydroponically grown bait tomatoes to examine the viability of the pathogen as described above. *Ss* was not detected from the bait root incubated with the pulp that was artificially inoculated with *Ss* and fermented for 6 months in greenhouse where the cumulative temperature was 2,689 ºC (Table 2). It was considered that low pH, anaerobic condition and effect of organic acids (see Table 2) accumulated during fermentation could be affected synergistically to extinguish *Ss* (Nakayama and Murai, 2012).

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POSTER

EVIDENCE OF *WHEAT YELLOW MOSAIC VIRUS* SPREAD IN LOCAL FIELDS

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Summary

Soil-borne pathogens can be spread with transportation of infested soil from one field to another by wind, water, machinery, and/or vehicles. Therefore, one of the important management practices to help prevent infection is minimizing soil movement. To visualize how the source of infection invades field, we investigated the distribution of wheat yellow mosaic virus (WYMV) in 3 fields by ELISA. Through our investigation we found 10 points were positive and 6 points proved to be suspected-positive for WYMV out of a total of 74 points. Furthermore, using a distribution map, it was possible to show that WYMV dispersal occurred indirectly. These results indicate that the source of infection is frequently carried from neighboring fields since WYMV was detected in fields where wheat was never cultivated.

Introduction

In Hokkaido, WYMV infections were detected for the first time in 1991, and have expanded through a wide area of this area (Fig. 1). It is transmitted by a soil-borne micro-organism known as *Polymyxa graminis*. Dormant spores of *P. graminis* can be spread through soil movement, by the wind or by mechanical movement occuring during field operations. One of the most important countermeasures for the spread of WYMV is the prevention of soil movement. However, to inhibit the spread of WYMV, farmers cannot prevent meteorogical phenomena like the wind, but they can prevent artificial spreading.

In order to lower the risk of WYMV dispersal, we are advising farmers to remove the mud on their farming equipment when moving them from one field to another. Therefore, to minimize soil movement by farm machinery and vehicles is critical, because it is impossible for farmers to control meteorological phenomena like wind.

Materials and Method

In 2009, we investigated the detailed distribution of WYMV in 3 fields (Field A to C) in eastern Hokkaido. The fields had been managed by the same farmers and had traceable cultivation backgrounds. Field A was diagnosed in 2008 when WYMV was detected in the center point of the field. At the time, it was suggested that other areas had already been infested. This field is located on a hill, and borders on the other farmer's field in the east while the other sides are surrounded by windbreaks or large forests. Field B is also located on the same hill and is surrounded by the other farmers' fields except for the north side. Field C is located in a lowland area and is surrounded by houses and by the other farmers' fields. Farmers in the area surrounding field C had never grown wheat.

Winter wheat cv. "Hokushin" was cultivated from September 2008 to July 2009 in the three fields and upper leaves were sampled in spring 2009 for ELISA. We tested a total of 74 points and made a distribution map of WYMV in these fields.

WYMV was detected in the upper leaves of the wheat by indirect-ELISA using an WYMV antibody (Uyeda *et al*., 1998). It was considered positive when its absorbance was more than two times that of the blank and was suspected-positive when the absorbance was between 1.5 and 2-fold higher than the blank.

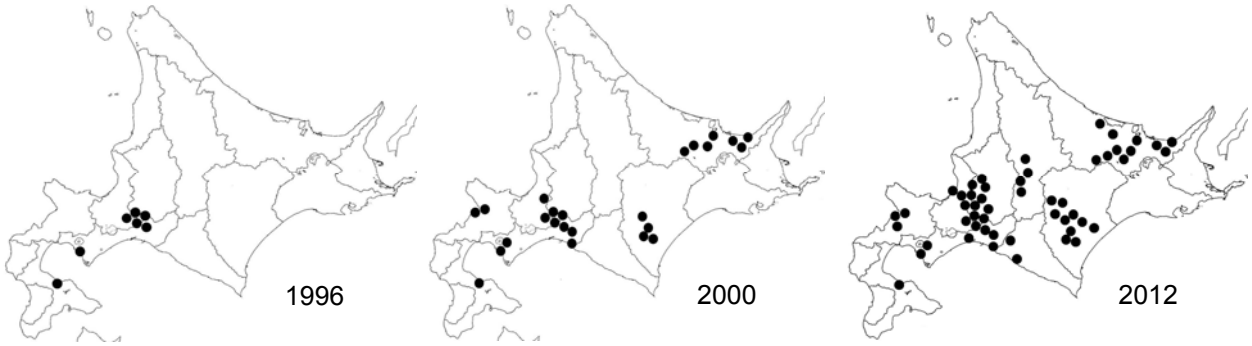

Figure 1 Spread of WYMV in Hokkaido (Horita *et al*., 2011 and Sasaki *et al*., unpublished)
Black dots show towns and villages where WYMV infections have occurred

Results and Discussion

Ten points proved to be positive and 6 points proved to be suspected-positive in a total of 74 field points tested by ELISA.

In field A, 4 out of 30 points proved to be positive and 2 points proved to be suspected-positive (Fig.2). The points that were found to be positive were adjacent to an operational path or farm road, except for one point which was adjacent to a site where WYMV had already been detected in 2008. The wind was not considered to be a major cause of the WYMV invasion because the points that were positive were located on the west end of the field bordering on the large forest. In addition, inflow of soil from adjacent fields by water was not considered to occur according to the configuration of the fields. Instead, our data suggested that the major factor causing the WYMV spread was the soil movement from other infected fields caused by the movement of farm machinery.

In field B, one of the 34 points was found to be positive and 3 were shown to be suspected-positive (Fig.3). This field was surrounded by other farmers' fields and was adjacent to general roads. Distribution of the infection in this field suggested the soil movement by wind and/or farm machinery.

In field C, 5 of 9 points were found to be positive and one was proven to be suspected-positive (Fig.4). The source of infection was considered to be carried from a distance because wheat had never been cultivated in this field and in the surrounding fields. For field C, there are two possible causes of infection; the soil movement by water from higher fields or by farm machinery.

By making a distribution map, it may be possible to determine the cause of WYMV dispersal by using the indirect evidence obtained in this study. However, it is necessary to test supplementary sites so as to truly

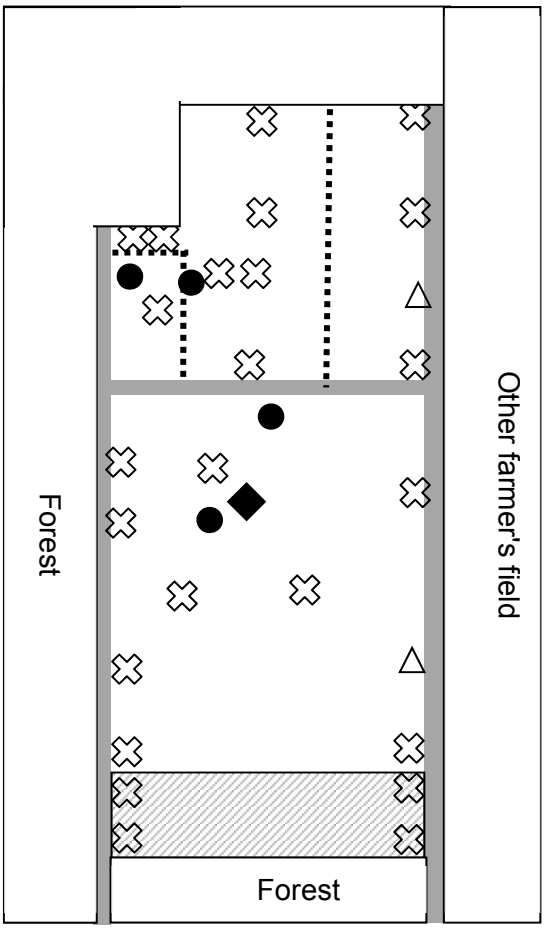

Figure 2 Distribution of WYMV in Field A (300m × 900m)
◆: First detected point in 2008
● : Positive △:Suspected-positive
× : Negative
: Farm road and general pathway
: Operational path
: Area where wheat had never been cultivated

identify the effects of removing soil from the farming machinery.

In conclusion, we detected WYMV in fields where wheat was never cultivated indicating that the source of infection is frequently carried from other neighboring fields. There is a risk of containing a dormant spore of *P*. *graminis* in the field where wheat has never been cultivated. Identically, other soil-borne pathogens (e. g. rhizomania, *Verticillium* wilt, potato powdery scab and potato-cyst nematodes) could spread just as easily. As our study shows, it is important to minimize soil movement as much as possible in order to maintain field sanitation.

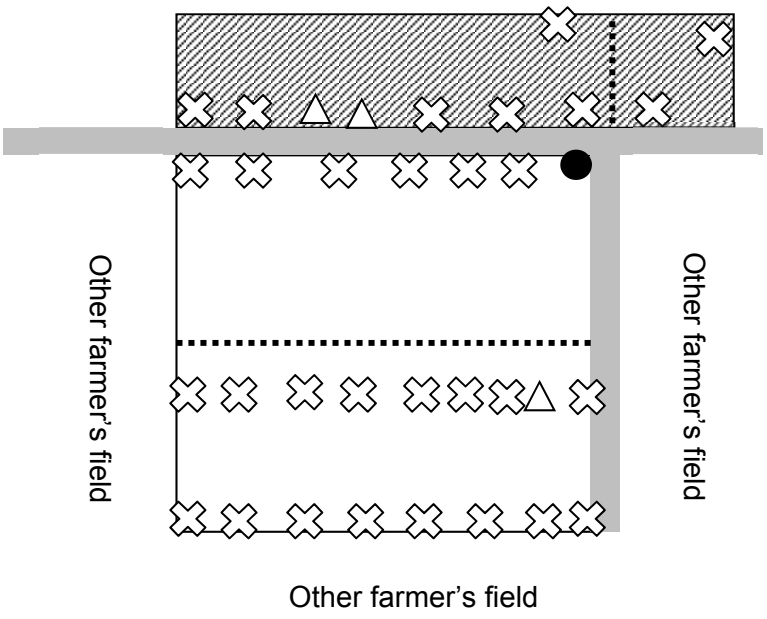

Figure 3 Distribution of WYMV in Field B (1000m × 640m)
◆: First detected point in 2008 ●: Positive △:Suspected-positive ×:Negative
: Farm road and general pathway : Operational path
: Area where wheat had never been cultivated

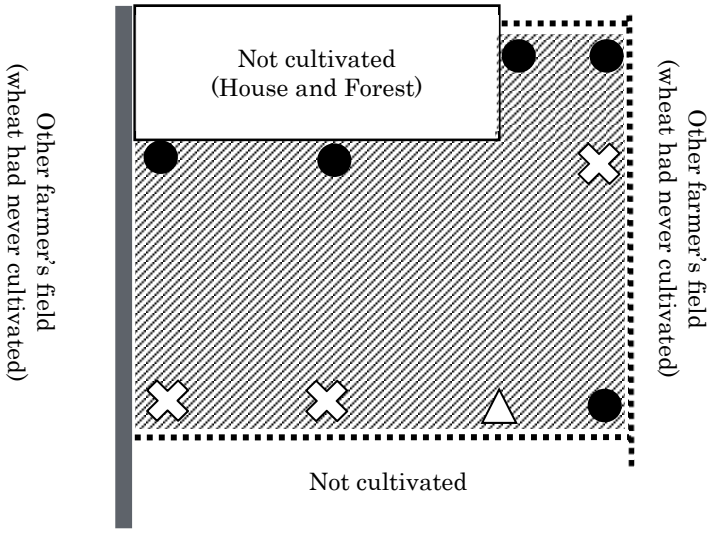

Figure 4 Distribution of WYMV in Field C (200m × 200m)
◆: First detected point in 2008 ●: Positive △:Suspected-positive ×:Negative
: Farm road and general pathway : Operational path
: Area where wheat had never been cultivated

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SUPPRESSION OF POTATO POWDERY SCAB CAUSED BY *SPONGOSPORA SUBTERRANEA* USING AN ANTAGONISTIC FUNGUS *ASPERGILLUS VERSICOLOR* ISOLATED FROM POTATO ROOTS

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Abstract

We isolated 508 soil fungi from potato root cultivated in the suspension of soils from 36 potato fields in Hokkaido. The fungi were screened in accordance with suppressiveness against root infection of *Spongospora subterranea* and also against disease severity of powdery scab caused by this pathogen. Finally we selected one antagonistic fungus that was identified as *Aspergillus versicolor* as a candidate of biological control agent. In the field experiment, *A. versicolor* Im6-50 suppressed powdery scab 54 to 70 in protection value when it applied directly on seed tubers whereas 77 to 93 with the synthetic fungicide fluazinam in successive three years. It was suggested that *A. versicolor* Im6-50 suppressed the disease by settling on the stolons of the inoculated potato plants and also in the rhizosphere because it was detected by polymerase chain reaction employing the specific primers from the surface of daughter tubers and from the soil in which the inoculated seed tubers were cultivated.

Powdery scab of potato caused by a protozoan *Spongospora subterranea* f.sp. *subterranea* (*Sss*) is one of the most important plant diseases for fresh and seed potato producers in Japan. In Hokkaido, the major potato production area of Japan, the disease occurs yearly around 10 % (ca. 5,000 ha) of total potato production fields. In order to establish an environmentally friendly protection measure, we focused on biological control using antagonistic microorganisms that were considered compatible with potato root.

In 2003, we collected the field soil samples from 36 potato fields in Hokkaido. Potato mericlone plantlets (cv. Irish Cobbler) were hydroponically cultivated with their roots submerged in the suspension of these soil samples for one month. We isolated 508 soil fungi from potato root by dilution plate method. Firstly, we screened the isolated fungi in accordance with suppressiveness against root infection of *Sss*. Each of the candidate fungi was then inoculated to the roots of potato mericlone plants by immersing the roots into the suspension of fungal mycelia at 22 ºC for 7 days. The pre-inoculated plants were transplanted and incubated in spore ball suspension for 1 day, and then in the fresh nutrient solution (Merz *et al*., 2004) at 18 ºC for 3 days. The relative amount of *Sss* infected on the roots of pre-inoculated potato plants was evaluated by using *Sss*-specific PCR as described previously (Nakayama *et al*., 2007). The fungi (60 isolates) suppressed the root infection compared to non-treatment control by less than 50% were selected. Secondly, these fungi were further screened by the suppressiveness against powdery scab. Seed tubers (cv. Irish Cobbler) were inoculated with the mycelia of candidate fungi followed by cultivation in plastic pots amended with *Sss* infested field soil for 3 months in green house. After the evaluation of disease severity of each treatment, eighteen fungi were considered as effective to suppress the disease (protection value ≥ 40; see the footnote of Table 1). Finally we selected one antagonistic fungus that was identified as *Aspergillus versicolor* following the test conducted in artificially infested field with *Sss* in NARO/HARC with the seed tubers inoculated with these candidates. *A. versicolor* Im6-50 suppressed powdery scab 54 to 70 in protection value when it applied directly on seed tubers whereas 77 to 93 with the synthetic fungicide fluazinam in successive three years in the field experiments (Table 1).

In order to clarify the compatibility of *A. versicolor* Im6-50 against potato and the mechanism of disease suppression, we tried to detect *A. versicolor* Im6-50 from daughter tubers and the ridge soil in potato field by PCR. DNA templates were prepared from the skin of the daughter tubers that were grown from the seed tubers inoculated with *A. versicolor* Im6-50 and the ridge soil where the inoculated seeds were cultivated basically as described in the literature (Nakayama *et al*., 2007). PCRs were conducted employing the isolate specific primers designed from the sequence of the intergenic spacer (IGS) region of rDNA of *A. versicolor* Im6-50 determined by following the method described by Appel and Gordon (1996). As we could successfully detected *A. versicolor* Im6-

50 by PCR from both the skin of the daughter tubers and the ridge soil, it was suggested that *A. versicolor* Im6-50 suppressed the disease by settling on the stolons of the inoculated potato plants and also in the rhizosphere.

Year	Treatment	Yield (kg)	Diseased tubers (%)	Disease sevrity[3]	Protection value[4]
2008	Control	9.44	42.9	27.5	
	Fungicide[1]	8.80	6.6	1.9	93.3
	Im6-50[2]	8.49	26.2	12.8	53.6
2009	Control	3.28	67.6	44.3	
	Fungicide[1]	6.59	21.7	10.0	77.4
	Im6-50[2]	5.03	32.6	19.7	55.6
2010	Control	12.76	49.6	20.5	
	Fungicide[1]	11.49	9.8	3.7	82.1
	Im6-50[2]	10.01	16.2	6.2	69.7

Figures in the table are average of 2 replications (14 plants per replication) (2008), 3 replications (8 plants per replication.) (2009), 3 replications (10 plants per replication) (2010).

1. Fluazinam (600g/10a、soil incorporation before planting), 2. Seed tubers were air-dried after dipping into mycelial suspension. Population density of myceliall suspension estimated by dilution plate method were 6.5×10^5 cfu/ml (2009) and 1.4×10^8 cfu/ml (2010), respectively. It was not determined in 2008. 3. Disease severity (DS)=ΣnNn×4N-1×100, n: disease index (0 to 4), Nn: number of tubers with index "n", N: number of tubers investigated. Disease index are as follows; 0: no disease symptom, 1: 1 to 3 lesions or less than 3% of the total surface covered with lesions, 2: 4 to 10 lesions or over 3 to 13% of the total surface covered with lesions, 3: 11 to 20 lesions or over 13 to 25% of the total surface covered with lesions, 4: over 20 lesions or over 25% of the total surface covered with lesions.
4. Protection value=100-(DS of treatment)×(DS of control)$^{-1}$×100

Table 1 Suppression of potato powdery scab by seed application of antagonistic fungus, *Aspergillus versicolor* Im6-50 in the field test conducted in NARO/HARC (2008-2010)

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DISCOVERY OF NEGATIVE-STRAND RNA VIRUSES IN FUNGI

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Summary

A major group of fungal viruses (mycoviruses) have double-stranded RNA genomes classified into six families while some have positive-sense single-stranded (ss)RNA or DNA genomes. Despite a growing number of mycoviruses identified, no negative-stranded (−)ssRNA mycoviruses have been reported. The current study provides two lines of evidence that suggests the presence of (−)ssRNA viruses in filamentous fungi. We conducted an exhaustive search for (−)ssRNA viral sequences using extant (−)ssRNA viruses as queries. The search showed (−)ssRNA virus sequences in the genome of a phytopathogenic obligate ascomycete, *Erysiphe pisi*. The integrated sequences showed moderate similarity to the L protein (RNA-dependent RNA polymerase) encoded by non-segmented (−)ssRNA viruses (members of the order *Mononegavirales*). A similar search for (−)ssRNA viral sequences in transcriptome shotgun assembly (TSA) libraries from fungi was performed. Two independent TAS libraries from *Sclerotinia homoeocarpa*, another phytopathogenic ascomycete, were shown to contain several sequences presumably covering the entire region of mononegavirus L gene, which likely originated from currently infecting (−)ssRNA viruses. These results strongly suggest both ancient and probably extant (−)ssRNA viruses infections in filamentous fungi.

Introduction

Fungal viruses (mycoviruses) comprise two groups: a major group of six families with double-stranded RNA genomes and a minor group with positive-sense single-stranded (ss)RNA genomes. Although many fungal viruses have been identified, no negative-stranded (−)ssRNA mycoviruses have been reported (Kondo *et al*., 2013a). Recently, molecular fossil records of (−)ssRNA virus infection in animals and plants were discovered in which homologous sequences to viral genomes have been detected in chromosome of diverse eukaryotic organisms (Horie *et al*., 2010; Katzourakis and Gifford, 2010; Chiba *et al*., 2011). Because non-retrovirus RNA viruses have long been believed not to be endogenized, these findings brought a huge impact in virology. In plant, particularly, sequences similar to cytorhabdovirus N (nucleocapsid protein) or varicosavirus CP (coat protein) were appeared to be wide-spread across over 9 plant families (Chiba *et al*., 2011). Thus, it is highly expected that such foot-print of (−)ssRNA virus infections in fungi may be retained in their genomes, even though no extant viruses are currently reported. Here we present two lines of evidence suggesting the presence of (−)ssRNA viruses in filamentous fungi based on an exhaustive search using extant (−)ssRNA viruses as queries.

Materials and Methods

Database searches were conducted using known (−)ssRNA virus sequences as queries in the NCBI website (nucleotide collection, nr/nt; genome survey sequences, GSS; high-throughput genomic sequences, HTGS; whole-genome shotgun contigs, WGS; non-human, non-mouse expressed sequence tags, EST; transcriptome shotgun assembly, TSA, and others). Detected sequences (e-value < 0.01) were confirmed for their presence by PCR when the materials were available. Deduced amino acid sequences of candidates were subjected to phylogenetic analyses (maximum likelihood, ML method) together with counterpart sequences of known (−)ssRNA viruses. Detailed procedure is described in a recent paper by Kondo *et al*. (2013b).

Results and Discussions

Negative-stranded (−)RNA virus-related sequences are present in the genome of a phytopathogenic fungus.

The survey revealed (−)ssRNA virus L protein (RdRp)-like sequences in the genome of a phytopathogenic obligate ascomycete, *Erysiphe pisi* (pea powdery mildew). These were termed *Erysiphe pisi* mononegavirus L protein-like sequences (EpMLLSs). In total, 8 classes of EpMLLS(1~8) were identified when a plant rhabdovirus (the family *Rhabdoviridae,* order *Mononegavirales*) L or an insect nyavirus (family *Nyamiviridae,* order *Mononegavirales*) L was used as a query. DNA fragments of the expected sizes were PCR-amplified on fungal genomic DNA of *E. pisi,* but not of *E. trifolii* (red clover powdery mildew). This suggests the endogenization event(s) had occurred after the divergence of two *Erysiphe* species (roughly estimated 5.2 million years ago) (Kondo *et al*. 2013b). Endogenization of (−)ssRNA viruses into the fungal genome might have occurred not only in the pea-associated powdery mildew fungus, but also in another powdery mildew fungus (*Golovinomyces orontii*) whose genomic information was recently opened (data not shown). These results suggest infections of ancient (−)ssRNA viruses in ancestors of some powdery mildew fungi.

(−)RNA virus-related sequences are also present in the transcriptome shotgun libraries from another phytopathogenic fungus.

A similar search for (−)ssRNA viruses in fungal transcriptome shotgun assembly libraries demonstrated that two independent libraries from *Sclerotinia homoeocarpa,* another phytopathogenic ascomycete (the dollar spot fungus), contained several assembled sequences covering the entire mononegavirus L gene. We identified at least three independent sequence contigs which were termed *Sclerotinia homoeocarpa* transcriptome shotgun assembly sequences 1~3 (ShTSA1~3). Interestingly, ShTSAs contained another two or three ORFs. Furthermore, a semi-consensus sequence was found in the putative intergenic regions between those ORFs including L gene. These are probably 'gene-junction' sequences, which are broadly conserved among mononegaviruses. Each of these ShTSAs is most likely from multiple transcripts corresponding to genes of an infecting virus, but not of host chromosomal origin since such sequences are absent in the genomic DNA database of *S. homoeocarpa*.

EpMLLSs and ShTSAs are relatives of the Nyavirus within the Mononegavirales.

A phylogenetic analysis of mononegavirus L proteins and L-like elements of fungal origin were conducted. Most known MLLSs in vertebrate and invertebrate genomes are placed into the modern mononegavirus clades (Horie *et al*., 2010; Katzourakis and Gifford, 2010). Similarly, the novel MLLSs from plant and insect genomes identified in parallel in this study form a cluster with the modern insect nyaviruses (Kuhn *et al*., 2013; Kondo *et al*.. 2013b) (data not shown). In contrast, EpMLLSs and ShTSA-L sequences were the closest relatives to each other, and these were distantly related to the modern mononegaviruses and related viruses, eventually forming an independent taxon in the order *Mononegavirales* as the fungal (−)ssRNA virus group (Fig. 1).

Overall, these results provide the first evidence for both ancient and extant (−)ssRNA virus infections in the kingdom Fungi (Kondo *et al*., 2013b). Further characterizations of these (−)ssRNA (actual) viruses are required to establish a new major taxon in the order *Mononegavirales*. The approaches taken in this study is useful to detect unforeseen viruses from unexpected host organisms. For instance, novel *Benyvirus*-related sequences were recently discovered as RNA (from host TSA libraries) and DNA (from host genome databases) forms in plants and insects, suggesting expansion of the genus *Benyvirus* and related new taxa (Kondo *et al*., 2013c). It is also possible that plant viral elements are present in the genome of fungal vectors if the viruses replicate in their fungal vectors during transmission.

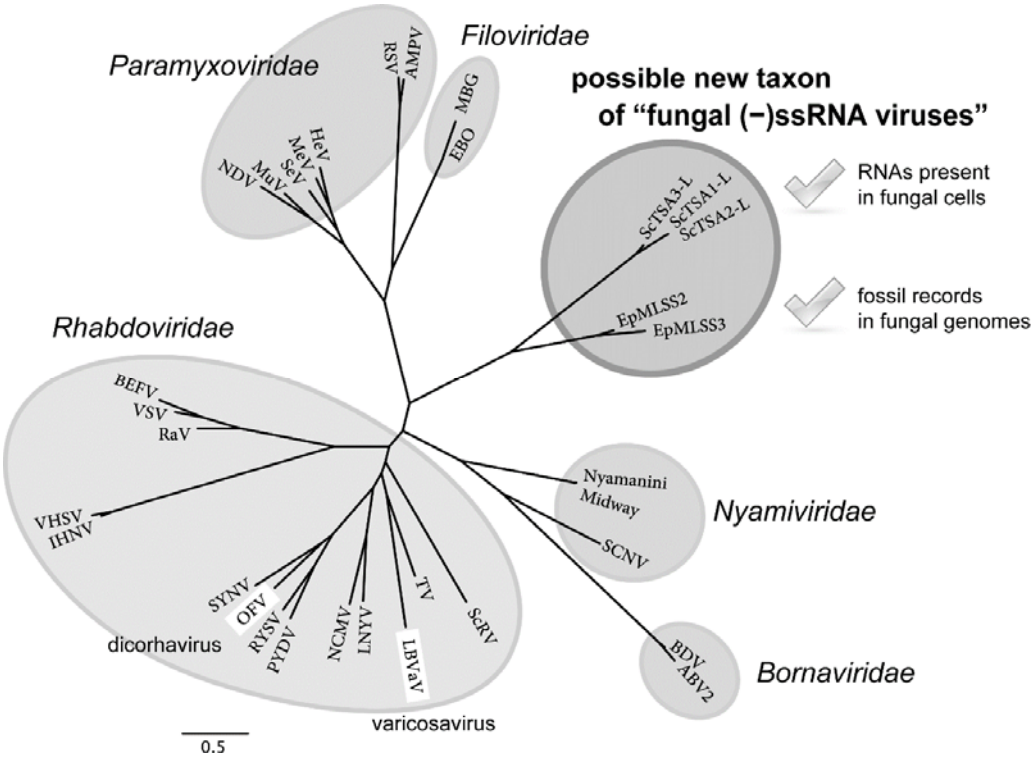

Figure 1 Phylogenetic analysis of fungal (−)ssRNA virus elements.

ML-phylogenetic tree was constructed based on L protein sequences of known (−)ssRNA viruses, novel MLLSs identified in this study, and ShTSA-derived L sequences

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