PROCEEDINGS OF THE FOURTH SYMPOSIUM OF THE INTERNATIONAL WORKING GROUP ON PLANT VIRUSES WITH FUNGAL VECTORS

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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 the chairperson. The goal of the working group is to provide a forum to facilitate international collaboration and multidisciplinary research on plant viruses with fungal vectors. Thus, topics at symposia have included a) biology of viruses with fungal vectors, b) biology of fungi that transmit plant viruses, c) interaction between these viruses and vectors, and d) epidemiology and control of diseases caused by plant viruses transmitted by soilborne fungi.

Symposia of the working group have been held at the Biologische Bundesanstalt (BBA) in Braunschweig, Germany (1990), McGill University in Montreal Canada (1993), The West Park Conference Centre, University of Dundee, Dundee, Scotland (1996), and most recently at the Asilomar Conference Center in Monterey, California. This volume serves as a record of material presented at this most recent meeting for use by members of the IWGPVFV and for those with an interest in the activities of the IWGPVFV.

As the IWGPVFV is a totally volunteer group, the success of its meetings is a result of the hard work and contributions of the local organizing committee and sponsors. Those responsible for the success of the most recent meeting are listed on the next page. The names and e-mail addresses of the current program committee are also listed. Please contact a member of the program committee if you wish to be included in any future mailings of the IWGPVFV. I have enjoyed serving as chairperson of this working group for the past six years and appreciate the effort of each participant in making the previous two meetings enjoyable and successful. The next symposium is scheduled for 2002.

John L. Sherwood
Chairperson IWGPVFV
(1993-1999)

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Detection, Etiology, Epidemiology and Management

GENETIC DIVERSITY IN BEET SOILBORNE MOSAIC VIRUS REVEALED BY SSCP ANALYSIS.

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Summary

Beet soilborne mosaic virus (BSBMV) and beet necrotic yellow vein virus (BNYVV) are closely related pathogens of sugar beet (*Beta vulgaris*). BSBMV often moves systemically and causes distinct foliar symptoms. It may be also associated with rhizomania-like root symptoms. Fifty-six BSBMV isolates from Colorado, Minnesota, Nebraska, Texas, and Wyoming were collected and overlapping RT-PCR products derived from BSBMV RNAs 2, 3, and 4 were compared by single-stranded conformational polymorphism (SSCP) analysis. Informative SSCP markers were identified on all RNAs examined. Results demonstrated that multiple viral isolates can be detected in a single field and that markers on any RNA can move in the viral population independently from other markers on the same or on other RNAs. These results suggest that genetic material has been exchanged between BSBMV isolates and the resulting genotypes have been observed over a wide geographical area. SSCP analysis suggests that BSBMV exhibits greater genetic diversity than BNYVV.

Introduction

Beet necrotic yellow vein virus (BNYVV) and beet soilborne mosaic virus (BSBMV) are two closely-related soilborne viruses that infect the roots of sugar beets (*Beta vulgaris*) by utilizing the fungus *Polymxya betae* as a vector. Both BNYVV and BSBMV possess a divided genome consisting of four, polyadenylated, single-stranded RNAs. Because of numerous distinct features, these viruses were recently separated from the Furovirus genus and placed in the new Benyvirus genus. BNYVV is the cause of rhizomania and has been identified in most sugar beet growing areas of the world. Relatively little genetic variability has been reported for BNYVV; the principal global populations comprising two 'strain groups' (Koenig et al., 1995, 1997; Kruse et al., 1994).

BSBMV has been confirmed only in the United States. BSBMV often moves systemically and causes distinct foliar symptoms that include chlorosis, necrosis and leaf crinkling. While the roots of most BSBMV-infected sugar beets appear normal, we have found numerous examples of plants that display rhizomania-like symptoms. Serological assays indicate that these plants are infected with BSBMV but not BNYVV suggesting that BSBMV may have the potential to cause rhizomania.

Our objectives for this study were: 1) determine the degree of genetic diversity in BSBMV isolates from a wide geographic area, 2) identify candidate genetic markers that may link to disease phenotypes; particularly those that include the roots, and 3) clarify the evolutionary relationship between BNYVV and BSBMV.

Materials and Methods

Sugar beets displaying foliar and/or root symptoms were collected from fields in Colorado, Minnesota, Nebraska, Texas and Wyoming. Fragments of symptomatic sugar beet leaves (0.8-1.0 gm) were homogenized in 4.5 ml TRIzol Reagent (Life Technologies, Gaithersburg, MD). Total RNA was isolated according to manufacturer's recommendations with the following modification. In order to minimize polysaccharides contamination, RNA was precipitated with 0.25 ml isopropanol followed by 0.25 ml of a high salt precipitation solution (1.2 M sodium citrate and 0.8 M sodium chloride) per 1 ml of TRIzol used for homogenization. Soil samples were air-dried and archived.

First strand cDNA was made from total RNA using a commercial kit (Life Technologies) under the following conditions: 0.5 µg oligo-dT in 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 10 mM DTT. Superscript II reverse transcriptase was used to synthesize cDNA at 42° C for 50 min. 1 µl aliquots of cDNA was used for the polymerase chain reaction (PCR) using unique and RNA-specific 18-mer oligonucleotide primers in

50 µl reaction volumes using Elongase DNA polymerase mixture (Life Technologies) and the 'hotstart' method (Chou et al, 1992). Typical cycling parameters were 94° C for 30 sec., 56° C for 30 sec., and 68° C for 1 min. 30 sec., for 35 cycles.

PCR products were analyzed by SSCP (single-strand conformation polymorphism) essentially as described (Orita et al., 1989; Koening et al., 1995). PCR products (typically 5 µl) were denatured for 5 min. at 70° C in an equal volume of formamide containing 20 mM EDTA, 0.1% bromophenol blue and 0.1% xylene-cyanol and immediately cooled on ice. Samples were loaded to 10% polyacrylamide gels and electrophoresed at room temperature overnight at 65 volts in a Hoefer Scientific SE600 apparatus with 0.5x TBE buffer (45 mM Tris, 45 mM borate, 1mM EDTA, pH 8.3). Initial buffer temperature was 4° C. After electrophoresis, the gels were silver-stained as described (Bassam et al., 1991).

Results and Discussion

In preliminary experiments, primer pairs that generated PCR products ranging in size from 166-740 were analyzed by SSCP. Under the conditions used for our analyes, PCR products of approximately 400 bp were most informative, although some larger PCR products, in particular, on RNA 2 were informative.

To date, we have analyzed 56 samples (19 from Colorado, 11 from Minnesota, 6 from Nebraska, 13 from Texas, and 7 from Wyoming) using primers that cover the majority of RNAs 2, 3 and 4. Our results clearly demonstrate that SSCP is a useful tool for detecting genetic variability evidenced by multiple migration patterns of single-stranded DNA fragments isolated from various BSBMV isolates. When this methodology was applied to BNYVV, two primary 'strain groups' were identified (Kruse et al., 1994; Koening et al., 1995). For BNYVV, the grouping was consistent, regardless of which RNA was being analyzed. If a PCR product derived from one BNYVV RNA was subjected to SSCP analysis, and gave rise to two groups, PCR products from one of the other BNYVV RNAs predicted the same two groups.

The tendency for viral isolates to fall into a limited number of stable groups was not observed for BSBMV. Instead, based on SSCP results, BSBMV appears to exhibit greater genetic diversity than BNYVV. More than two distinct migration patterns were observed with several of the PCR products analyzed, including products C and F on RNA 2, and products 1 and 4 on RNA 3. Figure I shows some of the SSCP patterns observed for PCR products derived from RNA 2.

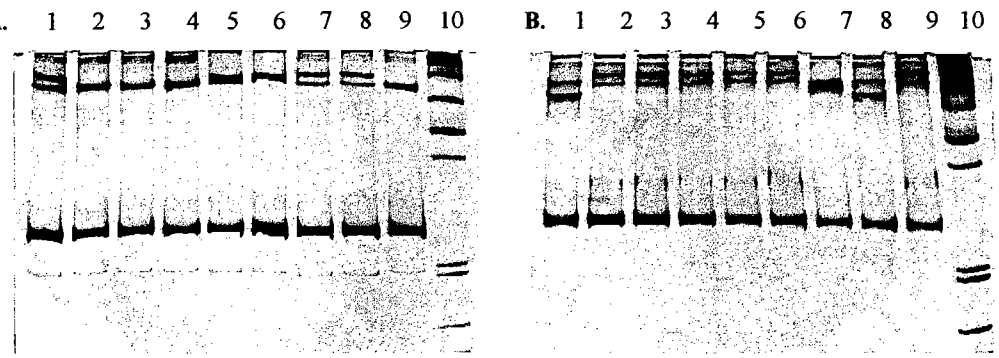

Figure 1. Representative SSCP gels of PCR products from beet soilborne mosaic virus RNA 2, product F (panel A.), and product C (panel B.). Numbered lanes correspond to the following samples: lane 1, EA-1; lane 2, PL-1; lane 3, RC-1; lane 4, RC-2; lane 5, MN9807-06; lane 6, MN9807-07; lane 7, MnX; lane 8, WY96-3B; lane 9, CO96-44, lane 10, DNA standards.

In Figure 1A, two patterns are readily identified. The first pattern, found in lanes 2, 3, 4, 5, 6 and 9, exhibits a closely spaced upper doublet that may appear as a solid band at the magnification shown in Figure 1. The second pattern, found in lanes 1, 7 and 8, exhibits a more widely spaced upper doublet. In Figure 1B, three

patterns are seen. In the first pattern, an upper doublet in seen in lanes 2, 3, 4, 5, 6 and 9. This group of samples corresponds to the first pattern seen in Figure 1A and includes samples from Texas (PL-1 in lane 2), Colorado (RC-1, RC-2 and CO96-44 in lanes 3, 4 and 9, respectively), Minnesota (MN9807-06, MN9807-07 and MnX in lanes 5, 6 and 7, respectively), and Wyoming (WY96-3B in lane 8, respectively). The second pattern, found in lanes 1 and 8, exhibits a more widely spaced and faster migrating doublet. The samples showing this pattern includes a sample from Colorado (EA-1 in lane 1) and Wyoming (WY96-3B in lane 8). The third pattern is a unique closely spaced doublet in lane 7, which is a sample labeled from Minnesota. Therefore, when we consider the two PCR products shown in Figure 1, we see two principle 'groups' with the exception of the isolate in Figure1B, lane 7.

The relatively simple grouping of viral isolates seen in Figure 1 is lost when we include samples from RNA 3 (Figure 2A). For this particular set of samples, we can also observe two distinct patterns. The first pattern consists of a closely spaced upper doublet and is found in lanes 1, 2, 3, 6, and 7. In addition to the upper doublet, the second pattern exhibits an additional band that is found in lanes 4, 5, 8, and 9. However, the RNA 3 markers bear no relation to the distribution of markers on RNA 2. Of particular interest is the fact that more than one marker is present in the same field. Lanes 3 and 4, illustrate the case of two RC samples that originated from the same field in Colorado. RC-1 in lane 3 exhibits only the upper doublet while RC-2 also exhibits the additional, uppermost band. Further variation is illustrated in Fig. 2B. In this case, banding patterns form an RNA3 3'per product show a grouping different from either 1A&B or 2A.

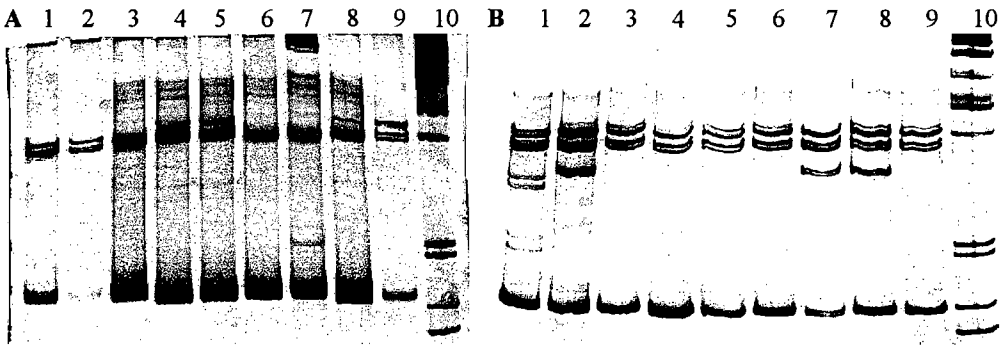

Figure 2. Representative SSCP gels of PCR products from beet soilborne mosaic virus RNA 3, product 4 (2A) and product 1 (2B). Numbered lanes correspond to the following samples: lane 1, EA-1; lane 2, PL-1; lane 3, RC-1; lane 4, RC-2; lane 5, MN9807-06; lane 6, MN9807-07; lane 7, MnX, lane 8, WY96-3B; lane 9, CO96-44, lane 10, DNA standards. Banding patterns in A&B suggest different isolate groupings.

Since similar markers are observed in viral isolates taken from widely separated locations, it is important to demonstrate that similar appearing markers are also highly similar at the nucleotide level. Therefore, the 408 base pair PCR product 4 from RNA 3 was subcloned and sequenced for five BSBMV isolates: PL-1 from Texas, RC-1 and RC-2 from Colorado, and MN9807-06 and MN9807-07 from Minnesota. These samples correspond to lanes 2-6 in Figure 2. The results clearly indicate that the presence or absence of the uppermost band is determined by five nucleotide changes (data not shown). For the isolates that exhibit the uppermost band, DNA sequencing demonstrated the substitution of G's for A's at positions 153, 156 and 321, while C's were substituted for T's at positions 276 and 297. Additional single base changes were observed in MN9807-06 at position 84 (a T for C substitution), as well as at position 329 (an A for G substitution). These results confirm that viral isolates from Texas, Colorado and Minnesota (PL-1, RC-1 and MN9807-07) exhibit essentially identical nucleotide sequence in the case of RNA 3, product 4. Viral isolates from Colorado and Minnesota (RC-2 and MN9807-06) also exhibit essentially identical nucleotide sequences.

This ongoing investigation has demonstrated that, in contrast to BNYVV, which is relatively stable from the genetic point of view, BSBMV isolates exhibit significant genetic diversity that can be identified using SSCP.

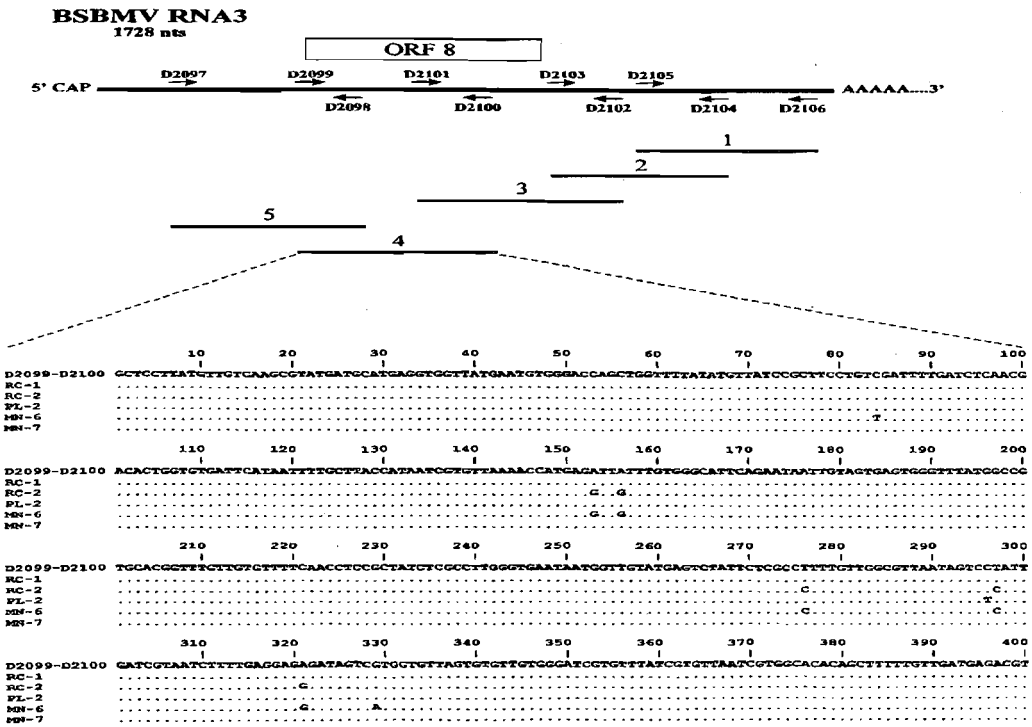

Figure 3. Schematic representation of BSBMV RNA 3. Nucleotide sequence of RT-PCR product 4 used in SSCP analysis, and sequence variation between BSBMV isolates determined by SSCP analysis.

Different BSBMV isolates possess a variety of genetic markers on different RNAs that can be found in many combinations. Rather than try and classify BSBMV into a limited number of 'strains' or 'strain groups' we propose to refer to BSBMV isolates as distinct genotypes. Different markers can be found in the same field and can also be found across wide geographic areas. The makers we have identified may be useful in attempts to obtain linkage between specific markers and disease phenotypes.

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MUTATION ANALYSIS OF *CIS*-ACTING SEQUENCES IN THE 3'- AND 5'-UNTRANSLATED REGIONS OF SATELLITE TOBACCO NECROSIS VIRUS STRAIN C RNA

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Summary

The putative, 3'-terminal stem-loop structure in satellite tobacco necrosis virus strain C (STNV-C) RNA constitutes an essential *cis*-acting structure for the promotion of (-)-strand RNA synthesis, but upstream sequences and a single-stranded tail are also important. The putative, 5'-terminal stem-loop structure in STNV-C RNA is not essential for productive RNA accumulation, but is required for optimal accumulation. Residues 2 and 3 are the minimal *cis*-sequences required for RNA synthesis and may constitute a 5'-terminal promoter. The RNA of chimeric mutants, which exchanged 3' and 5'-untranslated regions between STNV-C and helper tobacco necrosis virus strain D RNAs, accumulated in protoplasts implying similar replication mechanisms for both RNAs.

Introduction

Satellite viruses are small icosahedral RNA plant viruses, which are completely dependent on the presence of a specific helper virus to establish infection. By encoding their own coat protein (CP), satellite viruses differ from satellite RNAs, which are encapsidated by helper virus CP (Scholthof *et al.*, 1999). Satellite tobacco necrosis virus strain C (STNV-C) is a *Carmo*-like *Necrovirus* in the *Tombusviridae* family which has a monopartite, (+)-strand RNA genome of 1,221 nucleotides (nt) (Bringloe *et al.*, 1998) and is helped in its replication by tobacco necrosis virus strain D (TNV-D) (Coutts *et al.*, 1991). STNV-C RNA encodes a 22-kDa CP from a 606 nt open reading frame (ORF) which is preceded by a 5'-untranslated region (UTR) of 101 nt and followed by a 514 nt 3'-UTR (Bringloe *et al.*, 1998). Computer prediction of the secondary structure of the STNV-C RNA 3'- and 5'-UTRs suggest several stem-loop structures and pseudoknots which might contain essential *cis*-elements involved in template activity for respectively (-)- and (+)-strand RNA synthesis by TNV-D RNA-dependent RNA polymerase (RdRp) (Bringloe *et al.*, 1998). Using subclones of STNV-C and helper TNV-D cDNAs, for the transcription of biologically active RNA, we demonstrated that replication of the helper virus was essential for the accumulation of STNV-C RNA *in vivo* (Bringloe *et al.*, 1998).

Little is known about *cis*-elements needed for STNV-C RNA synthesis apart from a preliminary mutational analysis, which showed that deletions in either the 3'- or 5'-UTRs in most cases abolished accumulation (Bringloe *et al.*, 1998). These results suggested that, as with other *Carmo*-like viruses, a defined 3'-terminal stem-loop structure and a single-stranded (ss) tail are important for the initiation of (-)-strand synthesis (Song and Simon, 1995). While, similar to the satellite RNAs associated with a number of other viruses from the *Carmo*-like virus supergroup, including tomato bushy stunt virus (TBSV) (Chang *et al.*, 1995), cymbidium ringspot virus (CymRSV) (Havelda, *et al.*, 1995) and turnip crinkle virus (TCV) (Guan *et al.*, 1997), a 5'-terminal sequence of between 50 and 150 nt appears essential for the productive accumulation of RNA. Here we report the results of a mutational analysis of *cis*-acting sequences in the 3'- and 5'-UTRs of STNV-C RNA and identify regions important for accumulation.

Materials and Methods

Construction of mutant STNV-C RNA clones and synthesis of transcripts in vitro

All recombinant DNA techniques were as described (Bringloe *et al.*, 1998) or according to Sambrook *et al.* (1989). Full-length, infectious clones of wild-type (WT) STNV-C and TNV-D RNA (respectively pSTNVC and pTNVD) were used to construct cDNA mutants. All mutants were generated by reverse PCR-amplification with pairs of oligonucleotide primers (Bringloe *et al.*, 1998). Three chimeric mutants (SREP3'-T, SREP5'-T and SREP5'-3'-T), which respectively replaced the 3'-UTR, 5'-UTR and both UTRs of STNV-C RNA with the equivalent UTRs of the helper TNV-D were constructed by PCR-amplification of the respective genomic regions followed by ligation of the fragments and transformation into *Escherichia coli* DH5α. Plasmids (*ca.* 10μg) were linearised and transcripts synthesised using T7 RNA polymerase. PCR products (*ca.* 5μg) were purified by phenol/chloroform extraction and used directly for transcription. All transcripts were treated with DNase 1, extracted and resuspended in RNase-free water.

Protoplast isolation, inoculation, processing and Northern analysis

Nicotiana benthamiana leaf protoplasts (10^6/ml) were isolated, inoculated with RNA (5 μg each of transcripts of TNV-D RNA and WT STNV-C RNA or mutant STNV-C RNA) and incubated in continuous low light for 24 h (Bringloe *et al.*, 1998). After incubation the protoplasts were harvested, stored at –70°C and extracted for total RNA, which was dissolved in DEPC-treated water. RNA samples were then electrophoresed through denaturing formaldehyde-agarose gels and blotted to Hybond-

N membrane (Bringloe *et al.*, 1998). Blots were hybridised with riboprobes prepared by *in vitro* transcription of clones derived from pSTNVC (Bringloe *et al.*, 1998) using T7 RNA polymerase and [α-^{32}P] UTP. Blots were washed under conditions of high stringency at 68-70°C to reduce background. All experiments were repeated at least three times to ensure reproducibility of results and inoculation efficiency of every batch of protoplasts were measured by inclusion of WT STNV-C RNA controls.

Results

A single-stranded tail, a 3'-terminal stem-loop and two further upstream stem-loops are all required for accumulation of STNV-C RNA

The importance of the 3'-terminal ss tail, the sequence near the 3'-terminus, which can be folded into a stem-loop (H11) (Bringloe *et al.*, 1998), and two sequences further upstream, which can also be folded into stem loops (H10, H9), are important for RNA replication was investigated. To achieve this, a series of 3'-terminal deletions of 3 nt, which removed the -CCC terminal residues, 6 nt, which removed the ss tail, 30 nt, which removed H11 and 47 nt, which removed the two adjacent hairpins, H10 and H11 were made. As compared to WT STNVC, the accumulation of all but the first mutant was abolished. In two further mutations, internal deletions, which respectively removed stem-loops H9 and H10, abolished RNA accumulation. The production of (-)-strand RNA was monitored by a RT-PCR assay, which amplified a specific fragment of the STNV-C RNA (-)-strand. Only extracts from protoplasts inoculated with WT STNVC and the first mutant gave positive results in this assay. To characterize the 3'-termini of accumulating STNV-C RNA in more detail, progeny RNA was examined. First total RNA was reverse-transcribed. Following ligation of an anchor oligonucleotide to the cDNA and subsequent PCR amplification, fragments corresponding to the 3'-termini of STNV-C RNA were sequenced directly. Transfection of protoplasts with TNV-D and the mutant, which lacked the 3'-terminal -CCC residues of STNV-C RNA, gave rise to progeny RNA in which the original residues were restored in most cases (50%; 6 of 12) or were replaced by three other nucleotides (either -CAA or -CCA). These results demonstrate that these residues appear to be not essential for RNA accumulation and can be deleted but, that all other upstream deletions are lethal.

Importance of the 3'-terminal stem-loop for STNV-C RNA accumulation

To investigate whether secondary structure or sequence of the STNV-C RNA 3'-terminal stem-loop (H11) is needed for RNA replication, a number of mutations were introduced into H11. The mutations were designed to increase the probability of destabilization of the AU base pair in the upper part of the stem, or the lower GC base pair by creating non-Watson-Crick base pairing. Also compensatory mutations within the upper and lower parts of the stem were generated such that base pairs forming the stem were altered without significantly affecting thermodynamic stability, which was confirmed by refolding the RNA. These compensatory mutations, which restored base pairing in the stem but with a sequence different to that in WT STNV-C RNA, involved inverted base pairing within the upper or lower region of the stem or paired substitutions within the upper or lower region of the stem. The energetic status of the various mutants of H11 was calculated as described previously for the last 86 nt of WT STNV-C RNA sequence (Gultyaev *et al.*, 1995) including stem-loops H9, H10 and H11. Some of the mutations generated weaker or on occasion more stable structures. To determine whether the sequence of the loop in the 3'-stem-loop structure is important for replication a substitution mutant in the central two nt was also made.

When protoplasts were inoculated with any of three AU mutants together with TNVD, accumulation of both (+)-strand and (-)-strand RNA was reduced to barely detectable levels and all three mutations were lethal. By contrast mutations of the GC base pair in the lower region of the stem had modest or no effect on RNA accumulation In one mutant the stem of the stem-loop was thermodynamically weakened, as compared to WT STNVC, but maintained its structural integrity including the upper section and appeared to be a suitable template for TNV-D RdRp. In another mutant the structural integrity of the upper stem and/or base pairing of residues 5-7 from the 3'-end, and putative folding of the sequence into an alternative stem-loop, made it a poor template for helper RdRp. When protoplasts were inoculated with the loop mutant together with TNVD, accumulation of mutant RNA (+)-strand and (-)-strand were similar to the amounts found following inoculation with WT STNVC and TNVD.

Importance of the 5'-terminal stem-loop for STNV-C RNA accumulation

To determine whether sequences and/or the predicted stem-loop structure (H1) in the STNV-C RNA 5'-UTR are/is required for replication, mutants were made which replaced or deleted nt in the lower stem or replaced nucleotides in the upper stem. Further sets of mutants, which deleted or inserted residues into the loop, were also produced. Analysis of the ability of these mutants to replicate in protoplasts following co-inoculation with helper TNVD indicated that replication was often severely impaired when alterations to the 5'-UTR were made. Changes which altered the sequence and thereby the stability of the stem generally resulted in only trace amounts of the mutant STNV-C (+)-strand being detected. By contrast a mutant which removed the 5'-terminal G residue, accumulated to increased levels as compared to WT STNVC. The sequence of this mutant transcript is that of the natural WT according to the sequence of prototype STNV-C RNA (Bringloe *et al.*, 1998). Also a mutant which replaced the 5'-terminal G residue with an A residue accumulated to significant levels. Inclusion of an additional G residue in pSTNVC at the 5'-terminus of the virion RNA, for supposed increased T7 promoter activity during transcription actually decreased transcript accumulation in infected protoplasts. Insertion of additional 5' residues directly preceding the sequences of plant virus cDNAs, in full-length clones, generally reduces replication efficiency and this appears to be the case for STNV-C too. Replacement of the 5'-terminal G residue with an A residue increased the thermodynamic stability in the lower stem of the proposed stem-loop structure of RNA transcripts as compared to WT STNVC. Base pairing

between residues A1 and U43 achieves this as opposed to naturally forming a non-Watson-Crick basepair, G.U. These results demonstrate that residues 2 and 3 may be essential for STNV-C accumulation. Other mutations within the stem-loop structure, which destabilized of the upper stem caused reductions in accumulation, but did not abolish accumulation. Mutants, which were destabilized in the loop by nucleotide deletions or an insertion, behaved similarly. These results indicate that the proposed STNV-C RNA 5'-stem-loop structure is not essential for productive, plus-strand RNA accumulation but is required for optimal accumulation.

Productive accumulation is altered following truncation of the 5'- UTR of STNV-C RNA

The substitution analysis in H1 suggests that either the primary sequence at the base of the stem is essential, or that the essential information may be contained in another 5' *cis*-element. The region encompassing the structure was therefore subjected to progressive 5'-deletion analysis. Transcripts that contained either the complete WT STNVC stem-loop structure or the 5'- truncated mutants were co-inoculated together with helper TNVD in protoplasts. Northern blot analysis revealed that stepwise 3'-deletions of up to 66 nt upstream of the STNV-C 5'-terminus produced transcripts that still accumulated to detectable levels. Increases in mutant transcript accumulation were generally in direct proportion to increased lengths of the 5'-UTR upstream of the CP gene as compared to WT STNVC. This result confirms that the proposed stem-loop is not essential for replication, but is required for optimal accumulation and that STNV-C RNA residues 2 and 3 are the minimal *cis*-acting sequences required for RNA synthesis and might constitute a 5'-terminal promoter.

The STNV-C 5'- and 3'-UTRs can be functionally replaced by the equivalent UTRs of the helper virus, TNV-D

The accumulation of (+)-strand RNA of three chimeric mutants described earlier (SREP3'-T, SREP5'-T and SREP5'-3'-T) was detectable but reduced as compared to WT STNVC. These results show for transcription at least, both the 5'- and 3'-UTRs of STNV-C RNA and TNV-D RNA, which share respectively 47 % and 36 % sequence homology and 6 and 4 identical nt at their termini, are functionally interchangeable.

Discussion

The genomic and satellite RNAs of most viruses in the *Tombusviridae* family, which includes the *Carmovirus* and *Necrovirus* genera, are capable of forming one or more potentially stable stem-loop structures near the RNA 3'-terminus. Notably the genomic and satellite RNAs of TCV (Song and Simon, 1995), genomic and defective interfering (DI) RNAs of CymRSV (Havelda and Burgyan, 1995), TNV-D and STNV-C (Bringloe *et al.*, 1998) all possess similar stem-loops at their RNA 3'-termini. This suggests that these structures may be common RdRp recognition features in *Carmo*-like viruses that are required for initiation of (-)-strand RNA synthesis. The 3'-terminus of CymRSV RNA can be folded into three hairpins and mutational analysis of DI CymRSV RNA illustrated that none of the three hairpins could be deleted without abolishing replication. Mutations that disrupted base pairing of the stems also abolished replication, which could be partially restored by compensatory mutations that restored base pairing (Havelda and Burgyan, 1995). Similarly TCV genomic and satellite RNAs both contain conserved 3'-terminal stem-loop structures and, while a variety of 3'-terminal stem-loops of differing stability also act as efficient promoters *in vivo* (Stupina and Simon, 1997; Carpenter and Simon, 1998), a defined stem-loop and a ss tail are important for initiation of (-)-strand synthesis (Song and Simon, 1995).

Our results with STNV-C RNA suggest that the stability and sequence of the defined, 3'-terminal stem-loop (H11) is important for replication in establishing an efficient promoter *in vivo* and other sequences immediately downstream but not including the terminal -CCC residues are also important. It is likely that H11 contains 'core' promoter sequences that are recognised by the helper TNV-D RdRp. However the contribution of other upstream stem-loop structures (e.g. H9 and H10) concerning recognition of STNV-C RNA by TNV-D RdRp should not be discounted. The three, 3'-terminal –CCC residues on STNV-C RNA do not appear to be essential for accumulation and less than a full-length ss tail is recognised by the helper RdRp. Similar results have been reported for TCV satellite RNA (Song and Simon, 1995; Carpenter and Simon, 1996) and CymRSV RNA (Dalmay *et al.*, 1993). From our analysis of the sequence of the progeny STNV-C RNA D which accumulated following replication of one mutant it would appear that in the majority of cases the 3'-truncation of 3 nt is repaired *in vivo*. The 3'-terminal sequences of STNV-C RNA (Bringloe *et al.*, 1998) and TNV-D RNA (Coutts, *et al.*, 1991) are both -ACCC. Thus it is possible that, as found with genomic and satellite TCV RNAs, which both contain seven identical 3'-terminal residues primer-induced synthesis, with the helper virus supplying the primer, possibly through abortive RNA synthesis may repair 3'-terminal deletions (Nagy *et al.*, 1997).

In contrast to promoters directing the synthesis of (-)-strand or subgenomic RNAs, the promoters involved in (+)-strand synthesis of most plant RNA viruses are not well defined. In the *Carmo*-like viruses and *Tombusviridae* it is known that sequences in the 5'-UTR are important for replication. DI RNAs derived from the genomic RNAs of CymRSV (Havelda *et al.*, 1995), TBSV (Chang *et al., 1995)*, and TCV (Zhang and Simon, 1994) retain all or most of the 5'-UTR. In a mutational analysis of CymRSV it was found that small deletions were tolerated at some sites but that sequences from most of the 5'-UTR were necessary for replication (Havelda *et al.*, 1995). For STNV-C RNA by mutation analysis we have shown that the proposed 5'-stem-loop structure has an important quantitative effect on productive RNA accumulation but may not be essential. Further progressive deletion analysis revealed that a 5'-terminal sequence of only 3 nt is both sufficient and essential for basal levels of RNA accumulation.

No minimal *cis*-acting sequences were identified in the mutational analysis of the 3'-terminus of STNV-C RNA but the *in vivo* study reported here illustrates that minimal *cis*-acting sequences can operate in STNV-C RNA (-)-strand synthesis. Interestingly if the STNV-C RNA sequence is refolded with the three 5'-terminal residues deleted, a 5'-terminal stem-loop is

still predicted albeit with 3 basepairs less at the base (not shown). This observation further substantiates the essential nature of residues 2 and 3 in STNV-C RNA replication. However the role of these sequences in potentially directing the helper RdRp to the correct initiation sites within the STNV-C RNA 5'-UTR to initiate synthesis require further investigation. Recent *in vitro* studies with turnip yellow mosaic virus (TYMV) RNA have indicated that the minimal promoters for (-)-strand synthesis can be as small as 2 nt and that TYMV RdRp may have a preference for binding structured RNA, favoring initiation sites adjacent to structured RNA (Singh and Dreher, 1998). Furthermore it was suggested that such specificity might be used rather commonly in the replication of (+)-strand RNA viruses, including *Carmo*-like viruses (Singh and Dreher, 1998).

In the experiments with chimeric mutants, where the 3'- and 5'-UTRs of STNV-C RNA were exchanged for the equivalent regions of TNV-D RNA, RNA accumulation was observed in both single and double exchanges. This result suggests that the stem-loop structures at both ends of the genome of the satellite and the helper are functionally interchangeable and that the mechanisms underlying (-)-strand and (+)-strand synthesis may be the same for both viruses. These observations have implications for the biological interactions between STNV-C and TNV-D since it is known that the helper virus accumulation is suppressed by the presence of satellite virus (Scholthof *et al.*, 1999). It will now be possible to investigate whether this phenomenon is attributable to the helper RdRp selecting a more attractive template for amplification in a mixed infection.

Acknowledgements

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CHARACTERIZATION OF A SUGAR BEET (*BETA VULGARIS* L.) VIRUS CAUSED BLACK SCORCH SYMPTOM IN CHINA, A POSSIBLE NEW MEMBER OF *NECROVIRUS*

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Summary

A new sugarbeet virus disease, occurring together with rhizomania by BNYVV, was found in China, which infected 13 species of 4 plant families and caused black scorch on the leaves of sugarbeet, but no significant hair roots. The virus particle had an isometric shape of 30 nm and single capsid protein of 25.5 kDa. A RNA component of 4.0 kb existed in the isolates from different Provinces of China, but an extra smaller RNA of 0.8 kb was found in the isolate of Xinjiang (X). It was approved by RPA test that these two RNAs were not homologues. The larger RNA was infectious to indicating plants, but the smaller one was only co-infectious when mixed with the large one. The virus was transmitted by *Olpidium brasicae* in non-persistent manner. By serological tests, the virus reacted with antisera of TBRV and a willow tree isolate of TNV strains in rather distant relationships. According to these data and the homology of its partial nucleotide sequence with TNV strains, which of 3624 nts showed identity of 61.6% with TNV D strain, this virus was named as beet black scorch virus (BBSV), and its possible classification was discussed.

Introduction

Rhizomania disease caused severe damage on sugar beets in China and BNYVV was reported as the causal agent (Gao, 1983). During the investigation on rhizomania, however, a different type of symptom was found mixed with that caused by BNYVV and isometric virus particles were observed under electronic microscope(Cui, 1991). In the early stage of infection, brown lesions appeared on leaves of beet plants and the lesions will expand to fuse together, which caused the leaves to death with black scorch along the veins. The diseased plant shot up straightly and the leaves curled upwards like spoons. The vascular bundles of infected plants changed to brown color, but not much hair roots as rhizomania caused by BNYVV (Cai, 1993). The disease occurred in all areas of sugar beet plantation in China and, in particularly, widely expanded in the Provinces of Inner Mongolia, Ningxia, Xinjiang and Gansu. According to the investigation in Inner Mongolia and Ningxia, the disease occurred in 30% of the total planting area and caused heavy yield loss, especially when mixed with BNYVV. In this paper, the results of characterization are presented in the viral biology, serology and partial sequence.

Results

Inoculation and purification: By mechanical inoculation, 13 species of 4 families were infected with local lesions in *Chenopodium amaranticolor*, *C. quinoa*, *C. murale*, *Spinacia olerracea* and *Tetrogonia expensa*, or symptomless in *Phsalis florisana*, *Nicotiana tabacum* and *N. glutinosa*. In sugar beet, the infected leaves showed local lesions in dark-brown color one week post-inoculation. The virus was very stable for storage under 4°C, which was still infectious after 6 years. The virus was propagated by single lesion inoculation and purified by sucrose density gradient centrifugation. With the preparation, virus particles were observed that it had isometric shape of 30 nm in diameter (Fig. 1, A) and analyzed preliminarily by SDS-PAGE or agarose gel for its coat protein and nucleic acids, respectively. The results showed that the virus particle was capsided by single type protein subunits of 25.5 kDa (Fig 1. C), and a ssRNA of 4.0 kb (L) was contained in all isolates from different Provinces, while a subgenomic RNA of 1.7 kb was observed in purified virus occasionally. However, another ssRNA component of 0.8 kb (S) was found in an isolate from Xinjiang, in addition of the large one (Fig 1. B). In order to know the replicate function of viral genomes, inoculations with each viral RNA component recovered from agarose gels or RNA combination from different isolates were carried out with *C. amaranticolor* in greenhouse. In table 1, it was shown that the L-RNA infected the host independently and the S-RNA was not infectious by itself, except when it was recombined with the L-RNA either originated from same or different sources (Bo, 1996).

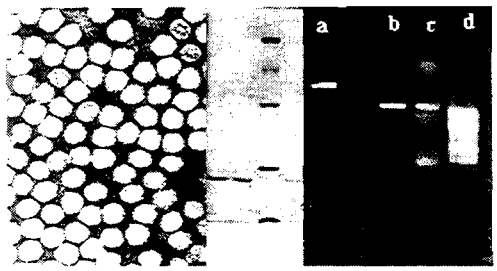

Fig.1. Electron micrograph of purified virus particles(A), coat protein (B) and viral RNA (C) assayed by electrophoresis. TMV and CMV RNA was used as markers (a,d); virus isolates from Ningxia (b) and Xinjiang (c) were detected.

Table 1. Plant infections by different combination of recovered viral RNA components

Items	Inocula				
	L-RNA of Xinjiang isolate	L+S-RNA of Xinjiang isolate	S-RNA of Xinjiang isolate	RNA(L) of Ningxia isolate	RNA(L) of Ningxia isolate + S-RNA of Xinjiang isolate
Symptom on *C. amaranticolor*	Necrotic lesion	Necrotic lesion	No	Necrotic lesion	Necrotic lesion
Symptom on *C. amaranticolor* reinoculated	Necrotic lesion	Necrotic lesion	No	Necrotic lesion	Necrotic lesion
No. of viral RNA components from inoculated plants	1	2	0	1	2
RNA size comparing to the original	Same	Same	-	Same	Same

Serological test: The purified virus isolated from Xinjiang and Ningxia Province was used for rabbit immunization, respectively, to prepare antiserum. By immunodiffusion tests (Fig.2), the virus showed very good antigenicity and no differentiation was found between the two isolates. Also, this virus reacted with antiserum against to tomato black ringspot virus (TBRV) as crossed precipitate lines, but not with those to CMV, CaMV, TRSV, CRSV and ToRSV used. A number of antisera against to different viruses or different strains of TNV was used for ISEM. The virus decorated strongly only by antiserum of a willow tree isolate of TNV in Germany, which did not react with A, B, D and some other TNV isolates, and weakly reactions with several other TNV strains. In this test, antiserum of willow tree isolate had a homologous titer with its own virus of 1: 6400, and heterogeneous titre was 1: 200 with the beet virus isolate. Antiserum to the beet virus reacted up to a dilution of 1:12800 with its own antigen, but if it reacted with TNV willow isolate the titer must be lower than 1:50. This results indicated rather distant relationships between the virus and TNV or TBRV.

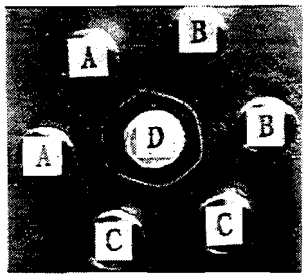

Fig. 2 Immunodiffusion test. Antisera against to TBRV (A), the Xinjiang isolate (B) and the Ningxia isolate (C) were used to react with the virus from Xinjiang.

Vector transmission: Since the virus titer in inoculated beet roots was much higher than in the leaves and virus transmission among plants could be inhabited significantly by treatments with fungicides to diseased soil or culture sands that were used as inocula, it was assumed that fungal vectors may be involved it. By inoculation to cowpea (*Vigna unguiculata* Wap.) roots that can not be infected by *Polymyxa betae*, *Olpidium brassicae* (Wor.) Dang was isolated as pure line from beet roots infected by the both fungi. When purified virus and zoospores of isolated *O. brassicae* were mixed, the infective efficiency among beet plants was much higher than virus only (Table 2). However, the efficiency would be decreased by treatments with fungicides or antisera against either to *O. brassicae* zoospore or the virus, when they were added respectively. Under electronic microscope, it was observed that the beet virus bound to on the zoospore surface after a preincubation and totally washing off. With these evidence, it was approved that the beet virus was transmitted by *O. brassicae* in a non-persistent manner (Adams, 1991; Campbeel, 1996).

A B

Fig. 3. Resting spore (A) and zoospore (B) of *O. brassicae*. Note that virus particles binding on the tail surface

Virus genome and nucleotide sequence: After polyadenylation, the viral RNA of Xinjiang isolate was used as template for synthesis of dsDNAs complementary to either L-RNA or S-RNA of the beet virus by reverse transcription. By northern blot and ribonuclease protection assay, it was shown that the L-RNAs of different isolates were highly conserved between one another and the S-RNA component of the Xinjiang isolate was heterologus from the L-RNAs from different sources. Based on this result and those of co-infection only with the L-RNA, the S-RNA was presumed as genome of a satellite RNA. Partial genomic

RNA of 3624 nucleic acids was sequenced. The result showed identity of 61.6% with that of TNV D strain.

Table 2. The virus transmission with *O. brassicae* vectors, detected by inoculation of *C. amaranticolor*

Inocula to beet plants	Purified virus				Purified virus+*O. brassicae*				*O. brassicae*				Buffer (50mM Glysine)			
Beet roots used for inoculation of *C. amaranticolor*	I	II	III	av.*	I	II	III	av.	I	II	III	av.	I	II	III	av.
No. of leaves inoculated	5	7	8	6.6	11	8	7	8.7	6	6	6	6	6	6	6	6
No. of lesions / leaf	1.4	8	3.2	4.2	36	114	78	76	0	0	0	0	0	0	0	0

* average.

Discussion

Stains of TNV, the type members of *Necrovirus*, had similarities in isometric particle shape, transmission by *O. brassicae* and organization of ssRNA genomes, but were highly heterogeneous for their serological relations and nucleic acids among different isolates. In some isolates, smaller particles of 16.8 nm in diameters were found as satellite TNV. However, a satellite-like ssRNA of 0.8 kb existed in the isolate of the beet virus from Xingjiang(Bo, 1996), but no coat protein or particle corresponding to that of satellite TNV(Francki, 1985) was found in the investigation since years. Combining with the distinct serological relationships and the low level of nucleotides homology to other TNV strains, it was suggested that the beet virus in China might be a new member of *Necrovirus*, named as beet black scorch virus currently. Before a final conclusion has been made for taxonomy of this virus, it is necessary to carry out further investigation on the relationships among species of *Necrovirus*.

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SEQUENCE ANALYSIS OF BYMOVIRUSES OF WHEAT AND BARLEY IN CHINA

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Summary

Between 1993-1999, barley and wheat leaves with typical mosaic symptoms of bymoviruses were collected from 24 sites in China. Partial sequence analysis of the 3'-terminal region (1.2 kb including the coat protein gene) of RNA1 indicated that all 14 wheat virus isolates from Anhui, Henan, Hubei, Jiangsu, Sichuan, Shandong, Shaanxi and Zhejiang provinces were wheat yellow mosaic bymovirus (WYMV) rather than wheat spindle streak mosaic bymovirus (WSSMV). Among 10 barley virus samples from Jiangsu, Shanghai and Zhejiang provinces, 7 were infected with barley yellow mosaic bymovirus (BaYMV) and 3 from Jiangsu (Haian, Rudong and Yancheng) were infected with a mixture of BaYMV and barley mild mosaic bymovirus (BaMMV). The complete nucleotide sequences of two isolates of WYMV (Yaan and Yangzhou, where differences in cultivar response occurred) and the Yancheng isolate of BaYMV were determined and compared with published sequences.

Introduction

Bymoviruses of wheat and barley occur extensively in Eastern China. Those on wheat have been variously described as wheat spindle streak mosaic virus (WSSMV) or wheat yellow mosaic virus (WYMV). Local experience suggested that cultivars sometimes responded differently to the viruses at different sites and therefore virus strain differences were suspected. On barley, the predominant virus is barley yellow mosaic virus (BaYMV) and virus strain differences have been detected (Chen et al., 1996). These experiments were part of studies to determine which viruses were present in different regions and to examine the variation between isolates. This may help explain differences in cultivar response and is an essential pre-requisite for the development of robust transgenic control strategies.

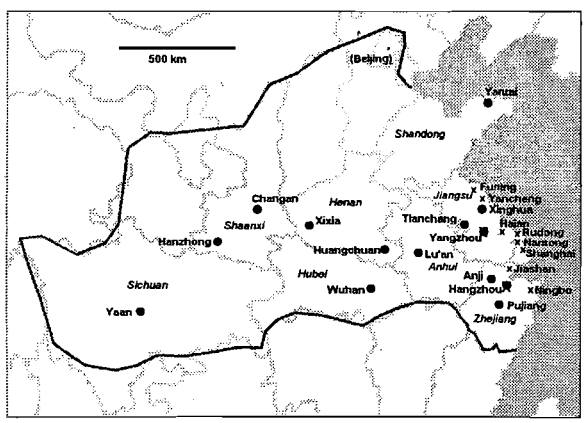

Fig. 1. Sites of wheat (•) and barley (x) bymovirus samples. Samples from both crops were obtained from Yangzhou and Hangzhou. Solid line shows region of winter wheat production

Materials and Methods

Between 1993 and 1999, virus isolates were obtained from wheat and barley from 24 different sites in China (Anhui, Henan, Hubei, Jiangsu, Sichuan, Shandong, Shanghai, Shaanxi and Zhejiang provinces; Fig. 1) and purified by the method of Chen et al. (1989). Viral RNAs were extracted from purified virus preparations by standard methods and genome fragments amplified

by RT-PCR, using primers designed to known sequences. To determine the terminal nucleotides, a DNA primer SP6R (5'-CCC CCC TAT AGT GTC ACC TAA AT-3') was ligated to the 3'-terminus of viral RNA and also to the 3'- terminus of first strand cDNA. Primer SP6 (5'-GAT TTA GGT GAC ACT ATA G-3'), complementary to SP6R, was then used in combination with primers designed to internal sequences to amplify, clone and sequence the extremities of the RNA. PCR products were cloned using the pGEM T-Easy vector system (Promega Inc., Southampton, UK) and competent cells JM109 were transformed. Plasmids were extracted using the QIAprep spin miniprep kit (Qiagen Ltd, Crawley, UK) and the clones were then sequenced on an ABI automated sequencer, using cycle sequencing with the ABI PRISM Dye terminator cycle ready reaction kit. The Wisconsin (GCG) package was used for sequence analysis and PHYLIP for phylogenetic analyses. Phylogenetic trees were constructed by a distance method (NEIGHBOR) using the original data set and 100 bootstrap data sets.

Results and Discussion

The nucleotide sequences of the 3'-terminal region (1.2 kb including the coat protein gene) of RNA1 were determined from 14 wheat virus isolates from Anhui, Henan, Hubei, Jiangsu, Sichuan, Shandong, Shaanxi and Zhejiang provinces. Genome comparisons indicated that all these were WYMV rather than WSSMV; for example, in the coat protein coding region, all isolates had 96-99% nucleotides identical to WYMV from Japan (Namba et al., 1998) but only 67-70% to WSSMV isolates. Amongst the predicted coat protein amino acid sequences, Anji, Huangchuan and Tianchang were identical and there were between 1 and 11 differences between the other pairs. These differences are shown in Table 1. Nucleotide and amino acid sequences were too similar for there to be many reliable sub-groupings in phylogenetic analyses and these are therefore not presented in detail. The only significant conclusions were that the Pujiang isolate grouped with one from Xinyang (Yu et al., 1995) and are the most different from the remaining isolates, that the isolates from Xixia and Lu'an grouped together and that two isolates from the same part of Shaanxi province (Changan determined in this study and Zhouzhi, acc. no. AJ240052) were very similar. Apart from this, there were no other obvious relationships between geographical proximity and molecular similarity.

Table 1. Numbers of differences between the coat protein amino acid sequences of WYMV isolates. Shaded areas show isolates that grouped significantly in phylogenetic analyses

	Xinyang	Pujiang	Changan	Zhouzhi	Yaan	Wuhan	Xinghua	Anji*	Hanzhong	Hangzhou	Yangzhou	Luotian	Xixia	Lu'an	Japan	Dengzhou
Xinyang	0	3	7	7	7	7	8	6	7	5	7	6	6	5	7	6
Pujiang		0	10	10	10	10	11	9	10	8	10	9	9	8	10	9
Changan			0	2	5	5	6	5	6	4	6	4	4	4	5	5
Zhouzhi				0	5	5	6	5	6	4	6	4	4	4	5	5
Yaan					0	4	5	4	5	3	5	5	5	5	5	4
Wuhan						0	1	1	2	2	2	3	3	4	4	1
Xinghua							0	2	3	3	3	4	4	5	5	2
Anji*								0	1	1	1	4	4	3	3	0
Hanzhong									0	2	2	5	5	4	4	1
Hangzhou										0	2	3	3	2	2	1
Yangzhou											0	5	5	4	4	1
Luotian												0	2	3	5	4
Xixia													0	3	5	4
Lu'an														0	4	3
Japan															0	3
Dengzhou																0

* Isolates from Huangchuan and Tianchang were identical to that from Anji

Field experiments had indicated that there were some differences in cultivar response at Yangzhou and Yaan (Table 2) and therefore the complete sequences of both RNAs of these two isolates were determined. Their organisation was identical to that reported for a Japanese isolate

Table 2. Percentage of plants of different cultivars infected by wheat yellow mosaic virus at the Yangzhou (YZ) and Yaan (YA) sites (means of 3 replicates; angular transformed data in parentheses)

Cultivar	Yangzhou 1997/8	Yangzhou 1998/9	Yaan 1998/99
Akakomugi	nt	29.8 (32.0)	0.0 (0.0)
Colfiorito	39.3 (38.5)	8.3 (15.9)	0.0 (0.0)
Colosseo	73.6 (60.1)	42.5 (40.5)	1.5 (5.8)
Ernie	nt	0.0 (0.0)	2.8 (9.2)
Haruyutaka	nt	0.0 (0.0)	11.6 (19.0)
Hokushin	nt	0.0 (0.0)	0.0 (0.0)
Italo	67.9 (56.8)	33.9 (34.7)	25.6 (30.2)
Newton	1.0 (3.3)	0.0 (0.0)	0.8 (3.0)
Pascal	0.7 (2.7)	0.0 (0.0)	0.0 (0.0)
Platani	100.0 (90)	70.0 (58.5)	0.0 (0.0)
Vona	58.2 (50.4)	39.2 (38.8)	96.3 (82.0)
SED*	8.34 (16)	8.42 (24)	7.22 (24)

* for angular transformed data, df in brackets; nt, not tested

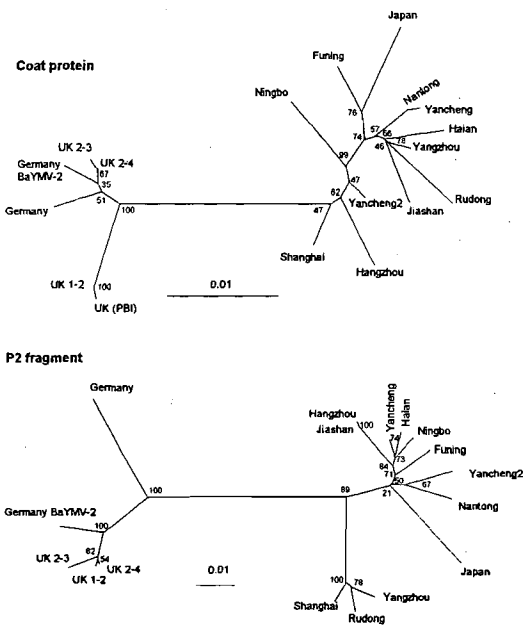

Fig. 2. Unrooted phylogenetic trees of the nucleotide sequences of the coat protein and P2 fragments of BaYMV isolates (NEIGHBOR analysis). Bootstrap values (100 replicates) are shown at the forks. Bars show distances as substitutions per base.

Table 3. Summary of amino acid differences between WYMV Yangzhou and Yaan isolates in the different putative protein products

	Numbers of amino acids		
	Total	Non-identical	Non-similar
RNA1			
P3	327	7	3
7K	66	1	0
CI	659	23	15
14K	124	5	3
NIa-VPg	187	4	0
NIa-Pro	220	12	8
NIb	528	7	4
CP	293	5	4
ORF1	2404	64	37
RNA2			
P1	255	4	1
P2	649	35	28
ORF2	904	39	29

(Namba et al., 1998) and they were very similar in size with cleavage sites similar to those already reported. The Yangzhou isolate was closer than the Yaan one to the Japanese isolate. Homologies between the viruses were usually greater in RNA1 than in RNA2, reflecting the greater variability of RNA2. The 3'UTRs had least variability and in particular the lengthy 3'UTR on RNA2 was highly conserved. In contrast, parts of the CI and NIa coding regions on RNA1 and the N-terminal part of the P2 coding region on RNA2 were particularly variable. Substantially conserved regions occurred in the 7K, one part of the CI (nucleotides 2026-2038) and parts of the NIb and coat protein. The amino acid sequences of their putative protein products (produced by cleavage of the polyproteins) were compared (Table 3). The most variable proteins were the P2 from RNA2, believed to be involved in fungus transmission, the NIa-Protease and the cylindrical inclusion (CI) protein. There were too many proteome differences to provide an indication where those associated with the differences in virulence are located, but there were no dissimilar amino acids in the 7K and NIa-VPg

proteins and only one in the P1 protein of RNA2, so it seems unlikely that these proteins are involved.

The nucleotide sequences of the coat protein coding region and about 700 nucleotides at the N-terminus of the P2 protein on RNA2 were determined for 10 Chinese BaYMV isolates from Jiangsu, Shanghai and Zhejiang provinces. Three of these samples also contained barley mild mosaic bymovirus (results not shown). Phylogenetic analyses showed that Asian isolates grouped separately from the European ones in both regions (Fig. 2), but there were no relationships between the Chinese isolates that could be linked to differences in cultivar response that have been reported (Chen et al., 1996). The complete sequence of a BaYMV isolate from Yancheng was determined. Except at the 3'-terminus of RNA1, its sequence was more closely related to that of a Japanese isolate than to one from Germany (Fig. 3).

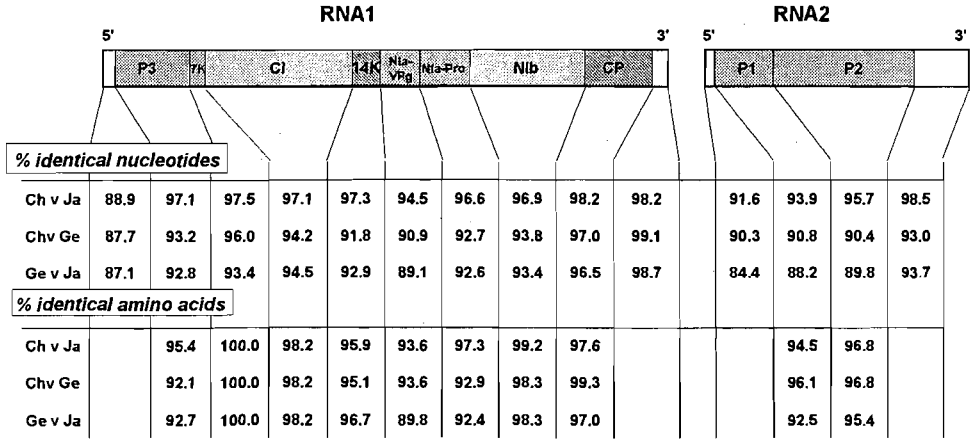

Fig. 3. BaYMV genome organisation and comparisons between isolates (Ch=China, Yancheng; Ja=Japan; Ge=Germany)

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CHARACTERIZATION AND DETECTION OF WHEAT BYMOVIRUSES

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Summary

Wheat spindle streak mosaic virus (WSSMV) and wheat yellow mosaic virus (WYMV) are two closely related bymoviruses which cause important wheat diseases. No molecular diagnostic protocol is available for either virus nor are serological methods able to discriminate them. Therefore an RT-PCR protocol was developed for their detection and discrimination. The sequence of the majority of the coat protein gene of three WSSMV and one WYMV isolates was determined. This data provided further evidence that the viruses are distinct species and was used with published sequence data to design specific primers for each virus. Using total RNA from thirteen WSSMV and five WYMV isolates in either one- or two-step RT-PCR, these primers were shown to reliably detect and discriminate the viruses. The primers were specific to each virus and no amplification was obtained with either soil-borne wheat mosaic virus, which is frequently associated with both diseases, or with other members of the genus *Bymovirus*. This new protocol will improve disease management by enabling accurate pathogen identification and earlier detection than is possible serologically.

Introduction

Both wheat yellow mosaic virus (WYMV) and wheat spindle streak mosaic virus (WSSMV) cause important diseases of wheat. The viruses are similar physically and morphologically and are transmitted by *Polymyxa graminis*. They also cause similar disease symptoms, *i.e.* chlorotic, spindle-shaped streaks on the leaves, reduced tillering and decreased yield. Both have a broad distribution and have been found in China, Japan, France, Germany, Italy, Canada and the USA (Brunt *et al.*, 1996). Until recently their taxonomic relationship was confused and they were considered to represent strains of the same virus (Brunt *et al.*, 1996). In general viruses of this type from North America were arbitrarily described as WSSMV, while those from China and Japan were described as WYMV. In Europe the position has been confusing with similar isolates being described as both WSSMV and WYMV, *e.g.* in France (Hariri *et al.*, 1996). However, by comparing the sequence of a French WSSMV isolate (WSSMV-F) and a Japanese WYMV isolate (WYMV-J), Namba *et al.* (1998) demonstrated that these viruses are indeed distinct species. Discrimination of WSSMV and WYMV is important practically as well as taxonomically. WSSMV has a broader host range than WYMV, infecting not only wheat but also durum wheat and rye (Brunt *et al.*, 1996). Cultivar resistance to these viruses is also likely to differ which is especially important since this is the only method of control. No molecular protocols have been published to discriminate the viruses, nor are either polyclonal antisera (Carroll *et al.*, 1995) or monoclonal antibodies (Hariri *et al.*, 1996) able to distinguish them.

Materials and Methods

Soils containing viruliferous spores of WSSMV (Montpellier-France, Arkansas-USA and New York-USA) or WYMV (Henan-China) were used to infect wheat cv. Thunderbird. Seedlings were incubated in a slurry of 40 g of soil at 20°C for 10-14 d before transplanting in sufficient sand to give a 1:9 dilution of the infected soil. The plants were grown for a further three weeks at 20°C before decreasing the temperature to 10°C and were tested periodically for infection by ELISA (Carroll *et al.*, 1995). Further isolates of WSSMV, WYMV, barley yellow mosaic virus (BaYMV), barley mild mosaic virus (BaMMV), oat mosaic virus (OMV) and soil-borne wheat mosaic virus (SBWMV) were also obtained *in planta* (Table 1). Total RNA was extracted from 200 mg of root or leaf tissue as described by Clover *et al.* (1999).

Table 1. Isolates of soil-borne viruses used during the study

Virus	Origin	Virus	Origin
WSSMV	Arkansas, USA	WYMV	Henan, China
	Indiana, USA		Henan, China
	Kansas, USA		Hubei, China
	New York, USA		Ibaraki, Japan
	Ontario, Canada		Iwate, Japan
	Montpellier, France	BaYMV	Hertfordshire, UK
	Seine-Maritime, France		Cambridgeshire, UK
	Loiret, France	BaMMV	Hertfordshire, UK
	Loir-et-Cher, France		Bedfordshire, UK
	Braunschweig, Germany	OMV	Monmouthshire, UK
	Parma, Italy		North Carolina, USA
	Bologna, Italy	SBWMV	Oklahoma, USA
	Rome, Italy		Wiltshire, UK

Degenerate primers were designed from published sequence (Sohn *et al.*,1994; Namba *et al.*, 1998) to complement the 3′ (WMVCPR: 5′GGTTAGCTCTGGRTGTCCATCAG3′) and 5′ (WMVCPF: 5′GCTGCGGACACACAAACWGACG3′) ends of the coat protein (CP) genes of WSSMV and WYMV, and these were used to amplify one WYMV isolate (Henan-China) and three WSSMV isolates (New York-USA; Arkansas-USA; Montpellier-France). RT was performed using 0.5 µl template RNA, 4 µl WMVCPR (5 µM) and 100 units MMLV reverse transcriptase (Promega) in a 10 µl volume at 37°C for 1 h. PCR was carried out using the Expand system (Roche Molecular Biochemicals) according to the manufacturers's protocol. Amplicons representing the CP gene were sequenced as described by Clover *et al.* (1999).

Multiple sequence analysis of these isolates together with published sequence (WSSMV: Accessions AJ237925, AJ237926, X73883 and AB010578; WYMV: Accessions AJ237924 and D86634; BaYMV: Accessions D01091 and X69757) was done using the Clustal V method from the MegAlign package (DNA Star). Areas of divergent sequence were identified and three primers were designed WSSMVF (5′CAGCAACCAAAGTYRCAGCAAC3′) which was

complementary to WSSMV, and WYMVF (5'CCTCCTTCAGGAACACAAGATTGTCA3') and WYMVF1 (5'ATGACAAGAAAGCCAGGGACC3') which complemented WYMV.

For two-step multiplex RT-PCR, cDNA was produced as described above using 2 µl Oligo-d(T)Not1 (5 µM). PCR amplification was performed in 50 µl reaction volumes containing 10 µl cDNA, 5 µl 10x *Taq* reaction buffer, 2.5 µl $MgCl_2$ (25 mM), 1 µl dNTP (10 mM), 2.5 units *Taq* DNA polymerase (Promega), 2 µl WSSMVF and 1 µl WYMVF (5 µM). The following thermocycle was used: 94°C for 2 min, then 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, followed by 72°C for 3 min. The Titan One Tube RT-PCR system (Roche Molecular Biochemicals) was used according to the manufacturers's protocol at an annealling temperature of 56°C. To each reaction, 1 µl template RNA, 3 µl $MgCl_2$ (25 mM), 2 µl Oligo-d(T)Not1 (5 µM) and 2 µl WSSMVF, WYMVF or WYMVF1 (5 µM) were added.

Results

Amplification of the CP gene of the three WSSMV isolates and one WYMV isolate produced products of the expected size, *i.e.* 879-882 nt. In this region of RNA 1, WSSMV-F and WYMV-J share only 68.2 % nucleotide homology and sequence alignment with these strains confirmed the taxonomic identification of the four isolates. The WSSMV isolates from Montpellier, New York and Arkansas had 99.4, 98.8 and 93.9 % nucleotide homology with the corrresponding region of WSSMV-F respectively. These substitutions were non-coding apart from those in the Arkansas isolate which coded for six amino-acid substitutions. The Henan isolate of WYMV shared 98.5 % nucleotide sequence homology with the corresponding region of WYMV-J, 4 of these 13 nucleotide substitutions coded for substitutions.

Both one- and two-step RT-PCR amplified a single product from all WSSMV isolates using the WSSMVF primer (982 nt) and all WYMV isolates using the WYMVF (544 nt) or WYMVF1 (1025 nt) primers. The products obtained using the two-step method were more intense and discrete than those from the one-step protocol. The WYMVF1 primer gave stronger bands than the WYMVF primer with all WYMV isolates using the Titan system but was unsuited for a one-step multiplex reaction because it amplified a product of similar size to that obtained with WSSMV. Both methods detected infection in all plants which gave positive ELISA results. There were no differences in the product size obtained from different isolates nor were any non-specific bands observed. No PCR products were obtained using RNA from healthy wheat or wheat infected with BaYMV, BaMMV, OMV or SBWMV. No samples were co-infected with WSSMV and WYMV but bands of the expected size were produced if RNA from both was used as a template in a two-step multiplex reaction. This protocol also detected infection three weeks before symptoms could be observed or infection diagnosed by ELISA.

Discussion

Sequence alignment of the CP gene of four bymoviruses isolated from wheat with the corresponding sequences of WSSMV-F and WYMV-J revealed that they fell into separate, homogenous groups representing each species, and confirmed their identity as WSSMV or WYMV. This data provides further evidence for the hypothesis of Namba *et al.* (1998) that WSSMV and WYMV are distinct species rather than strains of the same virus. This sequence data enabled the design of WSSMV and WYMV primers which were shown to be specific for

each virus using thirteen WSSMV and five WYMV isolates. Not only was this molecular protocol as reliable as ELISA, it was also more sensitive and could detect infection up to three weeks earlier, and could distinguish between WSSMV, WYMV and BaYMV. The protocol could be adapted for use in a one-step reaction with its innate advantages but each reaction was more costly and the reaction products less distinct.

From the results of this study, WYMV appears to be confined to China and Japan whereas WSSMV has a wider distribution and occurs throughout Europe and North America. Although four French isolates were tested, these were all WSSMV isolates and there was no evidence for the presence of WYMV despite earlier reports (Hariri *et al.*, 1996). It is anticipated that this protocol will improve WSSMV and WYMV testing for quarantine and breeding purposes, *e.g.* its increased sensitivity compared to ELISA will help to identify truly resistant breeding lines of wheat.

We are grateful to those researchers who donated the virus isolates and antisera used during this project, namely E. Milus, K. Perry, G. Bergstrom, P. Signoret, F. Gitton, M. Bonnefoy, W. Huth, C. Rubies-Autonell, V. Vallega, J. Yu, S. Kashiwazaki and Y. Ohto. This work was funded by the MAFF Plant Health Division, UK (PH0143) and carried out under Plant Health Licence No. PHF 1526A/1301/124.

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SEQUENCE ANALYSIS OF FUROVIRUSES OF CEREALS FROM CHINA AND EUROPE

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Summary

The complete nucleotide sequences of both RNAs of oat golden stripe virus (UK isolate) and two furoviruses from wheat in Shandong province (China) and in France have been determined. The genome organisation of all the viruses was similar to that of soil-borne wheat mosaic virus but there was only 62-70% nucleotide identity between them. The wheat viruses have been named Chinese wheat mosaic virus and European wheat mosaic virus respectively. Phylogenetic analyses of different genome regions supported the recognition of these viruses as distinct members of the genus *Furovirus*. Analysis of the coat protein readthrough domain on RNA2 of all four furoviruses, using several software packages, strongly predicted two mutually-compatible conserved transmembrane domains. The second of these domains is either absent or disrupted in the deletion mutants reported for these viruses. It is proposed that these domains may be functionally significant in fungus transmission.

Introduction

Soil-borne wheat mosaic virus (SBWMV), the type member of the genus *Furovirus*, has been reported from most winter wheat growing regions of the world including the USA, Brazil, France, Italy, Egypt, Japan and China. The complete sequences of the two RNAs of an isolate from Nebraska have been reported (Shirako and Wilson, 1993). Oat golden stripe virus (OGSV) is a serologically related furovirus that occurs on oats in Europe and the USA. We report here the complete sequences of OGSV and two wheat furoviruses isolates from France and China which have been named Chinese wheat mosaic virus (CWMV) and European wheat mosaic virus (EWMV) respectively.

Materials and Methods

Virus isolates were obtained from wheat (Yantai, Shandong, China and from near Poitiers, Poitou-Charente, France) and oats (Cranbrook, Kent, UK) and purified using the methods of Diao *et al.*, 1999 (CWMV, EWMV) or Adams *et al.*, 1988 (OGSV). Viral RNAs were extracted from the purified virus preparation by standard methods. Genome fragments were amplified by RT-PCR, using primers designed to conserved regions of the genome and these were cloned and sequenced. Primers designed from this sequence were then used in RT-PCR reactions to produce fragments extending towards the 5'- and 3'- termini. To ensure that the terminal nucleotides were included, a DNA primer SP6R (5'-CCCCCCTATAGTGTCACCTAAAT-3') was ligated to the 3'-terminus of viral RNA and also to the 3'- terminus of first strand cDNA. Primer SP6 (5'-GATTTAGGTGACACTATAG-3'), complementary to SP6R, was then used in combination with primers designed to internal sequences to amplify, clone and sequence the entire genome. PCR products were cloned using the pGEM T-Easy vector system (Promega Inc., Southampton, UK) and competent cells JM109 were transformed. Plasmids were extracted using the QIAprep spin miniprep kit (Qiagen Ltd, Crawley, UK) and the clones were then sequenced on an ABI automated sequencer, using cycle sequencing with the ABI PRISM Dye terminator cycle ready reaction kit.

The Wisconsin (GCG) package was used for sequence analysis and PHYLIP for phylogenetic analyses. Phylogenetic trees were constructed by a distance method (NEIGHBOR)

Table 1. Comparisons amongst four furoviruses showing the sizes of the coding regions with the percentage of identical nucleotides (upper part of each section) and amino acids (in bold italics in lower part of each section).

	nt	kDa	EWMV	OGSV	CWMV	SBWMV
RNA1						
ORF1 (Replicase)						
EWMV	3927	148.9	-	62.5	67.7	69.6
OGSV	4038	152.8	***63.3***	-	62.7	67.0
CWMV	4053	153.0	***73.7***	***62.7***	-	72.7
SBWMV	3963	150.0	***75.5***	***63.6***	***81.1***	-
Readthrough (RdRp)						
EWMV	1524	+58.9	-	73.0	73.8	74.5
OGSV	1524	+58.9	***79.3***	-	70.5	72.3
CWMV	1524	+59.3	***82.5***	***77.6***	-	78.9
SBWMV	1524	+59.2	***82.3***	***79.1***	***90.4***	-
Movement protein						
EWMV	981	37.2	-	55.9	64.6	67.3
OGSV	975	36.0	***57.3***	-	58.2	58.4
CWMV	990	37.4	***67.2***	***60.3***	-	71.2
SBWMV	984	37.1	***70.2***	***60.0***	***76.8***	-
RNA2						
Coat protein						
EWMV	531	19.5	-	68.7	66.5	72.9
OGSV	531	19.4	***72.2***	-	72.3	69.9
CWMV	531	19.2	***71.0***	***77.3***	-	71.0
SBWMV	531	19.3	***79.0***	***77.8***	***76.1***	-
CP readthrough domain						
EWMV	1734	+65.2	-	59.2	57.6	63.1
OGSV	1521	+57.1	***53.2***	-	55.9	59.6
CWMV	1749	+64.7	***50.3***	***53.8***	-	59.6
SBWMV	1734	+64.5	***59.9***	***56.0***	***58.4***	-
Cysteine-rich protein						
EWMV	498	18.0	-	62.8	64.8	70.3
OGSV	522	18.7	***64.0***	-	66.3	62.5
CWMV	522	18.8	***63.4***	***65.9***	-	63.4
SBWMV	525	18.8	***63.0***	***62.4***	***61.5***	-

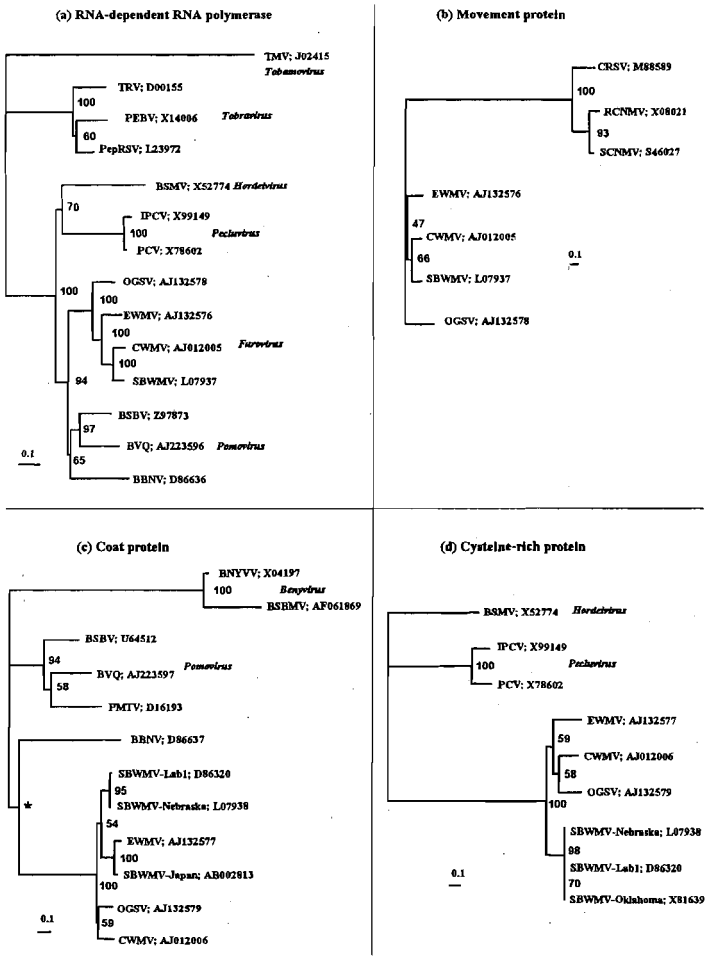
using the original data set and 100 bootstrap data sets. Transmembrane regions were predicted from peptide sequences by the programs HMMTOP (Tusnády and Simon, 1998), TMHMM (Sonnhammer *et al.*, 1998), TMPred (Hofmann and Stoffel, 1993) and TopPred 2 (von Heijne, 1992) and by considering likely α-helices using the scales of Chou and Fasman (1974) and of Deleage and Roux (1987). All the information was used to produce a consensus model.

Results and Discussion

The complete sequences of both RNAs of each of the virus isolates were determined and the sequences have been deposited in the databases with accession numbers AJ012005-6 (CWMV RNA1 and RNA2), AJ132576-7 (EWMV RNA1 and RNA2) and AJ132578-9 (OGSV RNA1 and RNA2). All virus isolates had two RNAs organised in a manner similar to SBWMV. An analysis of the nucleotide and amino acid homologies (Table 1) showed that there were substantial differences between the isolates. In the most conserved region (RdRp), nucleotide identity was 70-79%, while the least similar region was the coat protein readthrough domain where there were usually <60% identical nucleotides and amino acids. Phylogenetic analyses of the peptide sequences (Fig. 1) confirmed the view that these isolates should be regarded as separate viruses since the distances between them were of a similar magnitude to those between other pairs of viruses.

Evidence from other fungus-transmitted rod-shaped viruses indicates that the coat protein readthrough domain is incorporated into virions and located at one end of the particle and that the C-terminal half of the domain is important for fungus transmission. Spontaneous deletions in this region prevented transmission of BNYVV by *Polymyxa betae* (Tamada and Kusume, 1991) and of PMTV by *Spongospora subterranea* (Reavy *et al.*, 1998). In experiments with BNYVV, substitution of KTER (at amino acid numbers 553-556) by ATAR completely prevented transmission by *P. betae* (Tamada *et al.*, 1996). This motif does not occur in the readthrough domains of the other fungally-transmitted viruses that have been studied (except in the related BSBMV) and, although SBWMV has KTEIR at amino acids 466-469 this sequence does not occur in CWMV, EWMV or OGSV. Examination of the regions of the protein likely to be exposed on the surface (and which thus could be available for interaction with a receptor site on the vector)

Fig. 1. Phylogenetic trees of the peptide sequences of selected parts of the furovirus genomes and those of related plant viruses: (a) the RNA-dependent RNA polymerase (tree rooted with TMV); (b) the movement protein (tree rooted with dianthoviruses); (c) the coat protein (tree rooted with benyviruses) and (d) the cysteine-rich protein (tree rooted with BSMV). The values at the forks indicate the number of times out of 100 trees that this grouping occurred after bootstrapping the data.



showed little amino acid sequence conservation in the C-terminal part of the protein (data not shown). However, two highly conserved regions which are strongly predicted transmembrane domains were identified. Directional alignment of the two helices (Fig. 2) also shows evidence of compatibility between their amino acids, with groupings of amino acids that are either identical or in the same hydrophobicity group and evidence of possible fits between the small glycine residues on one helix and the larger aromatic ones on the other. The deletion in OGSV occurs from the middle of the second region and this virus isolate therefore has only one predicted transmembrane region. It is predicted that the region between the transmembrane domains would be on the inside of the membrane and therefore that the virus particle would be outside. It therefore seems possible that these regions are involved in attachment to the zoosporangial plasmalemma and assist virus particles to move between the cytoplasm of the plant host and that of the fungus vector. If this is so, the deletion in OGSV would probably eliminate fungus transmission, and this would be consistent with observations on other rod-shaped viruses transmitted by fungi.

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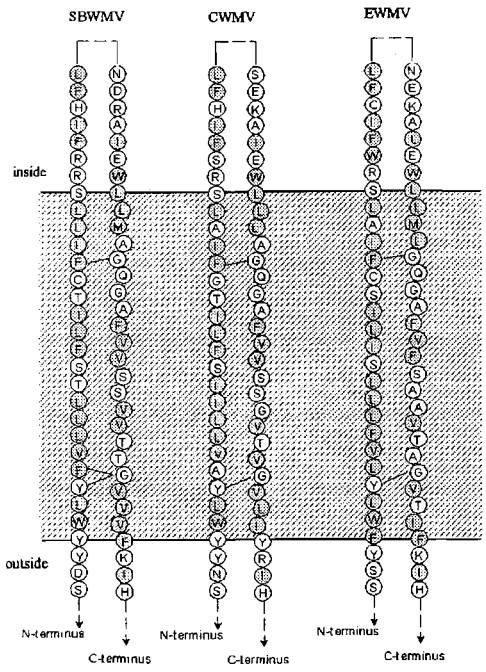
Fig. 2. Directional alignments of the two putative transmembrane regions of SBWMV, CWMV and EWMV showing their predicted alignment within the membrane. Highly hydrophobic residues are shaded and glycine-aromatic residue fits are marked by lines.



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WHEAT YELLOW MOSAIC VIRUS, THE ONLY BYMOVIRUS DETECTED IN WHEAT IN CHINA

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Summary

Based on RT-PCR method, bymovirus species were screened in wheat samples from six Provinces in China. With primer pairs specific to wheat yellow mosaic virus (WYMV) or wheat spindle streak mosaic virus (WSSMV) respectively, WYMV was identified in 25 of total 27 diseased wheat samples, but no WSSMV was detected. Thus, contrary previous reports of WSSMV, WYMV was the only bymovirus detected in wheat in China. Furthermore, five cDNA fragments corresponding to coat protein coding regions of WYMV isolates from China were cloned and sequenced. Deduced amino acid comparison of coat proteins among six Chinese isolates and one isolate from Japan showed very high sequence conservation, particularly in both the N- and C- termini. However, a highly virulent HC isolate from Henan Province showed 4 amino acid substitutions in highly conserved amino acid positions which might play a role in its pathogenic variation from others.

Introduction

The complete sequence of WYMV published recently confirmed that the wheat yellow mosaic virus (WYMV) and wheat spindle streak mosaic virus (WSSMV) are two distinct viruses within *Bymovirus* [Namba,1998], but not strains of the same species. However, since the bymovirus-related wheat diseases, which distribute along the valleys of more than two thousands kilometers in China, were described for symptoms on different wheat varieties, serological reactions with antisera to several bymoviruses and viral particle characteristics by different results [Chen,1993; Yu, 1986; Zhou, 1990], it is necessary to know whether there were any other possible causal agent responsible for the damage in the whole area, besides WYMV reported previously [Yu,1995]. Also, pathogenic differences among viral isolates were reported in China and Japan[Hou, 1993a], in which a set of wheat varieties, including resistant and highly susceptible ones, reacted to the infections by each isolate with different severity of symptoms, even in the opposite. Thus, an analysis of the viral species will give further insight into the relatedness of different WYMV isolates and may reveal variations among WYMV strains at molecular level.

Materials and Methods

27 wheat samples of different cultivars displaying either yellow mosaic or spindle streak mosaic on the leaves were collected from the fields of Provinces along the Yangtze River. Total RNA of infected wheat was extracted with phenol:chloroform and lithium chloride. Based on the nucleotide sequences of WYMV RNA1 [Yu, 1999] and WSSMV RNA1 [Sohn,1994], two pairs of oligonucleotides were designed to distinguish WYMV and WSSMV from one another by RT-PCR in the coding regions of two coat proteins (CP). The WYMV primers were 5'-GAGCTCATGGCAGCTGAC-3' and 5'-AGGGGAGTACTGGTTTAG GTTAGT-3' and the WSSMV primers were 5'-CGAGTACTGTTTGCATTACGGTGGTTGTGA-3' and 5'-CGAGTACTGATACAGATATACGACCAC-3', respectively. 2 μg total RNA of each sample was used for first strand of cDNA synthesis using the Riboclone cDNA Kit (Promega) according the manufacturer's instruction. First strand cDNA 2.5 μl was used as templates for PCR reactions. In the reactions, virus isolates of WYMV HC identified previously [Xing, 1994] and WSSMV NY [Carroll, 1995], two cDNA clones of pGWY30 [Yu, 1995] and Mol39 [Sohn, 1994] complementary to the RNA1 3' terminal regions of WYMV or WSSMV, respectively, were used as controls. The blunt-ended PCR products were ligated into pGEM-7Zf(+) (Promega). Sequences of the cDNA clones corresponding to the viral isolates from each Province were determined in a DNA autosequencer (Model 377A). The nucleotide and amino acid sequences were analyzed and compared using the Genetyx-Mac (Version 8.5).

Results

Species identification by RT-PCR: Approximately 0.9 kb DNA was amplified in 25 samples from all six Provinces by the primers of PY-1 and PY-2, except for two samples from Henan and Jiangsu (Table 1). However, no product was obtained with primers of PS-1 and PS-2 complementary to WSSMV, except for control templates of the NY isolate and the MoI39 cDNA. The primer pairs designed to amplify WYMV or WSSMV were highly specific, although, occasionally, a week band could be observed when the WYMV primers were used to detect the NY isolate. This result, combined with that of virion morphology, indicated that the filamentous virions in the wheat samples collected along the valley were WYMV, but not WSSMV from the same *Bymovirus* genus. Thus, the only virus detected in wheat in Anhui, Henan, Hubei, Jiangsu, Sichuan and Shaanxi Provinces was identified as WYMV and its distribution was shown in Fig.1.

Table 1. Detection of bymoviruses in wheats in China

Province	Sample number	RT-PCR detection	
		WYMV	WSSMV
Sichuan	1	1	0
Hubei	1	1	0
Shaanxi	7	7	0
Henan	9	8	0
Anhui	3	3	0
Jiangsu	6	5	0
Total No.	27	25	0

Sequence comparison of the coat proteins: The inserted fragments coding the CP gene of five virus isolates of WYMV-DZ (Henan), WYMV-LT (Hubei), WYMV-YZ (Jiangsu), WYMV-YA (Sichuan) and WYMV-ZZ (Shaanxi) were sequenced and compared with those of a Japanese isolate (JP) and another Chinese isolate (HC) reported previously [Namba, 1998; Yu, 1995]. The deduced WYMV CP consisted of 293 aa for most isolates, except for isolate DZ from Henan. Among the different isolates, the aa sequences showed very high identity of 97.6% to 99.3%, particularly in the regions of N terminus 110 aa and C terminus 55 aa. This conservation reflected that both ends of

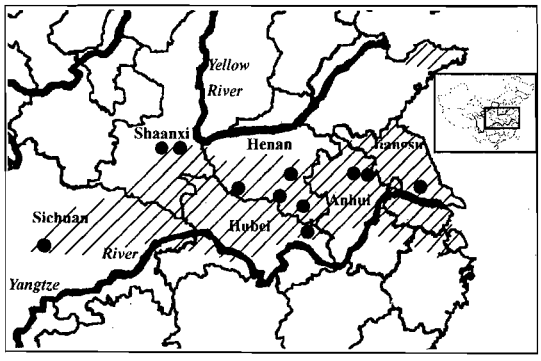

Fig.1 Distribution of wheat yellow mosaic bymovirus detected in wheat in China.
Black-dots indicated locations (Counties in each Province) where wheat samples were collected. The area where the occurrence of either wheat spindle streak mosaic virus or wheat yellow mosaic virus was reported previously were shadowed with italic lines.

WYM CP may be surface epitopes of viral particles, similar to that of potyviruses [Shukla, 1988]. However, most differentiation was found in the core region of less than 130 aa between the HC isolate and all other isolates (Fig. 2). The amino acid residues of valine at position 118 and phenylalanine at position 144 were changed to isoleucine, and leucine at position 208 and phenylalanines at position 238 were replaced by valines.

Discussion

In the mid- to lower-valleys of the Yangtze River expanding more than two thousand kilometers, semi-winterness wheat cultivars were grown under a similar climatic zone, where the lowest temperature in winter was about 0℃. In early Spring, wheat yellow mosaic disease may break out under low temperature

of 8℃ to 14℃. The yield loss caused by this disease was estimated about 20-30% in most years, and up to 50-70% occasionally. Since this disease appeared in Sichuan in the 1960's [Tao,1980], wheat viruses were reported from different locations in China and the causal agents had been identified as WYMV or, rather preferably named, as WSSMV [Chen, 1993;Yu, 1986; Zhou, 1990] . Based on this study, it is showed that WYMV was the only bymovirus present in the wheat production belt along the Yangtze Rive and the Yellow River in China.

Pathogenic differentiation was reported among isolates of bymoviruses in wheat in China [Hou, 1993a]. A WSSMV isolate from Henan Provinve, where the WYMV HC isolate was collected from the same location, showed stronger virulence than other isolates [Hou, 1993b]. By inoculating with viruliferous fungi in greenhouse, the WYMV HC isolate also showed stronger virulence than others in different wheat varieties tested (data not shown), which is similar as a WYMV isolate reported in Hokkaido [Kusume, 1997]. According to the symptom severity and the latent period after infection by different isolates, three pathogenic types were proposed for bymovirus isolates in China [Hou, 1993b]. Due to the variations in virulence, some wheat varieties were resistant only when they were grown within a restricted area. Interestedly, the mutated CP amino acid sequence of WYMV HC isolate coincided with the pathogenic differences (Fig. 2), which might represent a factor contributing to the pathogenic variations in WYMV.

```
HC  AADTQTDAQKEEARLAAATKKAADDADAARLRKVEADRVEAARVKKASDDKKARDLTATK  60
DZ  ++++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++  59
YZ  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++  60
YA  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++  60
ZZ  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++  60
LT  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++G+++++  60
JP  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++  60

HC  VDDGKIVADAGTKRTNAATKEKWSLPETKPVNAGLKLRISMDKLKSAPKSIVEHDNSVAL  120
DZ  ++++++++++++++++++++++++++++++++++++++++++++++++++++I++N++I+++  119
YZ  ++++++++++++++++G++++++++++++++++++++++++++++++++++I++N++I+++  120
YA  ++++++++++++++++++++++++++++++++++++++++++++++++++++I++++AI+++  120
ZZ  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++++I+++  120
LT  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++++I+++  120
JP  ++++++++++++++++++++++++++++++++++++++++++++++++++++I+++++I+++  120

HC  DSEVKKWSDAVRTSLGITTDEAWENALIPFLGWCANSGASDKHAENQTMQVDNGTGALTE  180
DZ  ++++++++++++++++++++++++I++++++++++++++++++++++++++++++++++++  179
YZ  ++++++++++++++++++++++++I++++++++++++++++++++++++++++++++++++  180
YA  ++++++++++++++++++++++++I++++++++++++++++++++++++++++++++V+++  180
ZZ  ++++++++++++++++++++++++I+T++S++++++++++++++++++++++++++++V+++  180
LT  ++++++++++++++++++++++++I++++++++++++++++++++++++++++++T+++  180
JP  +++++T++++++++++++++++++I+G++++++++++++++++++++++++++++++++++  180

HC  MSLSPFIVHARLNGGLRRILRAYSDETLLLLQEHKIVTKWAMKHGASAHAAYAFDFFFPR  240
DZ  ++++++++++++++++++++++++++++V++++++++++++++++++++++++++++++V++  239
YZ  ++++++++++++++++++++++++++++V++++++++++++++++++++++++++++++V++  240
YA  ++++++++++++++++++++++++++++V++++++++++++++++++++++++++++++V++  240
ZZ  ++++++++++++++++++++++++++++V++++++++++++++++++++++++++++++V++  240
LT  ++++++++++++++++++++++++++++V++++++++++++++++++++++++++++++V++  240
JP  ++++++++++++++++++++++++++++V++++++++++++++++++++N++++++++V++  240

HC  PWMNPQDIEVAKQARLAALGTGTYNTMLTSDTTNLRKTTNRRVLDTDGHPELT        293
DZ  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++        292
YZ  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++        293
YA  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++        293
ZZ  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++        293
LT  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++        293
JP  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++        293
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Fig. 2 Multiple alignment of predicated amino acid sequence of coat protein among different WYMV isolates. HC: Henan isolate (24), DZ: Henan isolate (AJ240048), YZ: Jiangsu isolate (AJ240051), YA: Sichuan isolate (AJ240050), ZZ: Shaanxi isolate (AJ240052), LT: Hubei isolate (AJ240049), JP: Japan isolate (11). + : identical amino acid; -: deleted amino acid. The shadowed residues indicated differences between HC and other isolates. The database accession numbers or the references of nucleotide sequences were indicated in parentheses.

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OCCURENCE IN FRENCH WINTER WHEAT CULTIVARS OF A VIRUS HAVING ROD SHAPED PARTICLES AND NO SEROLOGICAL RELATING WITH SBWMV.

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Summary

In a French area (Aube district) where soil borne wheat mosaic virus (SBWMV) is a common occurrence, we have found in one field two wheat cultivars, resistant to SBWMV, expressing mosaic symptoms and dwarfing. This virus was provisionally named Aubian wheat mosaic virus (AWMV). In the field infected plants were present only in one very large patch. In electron microscopy rod shaped particles were observed having from 50 to 600nm in length distributed in mainly two classes suggesting that the virus is multipartite. No serological relationships with SBWMV, SBRMV, OGSV and WYMV(WSSMV) have been observed. Neither *Polymyxa graminis* nor nematode species were found associated to infected roots.

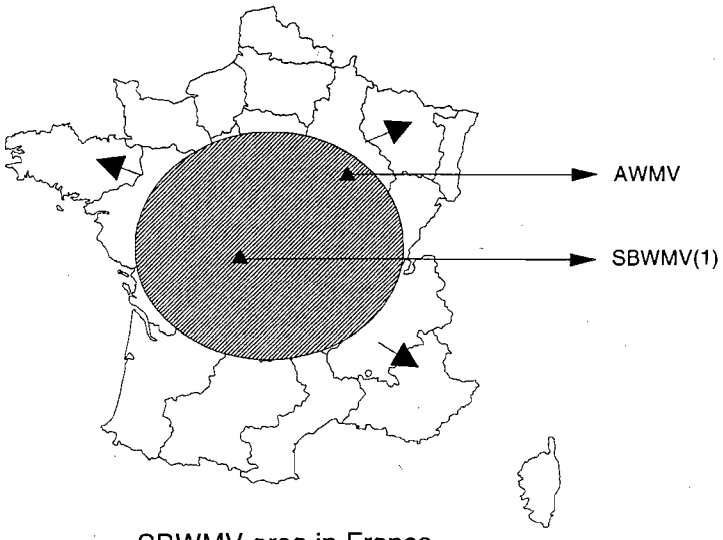
Introduction

Soilborne wheat mosaic virus (SBWMV) was first described in the USA. In France a SBWMV like virus (SBWMV-L) was discovered in the 70's (Hariri et al.,1987). Since then using a DAS ELISA test more than 2000 isolates have been detected, covering more and more areas mainly in the center of France (map). Several registered cultivars (cvs) are resistant to this virus. The resistance of the first described cvs has remained stable for more than 20 years.
Other viruses of SBWMV type in wheat have already been described in China and Japan which distinguish by their genetic sequences from the virus described in the USA (Diao et al., 1999[a]; Koenig & Huth, 1998). In the USA a variant of SBWMV has been described (Chen et al., 1996). In Great Britain there exists two serologically distinct forms of SBWMV-L (Clover et al., 1999) which could be similar to the situation in France (Table. 1).
Recently different European SBWMV are found: soilborne rye mosaic virus (SBRMV) in Germany (Koenig & Huth, 1998) and European wheat mosaic virus (EWMV) by Diao et al (1999[b]) in the south-west of France.
In 1999 in France in the Aube departement a Furo-like virus was isolated in a field, cultivated with different wheat cvs. Serologically it does not react with the French SBWMV and German SBRMV antisera. A first study has been undertaken to obtain some details on the virus characteristics found in the Aube departement.

Results

Purification of the Aubian wheat mosaic virus (AWMV)

Virus particles were purified from the leaves of natural infected wheat plants (*Triticum aestivum* cv.Sponsor) collected from a field near Bar-sur-Aube, France. Leaves freshly harvested were ground in 0.1 M citrate buffer pH 7.2 containing 1 M urea, 0.2% 2- mercaptoethanol, 2% Triton X-100 and Methylene chloride (v-w). After low-speed centrifugation, the supernatant was ultracentrifuged through a sucrose cushion at 200000 g for 90 min. The pellets were resuspended in the extraction buffer. Centrifugation through 7-30% sucrose density gradients in the distilled water was used as the final purification step.



SBWMV area in France

Infected soils always contains a high concentration of coarse sandy elements layed or mixed with a clay substrate. The same situation is found for AWMV.
(1) Site from which infected plants were collected to prepare an antisera

few infected fields

Table 1. Properties of wheat and oat furo-like viruses in the world

		SBWMV USA	SBWMV-L Italy	SBWMV-L France	SBWMV Japan	CWMV China	"Clover" virus UK	SBRMV Germany	OGSV UK	OGSV France	AWMV France	References
		(1)				(2)		(3)				
Sequence	NA (4)	100			72.2(cp)	71(cp)		53-73				
homology	AA (4)	100	80	80	81.9(cp)	78(cp)		80(cp)			80	
	NA (5)	96.7-99.1		96.7-99.1					96.7-99.1			
		+		+	+					+	0	(6)
Serology		+				+						(7)
		+						+	+			(8)
				0			+	(+)?				(9)
Host Range												
Wheat		+	+	+	+	+	+	(+)	0		+	
Rye		+		+				+				
Barley		+		0	+				0			
Oats			0	0					+	+		
References			(10)	(11)		(5)	(9)	(8)	(12)			
Seed transmissibility				0						0		(13)
							+					(9)

1 - Shirako & Brakke 1984
2 - Diao et al. 1999[4]
3 - Koenig & Huth 1990
4 - Koenig & Huth 1998
5 - Chen et al. 1996
6 - Unpublished data
7 - Chen 1993
8 - Huth & Lesemann 1990
9 - Clover et al. 1999
10 - Canova 1964
11 - Unpublished data
12 - Plumb et al 1977
13 - Unpublished data

In electron microscopy the rod shaped particles were observed having from 50 to 600 nm in length and distributed in mainly two classes suggesting that the virus is multipartite. The narrow central canal of the particles is typic of a furovirus and not of a hordeivirus.

Serological and PCR tests

Results of the immunologically tests using French and German SBWMV antisera and French wheat yellow mosaic virus (WYMV), tobacco mosaic virus (TMV) and oat golden stripe virus (OGSV) antisera are summarized in Table 2. No relationships were detected between "AWMV" and any other virus. Amplification by PCR using the universal Tobacco rattle virus (TRV) primers showed to be negative. However from a field sample of "AWMV" provided by us to Dr Huth a positive response was obtained using primers of SBRMV.

Table 2. Serological relationships of AWMV with different antisera

	Antisera									PCR
	SBWMV[a] France		WYMV[a] France		OGSV[a] France		SBRMV[a] German		TMV[b]	TRV[c]
Cultivars	ACP	DAS	ACP	DAS	ACP	DAS	ACP	DAS	France	
	0.12	0.11	0.08	0.11	0.08	0.1	0.09	0.12	0	0
	0.09	0.12	0.11	0.12	0.14	0.1	0.09	0.13	-	
Charger	0.08	0.1	0.11	0.1	0.09	0.1	0.1	0.12	-	
	0.13	0.11	0.12	0.13	0.06	0.12	0.1	0.14	-	
	0.09	0.12	0.09	0.11	0.07	-	-	0.12	-	
	0.13	0.11	0.07	0.13	0.14	0.12	0.08	0.11	0	0
	0.14	0.12	0.07	0.12	0.15	0.11	0.08	0.11	-	
	0.07	-	0.13	0.14	0.09	0.12	0.08	0.09	-	
Sponsor	0.13	-	0.11	0.09	0.08	0.1	0.09	0.13	-	
	0.07	-	0.13	0.12	0.13	-	-	0.09	-	
Healthy control	0.09	0.11	0.11	0.12	0.12	0.1	0.08	0.08	0	
Infected control[d]	2.4	1.5	2.8	2.7	1.1	1.6	1.7[e]	2.7[e]	+	+

a - DAS and ACP ELISA
b - Electron microscopy (SSEM)
c - RT PCR
d - Using homologous antigen
e - French SBWMV

Field Symptoms

Mosaic symptoms were observed from the month of march 1999 in an area cultivated with two cvs (Sponsor,Charger) resistant to French SBWMV-L. This contaminated area stopped net with 2 other different cvs (Tremie and Texel resistant and susceptible to SBWMV-L respectivelly). The symptoms on Charger lessened whereas in the contaminated area with Sponsor they progressed and were apparent until earing. In the contaminated area the two infected cvs didn't show any important signs of reduced plant height.

Vector Research

No nematodes of the *Trichodorus* type were detected in the soil (Marzin H, SRPV personal communication) .The first research for *Polymyxa* in the infected plant roots showed to be negative.

Conclusions

At this point of our investigation it is difficult to conclude on the status of the "AWMV". Is it a pathotype of French SBWMV-L overcoming the resistance to this virus? If yes it could be the first pathotype observed for this virus.
Serotype variants of SBWMV have been already found. Firstly Chen et al. (1997) have characterized epitopes which can distinguich serologically related variants of this virus from USA and another countries. A virus characterized by Clover et al. (1999) in Great Britain is faintly detected by only one of the three antisera he has checked. "AWMV" is not serologically related to French SBWMV-L however it could be a very distinct strain of this virus. How to conciliate the absence of serological relationships with French and German antisera and a high percentage of homology of the sequences of "AWMV" and all the others European SBWMV-L found by Huth (personal communication)? New investigations concerning the host range and the genome sequences are necessary to better identify this virus. The hight variability of the furovirus detected in cereals may be attributed to the limited movement of the virus in the soil and to the very specific relationships beetwen *Polymyxa* and their host plants. This type of viruses could be used to study the parameters influencing this variability.
Finally, even if its effects are limited in reducing wheat yield the virus from the Aube from an agronomical plan is a risk and should be taken into consideration.

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BURDOCK MOTTLE VIRUS HAS A HIGH GENOME SIMILARITY TO BEET NECROTIC YELLOW VEIN VIRUS

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Summary

The complete nucleotide sequences of burdock mottle virus (BdMV) RNA 1 and RNA 2 have been determined. The two RNAs terminate in a 3'-poly(A) tail. The RNA 1 is 7038 nucleotides in length and contains a single long open reading frame (ORF) encoding a 249 kDa polypeptide. Three distinct replication-associated domains of the ORF of BdMV RNA 1 are 62 to 81 % identical to the corresponding domains of beet necrotic yellow vein virus (BNYVV) RNA 1. The BdMV RNA 2 is 4315 nucleotides in length and contains six ORFs. The arrangement of the ORFs of RNA 2 is similar to that of BNYVV RNA 2: coat protein (CP, 20 kDa), CP readthrough protein (66 kDa), triple gene block (38, 12 and 13 kDa) and cystein-rich protein (13 kDa). The triple gene block proteins encoded by BdMV RNA2 are between 32 and 50 % identical to those of BNYVV RNA 2. The CPs of BdMV and BNYVV are 39 % identical. Thus, the genome organization of BdMV is very similar to that of BNYVV, and we propose that BdMV is a new species of the genus *Benyvirus*.

Introduction

Burdock mottle virus (BdMV) was first isolated from burdock plants (*Arctium lappa* L.) grown in a field near Kurashiki, Okayama, in 1970 (Inouye, 1973). In nature, the virus causes faint necrosis in small veins of leaves of burdock plants. Symptoms are usually mild, only faint leaf chlorosis or mottling. BdMV has a narrow host range, and is transmitted by sap inoculation to several plant species (Inouye, 1973): systemic infection in burdock, *Chenopodium quinoa*, *C. murale*, *Nicotiana clevelandii* and *N. rustica*, and local infection in *C. amaranticolor*, *Beta vulgaris*, *Spinacia oleracea*, *Tetragonia expansa* etc.

BdMV has straight tubular particles about 250 nm in length and about 17 nm in width (Inouye, 1973). Characteristic viroplasm-like inclusions are observed in the cytoplasm of infected plant cells (Inouye, 1973). The inclusions are round in shape and have no external membrane. Rod-shaped particles are usually arranged radially from the surface of inclusions to every direction. The particle morphology of BdMV is similar to that of fungus-transmitted rod-shaped viruses, but the mode of transmission in the field is not known. In this study, we have determined the complete nucleotide sequence of BdMV; analysis shows that it is a new member of the genus *Benyvirus*.

Materials and methods

Virus isolate and propagation: The S isolate of BdMV was originated from burdock leaves which were collected from a field of Soja, near Kurashiki, in 1970 (Inouye, 1973). The virus was propagated in *C. quinoa*.

cDNA synthesis and cloning: RNA was extracted from purified virus particles. cDNA was synthesized using cDNA Synthesis Module (Amersham) primed with random

hexanucleotides or a synthetic oligonucleotide $(dT)_{18}$ *Not* I. The double-stranded cDNAs were inserted into the Eco RV site of pZErO™-2 (Invitrogen). The plasmids were transformed into competent *Escherichia coli* strain TOP 10F'(Invitrogen). For cloning of the 5' and 3' terminal regions of both RNA 1 and RNA 2, cDNAs were also synthesized by RT-PCR. The 5'-extremities of RNA 1 and 2 were cloned using the 5'/3' RACE Kit (Boehringer).

Nucleotide sequencing: Nucleotide sequences of selected clones were determined by dideoxy chain termination using an Applied Biosystem 377 DNA sequencer (Perkin Elmer). Sequence data were assembled and analyzed by Auto Assembler™DNA Sequence Assembly Software (Version 3.0) and GENETYX-MAC/ATSQ (SDC).

Results

Sequence analysis of RNA 1

The RNA 1 sequence of BdMV is 7038 nucleotides (nt) in length and contains one large open reading frame (ORF) encoding a polypeptide of 249 kDa (P249) (Fig. 1). The 5' and 3' non-coding regions of RNA 1 are 151 nt and 233 nt, respectively (Fig. 1). The polypeptide contains an array of domain displaying sequence homology to domains of the putative RNA-dependent RNA polymerase of plus-strand RNA viruses and Sindbis-like plant RNA viruses: the putative methyltransferase domain, the NTP-binding/ helicase domain and the RNA-dependent RNA polymerase domain (Koonin and Dolja, 1993). Three distinct replication-associated domains of the ORF of BdMV are 62.1 % to 81.1 % identical to the corresponding domains of BNYVV RNA1(Bouzoubaa *et al.*, 1987)(Fig. 1), but are less identical (10 to 25 %) to those of other member of the genus *Furovirus*, *Hordeivirus*, *Tobamovirus* and *Tobravirus*. In addition, P249 contains a papain-like protease domain, which is 43 % identical to that of BNYVV, between the helicase and polymerase domains. This suggests that the RNA1 primary translation product could undergo post-translational cleavage (Hehn *et al.*, 1997). It is also noted that the P249 protein includes three additional sequences (41, 30 and 30 amino acids) at positions 1439 to 1592 (between the papain-like protease and polymerase domains), which is not present in the corresponding protein (P237) of BNYVV, and thus the coding-protein of BdMV is larger than that of BNYVV.

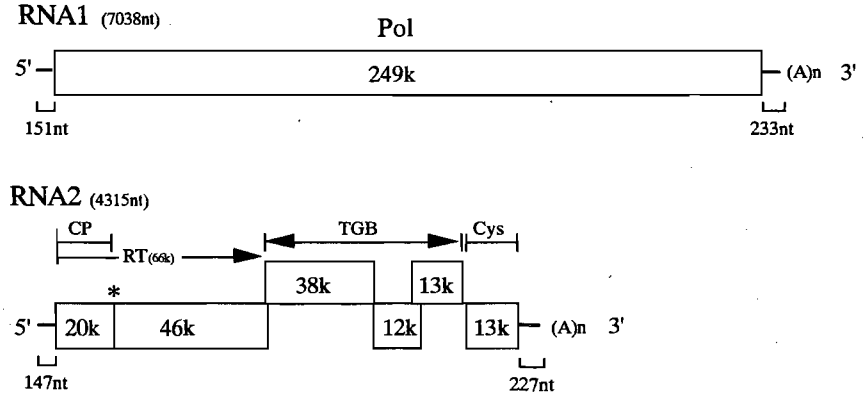

Fig. 1. Genome organization of BdMV.

Sequence analysis of RNA 2

The RNA 2 of BdMV is 4315 nt in length and contains six ORFs (Fig. 1). The 5' and 3' non-coding regions of RNA 2 are 147 nt and 227 nt, respectively (Fig. 1). RNA 2 encodes the 20 kDa coat protein at its 5' extremity followed by an in-phase 46 k ORF which is expressed by translational readthrough of the coat protein cistron amber termination codon (P 66 readthrough protein). Three following partially overlapping ORFs, encoding the proteins 38 kDa (P38), 12 kDa (P12), 13 kDa (P13), form a cassette of cell-to-cell movement proteins known as the triple gene block(TGB). The 3'-proximal ORF encodes a cysteine-rich 13 kDa protein (P13). The genome organization of BdMV RNA2 is similar to that of BNYVV RNA2 (Bouzoubaa *et al.*, 1986). The most similar regions of the polypeptides encoded by the RNA2 are P38 and P12 (TGB 1 and 2), which are 48 and 50 % identical, respectively (Table 1). The coat protein is 39 % identical, and other RNA2-encoded proteins are 20 to 32 % identical (Table 1). The similarities between the RNA 2 of BNYVV and BdMV are much lower than those between RNA 2 of BNYVV and beet soil-borne mosaic virus (BSBMV) (Rush *et al.*, 1996), both of which are 38 to 82 % identical (Table 1).

Table 1. Amino acid sequence identities of six proteins encoded by RNA2 of BdMV, BNYVV and BSBMV

Putative gene product		Identity (%)		
		BdMV/ BNYVV	BdMV/ BSBMV	BNYVV/ BSBMV
Coat protein		38	34	58
Readthrough protein		27	27	58
Triple-gene block	I	48	46	75
	II	50	53	82
	III	34	34	66
Cystein-rich protein		20	21	38

Comparison of the 5' and 3' non-coding regions

The first 8 nt (AAATTCAT) at the 5' non-coding regions of BdMV RNA 1 and RNA 2 are identical, but no sequence homology was detected between the following non-coding regions of both RNAs. It was noted, however, that the 5' non-coding regions of RNA 1 between BdMV and BNYVV are 62 % identical, and that of BdMV RNA 2 is 53 % and 51 % identical to that of BNYVV and BSBMV RNA 2, respectively.

The 3'-terminal 228 nt regions of BdMV RNA 1 and RNA 2 are 85.1% identical. Relatively high levels of identity (60 to 70 % identical) are also found in the 3'-terminal regions between BdMV RNAs and BNYVV RNA 1, BNYVV RNA 2, or BSBMV RNA 2. In particular, the 3'-proximal 70 nt of both the BdMV and BNYVV RNAs can be fold into a possibly similar stem-loop structure.

Discussion

In this paper, we present the complete nucleotide and deduced amino acid sequences of BdMV. On the basis of sequence similarities and genome organization, we propose that BdMV is a new species of the genus *Benyvirus* (Tamada, 1999). BdMV is the third member of the *Benyvirus*. The second member, BSBMV, which is widely distributed in the USA, is more closely related to BNYVV than BdMV(Rush *et al.*, 1996). In fact, BNYVV is serologically related to BSBMV, but not to BdMV. These relationships are also supported from sequence differences of the coat protein genes of three viruses. BNYVV usually contains RNA 3 and RNA 4, which are required for natural infection (Tamada, 1999), but such RNA molecules are not detected in BdMV. This suggests that the *Benyvirus* has intrinsically a bipartite genome. In the case of BdMV, characteristic viroplasm-like inclusions, which are not detected in BNYVV-infected cells, were found in the cytoplasm of plant cells infected with BdMV(Inouye, 1973), although the function and significance are unclear.

BdMV was so far isolated from only a restricted area (Okayama) and its vector is unknown (Inouye,1973). Genome organization of BdMV suggests the possibility that the virus is transmitted by the fungus. Our preliminary experiments showed that BdMV was not transmitted by *Polymyxa betae*, which is the vector of BNYVV and BSBMV, and the burdock plant is not a suitable host for *P. betae*; therefore, it seems probably that other species of the fungus is the vector of BdMV.

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MOLECULAR STUDIES ON ROD-SHAPED *POLYMYXA*-TRANSMITTED SUGAR BEET VIRUSES IN EUROPE AND KAZACHSTAN

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Summary

Beet soil-borne (BSBV) and *Beet Q* (BVQ) *pomoviruses* are widely spread in sugar beet-growing areas in Europe. Their genomes share about 70% nucleotide sequence identity. This means that they are about as distantly related between each other as with *Potato mop-top pomovirus*. Many nucleotide exchanges were found in the genomes of various sources of BSBV and even in the products of different PCRs done with extracts from plantlets grown in the same small soil sample. The great variability of the BSBV genome may be the reason for the conflicting reports on the pathogenicity of this virus. The genomes of the major strain groups of *Beet necrotic yellow vein benyvirus* (A type, B type), on the other hand, are very stable. PCR products obtained from beets grown hundreds of kilometres apart may show almost identical nucleotide sequences. - Sequence analysis revealed that a virus closely related to the P type of BNYVV occurs in Kazachstan. It also contained an RNA 5 typical for the P type. - Two isolates of BNYVV (an A type and a B type) which had been transmitted manually to local lesion hosts for many years were found to have small deletions in the KTER-encoding domain on RNA 2 which is essential for *Polymyxa* transmission.

Introduction

Three *Polymyxa*-transmitted viruses with rod-shaped particles are widely spread in sugar beet-growing areas Europe, i.e. beet necrotic yellow vein virus (BNYVV), beet soil-borne virus (BSBV) and beet virus Q (BVQ). Until recently they were considered to be furoviruses, however sequence analyses have shown that they have very different genome organisations. The genome of BNYVV consists of four or sometimes five RNA species. This virus has now become the type species of the genus benyvirus. We have determined the genome properties of BSBV and BVQ; they suggest that these two viruses belong to the genus pomovirus.

Materials and Methods

Viruses were trapped from infected plant material by means of antibodies and reverse transcription PCR was then done as described previously (Koenig et al., 1997b). PCR products were either subjected to single strand conformation polymorphism (SSCP) analyses (Koenig et al., 1997b) or they were purified from agarose gels using the Jetsorb Gel Extraction Kit (Genomed) and cloned into the pGEM-T vector (Promega) for sequencing. Most of the sequencing work was done by a commercial company (SeqLab, Göttingen). Sequences were analysed by the UWGCG software version 8 (Devereux *et al.*, 1984).

Results and Discussion

BSBV and BVQ were the first two pomoviruses for which the entire nucleotide sequences of their tripatite genomes were determined (Koenig *et al.*, 1996; 1997a and 1998; Koenig and Loss, 1997). BVQ was originally believed to be a serologically distantly related strain of BSBV (Wierthe strain; Lesemann *et al.*, 1989). However, since its genome shows only about 40 to 70 % sequence identity with BSBV, it is now regarded to be a separate virus (Koenig *et al.*, 1998). Conflicting reports exist on the pathogenicity of BSBV. Rhizomania-like symptoms have been reported by Prillwitz and Schlösser (1992), but other authors failed to see such symptoms (Kaufmann *et al.*, 1993; Hutchinson *et al.*, 1993). Single strand conformation (SSCP) and sequence analyses have now revealed that the genome of BSBV is rather variable which may explain the diverging reports on its pathogenicity. Even duplicate PCRs done with extracts from plantlets grown in the same soil sample yielded different sequences (Koenig *et al.*, manuscript submitted). The genome of BNYVV, on the other hand, seems to be rather stable. Within the three strain groups which have been detected in Europe, i.e. the A type which is mainly found in Southern and North Western Europe, the B type which is found mainly in Germany and France and the P type which up to now was found only around the French town of Pithiviers (Kruse *et al.*, 1994; Koenig *et al.*, 1995) there was very little variation, even when the samples originated from geographically widely separated countries. Thus almost identical sequences were found for A types from the Netherlands, Italy or Yugoslavia or for B types from Germany or France.

We have recently found that BNYVV from Kazachstan is closely related to the P type. Like the European P type (Koenig *et al.*, 1997b) it is associated with a fifth RNA species, whereas the genomes of A and B types in Europe contain only four RNA species. A detailed description of these results will be given elsewhere (Koenig and Lennefors, manuscript submitted).

Sequence analyses also revealed that the KTER-encoding domain in RNA 2 of BNYVV which as been found to be essential for *Polymyxa* transmission (Tamada *et al.*, 1996) may be partially or entirely lost in isolates which for many years have been transferred manually to local lesion hosts (Koenig, 1999). This happened with our

isolates Yu2P (an A type) and Rg 1 (a B type) which have RNAs 2 with deletions of 21 and 3 nucleotides around and in their KTER-encoding domains, respectively. A cDNA clone obtained from an isolate Yu2E, from which the deletion-containing isolate Yu2P was derived eleven years ago and which had been kept in a frozen state most of the time thereafter, had an intact KTER-encoding domain. In order to find out whether these two isolates may contain mixtures of intact and deleted RNAs 2 we have designed a primer which corresponds to most of the deletion area and another one which spans it. Together with an antisense primer derived from the sequence further downstream, the primer which corresponds to the deletion area gave a PCR product only with Yu2E whereas the primer which spans it yielded a PCR product only with Yu2P. This suggests that the deletion-carrying RNA 2 in Yu2P was probably not selected from a pre-existing mixture in the Yu2E isolate, but that the deletion had arisen *de novo* in the past eleven years and that the resultant deletion-carrying RNA 2 has then become dominant. In order to get some evidence whether or not populations of RNAs 2 with different deletions were present in the Yu2P and Rg1 isolates, we have analysed four clones for the Yu2P isolate which were derived from two different PCRs and three clones for the Rg1 isolate which were also derived from two different PCRs. They all had the same deletion typical for the isolate from which they were derived (Koenig, 1999).

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GENOME PROPERTIES OF A NEW FUROVIRUS WHICH IS WIDELY SPREAD IN CEREAL CROPS IN EUROPE

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Summary

A new furovirus named *Soil-borne rye mosaic virus* (SBRMV) (Koenig and Huth, 1998; Koenig *et al.*, 1999) has been found to be widely spread in several cereal crops in Europe. Its genome organisation is very similar to that of *Soil-borne wheat mosaic virus*, but the percentages of sequence identity in various parts of the genomes of the two viruses range only between 60 and 75% suggesting that that the new virus represents a distinct virus species.

Introduction

The occurrence of a furovirus in rye in Germany has been known for almost two decades (Proeseler *et al.*, 1982; Huth and Lesemann, 1990). However, only recently its economic importance and the fact that it is widely distributed have been recognised. Because of its serological affinities it was originally believed to be a strain of *Soil-borne wheat mosaic virus* (SBWMV), but the molecular studies described by Koenig and Huth (1998), Koenig *et al.* (1999) and in this paper indicate that it is not a strain of SBWMV, but a separate virus for which have proposed the name *Soil-borne rye mosaic virus* (SBRMV).

Materials and Methods

Originally, the nucleotide sequences were determined for three different sources of the virus either completely (source DE-G) or almost completely (sources DE-O and De-C). These virus sources originated from locations in Northern Germany (Lower Saxony) which are about 70 to 150 km apart from each other. Virus sources De-O and De-G came from infected rye plants grown in the Osnabrück and the Braunschweig areas, respectively, whereas source De-C came from an infected wheat plant grown in an experimental plot north of Hannover in a mainly rye-growing area. Later, further virus sources were obtained from infected rye, wheat or durum wheat plants in various parts of Europe. Nucleotide sequences were determined from unpurified viruses in naturally infected plants. Reverse transcription (RT)-PCR products were obtained using RNA from immunocaptured virus particles or denatured preparations of dsRNA as templates for cDNA synthesis (Koenig *et al.*, 1997; 1998;

Koenig and Loss, 1997). 5' and 3'terminal sequences were determined using magnetic bead technology as described previously (Koenig, 1997). For sequencing, PCR products were purified from agarose gels using the Jetsorb Gel Extraction Kit (Genomed) and cloned into the pGEM-T vector (Promega). Most of the sequencing work was done by a commercial company (SeqLab, Göttingen). Sequences were analysed by the UWGCG software version 8 (Devereux *et al.*, 1984). The secondary structures of the 3'UTRs of the viral RNAs were predicted using the genetic algorithm method of RNA folding as described previously and as implemented in the STAR package for RNA structure predictions (Gultaev *et al.*, 1995). The GenBank accession numbers are AF146278-80 for RNAs 1 of the SBRMV sources De-G, De-C and De-O, respectively, and AF146281-83 for RNAs 2 of the SBRMV sources De-C, De-G and De-O, respectively. The gene bank accession numbers for the partial sequences of RNA 1 of the sources De-Ma, De-Tr, Dk, F-A, F-B and I from various crops in various European countries are AF183160, AF183162, AF183163, AF183165, AF183168, AF183169, respectively, and for the partial sequences of RNA 2 of the sources De-Ma, Dk, F-A, F-B, F-A, I and PI they are AF183161, AF183164, AF183166, AF183167, AF183170 and AF183171, respectively.

Results and Discussion

SBRMV was found in many rye-growing areas in Northern Germany. Although, mechanically the virus was readily transmitted to wheat, in a field trial on an experimental plot near Braunschweig only 9 out of c. 120 000 wheat plants (i.e. 0.0075%) became infected. The infection rate for rye, on the other hand, varied between 20 and 70% on this field. In other areas it was >95%. These observations suggest that the *Polymyxa* populations in Northern Germany may be less adapted to wheat than to rye.

With most of the primers which we had designed on the basis of the sequence published for SBWMV (Shirako and Wilson, 1993) we failed to obtain PCR products with extracts from plants infected with the rye virus. PCR products were, however, obtained with several primers which were designed in parts of the SBWMV genome which are highly conserved also in the genomes of *Beet soil-borne* and *Beet virus Q* pomoviruses (Koenig and Loss, 1997; Koenig *et al.*, 1998) and other soil-borne viruses with rod-shaped particles. Those PCR products, which were obtained, were sequenced and on the basis of their sequences new specific primers were designed which allowed us to bridge up the gaps between the various PCR products. The complete nucleotide sequence was determined for the two RNAs of the De-G source. For the De-O and De-C sources the whole sequences except for very short primer regions at the 5' and 3'ends were analysed. For amplifying the 5'ends of the RNAs of the De-O and the De-C source, primers derived from the 5'ends of the respective RNAs of the De-G source could readily be used, whereas primers corresponding to the 5'ends of the two SBWMV RNAs failed. This suggests that the 5'ends of the RNAs of the De-C and De-O sources are very similar to those of the De-G source.

The genome organisation of SBRMV is virtually identical to that of SBWMV (Koenig *et al.*, 1999). A difference was found in the folding capacities of the 3'UTRs of the two viruses. With SBRMV both RNAs have an upstream pseudoknot domain (UDP) which has the ability to fold into seven pseudoknots whereas with SBWMV only RNA 1 can form such a large number of pseudoknots in its UDP. RNA 2 of SBWMV is much shorter and has a folding capacity for only three pseudoknots. The nucleotide sequences of the genomes of SBWMV and the various SBRMV sources and also of the recently described *Chinese wheat mosaic virus* (Diao *et al.*, 1999a) differ by 25% to 40%. The various sources of SBRMV are closely interrelated but not identical. There are no indications that the virus sources De-O and De-G from rye are more closely related amongst each other than to the wheat source DE-C.

Using several primer combinations which are either specific for SBRMV or SBWMV or which are able to detect both viruses we found that SBRMV is widely distributed in Europe and may infect several cereal species. In Northern Germany, Poland and Denmark it affects mainly rye and durum wheat (triticale), but in France and Italy it is wheat which becomes infected. Partial sequence analyses revealed that the various sources of SBRMV from different countries and different crops are all similar, but not identical (Koenig and Huth, manuscript submitted).

Very recently a new furovirus affecting wheat in France has been described by Diao *et al.* (1999b). It has been named *European wheat mosaic virus* (EWMV). The sequence of this virus is very similar to those which we have determined for the various sources of SBRMV. EWMV and SBRMV are thus obviously variants of the same virus and a renaming of one or both viruses will be necessary. The International Committee on Taxonomy of Viruses (ITCV) has been contacted on this matter.

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ANALYSIS OF INTERACTIONS OF BAYMV PROTEINS USING THE YEAST TWO-HYBRID SYSTEM

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Summary

In recent years, a powerful tool for the identification of protein protein interactions, the so-called yeast two-hybrid system has been developed. To better understand the interrelationships between different bymoviral proteins, we have initiated a study of pairwise interactions between different barley yellow mosaic virus (BaYMV)-encoded proteins in yeast cells. Using this approach, we find that the P1 protein (28kD protein) of RNA2 interacts with itself. Moreover, a part of the BaYMV polyprotein including the VPg region interacts with the coat protein in yeast.

Introduction

Bymoviruses possess a genome of two single-stranded, positive-sense RNAs of about 7.6 kb and 3.5kb. The genomic RNAs are translated into two polyproteins which are processed by virus-encoded proteinases to yield a number of mature proteins. Some functions are postulated because of sequence homology to known proteins. But despite of the coat protein and the P1-proteinase of RNA2, the functions of the bymoviral proteins are unknown. The aim of the work presented in this study was to collect more data about the BaYMV-encoded proteins. Proteins work not only as enzymes or structural components, they often realize a function via protein-protein interactions. An example are multicomponent complexes as they occur during replication. To understand and manipulate the function of a particular protein, it is generally useful to identify other proteins with which it associates. Therefore a yeast two hybrid system which has been shown to be a powerful tool to identify protein-protein interactions was used in this study.

Materials and Methods

Constructs

BaYMV proteins were amplified by PCR with plY29T7 or psY35T7 as template and cloned into the two-hybrid vectors pAS2, pAS2-1 (Harper et al.,1993) and /or pACT2 (Durfee et al., 1993, table 1). All the constructs derived from PCR were verified by sequencing.

Protein-protein interaction in yeast strains

The mating compatible yeast strains Y190 and Y187 (Harper et al., 1993) were used for hybrid protein expression. All constructs containing the BD-fusions were transformed in yeast strain Y187 and the AD-fusion constructs in strain Y190 according to Gietz and Schiestl (1995). Transformants were selected by plating on minimal media lacking leucine or tryptophan. In order to test the interaction of all the protein combinations, a mating procedure was performed as decribed by Finley and Brent (1994) resulting in yeast cells containing all

possible combinations of the produced constructs. Interactions of the hybrid proteins were tested by plating these yeast cells on media lacking leucine, tryptophan, histidine and containing 50mM 3AT. To confirm the interaction the expression of the *lacZ* gene as a second reporter gene was shown by a β-galactosidase assay as described by Chien et al. (1991).

Name	Sequence of the PCR-primers, 5´-3´	Vectors
P1	5´- CTC GCT **CCC ATG** GAG CAA ACC -3´ 5´- CTG CTG TGC TC**G GAT** CCT GAA GCA C -3´	pAS2, pACT2
P2	5´- ATA GTG **CCC ATG** GCT AGG AGC ACA GC -3´ 5´- CAC TAT CGC CAG CCT **GGA TCC** CAT AGC -3´	pACT2
CI+	5´- GCT ATG GGC TTC **CCA TGG** GCG ATA GTG CTG AG- 3´ 5´- CTA GCC TG**G AAT TC**A ATA ATA TC -3´	pAS2-1,pACT2
VPg+	5´- GGA CCT TGT TTC **CAT GG**T CTT CGT AAG -3´ 5´- CTT GTG ACA ATA AAA **CTG CAG** TAG AGG **C** -3´	pAS2-1
NIa	5´- ACA **CGG CCA TGG AGG** CCA GCT TAG CAA ACG C -3´ 5´- CAT CAG **AGG CCT CCA TGG CC**A TGA CGT CAG -3´	pAS2-1,pACT2
NIb	5´- CGT CAT TCC **CAT GG**A GGC TTC TG -3´ 5´- CTT GCA GGC **GAA TTC** CAT CAT CAC C -3´	pAS2-1,pACT2
CP	5´- GAT GAA ATT TC**C ATG** GAA GCA GCT GAT CC -3´ 5´- GGT TAG GT**G AAT TC**T GGG TGT CCA TC -3´	pAS2, pACT2
28	5´- GCT CTC AAA TTC TCC A**CC ATG** GCT ACT TCC AG -3´ 5´- CCG TTT GAA CCA T**GG ATC** CCA TTA AGC AAA TGC C -3´	pAS2, pAS2-1, pACT2
70	5´- GGG AT**G GCC GTG GCG GCC** GGT GAT GGT GAG CAC -3´ 5´- ACA **CGG CCA TGG AGG CC**A GCT TAG CAA ACG C-3´	pAS2, pACT2

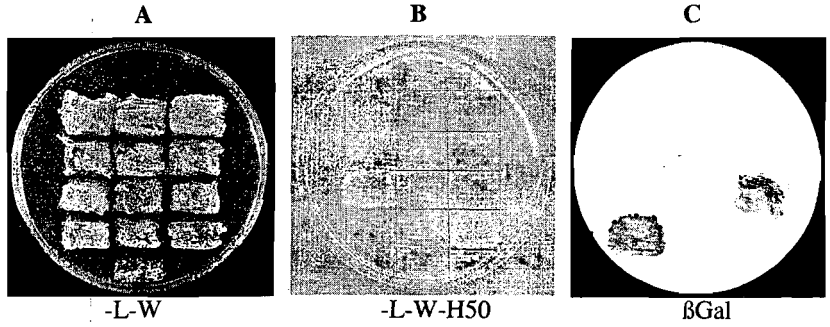
Table 1. List of PCR-primers which were used for the amplification of viral protein regions. Nucleotides displayed in bold are part of a restriction site and differ from the sequence of BaYMV-GER (X69757, Q01365).

Results

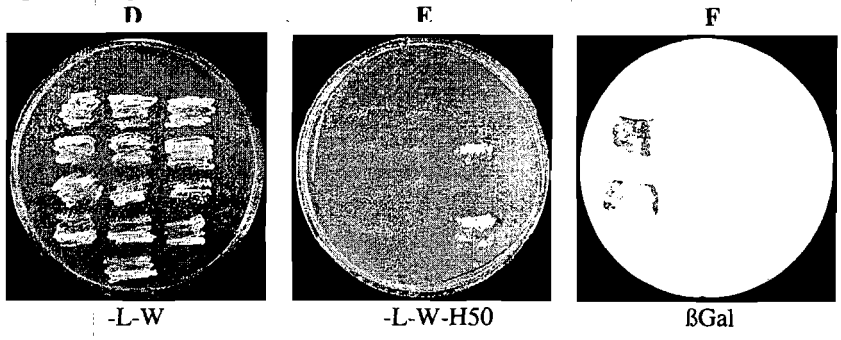
The combinations of fusion plasmids or control plasmids used for the interaction assay are shown in Fig. 1G. The expression of the fusion proteins (AD- or BD- with CI, NIb, CP, 28kD and 70 kD) was confirmed by immuno-western blot analysis using monoclonal antibody raised against the GAL4-AD or polyclonal antibodies against viral proteins (data not shown). The mated yeast cells were first plated on selection plates lacking leucine and trytophan so that only cells containing both plasmids coding for the fusion proteins could grow (Fig 1A, D). Interaction of two hybrid proteins was tested with the help of two reporter genes: *his* and *lacZ*. Transcription of these genes can be activated by two interacting fusion proteins. Thus, the growth on histidine-free medium and the β–galactosidase activity can be used as an indicator of interactions between fusion protein partners. Yeast cells carrying the CP- and the VPg+ protein or carrying the 28kD protein as BD- and AD-fusion could grow on medium lacking histidine (Fig 1B, E). The observed activation of the first reporter gene by interaction of these viral protein combinations could be confirmed by the activation of the second reporter gene *lacZ* in filter lift assays. Only the cells with the combination of CP/VPg+ or 28kD/28kD had significant β-galactosidase activity as indicated by the appearance of blue colonies (Fig 1C, F). In contrast yeast cells containing all other plasmid combinations, despite of the positive control, could not grow on histidine-free medium and did not show β–galactosidase activity. No reporter gene activity was found in the negative controls, in which the cells contained single fusion plasmids

together with the corresponding empty plasmid. This indicated that the detected activity of the reporter genes in the combinations of CP/VPg+ and 28kD/28kD resulted from a specific interaction. All the mating experiments were performed three times and the results were reproducible.

BD-28kD fusion protein as bait:



BD-VPg+ fusion protein as bait:



G

AD-P1	AD-P2	AD-CI
AD-NIa	AD-NIb	AD-CP
AD-28	AD-70	AD-Δ28
AD-SNF4	AD	SNF1/SNF4
	BD/AD	

Fig. 1: Yeast two-hybrid interaction analysis. Fig 1G shows the plating scheme of the mated yeast cells. The bait protein (28kD: A, B, C; VPg+: E, F, G) as binding domain (BD)-fusion is expressed together with the activation domain (AD)-virus protein mentioned in the scheme. The yeast proteins SNF1/4 are known to interact in yeast and are used as positive control. Combinations with AD-SNF4, ./AD, BD/AD are negative controls. The mated yeast cells were plated on medium lacking leucine (-L) and tryptophan (-W) for selection of cells containg both plasmids (A, D). Only cells expressing viral fusion proteins which interact could grow on medium lacking histidine and containing 50mM 3 AT (H50; B, E). Fig. C, F show the result of the filter lift assay (mirrored scheme G) for β–galactosidase activity (blue colonies). The results of the test for both reporter genes (*his*, *lacZ*) are consistent. Cells which grow on medium lacking histidine (B, E) display also β–galactosidase activity (C, F).

Discussion

In this study, the BaYMV-CP was found to interact in the yeast two hybrid system with the BaYMV-VPg+. The hybrid protein includes the VPg-part of the NIa region and approximately the half of the small 6K2 protein region, which is postulated by sequence analysis of putative cleavage sites of the NIa-proteinase. It has not been experimentally confirmed if and with which efficiency these sites are cleaved by the NIa proteinase and if intermediates exist. The existence of such a protein consisting of the 6K2 and the VPg or the NIa region could explain the observation that no interaction was found with the NIa region and the CP but with the VPg-part of the NIa and the CP. An other possible explanation could be that the whole NIa protein including the VPg region has such a conformation that the VPg-interacting domains are not accessible for interactions. Confirmation of the interaction by *in vitro* binding assays as well as identification of interacting domains are in progress. It has to be studied if this interaction occurs as well in virus-infected plant cells.

In this study, the 28kD protein (P1 of RNA2) was found to interact with itself. Homodimerisation or multimerisation has also been previously shown for the potyviral HC-Pro of PVA (Guo *et al.*, 1999) using the yeast two-hybrid system. A part of the potyviral HC-Pro shares significant aminoacid homology with the bymoviral RNA2-P1 (28kD) protein. The HC-Pro is a proteinase like the 28kD protein of BaYMV, which is involved of the processing of the viral polyprotein into mature proteins. The 28kD protein is part of crystal-like cytoplasmatic inclusions in infected plant cells occuring in huge amounts in an infected cell. It might be that the ability for homotypic interactions of this protein supports the building of these structures. The significance of 28kD protein multimerisation with respect to its biological functions remains unresolved.

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NUCLEOTIDE SEQUENCE OF BEET SOIL-BORNE MOSAIC VIRUS.

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Summary

The genome of beet soil-borne mosaic virus isolate EA (BSBMV-EA) [RNA1 (6.6 kb), RNA2 (4.6 kb), RNA3 (1.7 kb) and RNA4 (1.2 kb)] was cloned and sequenced. Computer analysis indicates that helicase and RNA-dependent RNA polymerase (RdRp) motifs are present in RNA1. Comparison of the sequence of RNA1 and the putative single long ORF (Mr 237, 539) revealed a 49.0% nucleotide and 79.8% amino acid sequence identity with RNA1 of beet necrotic yellow vein virus (BNYVV). The viral capsid protein (CP) cistron is located at the 5' terminal end of RNA2 and the CP has a calculated M_r 20,953. Following the CP cistron an in-phase coding region extends to encode a predicted readthrough translation product M_r 75,580. These two ORFs are analogous to the CP region and readthrough product of BNYVV. The remaining ORFs possess typical motifs of triple gene block proteins, which are also reported for BNYVV and several other plant viruses. Comparison of RNA2 and the putative ORFs revealed a 53.2% nucleotide sequence identity with RNA2 of BNYVV and amino acid identities of the ORF which ranged from 55.7 - 82.2 %. RNA3 and RNA4 contained single putative ORFs of M_r 28,624 and 13,536, repectively. Comparison of RNA3 and RNA4 and the putative ORFs revealed a 33.9% and 43.1% nucleotide sequence identity and amino acid identity of 22.8% and 40.5% with the respective RNAs and ORFs of BNYVV. Comparison of BSBMV with other furo-like viruses suggest that BSBMV is more closely related to BNYVV than to the other furoviruses. Thus, BSBMV may be related to BNYVV should be a member of the genus *Benyvirus*.

Introduction

Beet soil-borne mosaic virus (BSBMV), a proposed member of the genus *Benyvirus* (formerly *Furovirus* group), was identified in Texas (Liu & Duffus, 1987) as part of a complex of viruses associated with beet necrotic yellow vein virus (BNYVV). BNYVV is the causal agent of rhizomania in sugar beet (*Beta vulgaris* L.) and is characterized by massive lateral root proliferation, constriction of the main taproot, and stunting of the plant (Tamada, 1975). In contrast, the roots of BSBMV infected sugar beets are often asymptomatic (Rush & Heidel, 1995; Heidel *et al.*, 1997). Systemic foliar symptoms caused by BSBMV appear in field-grown sugar beets more frequently than do those caused by BNYVV. Both BNYVV and BSBMV are multipartite, rigid, rod-shaped viruses and are vectored by the soilborne plasmodiophoromycete fungus, *Polymyxa betae* Keskin. Partial characterization demonstrated that BSBMV was morphologically similar to BNYVV but serologically distinct (Wisler *et al.*, 1994).

Similar to BNYVV, the genome of BSBMV consists of four polyadenylated RNA species (Rush & Heidel, 1995; Heidel *et al.*, 1997). The sizes of the four RNA species, as determined by agarose-formaldehyde gel electrophoresis, were 6.6, 4.6, 1.8, and 1.2 nucleotides. A fifth RNA species was not observed. We present here the nucleotide sequence and genomic organization for BSBMV RNA1, RNA2, RNA3 and RNA4. The nucleotide sequence and genomic organization for BSBMV RNA2 was similar to that previously reported (Rush *et al.*, 1996). Comparison of BSBMV with BNYVV and other furo-like viruses may further clarify the taxonomic relationship of these viruses infecting sugar beets.

Materials and Methods

Virus propagation and purification. Sugar beets exhibiting typical foliar symptoms of BSBMV were collected from a field in Colorado (EA) and maintained in the greenhouse in beet culture (Heidel & Rush, 1994).

Symptomatic leaf tissue was tested for BSBMV and BNYVV by DAS-ELISA as previously described with some modifications. Sugar beet leaf tissue was ground in 0.1 M potassium phosphate buffer and the plant sap was mechanically inoculated onto Carborundum-dusted leaves of *Chenopodium quinoa* Willd., a local lesion host. BSBMV-EA was passaged no more than three times through *C. quinoa* before being purified. Virus was purified from *C. quinoa* leaves as described by Kendall *et al.* (1988) with several modifications (Heidel & Rush, 1997). BSBMV-EA RNAs were extracted from virions in purified preparations as previously described (Kendall *et al.*, 1988) and analyzed by electrophoresis through an agarose-formaldehyde denaturing gel in the presence of ethidium bromide.

cDNA library construction and sequencing. Standard molecular biology protocols or modifications suggested by commercial suppliers of the reagents were followed. Oligo(dT)$_{12\text{-}18}$, random or gene-specific oligonucleotide primers and a commercial cDNA synthesis kit (SuperScript Choice System, Gibco BRL) were used to synthesize double-stranded cDNA from viral RNAs extracted from virions in purified preparations. Size-selected ds cDNA using Sephacryl S-300 were ligated to *Eco* RI/*Not* I adapters and cloned into *Eco* RI site of the plasmid vector pBSKS+ (Stratagene). Independent clones with varying cDNA sizes to BSBMV were selected. Primer-based sequencing of both strands of all selected clones was performed at the Gene Technologies Lab (Texas A&M University) using the ABI PRISM Dye Terminator Cycle Sequencing Core Kit on the ABI 373 Automated Sequencer. At least two independent cDNA overlapping clones were sequenced in both orientations at least twice. Sequence data was compiled and analyzed using the LASERGENE 99 Suite (DNASTAR, Inc.) and the BLAST program (NCBI). Gene-specific oligonucleotide primers and a commercial 5' RACE system v.2.0 (Gibco BRL) was used to determine the 5' terminal sequences. The gene-specific oligonucleotide primer was used for first strand cDNA synthesis from total viral RNA, the first strand was subjected to homopolymeric dC-tailing, and then the 5' terminal sequence was amplified by PCR using the gene-specific primer and an abridged anchor primer.

Results and Discussion

Nucleotide sequence and genomic organization of BSBMV. The nucleotide sequence and genomic organization of BSBMV was determined and compared with other known furoviruses. BSBMV RNA1 is 6,675 nucleotides in length [excluding the poly(A) tail] and contains a single long open reading frame (ORF). Comparison of RNA1 nucleotide sequence with RNA1 of BNYVV revealed a 49.0 % nucleotide sequence identity. The RNA2 is 4,657 nucleotides in length [excluding the poly(A) tail]. Six putative ORFs, similar to that found in BNYVV, were identified. Comparison of the RNA2 nucleotide sequence with RNA2 of BNYVV revealed a 53.2 % nucleotide sequence identity and similar organization of the six ORFs. RNA3 is 1773 nt and RNA4 is 1216 nt in length [excluding the poly(A) tail]. Both contained a single putative ORF. Comparison of RNA3 with RNA3 of BNYVV revealed a 33.9 % nucleotide sequence identity. RNA4 shared a 43.1 % nucleotide sequence identity with BNYVV. A fifth RNA species has been described for some but not all BNYVV and was not observed for BSBMV. RNA5 is sometimes present in sugar beets expressing severe symptoms of BNYVV infection. The genomic organization of BSBMV is shown in Fig. 1. Comparison of the 5' and 3' untranslated regions (UTR) of BSBMV and BNYVV revealed strong sequence identity of the first 6 nucleotides and last 60 nucleotides of the 5' terminal sequences and 3' terminal sequences, respectively. Overall, the similarities between BSBMV and BNYVV lend further support that they represent members of the genus, *Benyvirus* (Torrance & Mayo, 1997).

BSBMV RNA1. The nucleotide sequence of BSBMV RNA1 is 6,675 nucleotides in length. Computer analysis of the RNA1 nucleotide sequence revealed a single long ORF with a potential to encode a polypeptide of M_r 273,539. RNA1 of BNYVV contains a single long ORF encoding a polypeptide of Mr 237,389 and shares amino acid homology with known viral RNA-dependent RNA polymerases, helicases, and methyltransferases (Bouzoubaa *et al.*, 1987). Comparison of the nucleotide sequence of RNA1 and the deduced polypeptide with the ORF1 of BNYVV revealed a nucleotide sequence and an amino acid sequence identity of 49.0% and 79.8%,

respectively (Table 1).

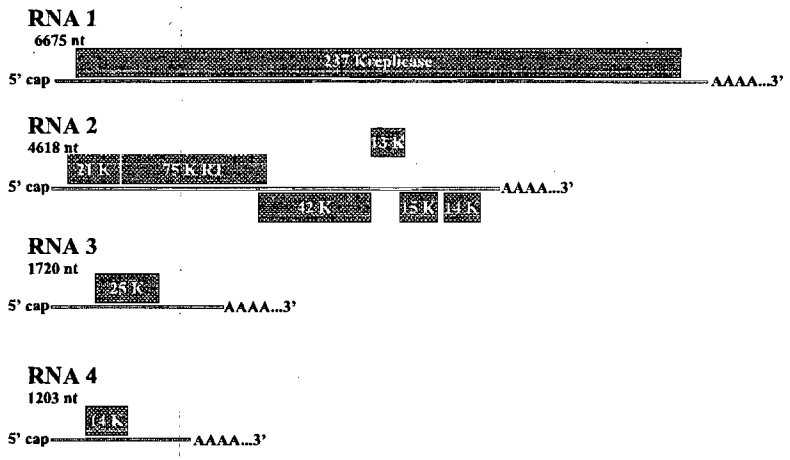

Fig. 1. Genomic organization of BSBMV.

Table 1. Percentage amino acid identity between specific BNYVV ORF regions and comparable regions of several related viruses.

BNYVV[a]	RNA1	RNA2					RNA3 [c]	RNA4 [c]
	237 kDa	21 kDa	75 kDa	42 kDa	13 kDa	15 kDa	25 kDa	31 kDa
BSBMV	79.8	56.4	55.7	74.6	82.2	65.2	22.8	40.5
BBNV	10.5	12.4	11.6	19.5	29.7	15.9	--	--
PMTV	NA[b]	18.2	14.3	21.4	30.5	15.9	--	--
SBWMV	11.1	15.3	14.4	--[d]	--[d]	--[d]	--	--
PCV	11.0	14.9	12.5	23.2	28.0	11.4	--	--
BSBV	10.9	18.9	11.8	22.4	32.8	14.4	--	--
IPCV	16.9	12.8	NA[b]	NA[b]	NA[b]	NA[b]	--	--

[a] Beet necrotic yellow vein virus (BNYVV). Beet soil-borne mosaic virus (BSBMV). Peanut clump virus (PCV). Potato mop-top virus (PMTV). Soil-borne wheat mosaic virus (SBWMV). Beet soil-borne virus (BSBV). Broad bean necrotic virus (BBNV). Indian peanut clump virus (IPCV)
[b] Sequence was not available for comparison.
[C] Comparable region to RNA3 & 4 found only in BSBMV and BNYVV.
[d] Comparable region not found.

BSBMV RNA2. The nucleotide sequence of BSBMV RNA2 was determined to be 4657 nt in length [excluding the poly(A) tail] (data not shown). This is comparable to the 4609 nt sequence of BNYVV RNA2 (Bouzoubaa *et al.*, 1986). Six putative ORFs were identified on BSBMV, which were nearly identical in size and position

to those found in BNYVV. Tthe nucleotide sequence and genomic organization for RNA2 of BSBMV was identical to that previously reported (Rush *et al.*, 1996), except for the 5' terminal sequence, which was 5'-AAATTCTAATTATT... compared to 5'-GGATTCTAATTATT... Comparison of specific regions on RNA2 with comparable regions on BNYVV revealed amino acid sequence identities ranging from 55.7 - 82.2 % (Table 1). However, comparison of regions on RNA2 with other closely related viruses revealed amino acid sequence identities as low as 6% to as high 34%. Since RNA1 and RNA2 are necessary and sufficient for infection (Quillet *et al.*, 1989; Tamada *et al.*, 1989) the high amino acid sequence identities may not be surprising. BSBMV was more similar to BNYVV than to any of the other viruses.

BSBMV RNA3 and RNA4. The nucleotide sequence of BSBMV RNA3 was determined to be 1773 nt in length [excluding the poly(A) tail] (data not shown). This is comparable to the 1774 nt sequence of BNYVV RNA3 (Saito *et al.*, 1996). A single putative ORF was identified on BSBMV with a predicted polypeptide of M_r 28, 624 which is slightly higher than the 25 kDa protein in BNYVV. The nucleotide sequence of BSBMV RNA 4 was determined to be 1216 nt in length [excluding the poly(A) tail] (data not shown). This is shorter than the 1465 nt sequence of BNYVV RNA4 (Saito *et al.*, 1996). A single putative ORF was identified on BSBMV with a predicted polypeptide of M_r 13,536 which is much smaller than the 31 kDa protein in BNYVV. An examination of the ORFs did not reveal frame shifts that could account for the smaller polypeptide but did reveal that the BSBMV polypeptide was truncated at the C-terminal end when compared to BNYVV. It is not clear at this time if the BSBMV RNA4 is a deleted species. RNA4 is essential for efficient transmission by the fungus, *P. betae*, whereas RNA3 may facilitate the spread of the virus in sugar beet roots (Tamada & Abe, 1989). Patterns of BNYVV RNAs have been shown to be different in sugar beet roots compared to leaves (Koenig *et al.*, 1986). Furthermore, shortened forms of BNYVV RNA3 and RNA4 have been detected when mechanically inoculated onto leaves of *C. quinoa*, even with as few as 1-2 passages (Bouzoubaa *et al.*, 1991). When these viruses are re-inoculated onto sugar beet plants through the fungal vector, the full-length RNA3 and RNA4 reappear. Comparison of the 25 kDa and the 31 kDa protein of BNYVV with the same region on BSBMV revealed an amino acid sequence identity of 22.8 % and 41.4 %, respectively. This indicates that more heterogeneity exists in RNA3 and RNA4 than for RNA1 and RNA2, which are sufficient and required for infection (Tamada *et al.*, 1989).

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SEQUENCE VARIATION OF BEET NECROTIC YELLOW VEIN VIRUS RNA5: THE EVOLUTION AND THE POSSIBLE ROUTE OF ITS SPREAD

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Summary

Beet necrotic yellow vein virus (BNYVV) RNA 5 was detected in virus isolates from Japan, China and France. Sequence comparisons of RNA 5 (nucleotides 327 to 1171) in 25 isolates showed that there are up to 8 % sequence differences, and that RNA 5 variants fall into three groups: group I contains most of the Japanese and Chinese isolates, group II two Japanese isolates, and group III four French isolates. The group I isolates fall into three small clusters. Comparisons of the coat protein gene of RNA 2 revealed that most of the Japanese isolates belonged to the A type strain, but some isolates were of the B type. French isolates (P type) were closely related to those of the A type. These results suggest that the three groups of RNA 5 variants separated from an original population a long time ago and, thereafter, the group I population diverged further into three clusters, which may have been associated with the A type strain rather than the B type.

Introduction

The genome of beet necrotic yellow vein virus (BNYVV) consists of five RNA components: RNAs 1 and 2 are required for viral RNA replication, assembly, virus movement and transmission, whereas RNAs 3 and 4 are needed for disease development and spread in nature (Richards and Tamada, 1992). RNA 5 is not present in all virus sources, but it has been reported in some Japanese and French isolates (Richards and Tamada, 1992; Koenig *et al.*, 1997). RNA 5 is 1342-1347 nucleotides in length and contains a single open reading frame (ORF) encoding a 26 kDa protein (Kiguchi *et al.*, 1996). It is associated with the severity of symptom development in sugarbeet roots (Tamada *et al.*, 1996). In this study, we have compared the sequences of RNA 5 present in 25 BNYVV isolates from Japan, China and France. In addition, the sequences of the coat protein gene (RNA 2) of the BNYVV isolates were compared. Detailed results are reported by Miyanishi *et al.* (1999).

Materials and Methods

Virus sources and detection of RNA 5: All BNYVV isolates used were derived from rhizomania-infested soil samples which were collected from Hokkaido, China, and France (Table 1). Sugarbeet seedlings were grown in special test tubes which contained a mixture of quartz sand and each soil sample. BNYVV infection in sugar beet roots was determined by ELISA. Detection of RNA 5 was conducted by northern blot hybridization using digoxigenin-labeled DNA probes and RT-PCR (Miyanishi *et al.*, 1999).

RT-PCR cloning and nucleotide sequencing: For sequencing of RNA 5 and the coat protein region of RNA 2, total nucleic acids were extracted by direct phenol methods from inoculated leaves of *Tetragonea expansa*. For RNA 5, two specific primers 5F (5'-GTTTTT CCGCTCGCAGCACAAG-3', nt 308 to 326) and 5R (5'-CGAGCCCGTAAACACCGCATA -3', complementary to nt 1172 to 1192) were used. For the coat protein region of RNA 2, three specific primers 2F (5'-CGAGTAATAAGTAGCCGCCGTC-3', nt 103 to 124), 2R (5'-CAAAAGGAAGACCATGTGAGGA-3', complementary to nt 916 to 937) and 2C

(5'-ACCCCGTTCCATTTATACCC-3', complementary to nt 2289 to 2308) were used. PCR products were ligated into a pGEM-T vector (Promega) and transformed into *Escherichia coli* strain XL1-Blue. The nucleotide sequences of selected clones were determined with an Applied Biosystems 377 DNA sequencer (Perkin Elmer). Sequence data were assembled and analyzed by GENETYX-MAC (SDC). Multiple alignments of nucleotide sequences were obtained using CLUSTAL W.

Results

Variation of RNA 5 sequences in 25 BNYVV isolates

Out of 194 Japanese BNYVV isolates, 88 (46%) were found to contain RNA 5. The RNA 5 is widely distributed throughout Hokkaido. All of the six isolates from Nei Menggu and Ningia in China were found to contain RNA 5. The two isolates derived from Pithiviers and Corbeille in France contained RNA 5, which accords with the results reported by Koenig *et al.* (1997). Nucleotide sequences of RNA 5 (nucleotide 327 to 1171 for D5) from 13 Japanese, 4 Chinese and 2 French isolates were compared with each other and thoses reported previously (Kiguchi *et al.*,1996; Koenig *et al*, 1997). There was a maximum of 8.3 % sequence variation between isolates at the nucleotide level. The RNA 5 variants are divided into three groups: group I contains 17 isolates from Japan and China, group II contains two Japanese isolates (Shari), and group III contains four isolates from France (Table 1 and Fig. 1 (a)). The isolates of the group I also fall into three small clusters except for one isolate U3. Cluster A contains seven isolates, cluster B, four isolates , and cluster C, seven isolates (Table 1 and Fig. 1(a)). The nucleotide differences within each cluster were very small, less than 0.6 % except for that of the U3 isolate, which was distinct from any of the three clusters, and perhaps represents another cluster. The isolates belonging to the same cluster were widely distributed throughout Hokkaido (Table 1). The Chinese isolates were included in clusters A and C. In the coding region of the 26 kDa protein (228 amino acids), there was a maximum of 1.5 % nucleotide difference (mean of 0.5%) when compared within each group. This corresponds to 10 nucleotide changes, resulting in 6 amino acid changes. The nucleotide difference between each group was a maximum of 5.3% (mean of 4.2%), which corresponds to 33 nucleotide differences, resulting in 17 amino acid changes.

Table 1. Origins of BNYVV isolates containing RNA 5 used in this study and reported previously, and the grouping of BNYVV and RNA 5 variants

Isolate	Location	Type of BNYVV[a]	Group of RNA 5[b]	Isolate	Location	Type of BNYVV[a]	Group of RNA 5[b]
D5[c]	Japan, Date		I-A	S16	Japan, Shari	A	I-B
D104	Japan, Date	A	I-A	S42	Japan, Shari	A	II
R83[c]	Japan, Rusutsu	A	I-B	S43	Japan, Shari		I-B
K79[c]	Japan, Kimobetsu		I-C	S44[c]	Japan, Shari	B	I-A
S110	Japan, Soubetsu	A	I-C	CH2	China, Hohhot	B	I-A
T101	Japan, Toya	A	I-C	CH3	China, Hohhot	B	I-A
SH1	Japan, Shimizu	A	I-A	CY1	China, Yinchuan	A	I-A
U2	Japan, Urahoro	A	I-C	CY3	China, Yinchuan	A	I-C
U3	Japan, Urahoro		I-?	FC	France, Corbeille	A	III
H48	Japan, Higashimokoto		I-C	FP	France, Pithiviers	A	III
S11	Japan, Shari	A	I-C	F28[d]	France, Pithiviers		III
S12	Japan, Shari	B	II	F72[d]	France, Pithiviers		III
S13	Japan, Shari	A	I-B				

[a]BNYVV type is based on the coat protein sequences shown in Fig. 1b.
[b]Grouping of RNA 5 is based on Fig.1a.
[c]Sequence data are from Kiguchi *et al.* (1996).
[d]Sequence data are from Koenig *et al.* (1997).

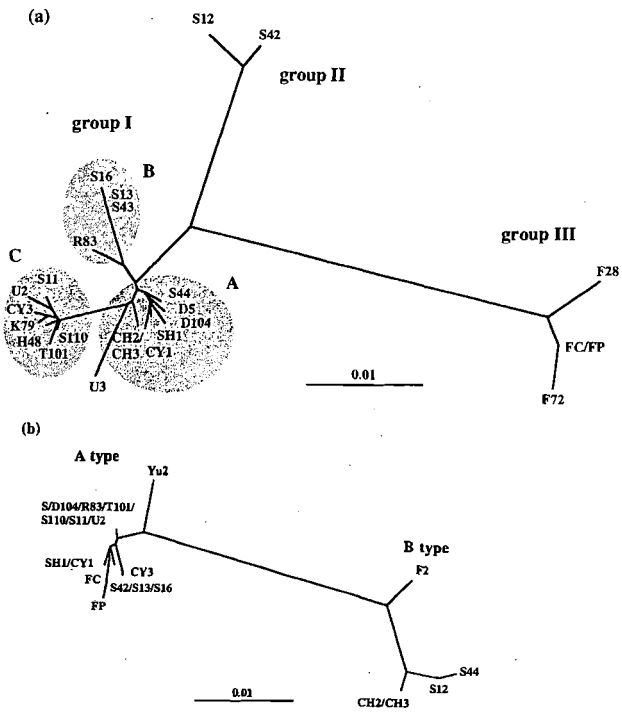

Fig.1. Phylogenetic trees obtained from sequence alignments of RNA 5 and the coat protein of BNYVV isolates using CRUSTAL W. The scale bars represent the number of nucleotide replacements per site. (**a**) Nucleotide sequences of the RNA 5 variants which were contained in the 25 isolates. The three small clusters (A, B and C) in the tree are shaded. (**b**) Nucleotide sequences of the coat protein gene of the 18 isolates which contain RNA 5 and the three isolates (S, F2 and Yu2) which lack RNA 5

Comparison of the coat protein gene for BNYVV isolates

Nucleotide sequences for the coat protein gene of the 18 isolates selected were determined. The coat proteins all consist of 188 residues, and there was a maximum of 4.6% difference at the nucleotide level. The results reveal that the coat protein sequences of the BNYVV isolates fall into two main groups: one contains 14 isolates and the other contains four isolates (Fig. 1(b)). This grouping corresponds well to the A type and B type in the classification of Kruse *et al.*(1994).

For the A type, the Japanese and Chinese isolates differed by only two nucleotides to each other, and no amino acid changes occurred. In addition, the sequences of the coat protein of the French isolates differed from those of the Japanese and Chinese isolates by four nucleotides. However, the nucleotide substitutions resulted in amino acid changes at only two positions, 17 (K to L) and 102 (I to V). These results indicate that the French isolates which were named the P type by Koenig *et al.* (1995) are very closely related to the A type.

For the B type, on the other hand, the coat protein sequences of the Japanese and Chinese isolates were very similar, differing by only two to four nucleotides and one or two amino acid changes occurred. Moreover, the sequences of these isolates differed from the F2 isolate (lacking RNA 5) by 7 to 12 nucleotides which led to one or two amino acid changes. However, these sequences differed from those of the aforesaid A type isolates by 20 to 27 nucleotides, which led to three to five amino acid changes. It should be noted that the A and B types can be discriminated by the same amino acid changes at three positions: 62 (T to S), 103 (S to N) and 172 (L to F).

Discussion

The nature and geographic location of BNYVV variants not only illustrates the evolution of BNYVV genomes but also the possible route of its spread to various regions. It is only in the last 30 years that BNYVV has developed into a serious problem in many sugar beet growing regions of the world. Here we present evidence for the existence of three groups of RNA 5 variants, which were detected in virus isolates from Japan, China and France. Most of the Japanese and Chinese isolates are included in group I, but two Japanese isolates are in group II, which is different from those in the French isolates (group III). The group I isolates fall into three small clusters. In the 26 kDa coding region of RNA 5, there was a maximum of 1.5% nucleotide sequence differences within the group and 8.4% nucleotide sequence differences between the groups.

To understand the evolutionary relationships among BNYVV isolates, the coat protein regions (RNA 2) were compared. The results showed that most of the Japanese and Chinese isolates belonged to the A type strain which contains the RNA 5 group I variants, but some isolates were of the B type and contain RNA 5 group II. Thus, mixed infections of the two types of virus and the two groups of RNA 5 were detected in a small area of Hokkaido, suggesting that at least two different BNYVV variants were once introduced into Hokkaido and have consequently spread from a single or a few locations. In addition, BNYVV might have been introduced into Japan and China by a similar route from at least two origins, because there is a very high similarity in the sequences between Chinese and Japanese isolates. The process of separation of the RNA 5 variants is unknown, but the fact that most isolates which contain RNA 5 belong to the A type virus suggests that ancestral RNA 5 was probably originally be associated with a virus ancestral to the A-like type virus rather than the B-like type. This is also based on evidence that the French isolates containing RNA 5 are closely related to the A type.

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EMERGENCE AND PARTIAL CHARACTERIZATION OF RICE STRIPE NECROSIS VIRUS IN THE AMERICAS

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Summary

A new disease of rice, known locally as "entorchamiento" (crinkling), was first observed in the Eastern Plains (Meta) of Colombia, in 1991. Symptoms included seedling death, leaf striping and severe plant malformation. Tissue extracts from "crinkled" rice plants, contained virus-like particles ca. 20 nm in diameter, with a bimodal length of 260 and 360 nm. Viral particle aggregates were also observed in the cytoplasm of infected rice leaf cells. Electrophoretic analyses of purified preparations and ds-RNA extracts, revealed a single protein subunit of M_r 22,500, and four ds-RNA bands ca. 6,300, 4,600, 2,700 and 1,800 bp in size. Preliminary work with the viral RNAs indicates that their 3' ends are polyadenilated, and that RSNV RNA-1 has significant sequence similarity to RNA-1 of *Beet necrotic yellow vein virus*. Cystosori, characteristic of plasmodiophorid fungal vectors of plant viruses, were consistently observed in the roots of diseased rice plants. A Western blot of tissue extracts obtained from 'crinkled' rice plants from Colombia, using antiserum against a West African isolate of rice stripe necrosis virus (RSNV), resulted in the detection of a protein band of approximate M_r 22,000. The RSNV antiserum recognized the Colombian virus isolate in serologically specific electron microscopy tests. These results confirm the presence of RSNV in the Americas, and suggest that RSNV is a member of the new *Benyvirus* genus.

Introduction

A new disease of rice (*Oryza sativa* L.) in Colombia, characterized by seedling death, severe plant malformation (Figure 1) and foliar striping (Figure 2), was first noticed in 1991, in the Eastern Plains of Colombia. By 1994, the disease, referred to as "entorchamiento" (crinkling), was already present in most of the rice-producing municipalities of the Meta department, causing yield losses of over 20% (Pardo and Muñoz, 1994). Intensive pesticide applications followed reports from two independent investigations citing aphids (Tapiero, 1994) and nematodes (Pardo and Muñoz, 1994) as the causal agents of the "crinkling" problem. Despite the use of highly toxic insecticides and nematicides applied at over five times the recommended doses, the disease continued to spread in the Eastern Plains and other rice-producing departments of Colombia. Subsequent investigations (Morales *et al.*, 1995) suggested that the "crinkling" disease of rice in Colombia was a soil-borne viral disease similar to rice stripe necrosis, described in West Africa as a disease of upland rice caused by a virus transmitted by the fungus *Polymyxa graminis* (Fauquet and Thouvenel, 1983; Fauquet *et al.*, 1988).

Materials and methods

Rice plants showing symptoms characteristic of the "crinkling" problem, were collected near Villavicencio, Meta, Colombia. The roots were washed, dried under vacuum, and then ground to a powder, to be used as inoculum. *Nicotiana benthamiana, Chenopodium album, C. amaranticolor, C. murale, C. quinoa, Datura stramonium, Oryza sativa* "Orizica 3", *Physalis floridana, P. peruviana, Sorghum bicolor* "Rio" and *Zea mays* "Sikuani", were mechanically inoculated with leaf tissue extracts from rice plants affected by the "crinkling" disease, using 5 ml of KPO_4 buffer, pH 7.5, per g of infected rice tissue. For the seed transmission tests, 1500 seeds harvested from "crinkled" Orizica 1 rice plants, were planted in a glasshouse in trays containing sterilized soil.

Leaf extracts and partially purified preparations from infected rice plants were negatively stained in 2% uranyl acetate, pH 3.7, and examined with a transmission electron microscope. Leaf tissue of symptomatic rice plants was prepared for cytology as described earlier (Morales *et al.*, 1990). Scanning electron microscopy of fungal structures was performed according to the method of Wynn (1976). Roots of symptomatic rice plants were washed with sterile water, and then stained for 15 min in 0.05% cotton blue in lactophenol. For coat protein analysis, partially purified virus

preparations were analyzed in 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS) as described by Weber and Osborn (1969). For the analysis of ds-RNA, healthy and symptomatic rice plants were used as control and source of viral RNA, respectively, according to the procedure of Dodds and Bar-Joseph (1993).

An antiserum to the West African isolate of rice stripe necrosis virus (Fauquet and Thouvenel, 1983)) was kindly provided by Dr. Jean-Loup Notteghem, Plant Pathology Laboratory, CIRAD, Montpellier, France. Serologically specific electron microscopy (SSEM) tests were conducted as described by Derrick (1973). The RSNV antiserum was also used in Western blotting, using the trans-blot kit produced by Bio-Rad (Hertfordshire , England HP2 7TD).

Results

All rice plants showing "crinkling" symptoms, were shown to contain rod-shaped particles ca. 20 nm in diameter (Figure 3) with a bimodal length distribution of 260 and 360 nm. Only *Chenopodium quinoa* developed local lesions in the mechanical inoculation tests. None of the 1,500 rice seedlings grown from seed harvested from "crinkled" mother plants, developed symptoms. In transmission electron microscopy examinations, the cytoplasm of infected rice cells contained particle aggregates observed in longitudinal or cross section (Figure 4). The examination of the root system by light microscopy revealed the presence of abundant cystosori (Figure 5) containing irregular aggregates of spores characteristic of plasmodiophorid fungal vectors of plant viruses (Barr, 1979; Adams, 1991). SDS-PAGE analysis of partially purified virus preparations revealed the presence of a single protein species of ca. M_r 22,500. Four distinct bands of ca. size 6,300, 4,600, 2,700 and 1,800 bp, were observed in 5% acrylamide gels of ds-RNA extracts from diseased rice plants (Figure 6).

In SSEM tests, the use of the RSNV antiserum resulted in a 16.5-fold increase in the average number (313) of virus particles trapped, with respect to the average number (19) of virus particles observed per mesh without treating the grid with antiserum. The Western blotting analysis resulted in the detection of a similar protein molecule of M_r 22,000, corresponding to the capsid protein of the Colombian virus isolate.

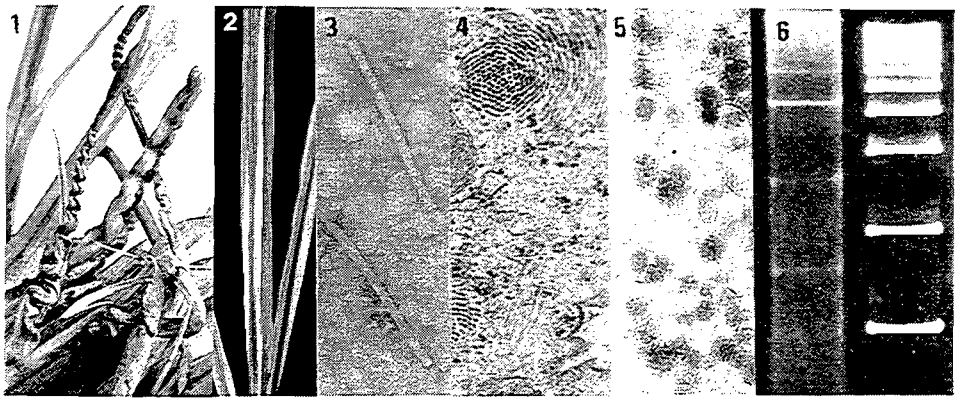

Fig. 1. Severe plant malformation and **Fig. 2.** Foliar striping characteristic of the (crinkling) disease of rice in Colombia. **Fig. 3.** Virus particles observed in leaf extracts obtained from rice plants affected by the "entorchamiento" disease of rice in Colombia. **Fig. 4.** Aggregates of virus rods (V) in the cytoplasm of rice cells infected by rice stripe necrosis virus. **Fig. 5.** Resting spore clusters (cystosori) in the rootlets of rice plants affected by rice stripe necrosis. **Fig. 6.** Analysis of dsRNA from rice plants affected by "entorchamiento". Lane 1, dsRNA bands (marked) extracted from diseased rice plants; lane 2, 1-kb ladder.

Discussion

The symptoms of the "entorchamiento" disease of rice in Colombia, are identical to those described for RSNV in West Africa (Louvel and Bidaux, 1977). The results obtained in this investigation, show that the "crinkling" disease of rice is associated with RSNV and the fungus vector *Polymyxa graminis*. The morphological characteristics of the Colombian isolate of RSNV, are also similar to those of the African isolate of RSNV, particularly their longer particle lengths (260-270 nm and 360-380 nm). These particle lengths differ from those reported (150-170 nm and 240-300 nm) for most furoviruses (Brunt and Richards, 1989; Putz, 1977). Unlike the predominantly bipartite *Furovirus* genus, *Beet necrotic yellow vein virus* (BNYVV) and *Beet soilborne mosaic virus* (BSBMV) have been shown to have a multipartite genome consisting of four or five RNA species (Bouzoubaa *et al.*, 1985; Bouzoubaa *et al.*, 1986; Bouzoubaa *et al.*, 1987; Heidel *et al.* 1997; Putz, 1977). BYNV and the Colombian isolate of RSNV also differ from other furoviruses (Brunt, 1988) in having longer particle lengths (260-265 and 360-390 nm). In this investigation, the two larger RNA species of the Colombian RSNV isolate, were similar in size to the corresponding RNA species of BNYVV and BSBMV (Bouzoubaa *et al.*, 1985; Bouzoubaa *et al.*, 1986; Heidel *et al.* 1997; Putz, 1977; Putz *et al.*, 1983), but some differences are apparent for the two smaller RNA species (Putz, 1977; Koenig *et al.*, 1986; Kuszala *et al.* 1986; Heidel *et al.*, 1997;). However, similar differences in the size of RNAs 3 and 4, have been reported for different BNYVV isolates and hosts (Bouzoubaa *et al.*, 1985; Burgermeister *et al.*, 1986; Heidel *et al.*, 1997; Koenig *et al.*, 1986; Kuzsala *et al.*, 1986). These results suggest that RSNV, BNYVV and BSBMV have a similar genomic organization and, thus, RSNV might be a species of the new *Benyvirus* genus (Pringle, 1998). Benyviruses have other characteristic properties, which differentiate them from other viruses previously assigned to the *Furovirus* genus. One of these characteristics is that the 3' end of their RNAs is polyadenylated. In preliminary work with the Colombian isolate of RSNV, we confirmed the presence of a poly-A tail in RSNV.

Following the identification of RSNV in the Eastern Plains of Colombia, the virus and associated fungus vector have been shown to be widely distributed in the main rice growing departments of Colombia. Recently, RSNV has been detected in Panama. Thus, it is possible that the distribution of RSNV in Latin America is broader than presently recognized. The main factor responsible for the relatively rapid spread of the "crinkling" disease in Colombia, seems to be the use of contaminated seed and agricultural machinery shared by different rice growers. The dissemination of RSNV in West Africa has been comparatively slower, probably due to the lower degree of mechanization of rice in Africa, and to the cultivation of other rice species, such as *Oryza glaberrima,* which is resistant to RSNV. The emergence of rice stripe necrosis in South America, is a serious threat to most rice producing countries both in tropical and temperate regions of the Americas. The development of RSNV-resistant cultivars is probably the most sustainable disease control strategy (Brunt and Richards, 1989).

Acknowledgements

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VARIABILITY WITHIN READ-THROUGH REGION IN POTATO MOP-TOP VIRUS (PMTV) RNA 3 AMONG SCANDINAVIAN ISOLATES AND DELETION VARIANTS DETECTED IN TEST PLANTS AND POTATO TUBERS.

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Summary

Sequence of RNA 3 was determined from a Swedish, Danish and Finnish isolate of *Potato mop-top virus* (PMTV). A high amino acid similarity was found in the coat protein region of the three isolates. In contrast, considerable sequence variability was found within the read through (RT) region, but also highly conserved parts. The size of RNA 3 was examined in infected roots of bait plants (*Nicotiana debneyi*) and in mechanically infected leaves (*N. benthamiana*). Viral sequences were identical until three transmissions at two weeks intervals, after which a shorter form of RNA 3 appeared in infected leaves. This shorter sequence lacked some of the variable part of the RT region. A similar deletion was also found in RNA 3 examined from a naturally infected potato tuber.

Introduction

Potato mop-top virus (PMTV) was first identified and described in Scotland (Calvert and Harrison, 1966). PMTV is soil-borne and transmitted by *Spongospora subterranea* (Arif et al., 1995). The three RNA species of PMTV have sizes of 6.0 kb (RNA 1; Savenkov et al., 1999) 3,0 kb (RNA 2; Scott et al., 1994) and 2.5 - 2.9 kb (RNA 3; Kashiwasaki et al., 1995; Reavy et al., 1998). RNA 3 contains one open reading frame encoding the virus coat protein (CP) of 20 kDa terminated by an amber (UAG) codon. This is followed by an in-phase coding region for an additional 47 - 67 kDa read through (RT) domain, depending on isolate (Kashiwasaki et al., 1995; Reavy et al., 1998).

Results and discussion.

Sequences of Scandinavian isolates of PMTV were obtained from root sap from bait-plants (*Nicotiana debneyi*) by immuno capture RT-PCR. The complete sequence of RNA 3 was determined from a Swedish isolate of PMTV, and parts of RNA 3 of a Danish and a Finnish PMTV isolate. Comparisons were made with published sequences of Scottish isolates, PMTV-T and PMTV-S (Kashiwasaki et al., 1995; Reavy et al., 1998).

In accordance to previous studies (Mayo et al., 1996: Reavy et al., 1997), a high amino acid identity was found in the coat protein region of the three Scandinavian isolates. Different from the CP region, the RT region contained parts with considerable sequence variability, as well as conserved parts with few amino-acid exchanges, and one region where only one nucleotide differed between the Swedish and the Scottish isolates.

The distribution of exchanges between the Swedish (PMTV-Sw) and the Scottish isolate (PMTV-S) in the RT region can be described as follows: Synonymous changes of nucleotides were uniformly scattered through ca. three fourths of the RT-region, starting from the beginning of the RT-region. Within the first 678 nucleotides, two non-synonymous nucleotide changes occurred, A more variable region was found within the following 861 nucleotides, where fifteen non-synonymous nucleotide changes occurred.

The C-terminal part of the RT-region of PMTV-Sw, contained only one nucleotide change (non-synonymous), located within the 298 last nucleotides of PMTV-S. The Swedish, the Finnish and Danish PMTV isolates contained an additional, and identical sequence of 109 nucleotides completing the RT-ORF which have not been reported in the Scottish isolates. PMTV-Sw therefore encodes for an 91 kDa RT-protein, compared to the 87 kDa calculated for PMTV-S (Reavy et al., 1998).

Mechanical inoculation is one means by which deletions might be generated in viral sequences. The mechanism behind this is thought to be a lack of selection pressure, for instance by vector transmission, on certain parts of the genome. Since part of the variable region is lacking in PMTV-T, which has been maintained through serial mechanical inoculations over years (Reavy et al., 1998)., we started an investigation to find out if this region will be deleted following serial mechanical inoculations. In two of the four Scandinavian isolates, a deletion of 941 nucleotides within the variable region was found after the third mechanical inoculation. In comparison, samples from four tubers naturally infected in the field, were investigated the same way. In one of these tubers, a deletion of 558 nucleotides was detected within the RT region, starting only 18 nucleotides downstream from the deletion of 941 nucleotides.

The variable region is proposed to be involved in vector transmission since the isolate PMTV-T that has a deletion within this region, cannot be transmitted by the *vector Spongospora subterranea,* whilst the other Scottish isolate, PMTV-S, is vector transmissible (Reavy et al., 1998). More knowledge about any involvement of this region in vector transmission, might as well give reasons why it shows such a high degree of variability between isolates from different geographical regions.

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ANALYSIS OF THE GENOMIC STRUCTURE AND PHYLOGENY OF POTATO MOP-TOP VIRUS (PMTV)

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Summary

The fungal-transmited rod-shaped viruses (former furovirus group) are grouped to the genera *Furovirus, Pomovirus, Pecluvirus* and *Benevirus.* They also share similar genomic structures with other genera of tubular viruses*: Hordeivirus, Tobravirus and Tobamovirus. Potato mop-top pomovirus* is economically one of the most important viruses in the Nordic countries. RNA-dependent RNA polymerase sequences are among the most important features used in molecular taxonomy of viruses. Therefore, it is of crucial importance to characterise the RNA 1 sequence from PMTV, the type member of the genus. The complete nucleotide sequence (6043 nt.) of RNA1 from PMTV was determined. The first ORF from the 5'-terminus (ORF1) encodes a protein with a predicted Mr of 148 kDa. ORF2 extends immediately through the opal stop codon of ORF1 to give a protein with a predicted molecular mass of 206 kDa. Comparison of the nucleotide and amino acid sequences indicates similarites between PMTV RNA1 and the corresponding RNAs of three other pomoviruses and the furoviruses SBWMV and CWMV.

Introduction

The fungal-transmitted rod-shaped viruses are taxonomically grouped to the genera *Furovirus, Pomovirus, Pecluvirus* and *Benevirus.* PMTV is the type member of the genus Pomovirus. The pomoviruses are a genus of positive-strand RNA viruses whose genomes are divided among three messenger RNAs. These RNAs were designated as RNA1, RNA2 and RNA3 according to the size. PMTV has fragile, tubular, rod-shaped particles and is transmitted in soil by the plasmodiophorid protist *Spongospora subterranea.* PMTV particles contain three species of single-stranded RNA, thus PMTV has a tripartate genome. The complete nucleotide sequences of PMTV RNA 2 and RNA 3 are available from the isolate PMTV-T from Scotland (Scott et al., 1994; Kashiwazaki et al., 1995). RNA 2 of PMTV encodes four putative proteins. The first three proteins form the triple gene block (TGB) . The 5' cistron of RNA2 encodes protein with a helicase domain. The second and third cistrons encode two hydrophobic proteins. The function of the fourth protein (8K) is unknown. RNA 3 of PMTV encodes the coat protein (CP) and a read-through protein.

The sequence from RNA 1 of PMTV was not available. It is estimated to comprise approximately half of the PMTV genome. RNA 1 encodes an RNA-dependent RNA polymerase (RdRp) in the three other known pomoviruses. RdRp sequences are among the most important features used in molecular taxonomy of viruses. Therefore it was of intrinsic scientific importance to characterise the RNA 1 sequence from PMTV, the type member of the genus.

Materials and Methods

Materials and methods have been described in details by Savenkov *et al.* (1999).

Results and Discussion.

Thirteen clones covering a sequence of the RNA 1 were sequenced and aligned to provide the complete 6043 nt sequence of RNA 1 of PMTV-Sw (Fig 1; EMBL Sequence Database accession no. AJ238607). Computer assisted analysis revealed the presence of two ORFs (Fig. 1). ORF1 (3909 nt) starts at the first AUG codon at position 115 and terminates with a UGA stop codon at position 4024. The putative polypeptide encoded by this ORF has calculated molecular mass (M_r) 148 kDa. An in-frame coding sequence extends immediately after the opal codon and creates ORF 2. It ends at a UGA (5551) and has a polypeptide coding capacity of 509 amino acids (M_r 58 kDa). The predicted translational fusion protein from ORF 1 and 2 has M_r of 206 kDa. The 5'-untranslated region (5'-UTR) and 3'-UTR of RNA 1 are 114 nt and 489 nt long, respectively. Thus, ORFs 1 and 2 account for 89.9% of the RNA 1 sequence.

206K protein has a methyltransferase, a helicase and a polymerase motif, respectively, resembling those of other positive-sense RNA viruses. The arrangement of the conserved motifs and ORFs in the RNA 1 of PMTV was similar to the three previously characterized pomoviruses, BSBV (Koenig & Loss, 1997), BVQ (Koenig *et al.*, 1998) and BBNV(Lu *et al.*, 1998). The putative capping, helicase, and polymerase domains are conserved with nonstructural proteins of many other positive stranded RNA viruses. Together, these viruses comprise the alphavirus-like superfamily (Koonin & Dolja, 1993).

Multiple alignments and phylogenetic analyses of the RdRp sequences from PMTV, BBNV, BSBV, and other representatives of tubular viruses revealed significantly greater sequence similarity between pomoviruses and furoviruses, than between pomoviruses and the other viruses analysed (Fig. 2). These findings indicated that the RdRps of pomoviruses and furoviruses are closely related, forming a distinct cluster (Fig. 2). On the other hand, the RdRps of hordeiviruses are closely related to pecluviruses, forming another cluster (Fig. 2). These data are consistent with the previous reports that PCV is more closely related to the hordeiviruses than to the pomoviruses based on comparison of

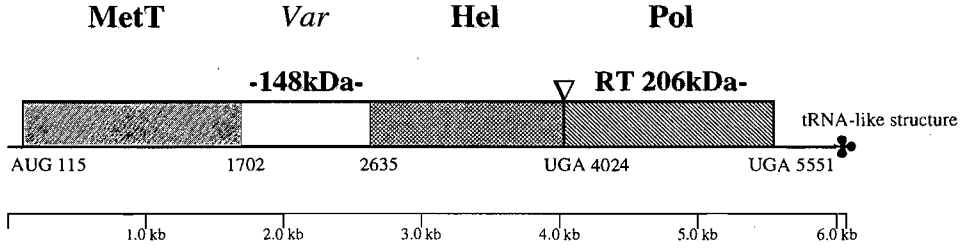

Fig. 1. Genetic organisation of RNA 1 in PMTV-Sw. The large rectangle represents the ORFs encoding the 148K and the 206K proteins. Shaded regions within these ORFs indicate the location of the methyltransferase (MetT), helicase (Hel), and polymerase (Pol) domains. ∇, the opal (UGA) stop codon which interrupts internally the ORF for 206K protein and terminates the coding region for 148K protein; *Var*, region variable among the viruses transmitted by fungi; RT, readthrough protein.

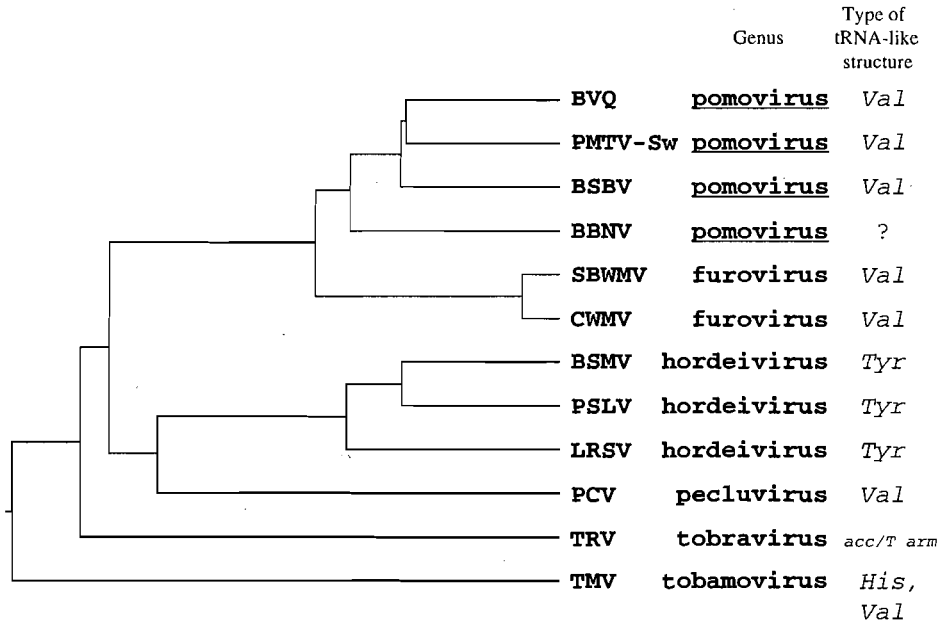

Fig. 2. Phylogenetic analysis of the deduced protein sequences of RdRps from different tubular viruses (on the left) and type of tRNA-like structure (on the right). *acc/T arm,* TRV RNAs do not posses a tRNA-like structure capable of aminoacylation but do have a 3'-pseudoknotted acceptor/T arm mimic and can be 3'-adenylated by tRNA-nucleotidyl transferase (CCA-NTase) (Goodwin & Dreher, 1998).

the RdRps, TGB proteins, coat proteins and cysteine-rich proteins (Herzog *et al.*, 1994; Solovyev *et al.*, 1996; Lu *et al.*, 1998; Savenkov *et al.*, 1998).

Since pomovirus genomes are segmented, it seems likely that cis-acting regions that are important for many viral processes might be conserved among the genomic RNA. Indeed, the starting sequence GUAUUU in RNA 1 is also found in RNA 2 and RNA 3 of PMTV, as well in the RNAs of many other tubular viruses that start with the sequence GUA.

The 3'-UTR of RNA 1 is 489 nt long in PMTV-Sw. The 80 3'-terminal nucleotides can be folded into a tRNA-like structure. The tRNA-like structures of RNA 1, RNA 3 and RNA 2 are of equal length and are identical in nucleotide sequence. Among the (+) strand RNA viruses, the variety of terminal structures is far greater than that of their hosts' mRNAs. Almost all host RNAs are polyadenylated. On the other hand, positive strand viral 3'-termini are either poly(A) tails, or some other type of heteropolymeric sequence. It is intresting that the different types of 3' termini are not exclusively clustered based on the sequences of the viral RdRp (Fig. 2), although tRNA-like structures are ristricted to proposed supergroup 3 viruses and poly(A) tails are absent from the supergroup 2 viruses (Koonin & Dolja, 1993).

The tRNA-like structures of PMTV, like in those other fungal-transmitted viruses have an anticodon (GAC) for valine. Indeed, these 3'-ends mimic tRNA in several respects. They can be specifically charged *in vitro* (and *in vivo* as was demonstrated for

BMV) with valin by valyl-tRNA synthetases. PMTV RNAs are also recognised by other tRNA-interacting enzymes such as tRNA-nucleotidyl transferase and elongation factor EF-1α (Goodwin & Dreher, 1998). tRNA-nucleotidyl transferase adds the 3'-terminal C and A residues to the 3' ends, maintaining the integrity of the viral 3' end in a manner comparable to a telomerase. In contrast, the tRNA-like structures in hordeiviruses have an anticodon sequence for tyrosine and are capable of tyrosilation (Agranovsky *et al.* 1981; 1992; Goodwin & Dreher, 1998).

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PRELIMINARY MOLECULAR CHARACTERIZATION OF ITALIAN ISOLATES OF BEET NECROTIC YELLOW VEIN BENYVIRUS (BNYVV).

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Summary

Molecular characterization of beet necrotic yellow vein benyvirus (BNYVV) was carried out in order to identify the possible presence in Italy of the two strains widespread in Europe (type A and type B). During 1997 and 1998, about 70 soil samples collected from representative Italian areas most severely affected by rhizomania tested positive by ELISA for BNYVV. For the molecular analysis of each of these isolates, susceptible sugar beet seeds were sown in infected soil samples. mRNA was extracted from infected roots and cDNA corresponding to two different regions of the viral genome was amplified through reverse-transcriptase-PCR (RT-PCR). Specific RT-PCR products were analyzed for restriction fragment length polymorphism (RFLP). This report highlights the presence of strain type A in the main sugar beet production areas in Italy. Molecular characterization of samples from of other highly infected European regions is also reported.

Introduction

Rhizomania is a worldwide devastating disease of sugar beet caused by beet necrotic yellow vein benyvirus (BNYVV). It was first described in Italy in the mid 1950's (Canova, 1959) and was initially widespread in the Po Valley (Bongiovanni, 1964). BNYVV was afterwards detected in new sugar-beet growing areas in Central and Southern Italy (Rana *et al.*, 1978; Di Franco and Russo, 1978; Rubies-Autonell *et al.*, 1995). In the most recent survey of soil-borne virus diseases of sugar beet, BNYVV, alone or in mixed infection with beet soil-borne potamovirus (BSBV), was detected in 93% of infected soil samples collected from 126 sites representative of the Italian sugar-beet growing areas (Turina *et al.*, 1996). So far, resistant cultivars are the only economical option to control rhizomania. However there is considerable difference in reactions of sugar beet genotypes to infections by BNYVV grown in different areas possibly because of the occurrence of different strains of BNYVV. Kruse *et al.*, (1994) showed that the majority of European virus isolates may be classified into two major groups by restriction fragment length polymorphism (RFLP) analysis of reverse transcription RT-PCR products. Koenig *et al.*, (1995) have confirmed the result of the previous analysis by single strand conformation polymorphism (SSCP) and suggested that additional types of BNYVV variants may exist. The aim of the present work is the characterization of Italian isolates of BNYVV from representative areas infected by rhizomania by means of RFLP analyses of RT-PCR products.

Materials and Methods

Soil sample collection. During 1997 and 1998, soil samples were collected from 90 sites representative of Italian sugar beet growing areas. Some soil samples from other parts of Europe (France, Spain) were included. All the samples were assayed for the presence of BNYVV with a double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) carried out according to protocols previously described (Turina *et al.*,1996). Molecular analysis was carried out on the samples which tested positive in DAS-ELISA (68 samples from Italian fields, 6 from Spain and 3 from France).

Preparation of RNA extracts from sugar beets samples. RNA extracts for the synthesis of RT-PCR products were obtained directly from 0,2 g of infected sugar beet roots grown for 3-4 weeks on BNYVV infected soils as described by Turina *et al.*, (1996). Two different methods of RNA extraction from roots were tested. Total RNAs were obtained by maceration in mortar of root tissues in 0,6 ml of 2x STE buffer (10 mM Tris-HCl, pH8; 100 mM NaCl, 1 mM EDTA), 1%SDS, 25 µl 0,1M dithiothreitol (DTT) and 0,6 ml of phenol-cloroform-isoamyl alcohol (25:24:1) [Ø $CHCl_3$ -IAA]; upper aqueous phase was back extracted with Ø $CHCl_3$, precipitated with LiCl and washed with 70% ethanol as previously

described (Jones *et al.*, 1990). Alternatively, mRNA extracts from roots were prepared by the Quick Prep Micro mRNA purification kit (Amersham-Pharmacia-Biotech, USA) according to the protocol suggested by the manufacturer.

RT-PCR. Reverse transcriptase Superscript II (Gibco-Life Technologies, Rockville, MD) was used for oligo(dT)-primed cDNA synthesis in a total reaction volume of 20 µL, and DyNAzyme™ II DNA polymerase (Finnzymes Oy, Finland) for PCR. For the amplification of two different regions of the viral genome the primers pairs (Gibco-Life Technologies, Rockville, MD) were designed on the basis of the nucleotide (nt) sequence data of Kruse *et al.*, (1994): for RNA2, 5'-CCATTGAATAGAATTTCACC-3' (nt 19 to 38) and 5'-CCCCATAGTAATTTTAACTC-3' (nt 1088 to 1069); for RNA3 5'-GTGATATATGTGAGGACGCT-3' (nt 50 to 69) and 5'-CCGTGAAATCACGTGTAGTT-3' (nt 1251-1268). PCR reactions (50µl) contained 3µl of cDNA mixture and 40 pmoles of each primer. Amplification was carried out for 40 cycles, each of 1 min at 94°C for denaturation, 1 min annealing at 52°C and synthesis for 1,5 min at 72°C (DNA thermal cycler 480, Perkin Elmer). Amplification of fragments was analyzed by electrophoresis through 1% agarose gel in TBE buffer (0,089 M tris-borate; 0,089 M boric acid; 0,002M EDTA) stained with ethidium bromide. Some samples required two cycles of PCR amplification in order to obtain nucleic acids amounts sufficient for the restriction digestions.

RFLP analyses of RT-PCR. Specific RT-PCR products (5 µl) were treated for 1h at 37°C by four restriction endonucleases: *Sca*I, *Eco*RI, *Xma*I and *Bam*HI (Gibco BRL). In preliminary experiments the amount of restriction endonucleases necessary to achieve the cleavage of the PCR products were determined. The differentiation of RFLP patterns is based, as indicated by Kruse *et al.*, (1994), on the detection by electrophoresis through a 1,8 % agarose gel of the following cleavage sites: *Sca*I (nt 136 to 141 and 451 to 456), *Eco*RI (nt 561 to 566) and *Xma*I (nt 635 to 640) for RNA 2 and *Eco*RI (nt 377 to 382) and *Bam* HI (nt 751 to 756) for RNA 3. Positive controls for group A (isolates O-11, T-101) and group B (isolate GW) were kindly supplied by Dr. T. Tamada (University of Okayama).

Results

The best results for RNA extraction from roots were obtained with the Quick Prep Micro mRNA purification kit, which was faster and resulted in an overall more sensitive RT-PCR amplification when compared to the phenol-chloroform extraction, lithium chloride precipitation method. RT-PCR amplification products of 1069 bp for RNA2 and 1218 bp for RNA 3 were obtained (Fig. 1). An example of the cleavage patterns obtained after treating RT-PCR products of various origins is given in Fig 2. Each sample classified as group A was consistently cleaved by *Eco*RI and *Sca*I for RT-PCR products of RNA 2 while *Xma*I could not cleave them. RT-PCR products of RNA3 could not be cleaved by *Eco*RI and *Bam*HI.

Gel bands of sizes corresponding to partially cleaved PCR product were also present probably due to the high rate of mutations occurring at the restriction sites during PCR amplification with Taq polymerase.

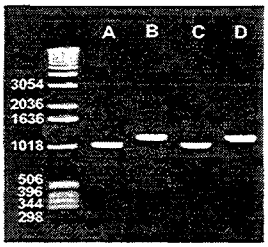

Figure 1. Ethidium bromide staining of RT-PCR products from RNA 2 (A, C) and RNA 3 (B, D) from an Italian isolate (A, B) and the French isolate from Caroy (C, D).

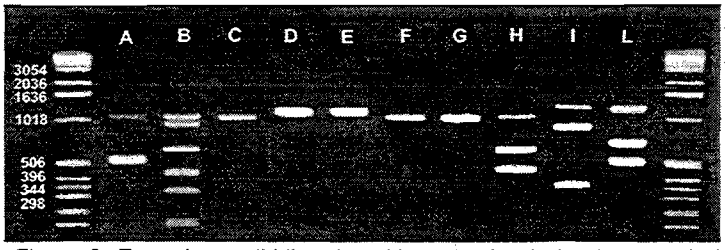

Figure 2. Example to ethidium bromide stained gel showing a typical RFLP pattern obtained with RT-PCR products of RNA 2 (A, B, C, F, G, H) and RNA 3 (D, E, I, L) from an Italian isolate (A-E) and the French isolate from Caroy (F-L). Cleavage patterns was obtained after treatment by four restriction endonucleases: *EcoRI* (A, F, D, I), *Sca*I (B, G), *Xma*I (C, H) and *Bam*HI (E, L).

The two strain groups type A and type B were easily differentiated by the presence or absence of cleavage products. The results of RFLP analyses for the Italian isolates are summarized in Fig. 3. All of the 68 Italian BNYVV isolates were classified as type A. Moreover, one isolate from the North France (Pithivier), and 6 from North Western Spain (Valladolid province) fell into group type A while two isolates from North France (Artenay and Caroy) showed a restriction digest pattern typical of type B isolates.

Moreover, soil samples from Abruzzo region, where rhizomania has been absent so far, tested positive for BNYVV.

Figure 3. Distribution of type isolates of BNYVV in sugar beet growing areas in Italy.

Discussion

The RFLP analyses by four restriction endonucleases (*Sca*I, *Eco*RI, *Xma*I and *Bam*HI) of RT-PCR products of only two regions of the BNYVV viral genome proved to be both simple and reliable for classification of the Italian isolates into the existing type groups established by Kruse *et al.*,(1994).

Our data show the widespread diffusion of strain group A in all Italian rhizomania disease areas: in particular no difference was detected with this method between the isolates in the traditional sugar beet growing areas of the Po Valley and the areas where rhizomania was only more recently detected (Abruzzo, Friuli-Venezia Giulia, Marche, Sardegna, Umbria regions). Our analysis showed that all samples were cleaved by *Eco*RI and *Sca*I for RT-PCR products of RNA 2 and could not be cleaved by *Xma*I: previous results by Kruse *et al.*, (1994) showed that three isolates collected in the Po Valley were tentatively assigned to group A: one of them could not be cleaved by *Eco*RI in the cDNA derived from RNA 2 and for a second isolate no restriction pattern was shown for cDNA derived from RNA 2.

Our data place the 6 Spanish BNYVV isolates in group A, supporting data recently published for samples collected in the same areas (Suarez *et al.*,1999). Two French isolates showed a restriction pattern typical of isolates belonging to group B; previous results showed the presence in the same areas of isolates of both groups (Kruse *et al.*,1994). Our data confirm the relative uniformity and the prevalence of one of the groups on wide areas and the phylogenic implication previously suggested (Kruse *et al.*, 1994). A limitation of this technique and of the classification derived from it, is suggested by the fact that it can not distinguish among biological and pathogenetical differences present in Italian sugar beet growing areas. Future studies will explore the possibility of using single-strand conformation polymorphism (SSCP) to study the existence of other strains and/ or variants of beet necrotic yellow vein benyvirus (BNYVV) (Koenig *et al.*, 1995; Koenig, 1996) or finer molecular mapping in order to correlate molecular and pathogenicity data.

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THE INTRAMURAL DECORATIONS IN THE RESTING SPORE OF OLPIDIUM BRASSICAE

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Summary

The ultrastructural study of the resting spore of *Olpidium brassicae* has disclosed two types of intramural decoration on the surface of the cytoplasm and on the inner surface of the wall of the resting spore: vitz. 1) a raspberry-like sculpture with a granular formation, 300 - 400 nm in diameter, 2) a rod-like formation, 500 - 2000nm x 250nm. Their structural significance has not been determined.

Introduction

While *Olpidium brassicae* is one of the best known fungal vectors of plant viruses, our understanding of the ultrastructure of the resting spore is limited. This paper reports the results of our observations on the ultrastructure of intramural decorations found after subjecting the resting spore of *O. brassicae* to a freeze fracture treatment.

Materials and methods

Roots of healthy tobacco (*Nicotiana tabacum* L. cv. Bright Yellow) seedlings germinated in moist sand at 25°C for 6 to 10 days were inoculated in concentrated zoospore suspensions (10^3 zoospores/ml) (Hiruki, 1999). The roots washed twice with distilled water were fixed in 2% formaldehyde plus 2% glutaraldehyde followed by post-fixation in 1% osmium tetroxide. Roots used for a freeze-fracture treatment were fixed for 4 to 6 hrs at room temperature in 0.1M phosphate buffer pH7.0 containing 2% glutaraldehyde plus 2% formaldehyde and after post-fixation in 2% aqueous osmium tetroxide for 2 to 4 hrs. Fixed roots were frozen rapidly in Freon 13 and fractured with a pre-cooled scalpel. The fractured roots were transferred into liquid nitrogen, freeze-dried. Dried specimens were fixed to stubs using low resistance contact cement (Fullam Inc., New York) and coated with 50Å of carbon and 50Å of gold in an Edwards Vacuum Evaporator. Coated specimens were examined with a Cambridge Stereoscan S4 operating at 28kV and 15-30 tilt.

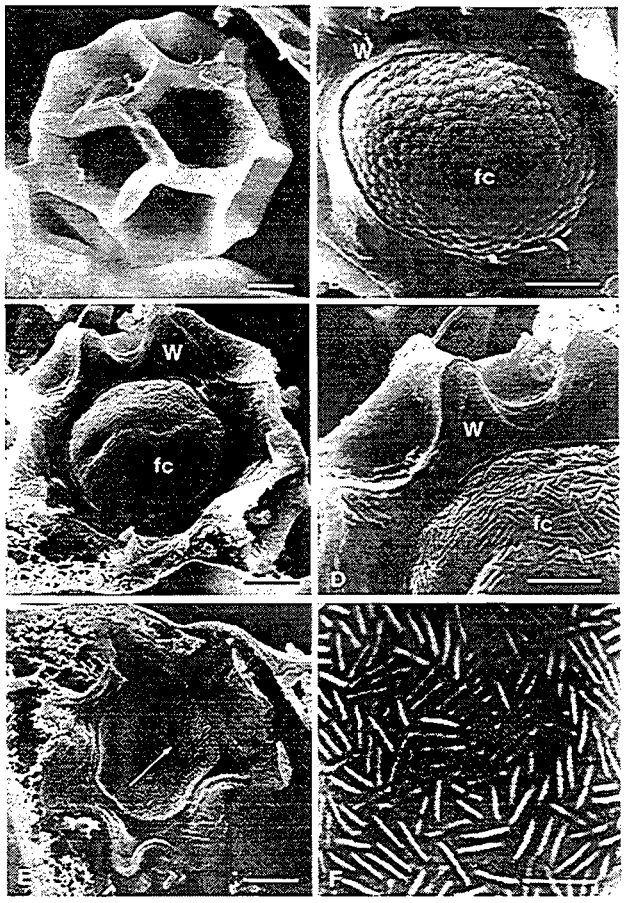

Fig. 1. Scanning electron micrographs of the intramural decorations in *Olpidium* resting spores in tobacco seedlings.

A, an unfractured resting spore with ridge making up five and six-sided facets. Bar, 2 μm; B, a resting spore with fractured walls. Note the thick walls (w) surrounding the fungal cytoplasm (fc) which has a sculptured surface with a raspberry-like appearance. Bar, 2 μm; C, a fractured resting spore with a sculpture of rod-like depressions on the surface of the cytoplasm (fc). Bar, 3 μm; D, An enlarged detail of C which shows the layers of the wall and the rod-like depressions on the surface of the cytoplasm (fc). Bar, 2 μm. E, a paired fracture surface of the resting spore in C. Note the rod-like raised areas (arrow). Bar, 1 μm; F, an enlargement of E which shows the rod-like raised area. Bar, 3 μm

Results

The wall of individual resting spores possessed distinct ridge which outlined five- and six-sided facets (Fig. 1- A). The ridged wall of a freeze-fractured resting spore was composed of several layers (Fig. 1-B, -C) and enclosed the fungal cytoplasm which had a sculptured surface. Two types of sculptured surface observed were: one with raised area giving a 'raspberry-like' appearance consisting of numerous granule-like formations each one measuring about 300 to 400 nm in diameter (Fig. 1-B); and the other with rod-like depressions, 1500 - 2000 nm X 250 nm (Fig. 1- C, -D). A fractured surface paired to that shown in Fig.1-C had rod-like raised areas of a comparable configuration and size (Fig. 1-E, -F). In some cells host material was associated with the surface of the resting spore (Fig. 1-C, -E).

Discussion

This study has confirmed and extended the previous studies on the resting spores of *O. brassicae* (Hiruki, 1987, 1990, 1991). The persistence of tobacco stunt virus (TSV) in *Olpidium*-infested soil is explained by the internal association of TSV with stable resting spores (Hiruki, 1987,1994). Moreover, TSV in the resting spores has been shown to withstand not only unfavorable environmental conditions but also certain drastic treatments such as strong acid, alkali, and UV irradiation (Hiruki, 1987). The significance of the intramural decorations, observed in this study in the formation and perhaps in the development also, of resting spore wall of *O. brassicae*, remains unclear. It is conceivable that the formation of such extradecorations is closely connected with the internal cytological development of the fungus. A carefully programmed time-course study on the formation and development of *Olpidium* resting spores is expected to yield some useful information to fill the gap in this respect. Such a study is in progress.

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CHARACTERISATION OF *POLYMYXA* TRANSMITTING RICE STRIPE NECROSIS VIRUS IN COLOMBIA

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Summary

Isolates of *Polymyxa* transmitting rice stripe necrosis virus have been established in sand culture and are being characterized. The nutrient pH was not a major influence on root infection. The fungus could be detected in inoculated rice, oats, Sorghum and maize but not in barley or wheat. At 25-30°C, large numbers of zoospores were produced about 2 weeks after inoculation. A region of approximately 1.1kb of ribosomal DNA, consisting of 600bp at the end of the 18S gene, the 5.8S DNA and the two internal transcribed spacers, was obtained from two independent isolates and compared with those from other isolates of *Polymyxa*, and some other plasmodiophorids. The two sequences were identical and most closely resembled *Polymyxa graminis* type II isolates in phylogenetic analyses.

Introduction

Rice stripe necrosis virus (RSNV), believed to be transmitted by *Polymyxa graminis*, has become rapidly established as a major disease of rice in Colombia (see Morales et al., this volume). The experiments reported here were done to characterize isolates of the vector from Colombia in order to determine the optimal conditions for infection and clarify its taxonomic status.

Materials and Methods

A hydroponic system similar to that which has been used successfully for many years to grow *Polymyxa graminis* in temperate cereals, especially barley (Adams *et al.*, 1986) was adapted for rice by using a coarser grade of sand (coarse industrial sand), a nutrient solution recommended by CIAT and an air temperature of about 25-32°C with supplementary light and humidification. Rice seedlings (usually cv. Oryzica 3, which is susceptible to the disease in Colombia) were pre-germinated and inoculated at planting (when about 5 days old) using either crushed dried roots supplied by CIAT or (in an attempt to establish pure isolates) selected resting spore clusters. These clusters were selected by hand under a stereomicroscope, transferred to plugs of water agar and plugs with around 20 resting spore clusters used to inoculate rice seedlings. Plant growth was monitored and roots were examined periodically under the light microscope for the presence of zoosporangia or resting spores of *P. graminis*.

Two root samples obtained from "crinkled" rice plants collected in the departments of Meta (sample 1) and Tolima (sample 2), were analyzed by molecular techniques. For sample 2, DNA was prepared from infected roots (freeze dried and ground) using the method of Lee and Taylor (1990), but including an additional RNase digestion. For sample 1 root extracts were used; DNA was released from a 1cm long infected root by crushing it in an Eppendorf tube, adding 100 µl of 10mM Tris pH8 and boiling for 10 minutes. Molecular characterization of the isolates involved analysis of their ribosomal DNA after amplification of the region between primers NS7 (5' AACTTAAAGGAATTGACGGAAG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') by PCR. Experimental conditions and procedures for sequencing and alignment and phylogenetic analysis (using the programs in PHYLIP) were as described by Ward and Adams (1998). Phylogenetic trees were constructed using NEIGHBOR from the original data set and 100 bootstrap data sets.

Results and Discussion

The growth of *P.graminis* and of rice was compared in nutrient solutions of pH 4, 5, 6, 7 or 8 after inoculation either with root powder or selected cystosori. After inoculation with root powder, distorted leaves and yellow stripes developed on plants in all pH treatments and *P. graminis* zoosporangia were seen in their roots. Electron microscopy of leaf sap from affected leaves showed the presence of rod-shaped virus particles typical of RSNV. These plants soon stopped growing and subsequently died. Some plants inoculated with selected cystosori and growing at pH 4, 5 and 8 also developed RSNV symptoms and *P. graminis* zoosporangia were found in their roots but the plants continued to grow (Fig. 1). It appears that pH has relatively little effect on the development of this isolate of *P. graminis*.

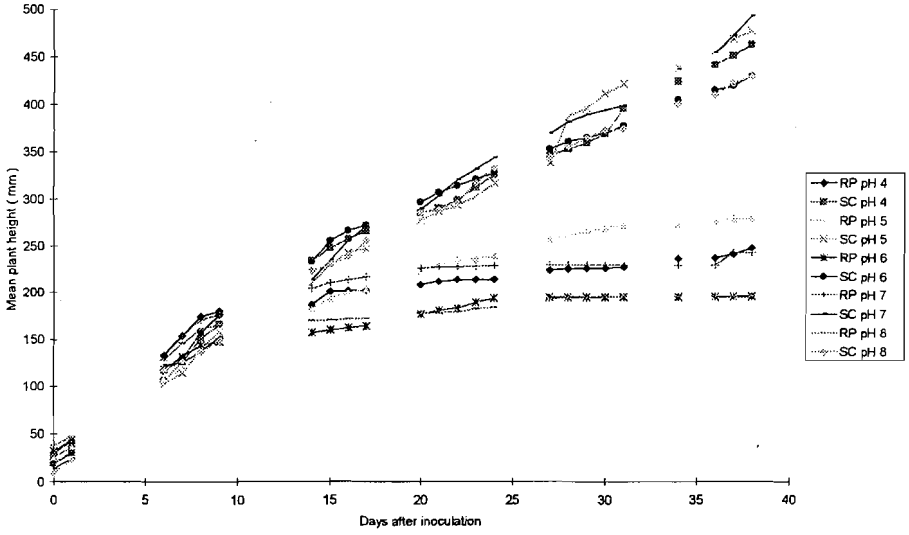

Fig. 1. Mean height of rice plants at different times after inoculation with root powder (RP) or selected cystosori (SC) and growing in media of different pH values

The progress of infection in rice was studied by estimating zoospore production at different times after inoculation. Seedlings were inoculated by dipping their roots in infected root powder prior to planting. At daily intervals starting 7 days after inoculation, two pots per day were removed from the hydroponic tank and the sand allowed to dry out overnight. The roots were then quickly rinsed free of sand and submerged in a dilute nutrient solution containing bovine serum albumen at 15°C for 1 hour. The zoospores released were then fixed by adding a few drops of KI solution and their concentration estimated using a haemacytometer. Plant and root fresh weights were also recorded. Large numbers of zoospores were obtained between 2 and 3 weeks after inoculation, with the greatest numbers 15 days after inoculation (Fig. 2). After this time, increasing numbers of resting spores were produced.

In three identical experiments, four different watering regimes were compared for their effect on the development of *P. graminis*. The regimes were (1) continual flooding, (2) normal (6h flooding : 6h drying), (3) normal with none on alternate days and (4) minimum hand watering. In the first experiment, zoospore production was again used as a measure of the development of *P.*

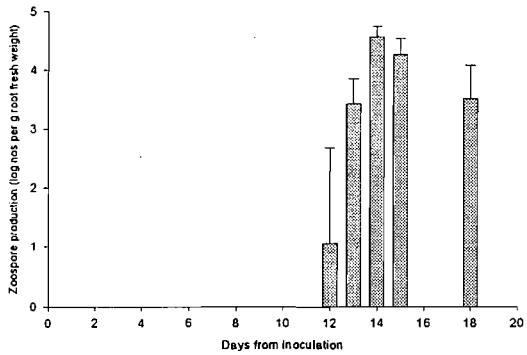

Fig. 2. Production of zoospores from rice plants at different times after inoculation

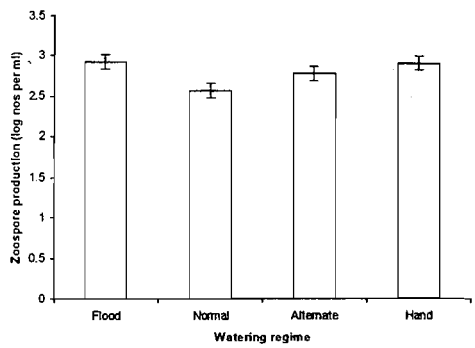

Fig. 3. Zoospore production from roots of rice plants maintained under four different watering regimes for 16 days after inoculation

graminis and the results (Fig. 3) showed little difference between the treatments although slightly less in the normal watering regime. In the second and third experiments very little *P. graminis* developed in any treatment.

In four experiments, seedlings of various cereal species were inoculated with infected root powder and the development of *P. graminis* assessed microscopically, usually about 3-4 weeks after inoculation. Although *P. graminis* developed only poorly in these experiments, the fungus was seen not only in roots of rice (cv. Oryzica 3) but also in those of oats (cv. Peniarth), Sorghum (ET3491) and forage maize. No evidence of fungus development was seen in barley (cv. Maris Otter) or wheat (cv. Galahad).

Two independent clones of plasmodiophorid rDNA from the Meta sample and three from the Tolima sample were obtained and analysed. They all showed the same gel patterns after digestion with restriction enzymes and the patterns were slightly different from those of other *Polymyxa* isolates. The sequences of all clones (between NS7 and ITS4) were identical and have been deposited in the EMBL database with accession no. AJ010424. The sequence of a *Ligniera* isolate was also completed (we previously reported only the sequence between ITS4 and ITS5) to use in the phylogenetic analyses and was deposited in the EMBL database with accession no. AJ010425.

The NS7-NS8 and ITS5-ITS4 regions were used separately in the phylogenetic analysis since complete NS7-ITS4 sequences are not available for some of the organisms used in the comparisons. The phylogenetic tree using all the known plasmodiophorid ITS4-ITS5 sequences and with *Olpidium brassicae* used as the outgroup (Fig. 4) strongly supports the identity of the Colombian RSNV-associated fungus as *Polymyxa graminis*. It groups with Type II *Polymyxa graminis* isolates originating from barley and oats (Ward & Adams, 1998) with a high bootstrap value (96%). As in earlier phylogenetic analyses there is some support for the separation of *Polymyxa* species (*P. betae* and *P. graminis*), but this is not unequivocal, the bootstrap value for the *P. graminis* node is only 43%. Evidence for the *Polymyxa* genus grouping is greater, the bootstrap value for this node is 96%. *Ligniera* is *Polymyxa*'s closest known relative and *Plasmodiophora* and *Spongospora* form a separate, well supported grouping. For the NS7-NS8 sequence analysis a much wider range of eukaryotic taxa were used including fungi, protozoa, plants and stramenopiles (see Ward and Adams, 1998). The results of the new analyses (including

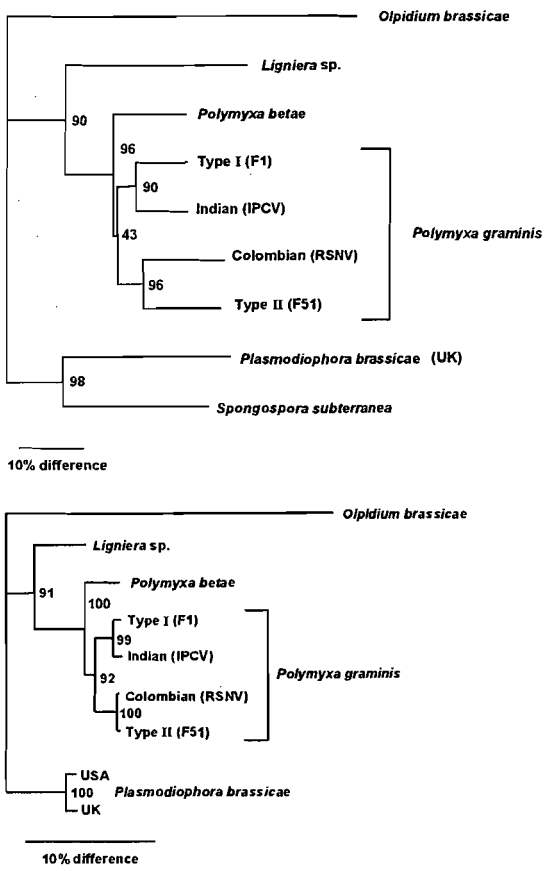

Fig. 4. Phylogenetic (NEIGHBOR) trees of (top) the ITS4-ITS5 and (below) the NS7-NS8 rDNA regions of plasmodiophorid fungi, with *Olpidium brassicae* as the outgroup

the Colombian *Polymyxa* isolate) using NEIGHBOR, was essentially the same as that reported previously (Ward and Adams, 1998). The plasmodiophorid grouping was well defined, with 100% bootstrap value, but the plasmodiophorids did not group with any other taxa. The groupings within the plasmodiophorids (Fig. 4) were identical to those for the ITS4-ITS5 region although *Spongospora* was not included as its sequence in this region is not available. In this analysis, the support for the separation of *Polymyxa* species was much higher.

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SEROLOGICAL DETECTION AND QUANTIFICATION OF *OLPIDIUM BRASSICAE*, AN IMPORTANT VIRUS VECTOR

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Summary

A polyclonal antiserum has been produced against the zoospore stage of *O. brassicae*. This has been shown to be very sensitive in detecting zoospores in ELISA and was found to cross-react with the resting spores of *O. brassicae*. The minimum detection limits for these two stages have been determined. Cross-reactivity with a range of other organisms is being evaluated. The antiserum has given very sensitive detection of infection of lettuce roots by *O. brassicae*. Direct tests on spiked soils have failed to demonstrate detection of resting spores, so far. However, indirect tests performed by baiting spiked soils with lettuce seedlings and subsequently testing the roots of the lettuce plants by ELISA has given very sensitive detection. Using the antiserum, it has been possible to visualise *O. brassicae* and infected cells in plant roots by immunofluorescent microscopy. The antiserum is currently being further evaluated. *O. brassicae* transmits lettuce big-vein virus (LBVV). We have also recently produced a monoclonal antibody to LBVV-associated particles. This monoclonal antibody is in the process of being assessed and characterised.

Introduction

Olpidium brassicae is a soil-borne obligate zoosporic fungal parasite of plant roots. It is a member of the Chytridiomycetes and is important as the vector of a number of plant viruses. In the UK, the most important of these viruses is lettuce big-vein virus (LBVV). LBVV is also an important disease of lettuce in Spain where much of the lettuce for northern Europe is grown in winter. The main symptoms of the disease are vein-banding accompanied by crinkling and distortion of leaves. It can prevent or delay head formation, decrease head size and reduce the proportion of harvestable heads (Ryder, 1980). Control of LBVV is virtually impossible once *O. brassicae* is established in lettuce fields. The resting spore stage has been shown to remain viable for in excess of 20 years (Campbell, 1985). Attempts to eradicate *O. brassicae* by soil sterilisation have proven mostly ineffective.

We are developing an integrated control strategy for big-vein. This is based on good hygiene and the use of disinfectants during propagation (in order to provide healthy planting material), deployment of biological control agents, development of resistant lettuce and systems for disease avoidance, based on detection of *O. brassicae* and LBVV in field soils. This paper describes the serological detection and quantification of *O. brassicae* and discusses the potential of vector and virus detection as components of an integrated strategy for controlling LBVV.

Materials and Methods

A polyclonal antiserum has been produced against the zoospore stage of *O. brassicae*. It has been tested in a number of ELISA formats for its ability to detect the different stages of the fungal life cycle released from lettuce roots. Dilution series of zoospores and resting spores were made and tested by ELISA in order to establish minimum detection limits. The antiserum has also been used in immunofluorescent microscopy of lettuce roots. The ability of the antiserum to detect and quantify *O. brassicae* in infected plant roots and in soil has been evaluated.

By growing lettuce under optimum conditions for big-vein symptom expression (Walsh, 1994), we have been able to induce symptoms all year round. This has allowed us to purify LBVV particles and produce a monoclonal antibody to them. The monoclonal antibody is currently being characterised and assessed for its ability to detect LBVV.

Results

The polyclonal antiserum to *O. brassicae* gave very sensitive detection of zoospores in ELISA and was found to cross-react with *O. brassicae* resting spores. The minimum detection limits for these two stages have been determined. Cross-reactivity with a range of other organisms is being evaluated. The antiserum has given very sensitive detection of *O. brassicae* infection in lettuce roots. Direct tests on spiked soils have so far failed to demonstrate detection of resting spores. However, indirect tests performed by baiting spiked soils with lettuce seedlings and subsequently testing the roots of these lettuce plants by ELISA, has given very sensitive detection. It was possible to visualise *O. brassicae* and infected cells in plant roots by immunofluorescent microscopy. The antiserum is currently being further evaluated.

In conjunction with immuno-sorbent electron microscopy (ISEM), the monoclonal antibody has been shown to specifically decorate virus-like particles reported by others (Kuwata *et al.*, 1983; Vetten *et al.*, 1987; Huijberts *et al.*, 1990) to be the causal agent of, or associated with big-vein. The antibody has failed to reveal such particles in healthy lettuce plants. When tested in ELISA, infected leaves with typical big-vein symptoms gave high absorbences, whereas leaves from healthy lettuce plants gave very low absorbences close to zero.

Discussion

If sensitive and accurate serological detection of *O. brassicae* and LBVV can be achieved, this will allow screening of lettuce and related germplasm for resistance to both the fungus and the virus to commence. Formerly, resistance selection has been based on screening for resistance to symptom development. Using the antisera to test soil samples from lettuce fields, or plants baited in such soil samples during the winter months in the UK, or the summer months in warmer regions of the world where lettuce production occurs in the winter months, it should be possible to quantify inoculum levels. It will then be possible to advise farmers on infestation levels so that they can

reduce the risk of big-vein infection by practising disease escape. Where low infestation levels are detected, it may be possible to produce symptomless crops by deploying suitable control measures.

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CHARACTERIZATION OF NATURAL CUCUMBER NECROSIS VIRUS MUTANTS DEFICIENT IN FUNGUS TRANSMISSION

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Summary

Previous work has demonstrated that the CNV coat protein (CP) contains determinants that specify transmission by *Olpidium bornovanus*. In addition, *in vitro* binding studies have suggested that zoospores contain specific receptors for virion attachment. This study identifies six naturally occurring CNV transmission mutants. In each of the mutants a single amino acid substitution in the CP was found to be responsible for the reduction in transmission. Four mutants mapped to the CP protruding domain and two others mapped to the shell domain. Interestingly, mutations in three of the protruding domain mutants affect amino acids which are adjacent to each other in the particle tertiary structure and one of the shell domain mutations is in the proximity of a previously identified shell domain transmission mutant. *In vitro* binding studies showed that each transmission mutant binds to zoospores at reduced efficiency compared to wild type (WT) CNV particles. These studies suggest that specific regions of the CNV CP serve as viral attachment sites for binding to a specific zoospore receptor.

Introduction

Cucumber necrosis virus (CNV) is one of several viruses in the *Tombusviridae* transmitted by *Olpidium* spp. (Campbell, 1996; Adams, 1991). Transmission occurs following independent release of zoospores and virus into the soil and subsequent adsorption of virus particles onto the surface membrane of motile zoospores. Transmission of virus to plants occurs upon encystment of zoospores on host roots.

Previous work has shown that the CNV coat protein contains determinants that specify transmission by *O. bornovanus* zoospores (McLean *et al.*, 1994). The CNV particle is spherical and consists of 180 identical 41 kDa coat protein subunits. By analogy to the TBSV coat protein, the CNV coat protein consists of three major structural domains: an inward facing RNA binding domain (R), the shell (S) and outward facing protruding domain (P).

Robbins *et al.* (1997) showed that a single amino acid substitution (Glu to Lys) in the CNV coat protein S domain was responsible for the loss of transmission of a naturally occurring CNV mutant (LL5). *In vitro* binding studies demonstrated that LL5 binds to zoospores with reduced efficiency suggesting that the Glu to Lys substitution affected attachment of CNV virions to a putative zoospore receptor. We wished to characterize other CNV coat protein transmission mutants to determine if other regions of the CNV coat protein are involved in attachment to zoospores.

Materials and Methods

Isolation of CNV transmission mutants. Isolation of CNV transmission mutants was essentially as previously described (Robbins *et al.*, 1997). CNV was mechanically passaged 12 times through the systemic host, *Nicotiana clevelandii.* Leaves from the final passage were inoculated onto cucumber and individual local lesions were then further "purified " by a second passage

through cucumber. Purified virus was then multiplied in *N. benthamiana* and virus was tested for transmission by *O. bornovanus*.

Transmission assay. Virions were assessed for transmission *by O. bornovanus* (SS196 isolate) as previously described (McLean *et al.*, 1994). Specified amounts of virions were incubated with zoospores (1 X 10^4) for 15 min and then poured into sand containing cucumber seedlings. Roots were assessed for virus five days post-inoculation using DAS-ELISA.

Cloning and sequence analysis of transmission mutants. The coat protein gene of putative CNV transmission mutants was amplified from total leaf RNA extracts or directly from virions using RT-PCR and oligonucleotide primers that flank the CNV coat protein gene. The coat protein gene was then cloned in place of the wild type CNV coat protein gene in an infectious CNV cDNA clone. Transcripts of the clone were inoculated onto *N. benthamiana* and virus obtained from infected plants was tested in a transmission assay to confirm reduced transmission. Once confirmed, the coat protein gene insert was sequenced to determine the location of the mutation.

In vitro binding assay. The assay used was modified from a previous binding assay (Robbins *et al.*, 1997) described in more detail by Robbins and Rochon. (this Proceedings). Briefly, 100 ug of virions was mixed with 5 X 10^5 zoospores in 1 ml of 50 mM $NaPO_4$, pH 7.6 for 1 hr at room temperature. Zoospores (and any bound virus) were pelleted by low speed centrifugation, washed and then detected and quantified by Western blot analysis or slot blot analysis using a CNV monoclonal antibody.

Results

Identification and characterization of CNV mutants deficient in fungus transmission

Virus obtained from single local lesions was analyzed for transmission using a previously described transmission assay (McLean *et al.*, 1994). Of 87 local lesions examined (see Materials and Methods) virus from six lesions was found to consistently show reduced transmission (Table 1). LLK8, LLK10 and LLK63 transmitted at a low efficiency (i.e., 21%, 27% and 14% that of wild type CNV, respectively) whereas LLK82, LLK84 and LLK85 transmitted at a higher efficiency (75%, 50% and 85% that of CNV).

TABLE 1

Transmission efficiency of several CNV variants

Virus/Mutant	Transmission frequency[1]	% transmission[2]
WT CNV	49/51	96
LLK8	3/14	21
LLK10	4/15	27
LLK63	3/21	14
LLK82	15/20	75
LLK84	10/20	50
LLK85	17/20	85

[1]Refers to the number of plants showing transmission versus the number of plants tested.

[2]Calculated from the transmission frequency.

The coat protein gene of each of the cloned transmission mutants was sequenced to determine the location and nature of the mutation. A single amino acid change in each of the mutants was found to be responsible for the reduced transmission (Fig.1). Two of the transmission mutants, LLK85 and LLK84, contained amino acid substitutions in the coat protein shell domain whereas the remaining (LLK8, LLK10, LLK63 and LLK82) contained substitutions in the coat protein protruding domain. Interestingly, the mutations in LLK8 and LLK10 are immediately adjacent to each other in the primary sequence of the coat protein. Moreover, structural analysis of the CNV coat protein subunit indicates that the amino acid substitution in LLK82 is located near those of LLK8 and LLK10 in an adjacent antiparallel B sheet. The mutations in the previously described LL5 transmission mutant (Robbins *et al.*, 1997) and LLK84 are also juxtaposed in the three dimensional structure of the CNV coat protein. In addition, both are located in loops in regions of the CNV coat protein known to be involved in trimer subunit contacts. The mutation in LLK63 is located in a loop at the tip of the protruding domain (not shown).

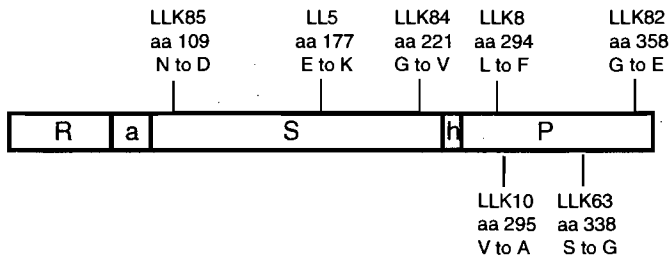

FIG. 1. Location of amino acid substitutions in the coat protein of several CNV transmission mutants. The CNV coat protein along with individual domains (R, a, S, h, and P) are shown diagrammatically. The location of amino acid substitutions in the coat protein as well as the amino acid change is indicated.

In vitro binding assays.

LL5 was previously shown to bind zoospores with reduced efficiency in an *in vitro* binding assay (Robbins *et al.*, 1997) suggesting that the loss of transmission of this mutant was at least partially due to its reduced ability to bind zoospores. Table 2 summarizes the results of *in vitro* binding assays conducted with the six transmission mutants identified in this study. Each of the mutants was found to show reduced binding (ranging on average from approximately 30-90% of WT binding). In addition, an artificially constructed double mutant LL5K8 that contains the amino acid substitutions of both LL5 and LLK8 shows only about 25% of WT CNV binding (see Table 2), less binding than is observed for either single mutation. These data suggest that the reduced transmission of the mutants described in this study is due, at least in part, to reduced ability to bind zoospores.

TABLE 2

Binding efficiency of transmission mutants

Virus/Mutant	% of WT CNV binding
WT CNV	100
LLK8	74 +/- 7
LLK10	44 +/- 16
LLK63	25 +/- 17
LLK82	68 +/- 16
LLK84	86 +/- 26
LLK85	49 +/- 7
LL5K8	25 +/- 7

Discussion

The six transmission mutants identified in this study all affect binding of particles to zoospores raising the suggestion that the mutated sites represent virus attachment sites for a putative zoospore receptor (see Robbins *et al.*, this Proceedings). It is possible that there are two or perhaps three main attachment sites, two of which involve more than one amino acid. One virus attachment site is proposed to be located in the coat protein shell domain and includes the mutated sites identified in LL5 and LLK84. The other is proposed to be located in the protruding domain and includes mutated sites identified in LLK8, LLK10 and LLK82. A possible third site is the one identified by LLK63. A more extensive site-directed mutational analysis of these regions may help to elucidate the contributions these regions make to zoospore attachment.

Acknowledgments

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Heterologous expression of the coat protein and P39 genes of *Indian peanut clump virus*

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Summary

Of the proteins of *Indian peanut clump virus* (IPCV), the prime candidates for involvment in its transmission by *Polymyxa graminis* are the coat protein and P39. Genes of both proteins have been cloned for use in a prokaryotic expression system, in order to purify the proteins by affinity chromatography and to produce specific antisera.

Large amounts of coat protein were produced by expression of the CP gene alone. However, co-expression of CP + P39 suppressed the production of CP. These results are the first steps of a programme aimed at understanding the role of these two proteins in the assembly of the capsid and in the transmission of the virus.

Introduction

Indian peanut clump virus (IPCV) (genus *Pecluvirus*) is responsible for clump disease of groundnut in the Indian Subcontinent, where it causes annual losses of up to 38 million US dollars (Reddy *et al.,* 1999).

The genomes of IPCV and *Peanut clump virus* (PCV) have been sequenced (Herzog *et al.,* 1994, Miller *et al.,* 1996, Naidu *et al.,* 1996), as have those of other former furoviruses. To evaluate the potential of a coat protein-mediated resistance strategy, transgenic *Nicotiana benthamiana* plants have been obtained that express sequence from the 5' region of RNA-2 of IPCV-H that contains the translatable sequence of the coat protein (CP) gene (Wesley, 1993; Mayo & Naidu, 1996).

Several lines showed some resistance and expressed levels of proteins detectable by ELISA and immunoblotting. When extracts of some of the transgenic plants, or extracts of *Escherichia coli* transformed so as to express CP were examined, virus-like particles (VLP) were observed (Bragard *et al.,* 1998). A challenge inoculation of the aforementioned transgenic *N. benthamiana* with a different strain of IPCV resulted in the formation of hetero-encapsidated particles (Bragard *et al.,* 1999).

In order to understand the phenomenon better, the IPCV coat protein gene and the next downstream gene (P39) have been cloned and expressed in *E. coli*.

Materials and methods

RT-PCR, cloning and expression

The IPCV coat protein and P39 genes were obtained by RT-PCR. Specific primers were designed to amplify the targetted sequence. Taq polymerase-amplified PCR products were inserted into a T-overhang cloning and expression vector (pBAD TOPO TA, Invitrogen, The Netherlands). The vector pBAD allows the production of a recombinant fusion protein including the inserted gene plus a V5 epitope tag and a C-terminal polyhistidine (6X His) region. This should allow the detection by the use of antibodies to V5 (Invitrogen) or to hexa-histidine. A pET vector that contained the CP gene of IPCV (Naïdu, unpublished) was also used.

For expression, manufacturer's instructions or protocols described elsewhere (Bragard *et al.*, 1999) were followed.

Electron microscopy

For electron microscopy, frozen suspensions of partially purified *E. coli* were thawed on ice and centrifuged at 4°C at 6000 x g for 10 min to remove insoluble material. The remaining solution was kept on ice and sonicated. ISEM and antibody coating (AC) of antibody-trapped VLP were done as described by Roberts (1986). Antibodies to IPCV were diluted in 0.06M phosphate buffer, pH 6.5. Grids were negatively stained with 2% sodium phosphotungstate, pH6, and examined in a JEOL SX-100 operating at 60 kvolts.

Results

Six different plasmids were obtained. They contained the coat protein gene, the P39 gene or both. For each of these three fragments, clones were prepared by suppressing the stop codon so as to add an histidine tag or not. Coat protein could not be detected by SDS-PAGE of total *E. coli* extracts, but was found by immunoblotting. The expression level was tightly controlled by using the L-arabinose regulation system of pBAD. The duration of the induction period and the level of β-mercaptoethanol used in the system affected the results (Fig. 1). No VLP were detected in extracts of cells expressing IPCV-L CP, in contrast to results with constructs containing IPCV-H CP (Fig. 2).

The 39 Kda protein was not readily detectable, either by SDS-PAGE or immunoblotting. Nevertheless, by using larger culture batches of the histidine-tagged clones, affinity chromatography purification and a concentration step, a protein possibly corresponding to the 39K was detected in expression products of both pIPCVP39HIS and pIPCVCPP39HIS.

Discussion

Expression in a prokaryotic system resulted in the production of large amounts of IPCV CP. Coat protein production was successfully regulated by L-arabinose. The IPCV-H CP produced was functional as it was able to interact to form multimers and

to assemble into VLPs detectable by ISEM, as previously described (Bragard *et al.*, 1999).

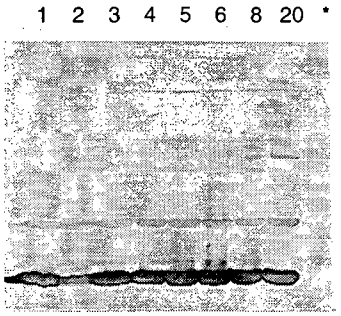

Fig. 1 : Detection of the induced IPCV-CP in *E. coli* by immunoblotting. Bacterial cells were grown for 4 hours before induction. Fractions collected after 1hr , 2hrs, 3hrs, 4hrs, 5hrs, 6hrs, 8hrs and 20 hrs respectively were electrophoresed (SDS-PAGE). Samples were transferred onto a nitrocellulose membrane and a IPCV rabbit polyclonal antiserum was used to detect the CP. Purifed IPCV-H was used as a positive control (lane *).

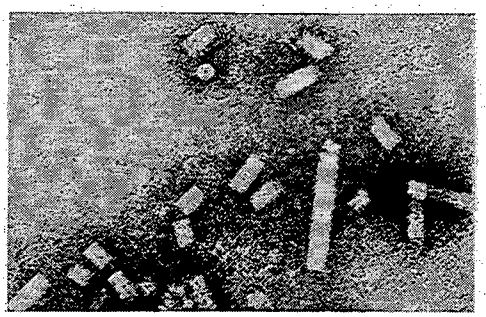

Fig. 2 : Electron micrographs of virus-like particles trapped on grids with antibodies to IPCV-H, from extracts of *E. coli* expressing pET containing the IPCV-H CP gene.

However, the behaviour of IPCV CP may not be like that of other plant viruses transmitted by fungi because the CP genes of pecluviruses are unusual in lacking an open reading frame (ORF) downstream of the CP gene that is expressed by readthrough suppression of the coat protein gene termination codon. A leaky scanning mechanism has been proposed for the expression of the PCV P39 gene (Herzog *et al.*, 1995). Such a gene might be involved in particle assembly as well as in the transmission process, as for RT protein of other similar viruses.

These results raise many questions regarding I) the presence of an origin of assembly (OAS) or self-endoreplicating fragment (SERF) within the coat protein

gene sequence, II) possible genomic masking or III) implications for transmission by *Polymyxa*, the putative vector.

Acknowledgements

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COMPARATIVE ANATOMY, TAXONOMY AND BIOLOGY OF *POLYMYXA BETAE* AND *POLYMYXA GRAMINIS:* AN OVERVIEW

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Summary

A brief history of the taxonomy of this group of organisms is presented. Also included is an overview of their biology, including their host ranges and the viruses they transmit; emphasis is placed primarily on their anatomy, developmentally and at maturity.

The genus Polymyxa, with *P. graminis* as the type species, was establishd by Ledingham (1939). *P. betae,* the only other species known, was later identified from roots of sugar beet (Keskin, 1964). The genus has commonly been classified as a fungus within the Class Plasmodiophororomycetes by most mycologists. However, because they share numerous anatomical, biological and biochemical properties with other groups of organisms, they are now more often regarded not as fungi, and are placed instead into Phylum Plasmodiophoromycota or Myxomycota of Kingdom Protista (Alexopoulos *et al.*, 1996; Beakes, 1998). The Plasmodiophoromycota, commonly referred to as "plasmodiophorids", are however still discussed in many mycology and plant pathology texts, e.g. Alexopoulos et al. (1996), where they are recognized as a group closely related anatomically, pathologically and economically to fungi. The major importance of *P. betae* and *P. graminis* is not as plant pathogens *per se,* rather as vectors for several important plant pathogenic viruses. Although several biological and biochemical properties tend to differentiate the two species, many common anatomical properties tend to unify them. The two species, as well as some strains of *P. graminis,* can be differentiated by their ribosomal DNA profiles (Legrève, et al., 1996; Ward & Adams, 1996,1998) and optimum temperature requirements for growth (Delfosse, et al., 1996). *P. betae* infects numerous dicot hosts (Barr & Asher, 1992); *P. graminis* infects mostly grass and cereal hosts (Barr, 1979). Both are obligate parasites that invade and reside in host root cortex tissue. *P. betae* is known to transmit four viruses, whereas *P. graminis* transmits at least twelve (Table 1). Of the viruses transmitted by *P.betae,* beet necrotic yellow vein virus is the most important, causing the disease rhizomania in many sugar beet producing regions of the world. Several of the viruses transmitted by *P. graminis* are very important economically, but are primarily of just regional importance.

Table 1. Viruses transmitted by *Polymyxa betae* and *P. graminis*

P. betae:
- Beet necrotic yellow vein virus
- Beet soil borne virus
- Beet soil borne mosaic virus
- Beet virus Q

P. graminis:
- Wheat soil-borne mosaic virus
- Wheat spindle streak mosaic virus
- Wheat yellow mosaic virus
- Chinese wheat mosaic virus

P. graminis (continued):
- Barley mild mosaic virus
- Barley yellow mosaic virus
- Oat golden stripe virus
- Oat mosaic virus
- Rice necrosis mosaic virus
- Rice stripe necrosis virus
- Indian peanut clump virus
- Peanut clump virus

The life cycle of *Polymyxa* spp. is complex (Fig. 1). Following initial infection newly formed plasmodia may differentiate into either: (a) asexual zoosporangia that produce zoospores, providing secondary inoculum for repeated infections during the same growing season, or (b) thick walled, presumably sexual stage resting spores borne in clusters (sporosori) that can remain viable for many years in soil; upon germination resting spores produce zoospores that function as primary inoculum (Karling, 1968; Teakle, 1969).

Anatomically *P. betae* and *P. graminis* are indistinguishable both during development and at maturity. Following is a summary of anatomical properties of the life cycle, drawn from both species, based on light and electron microscopy (Barr, 1979; Barr & Asher,1996; Chen, et al., 1998; Dahm & Buchenhauer, 1993; Ledingham, 1939; Littlefield et al., 1997,1998). Germination of resting spores to form primary zoospores has not been studied in detail, but the presence of specialized penetration organelles, e.g. the stachel and rohr in secondary zoospores (Barr, 1988; Keskin & Fuchs, 1969), suggests a host penetration mechanism similar to that in *Plasmodiophora brassicae* (Aist and Williams, 1971). Following host cell penetration small aggregates of plasmodia are formed (Figs. 2,3), followed by development of larger plasmodial masses. The latter can differentiate to form either zoosporangia (Figs. 4,5) or sporosori (Figs. 7-10). Zoosporangia are enclosed by thin cell walls; sporogenic plasmodia in host cells are enclosed only by a membrane. Zoosporangia produce several hundred zoospores (Fig. 6) that are released through sporangial exit tubes (Fig. 5). Sporogenic plasmodia (Fig. 7) cleave synchronously, forming masses of thick walled resting spores aggregated into sporosori (Figs. 8-10). Resting spores are surrounded by four layers of cell wall material plus a fifth layer, the apical cap layer, that is restricted to the end of the spore facing the periphery of the sporosorus (Fig. 11). See Littlefield et al. (1998) for a more complete list of references dealing with light and electron microscopy of *P. betae* and *P. graminis*. Beet necrotic yellow vein virus (Dubois, et al., 1994; Rysanek, et al., 1992; Scholten, et al., 1994) and barley mild mosaic virus (Jianping, et al., 1991) have been identified in their vectors and/or host roots by immunolabeling.

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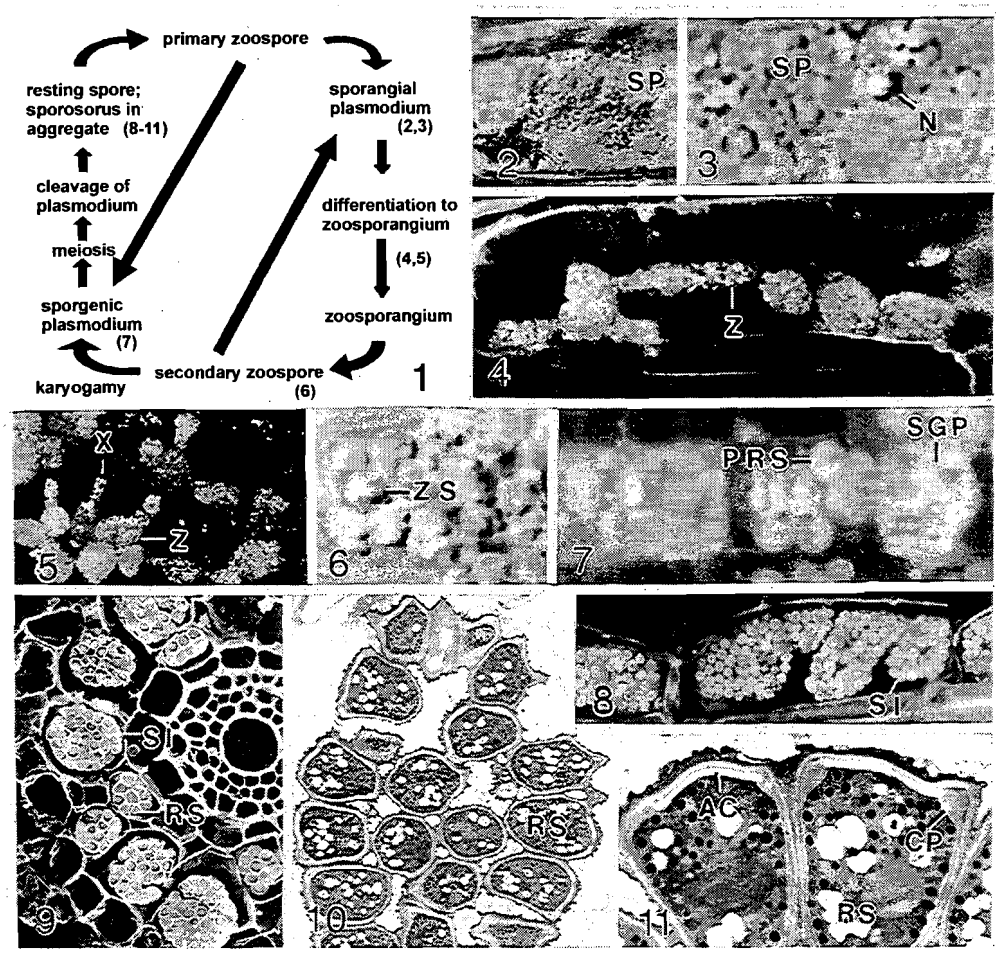

Fig. 1. Abbreviated life cycle diagram for *Polymyxa* spp. Numbers indicate corresponding figures. **Figs. 2-11.** Light- (LM), laser confocal- (LCM), scanning- (SEM) and transmission electron (TEM) micrographs of several developmental stages of *P. graminis*. **2.** & **3.** Zoosporangial plasmodia (SP); N = nucleus; LM. X300 & X1600, respectively. **4.** Elongated, immature zoosporangium (Z) in root cell, not yet differentiated to form zoospores; LCM. X680. **5.** Fully developed, multi-lobed zoosporangia (Z), with zoospore exit tubes (X) extending from lobes; LCM. X340. **6.** Zoospores (ZS) in mature zoosporangium; LM. X1460. **7.** Sporogenic plasmodium (SGP) yet undifferentiated into resting spores, and protoplasts of mature resting spores (PRS); walls of resting spores are not visible at this magnification in LCM. X1040. **8.** Host cortical cells containing sporosori (SI) comprised of many resting spores; LCM. X380. **9.** Cross section of host root containing sporosori (SI), each comprised of aggregated resting spores (RS); SEM. X400. **10.** Cross section of a sporosorus, showing numerous, partially fused resting spores (RS); TEM. X2400. **11.** Longisection of two resting spores (RS); apical cap layer (AC) of the multilayered cell wall and typical conical projections (CP) located on the apical periphery of the spores are clearly visible; TEM. X7900.

THE DEVELOPMENT OF A RECOMBINANT ANTIBODY TO *POLYMYXA BETAE* AND ITS USE IN RESISTANCE SCREENING.

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Summary

Antibodies to the obligate sugar-beet root parasite *Polymyxa betae* have been produced using a novel approach that selects for fungal gene sequences encoding proteins that are highly expressed *in planta* during infection. Recombinant fusion proteins expressed from these sequences *in vitro* were then used as immunogens to raise antibodies in animals. This approach resulted in the production of precisely targeted antibodies that specifically cross-reacted with *P. betae* in sugar-beet roots without the need for further purification. Subsequent tests also showed cross reactions with the closely related *Polymyxa graminis* in barley roots and *Plasmodiophora brassicae* in cabbage roots. In all cases, there was no interaction with proteins from host plants, or from other microorganisms commonly found in association with sugar-beet, barley or cabbage roots. Western blot analysis was used to differentiate inbred *P. betae*-resistant and susceptible hybrid sugar-beet lines inoculated in a standard glasshouse test. An ELISA-based detection system for *P. betae* is now being developed for routine high throughput screening of resistant germplasm.

Introduction

Polymyxa betae Keskin is an obligate parasite of sugar beet roots and the vector of beet necrotic yellow vein virus, the causal agent of rhizomania disease (Asher 1993). It is therefore a pathogen of significant economic importance but its obligate parasitic nature makes it difficult to work with. Despite this, nucleic acid-based molecular diagnostic methods have been developed for detecting the fungus in roots (Mutasa *et al.,* 1996) and for distinguishing fungal isolates (Ward & Adams, 1998). Consequently, the molecular resources and techniques for manipulating *P.betae* have become well established and now provide the opportunity to selectively clone, characterise and exploit fungal genes. Here, we describe a recombinant DNA approach to preparing *P. betae* immunogens free from host plant material. Unlike methods that depend on the use of infected plant tissue, this approach has the advantage of allowing the selection of strongly expressed fungal genes, whose products are present at high levels in infected plants and are therefore readily detected. It also provides the opportunity for rigorous screening and characterisation of potential immunogens at the nucleic acid level by Southern hybridisation, Northern hybridisation and sequencing before selecting the best candidates for animal immunisation. The polyclonal antiserum produced using this technique allowed the selection of *P. betae* resistant sugar-beet lines by western blotting, without the need for further purification.

Materials & Methods

Fungal culture & cDNA library construction. 10-day old sugar beet seedlings *(Beta vulgaris* cv. Hilma) were inoculated with *P. betae* as previously described (Mutasa *et al.*, 1993) and grown in sand culture (Adams *et al.*, 1986). RNA was extracted from infected sugar beet roots according to the protocol previously described by Chirgwin *et al* ., (1979). Two µg of fractionated mRNA was then used to make a ZAP-cDNA library (Stratagene Europe, Amsterdam, The Netherlands).

Isolation & characterisation of candidate gene sequences. Abundant fungal cDNA sequences were selected by plus/minus differential screening of the cDNA library using total RNA probes from infected and uninfected sugar-beet roots and confirmed by Southern blot hybridisation. Northern blot analysis was then used to determine expression in *P. betae* at different stages of development within plant roots. Sequences expressed at all stages of fungal growth were selected and sequenced.

cDNA expression, protein purification & animal immunisation. Selected sequences were expressed as N-terminal histidine tagged fusion proteins in *E. coli* JM109, using the Xpress Protein Expression & Purification System (Invitrogen BV, Groningen, The Netherlands). 50µg of purified protein emulsified in PBS and Freunds complete adjuvant (Sigma Chemical Co., Poole, Dorset. UK) was used to immunise a female Dutch rabbit. Four weeks later the rabbit was again immunised with 50µg of antigen emulsified in Freunds incomplete adjuvant. Blood was collected 14 days after the last immunisation and at three weekly intervals thereafter until a total of 10 bleeds had been taken. The serum fraction was collected and stored at -20°C until required.

Western blot analysis by standard protocols (Sambrook *et al.*, 1989) was used to determine specificity of antibodies by testing against total protein extracts from infected and uninfected plants. The utility of the antibodies as a tool for selecting for *P. betae* resistance in young plants was then assessed by screening inbred lines generated by hybridisation of susceptible cultivars with resistant wild *Beta* sources.

Results & Discussion

Plus/minus differential screening proved to be a very powerful technique for selectively isolating gene sequences from *P. betae* and is applicable to any obligate pathogen-host system. Sequence data analysis revealed that one of the *P. betae* genes selected as being highly expressed *in planta* encoded a glutathione-s-transferase (Accession number: AJ132355), and was expressed at all stages of fungal development (fig 1). This is particularly important for *P. betae* which has distinct morphological structures at different stages in the life cycle, with very different surface properties (Keskin 1964; D'Ambra & Mutto 1977). It is therefore very likely that conventional antibodies raised against one structure would fail to cross react with another (Thornton & Dewey 1996).

Glutathione-s-transferase (gst) and related enzymes have a well recognised role in overcoming the host plant's defence mechanism, by detoxification of reactive oxygen produced during the hypersensitive response in plants (Levine *et al.*, 1994; Heath 1996).

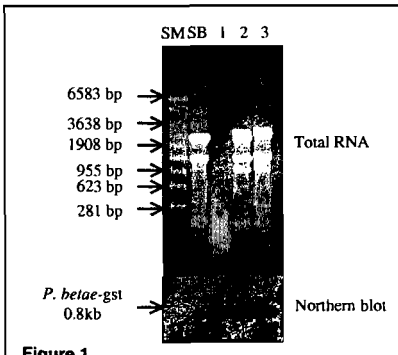

Figure 1

P. betae-gst is expressed at all stages of fungal development. RNA from old, dry resting spores was too degraded to give a signal.
SM= size marker; SB= control roots; 1= dry resting spores; 2= zoosporangia; 3= fresh resting spores.

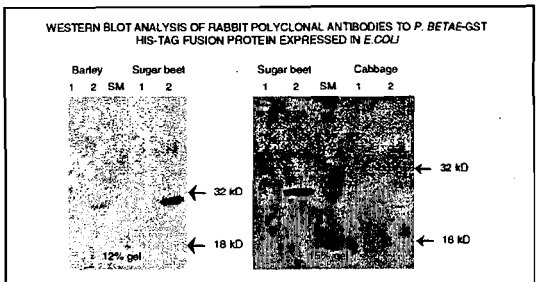

Figure 2

Western blot of sugar-beet, barley and cabbage roots uninoculated (1) or inoculated (2) with *P. betae, P. graminis,* and *Plamodiophora brassicae* respectively. SM= size marker.

The *P. betae* gst protein sequence was therefore a good candidate for expression and antibody production. Figure 2 clearly shows that rabbit polyclonal antibodies raised against the *P.betae*-gst histidine tagged fusion protein specifically cross reacted with *P. betae* in sugar-beet roots, and was also able to react with the closely related *Polymyxa graminis* and *Plasmodiophora brassicae* in their respective barley and cabbage plant hosts. In all cases, the antiserum reacted only with protein extracts from the pathogen and not the plant host or contaminating microorganisms. Our method therefore produced highly specific and precisely targeted polyclonal antibodies without the

need for further purification.

In other systems where antiserum against plant pathogenic bacteria or fungi has been developed, similar levels of specificity have been achieved only with monoclonal antibodies (Thornton *et al.*, 1997; Griep *et al.*, 1998). This often requires extensive screening of many hybridomas, particularly in the case of obligate pathogens (Murdoch *et al.*, 1998), and retrospective confirmation that antibodies are cross-reacting with fungal epitopes, often by electron microscopy. Thus, although specificity can be achieved by currently available methods, they are more costly and require sophisticated equipment and animal tissue culture facilities.

On western blots, the antiserum was capable of detecting *P. betae* at levels normally encountered in naturally infested field soil. The utility of the antiserum for screening *P. betae* resistant sugar beet was demonstrated by the clear differences in susceptibility detected in 9 inbred lines derived from sugar beet x *P. betae* resistant *Beta maritima* hybrids (figure 3). Unlike PCR based methods, antibodies detect the actual amount of antigen without amplification and, in addition, differentiate between living and dead fungal biomass. Immunodetection also allows for more cost effective, robust, high throughput ELISA-based assays (currently under development), simplifying the screening of large breeding populations.

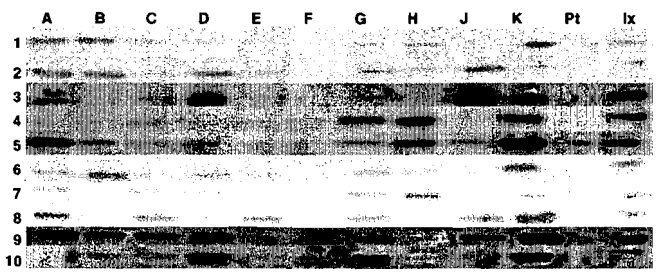

Figure 3

Western blot of total protein extracts from seedling roots of 10 plants from each of 9 inbrec families (A-J) derived from sugar-beet x *P.betae*-resistant *Beta maritima* hybrids. K= standarc sugar-beet cultivar, Pt= *Beta patellaris* (resistance control); Ix = infected roots (susceptible control).

E and F appear to be composed of predominantly resistant plants.

The development of antibodies specific to both *P. betae* and *P. graminis* allows detection and quantification of both of these important fungal vectors for the first time. These antibodies will also permit detailed analysis of the fungi in association with their plant hosts and associated viruses, through immuno-labelling technology. In addition to obligate pathogens however, there may also be a benefit to using this approach with culturable plant pathogens because it targets genes that are expressed by the pathogen *in planta*, and which may not necessarily be expressed *in vitro*.

Acknowledgements

The work was funded by the UK Sugar Beet Research & Education Fund. The authors are also grateful to Dr John Walsh for cabbage plants infected with *Plamodiophora brassicae* and Dr Mike Adams for isolates of *Polymyxa betae* and *Polymyxa graminis*. We would like to acknowledge Dr Berthold Göttgens for technical advice on protein expression, purification and analysis and for assistance with sequence data analysis.

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EVIDENCE THAT FUNGAL ZOOSPORES CONTAIN SPECIFIC RECEPTORS FOR TRANSMISSION OF CUCUMBER NECROSIS VIRUS

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Summary

The acquisition phase of CNV transmission by *Olpidium bornovanus* is believed to involve specific attachment of virus particles to the zoospore plasmalemma and axonemal sheath. An *in vitro* binding assay was developed to examine the interaction between CNV and *O. bornovanus* zoospores. Binding to zoospores was found to be specific; i.e., CNV, melon necrotic spot virus (MNSV) and cucumber leaf spot virus (CLSV), which are each transmitted by *O. bornovanus,* bound *O. bornovanus* zoospores to a higher level than tobacco necrosis virus (TNV) which is transmitted by *O. brassicae.* Conversely, TNV bound *O. brassicae* zoospores to a higher level than CNV, MNSV or CLSV. Binding of CNV *to O. bornovanus* zoospores was found to be saturable at high concentrations of virus. In addition, binding of MNSV and TNV could be competitively inhibited by excess CNV. Together, these data suggest that a specific receptor or receptor-like molecule mediates binding of CNV to *O. bornovanus* zoospores.

Introduction

CNV is one of several small icosahedral viruses in the *Tombusviridae* known to be transmitted by *Olpidium* spp. (Campbell, 1996). Transmission occurs following independent release of zoospores and virus into the soil and subsequent adsorption of particles onto the surface membrane of motile zoospores. Virus gains entry into root cells following encystment of fungal zoospores. Electron microscopy studies have suggested that adsorption of virus to the zoospore plasmalemma is very specific. Tobacco necrosis virus (TNV), which is transmitted by *O. brassicae* (but not *O. bornovanus*) binds *O. brassicae* zoospores (but not *O. bornovanus* zoospores) and CNV which is transmitted by *O. bornovanus* (but not *O. brassicae*) binds *O. bornovanus* zoospores but not *O. brassicae* zoospores (Temmink *et al.*, 1970). In addition, biological studies have shown that there are strains of *Olpidium* which vary in their ability to transmit a given virus (Temmink *et al.*, 1970; Campbell *et al.*, 1991; Adams, 1991) The specificity of interaction has suggested that both virus and zoospores possess specific components that determine transmissibility.

Previous work has shown that the CNV coat protein contains determinants for transmission by *O. bornovanus* (McLean *et al.*, 1994). In addition, studies of naturally occurring CNV mutants obtained by serial mechanical passage indicate that virions contain specific amino acids or sites which facilitate transmission and binding of particles to zoospores (Robbins *et al.*, 1997; Kakani *et al.*, this Proceedings). In this study, we wished to assess whether acquisition of CNV by *O. bornovanus* involves specific zoospore receptor or receptor-like molecules.

Materials and Methods

In vitro binding assay. *O. bornovanus* or *O. brassicae* zoospores (4 X 10^5 to 1 X 10^6) were incubated with virus in one ml of 50 mM sodium phosphate buffer, pH 7.6 for 1 hour. Following binding, zoospores and any bound virus were pelleted by centrifugation at 2,000 X g for 7 min and then washed in buffer. The zoospore pellet was assessed for the presence of virus using Western blot analysis and virus-specific antisera. The quantity of bound protein was determined by densitometric analysis.

Results

Three major criteria for viral recognition sites as receptors are specificity, saturability and ability to be competitively inhibited (Tardieu *et al.*, 1982). The following outlines studies that were conducted to assess if binding of CNV to *Olpidium* zoospores fulfills these criteria.

In vitro binding of virus to Olpidium zoospores is specific. Previous work has shown that specific interactions occur between *Olpidium* species and the viruses they transmit (Temmink *et al.*, 1970; Campbell, 1996) CNV, cucumber leaf spot virus (CLSV), and melon necrotic spot virus (MNSV) are each transmitted by *O. bornovanus* but *not O. brassicae* and TNV is transmitted by *O. brassicae* but not *O. bornovanus*. We wished to assess whether the specificity of transmission relies on the ability of these viruses to recognize and bind a putative zoospore receptor. To do this, each of these viruses were subjected to a *in vitro* binding assay using either *O. bornovanus* or *O. brassicae* zoospores. Fig. 1A shows that CNV, MNSV and CLSV each bind *O. bornovanus* zoospores and that binding of these viruses is greater than that obtained with TNV. The level of binding of CLSV was the greatest being approximately five fold higher than that of either CNV or MNSV. Binding of

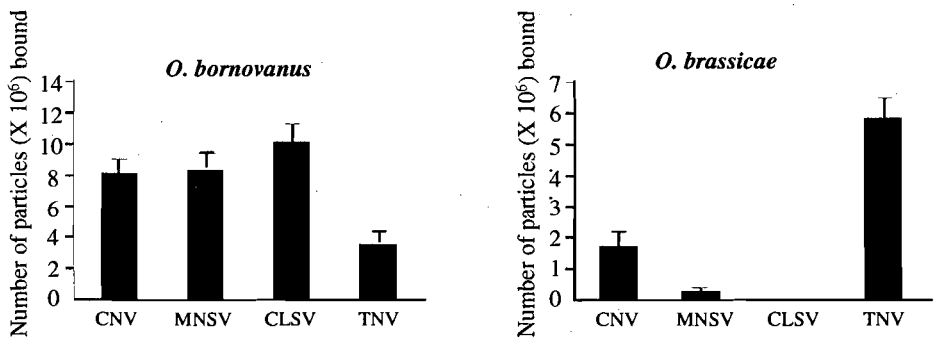

FIG. 1. Specificity of binding of several viruses to *Olpidium* zoospores *in vitro*. An *in vitro* binding assay was conducted with 100 ug of CNV, MNSV, CLSV or TNV and 1 X 10^6 *O. bornovanus* (A) or *O. brassicae* (B) zoospores. Values are the average of triplicate treatments from two separate experiments. The actual value for CLSV in (A) is five times greater than that shown. The amount of bound virus was determined using Western blot analysis followed by densitometry.

CNV and MNSV was more than twice that of TNV. In the reciprocal experiment (Fig. 1B), where each virus was bound to *O. brassicae* zoospores, the level of binding of TNV was greatest and was approximately three times greater than that of CNV and 18 times greater than that of MNSV. We were unable to detect binding of CLSV to *O. brassicae* zoospores. The differential binding of viruses to *Olpidium* zoospores suggests that natural transmission of these viruses involves specific interactions between virus particles and receptor or receptor-like molecules on zoospore membranes.

Binding of virus to zoospores is saturable. Increasing amounts of CNV were added to fixed amounts *of O. bornovanus* zoospores in an *in vitro* binding assay to determine whether binding to zoospores is saturable. Fig. 2 shows that binding of CNV to zoospores is saturable at high concentrations of CNV. Similar experiments using TNV and *O. brassicae* zoospores indicated that TNV binding to *O. brassicae* zoospores is also saturable but that binding of CNV to *O. brassicae* is not (data not shown). These studies reinforce the specificity studies described above suggesting that binding of virus to zoospores involves a specific receptor.

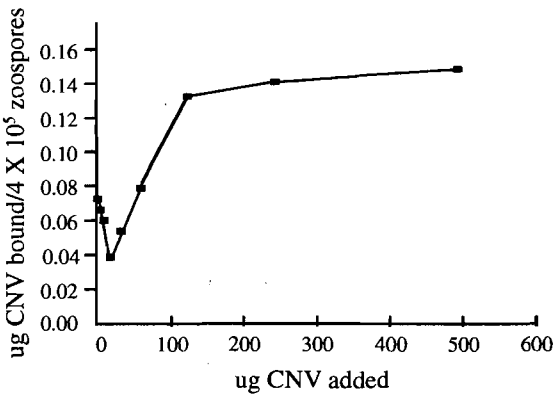

FIG. 2. Saturation binding of CNV to *O. bornovanus* zoospores. Increasing amounts of CNV were added to 4 X 10^5 *O. bornovanus* zoospores and subjected to an *in vitro* binding assay. The amount of bound virus was determined by Western blot analysis followed by densitometry.

CNV can compete with MNSV and TNV for binding sites. Increasing amounts of CNV were added to MNSV and the amount of bound MNSV was determined. Fig. 3A shows that a ten-fold excess of competitor CNV results in about 50% reduction in the level of bound MNSV. Similar results were obtained when CNV was used to competitively inhibit binding of TNV (Fig. 3B). In this case, a ten-fold excess of competitor CNV resulted in a 70% reduction in the binding of TNV.

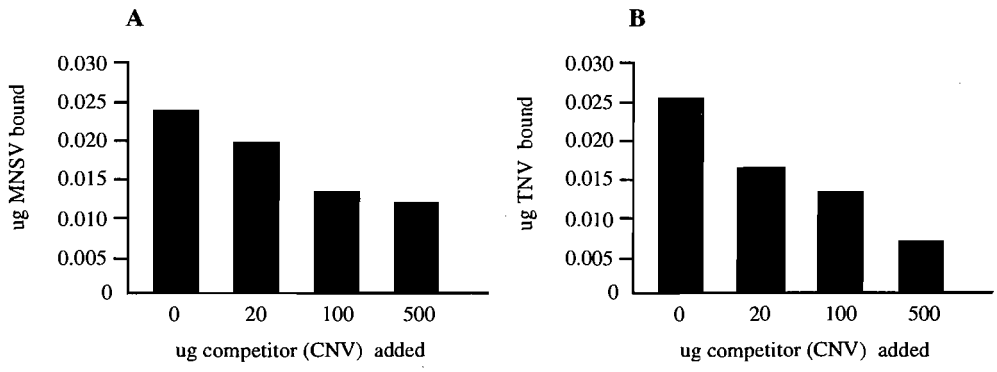

FIG. 3. Competitive inhibition of MNSV and TNV binding with CNV. Increasing amounts of CNV were added concurrently to either 50 ug of MNSV (A) or 50 ug TNV (B) and 4 X 10^5 O. bornovanus zoospores. The amount of virus MNSV or TNV bound was determined using Western blot analysis followed by densitometry.

Discussion

Although we have provided biochemical evidence that *O. bornovanus* zoospores contain specific receptors for CNV attachment we do not yet know the nature of the receptor molecule. Virus overlay assays may help to identify a putative protein receptor. Alternatively, or in conjunction, pretreatment of zoospores with specific enzymes prior to binding assays may aid in the identification of the receptor molecule.

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OCCURRENCE OF SOIL-BORNE WHEAT MOSAIC VIRUS IN THE UK

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Summary

During April 1999, soil-borne wheat mosaic virus (SBWMV) was observed for the first time in the UK. Affected crops had characteristic symptoms and the diagnosis was confirmed by electron microscopy, ELISA, RT-PCR and sequencing. Serological and molecular studies revealed that the UK isolate is most closely related to European SBWMV and soil-borne rye mosaic virus. Five fields, planted with winter wheat cvs. Consort, Equinox and Savannah were infected at the outbreak site in Wiltshire but the virus has not been detected elsewhere in the UK. Trials at the site revealed that infection substantially reduced the yield of all three cultivars and losses of up to 51 % were observed. The importance of the wheat crop in the UK and the yield reductions recorded at the outbreak site suggest that SBWMV could cause substantial economic losses in the UK were it to become widespread.

Introduction

Soil-borne wheat mosaic virus (SBWMV) causes a serious disease in many cereal species including winter wheat, durum wheat, barley, rye and triticale (Brakke, 1971). Symptoms on susceptible cultivars include a pale green-yellow mosaic or streaks on leaves and leaf sheaths, moderate to severe stunting and a decrease in yield. For example, losses of 40-50 % occur in infected areas of commercial fields in Florida, USA (Kucharek & Walker, 1974). Affected plants tend to occur in pale green or yellow patches which correspond to the distribution of the protist vector of the virus, *Polymyxa graminis* (Brakke 1971). The disease was first observed in 1919 in the American states of Illinois and Indiana but has now spread to at least 16 states and probably occurs throughout the winter wheat area. Elsewhere the disease has been reported from Canada and Brazil in the Americas, China and Japan in Asia, Zambia in Africa, and France, Germany, Italy and Poland in Europe. Until recently SBWMV had not been observed in the UK despite surveys of winter wheat in 1994 and 1995 by the Central Science Laboratory (CSL). However, characteristic SBWMV symptoms were observed in two crops of winter wheat cv. Equinox, near Trowbridge, Wiltshire during April, 1999 (Clover *et al.*, 1999b). This paper describes the research that has subsequently been carried out to identify and characterize the virus, to assess its effect on yield and identify the area infected by the disease.

Materials and Methods

An initial survey of the suspected SBWMV-infected site in Wiltshire by the Plant Health and Seeds Inspectorate (PHSI) revealed that five fields on the farm contained patches of affected plants. Leaf samples were collected from 60-170 plants per field and were tested for the presence of SBWMV in both DAS- and TAS-ELISA using antisera and antibodies supplied

by Dr. Huth, BBA-Braunschweig and Dr. Torrance, SCRI-Dundee respectively. The samples were also tested for the presence of wheat spindle streak mosaic virus (WSSMV) by DAS-ELISA using antisera from Dr. Huth. Transmission electron microscopy, including immunosorbent electron microscopy, was used to investigate the presence of virus particles in representative samples (Clover *et al.*, 1999a).

Total RNA was extracted from two infected leaf samples from each field (Clover *et al.*, 1999a). Degenerate primers were designed to complement 4060-4080 nt (SBWMVF: 5'AATGACGGTTTGGGTCVAAGT3') and reverse complement 5133-5152 nt SBWMVR: 5'ATCAGTGAATCATCACCRCC3') of the replicase gene of SBWMV RNA1 (Accession L07937; Shirako and Wilson, 1993) and were used to amplify viral RNA from infected plants. RT was performed using 1 µl template RNA, 2 µl SBWMVR (5 µM) and 100 units MMLV reverse transcriptase (Promega) in a 10 µl volume at 37°C for 1 h. PCR was carried out using the Expand system (Roche Molecular Biochemicals) according to the manufacturers's protocol and amplified products were sequenced. Nucleic acid extracted from SBWMV-infected wheat from Nebraska-USA was used as a positive control in RT-PCR. The sequence of homologous areas of a SBWMV isolate from Ozzano-Italy and a soil-borne rye mosaic virus (SBRMV) isolate from North West Zealand-Denmark were determined for comparison.

The yield effect of the disease at the suspected-SBWMV site in Wiltshire was determined by comparing healthy and infected areas. Plots were marked out in late May and measured either 1 m^2 or 82.8 m^2 for hand- or combine-harvesting respectively. Five leaf samples were collected from each plot and tested for SBWMV infection by ELISA. In total, 100 hand-harvested plots were positioned along transects through healthy and infected areas of the five fields. Crop height, fertile tiller number, grain moisture, specific weight, grain yield (15% moisture) and 1000-grain weight were measured in each plot. Two fields, planted with cvs. Equinox and Savannah, provided areas suitable for combine harvesting and grain yield (15% moisture) from four plots in healthy or infected areas in both fields was recorded. Data from these experiments were analyzed by regression analysis.

Following the confirmation of SBWMV at the outbreak site in Wiltshire, the PHSI, farmers and consultants were provided with a description of the disease symptoms and requested to send plants with suspicious symptoms to CSL for diagnosis. The PHSI also specifically inspected wheat crops around the outbreak site and examined selected fields throughout the UK as part of an ongoing survey of field crops.

Results

Infected plants in the five affected fields in Wiltshire exhibited distinctive symptoms of SBWMV. The plants were severely stunted and had distorted leaves covered in bright yellow, parallel streaks which extended onto the leaf sheaths. Affected plants were generally found in discrete, elliptical patches which ranged in size from a few square metres to almost a hectare and constituted between a few percent to more than half of the field. All leaf samples from symptomatic plants were positive for SBWMV when tested using both DAS- and TAS-ELISA while those from asymptomatic plants were negative. The TAS-ELISA showed that the virus had a closer serological affinity to European SBWMV isolates than those from the USA. WSSMV was not detected in any leaf sample collected from the site. Electron microscopy of leaves from infected plants revealed the presence of SBWMV-like virions. The particles were

straight, rod-shaped with a distinct axial canal, 20 nm wide and of two modal lengths, either *c.* 150 nm or 300 nm. In immunosorbent election microscopy, these particles were decorated using SBWMV-specific antisera and labelled using protein-A gold.

Amplification of RNA from infected plants collected at the Wiltshire outbreak site using SBWMV RNA 1 primers produced a single product of the expected size (1093 nt). Amplicons of the same size were produced using SBWMV isolates from Nebraska-USA, Ozzano-Italy and a SBRMV isolate from North West Zealand-Denmark. Comparison of these sequences showed that the Wiltshire isolate shared the greatest nucleotide (and amino acid) identity to SBWMV isolated in Italy. The Wiltshire isolate was also closely related to SBRMV isolates from Germany and Denmark but had least homology with the American SBWMV isolate (Table 1).

Nucleotide/ Amino acid identity (%)		SBWMV		SBRMV	
		Italy	USA	Germany	Denmark
SBWMV	UK	97.8/99.1	74.6/83.1	96.0/98.0	95.8/97.4
	Italy	--	75.3/83.7	95.9/97.7	95.6/97.1
	USA (L07937)	--	--	74.7/84.3	75.2/84.6
SBRMV	Germany (AF146278)	--	--	--	96.9/99.1
	Denmark	--	--	--	--

Table 1. Comparisons of nucleotide and amino-acid sequences of part of the replicase gene of SBWMV and SBRMV isolates (accession numbers in parentheses).

Hand-harvesting at the outbreak site showed that SBWMV reduced the yield of all three cultivars by approximately 40 % (range 35-47 %). This reduction was due to decreases in both the tiller number and grain weight (Table 2). Infected plants were an average of 12.5 cm (S.E. 2.3, $P<0.001$) shorter, a reduction of 16 %, but the virus did not significantly affect moisture content or specific weight. Similar results were obtained by combine harvesting larger areas; infection reduced grain yield in these areas by 51 (field 13) and 49 % (field 19).

Cultivar (field number)	Tiller number (per m^2)			1000-grain weight (g)			Grain yield (t ha^{-1})		
	Healthy	SBWMV	S.E.	Healthy	SBWMV	S.E.	Healthy	SBWMV	S.E.
Consort (3)	375	219	77^{NS}	39.7	30.8	3.4*	7.6	4.6	1.2*
Savannah (13)	433	305	32**	48.6	41.4	1.1**	10.4	5.9	0.6**
Equinox (6)	475	448	66^{NS}	47.5	38.0	1.3**	11.5	7.5	0.8**
Equinox (16)	529	410	101^{NS}	49.4	42.2	3.1*	11.5	7.3	1.9^{NS}
Equinox (19)	447	242	25**	46.8	39.3	0.8**	11.2	5.9	0.4**
MEAN	452	325	32**	46.4	38.3	0.5**	10.4	6.2	0.4*

Table 2. The effect of SBWMV on winter wheat yield in healthy and infected hand-harvested plots in Wiltshire (S.E., standard error; *, $P<0.001$; **, $P<0.05$; ^{NS}, Not significant).

Following the identification of SBWMV in Wiltshire, twenty wheat samples showing suspicious symptoms were received from twelve other sites. Three of these sites were in the

south-west close to the outbreak site, four from the south-east and the remainder from East Anglia. Neither SBWMV nor WSSMV were detected in these samples and the symptoms could be attributed to infection by other pathogens or factors such as soil compaction.

Discussion

The presence of SBWMV at one site in the UK has been confirmed. The origin of the virus is unknown but consultation with the farmer involved has revealed that symptomatic plants have been seen in several recent years. The most likely means of introduction into the UK is as a soil contaminant, *e.g.* on imported nursery stock or seed potatoes. Serological and molecular studies have shown that this isolate is most closely related to European SBWMV isolates and the recently demarcated species, SBRMV (Koenig *et al.*, 1999). Future research may conclude that the Wiltshire isolate is better described as an isolate of SBRMV.

By reducing both the number of grains and their individual weight, SBWMV decreased the yield of the three infected wheat cultivars by up to 51 %. One of these, cv. Consort, is the most widely grown cultivar in the UK and the three cultivars have more than 40 % of the market share in the UK. The only method of controlling the disease is by growing tolerant cultivars. Although such varieties are available in France (*e.g.* cv. Trémie), little is known about the tolerance of UK varieties and this subject will be studied in the future. The results of an initial survey indicated that no further farms have become infected and measures have been imposed to reduce the risk of future dispersal. However, bearing in mind the value of the UK wheat crop (*c.* £1.5 billion per annum) and the yield reductions caused by the disease, SBWMV clearly has the potential to cause significant economic losses if it were to spread extensively throughout the wheat growing area.

We are indebted to Dr. W. Huth and Dr. L. Torrance who provided antisera and antibodies used during this research. This work was funded by MAFF Plant Health Division (Plant Health Licence No. PHF 1526A/1301/124).

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GEOGRAPHICAL DISTRIBUTION OF THE INDIAN PEANUT CLUMP VIRUS (IPCV) IN RAJASTHAN: SOIL CHARACTERISTICS AND FARMING PRACTICES INFLUENCING THE DISEASE OCCURRENCE.

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Summary

Surveys were conducted along roads in Rajasthan in areas infested with IPCV, a soil-borne and seed-transmitted virus. Furthermore, farmers in Rajasthan were sent a questionnaire illustrated with pictures of typical symptoms. Over 500 questionnaires were sent and 72 answers were received covering the districts of Jaipur, Dausa, Tonk and Sikar. It is apparent from the survey that the disease occurs mainly in sandy and sandy-loam soils. The majority of the farms were irrigated and showed a disease incidence ranging from 10 to 20 %, which in most instances, extended over the entire farm. The problem is not recent but seems to have extended during the last 5 years. The longer the farmers had been growing wheat or barley the higher was the incidence. If groundnut, millet and maize produced on a farm are reused for seed, it increases the disease incidence on the farm. About 250 000 ha are under groundnut cultivation in Rajasthan and peanut clump disease is considered to be the second-most limiting factor to groundnut production in the state, the major problem being damage caused by white grubs.

Introduction

Peanut clump viruses are among the most damaging soil-borne pathogens of groundnut, causing crop losses estimated at over US$ 38 millions per annum world-wide. The viruses occur in semi-arid areas of western Africa and the Indian subcontinent. In India, the virus is *Indian peanut clump virus* (IPCV). IPCV has been shown to be transmitted through seeds of groundnut and such cereals as, pearl millet, finger millet, foxtail millet, maize and wheat . It is also transmitted in a persistent way by the root obligate endoparasite, *Polymyxa graminis* Led. Resistance to clump could not be identified in groundnut germplasm. Biocides, though effective in reducing disease incidence, are hazardous and not economical. The two main management options left are to devise cultural practices which lead to reduction of IPCV incidence or to induce host-plant resistance by non-conventional approaches (Reddy *et al.*, 1999). In India, the disease was reported from the states of Andhra Pradesh, Gujarat, Punjab and Tamil Nadu, and it is considered to be one of the major constraints to groundnut production in sandy soils in the state of Rajasthan (Mathur & Sobti, 1993). The disease in Rajasthan has been known since the late 60's but at that time it was identified as "rosette" disease (Mathur *et al.*, 1971, L.C. Sharma, personal communication). During the last 5 years, farmers and agricultural extension officers regularly reported the problem to scientists at the Durgapura Agricultural Research Station, Jaipur. Little was known about the actual spread and severity of clump disease in Rajasthan where over 250 000 ha are under groundnut cultivation each rainy season. Therefore systematic surveys were conducted in Rajasthan to determine the economic importance of clump disease, to identify farming practices

that could be responsible for high disease incidence, and to inform Rajasthan farmers about the causal agent and the methods available to date to contain the spread of this pernicious soil-borne disease.

Materials and Methods

Systematic surveys were conducted by road in Rajasthan in groundnut growing areas during the rainy seasons 1994 to 1997. The districts covered by the surveys and the respective areas under groundnut cultivation in 1997 were: Bikaner (23 200 ha), Bhilwara (19 100 ha), Chittorgarh (48 754 ha), Dausa (16 200 ha), Jaipur (20 700 ha), Swaimadhopur (18 390 ha), Tonk (20 460 ha), Sikar (2 660 ha) and Churu (2 000 ha). Districts are divided into "tehsils" which group 5-10 villages and each tehsil is under the supervision of one agricultural extension officer (AEO). On average about 4 tehsils regarded as important groundnut growing areas, were surveyed in each district. In every tehsil, at least 3 villages identified by the local farmers as clump problematic areas and accessible by road were visited. Finally in each village, 3 fields were scored for IPCV incidence. Groundnut samples were collected and tested for IPCV presence by ELISA. The disease sites were mapped to illustrate IPCV importance and distribution in the state of Rajasthan.

Furthermore, farmers in Rajasthan were sent a questionnaire, through AEO, written in Hindi and illustrated with color photographs of typical symptoms. Over 500 questionnaires were distributed. In most of the case, the questionnaire was filled with the help of the AEO of the region. Some were also filled during the aforementioned surveys. Data from the inquiry were arranged in summary frequency tables and a correlation matrix (Pearson correlation coefficient) was established between IPCV incidence and the various factors studied.

Results

Surveys conducted by road. A map of Rajasthan showing all the disease sites observed during the surveys is presented in Fig. 1. It is apparent from the surveys that peanut clump disease is widely spread in groundnut growing areas of Rajasthan. Most of the crops surveyed were sown in June-July and most of the farms were provided with irrigation facilities (ground water). In the Indira Gandhi Nehar Project (IGNP, Bikaner district), crops were sown in April-May under irrigation provided by a canal. Groundnut leaf samples collected during the survey reacted positively with a polyclonal serum raised against the Durgapura isolate of IPCV (IPCV-D). One sample from Ranoli (Dausa district) did not react with any of the sera available for IPCV detection.

Survey conducted with the help of a questionnaire. Seventy two farmers replied to the questionnaire. This represents a participation of about 15%. Farmers originated from Dausa (36%), Jaipur (32%), Tonk (18%) and Sikar (14%) districts. According to the answers, most of the farms were located in sandy (33%) and sandy-loam (55%) soil areas, others (12%) were in loamy soil. Irrigation facilities were available in most of cases (71%) and the farms were generally of small size. Forty one (57%) had a surface not exceeding 2 ha, 23 farms (32%) had a surface of between 2 and 5 ha and 6 farms (8%) had a surface of 5 to 15 ha. The numbers of field on the farms of 53 farmers (74%) were less than 5. To the question "What are the major post-rainy season crops that you grow?", mustard was quoted in 54 cases (75%), wheat in 52 (72%), barley in 36 (50%), and chickpea in 35 (49%). Other crops with minor importance were

various vegetables including pea (30%). The major rainy season crops quoted by Rajasthan farmers were groundnut (93% of the answer), pearl millet (58%), cluster bean (*Cyamopsis tetragonolobus*) (36%), maize (28%), and sorghum (10%).

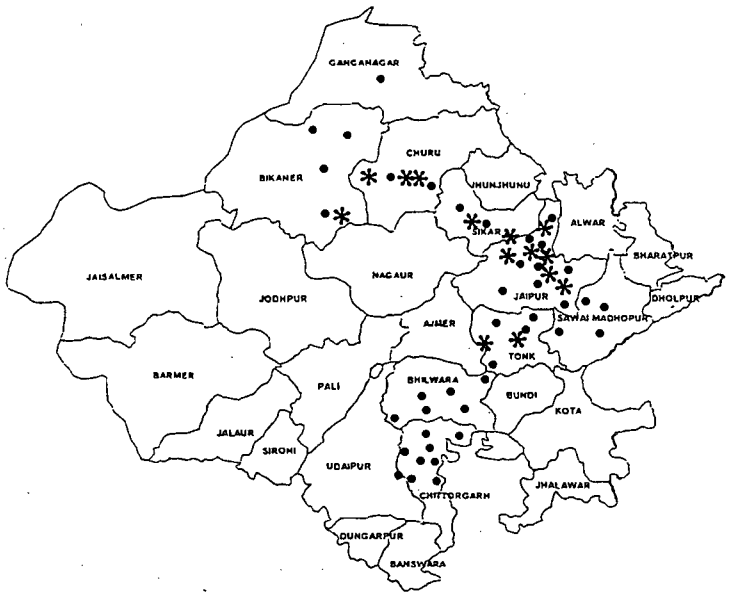

Figure 1. Peanut clump distribution in Rajasthan. Disease sites observed during the 1994 to 1997 rainy seasons in major groundnut growing areas. Incidence: (•) below 20%; (⁎) 20-70%

Regarding peanut clump disease, usually all the fields of a farm were infested (43 farms representing 60% of the answers). Farms located in loamy soil showed the lowest spread of IPCV on the farm (percentage of infested field). According to the farmers, IPCV incidence on their farm ranged from 10-20% in 56% of the case, and from 20-50% in 32%. A minority reported incidences of 0-10% (7%) and 50-100% (5%). The problem is not recent but seems to have extended during the last 5 years. Indeed, most of the farmers (67%) noted peanut clump as a problem that had affected their farm in the course of the last five years. Twelve farmers (17%) had encountered clump disease for the last 20 years and few (8%) noticed the disease about 5 to 10 years ago. The longer the farmers had been growing wheat or barley the higher was the incidence. If groundnut, millet and maize produced on a farm had been reused for seed, it increased the disease incidence (Pearson $r = 0.334$, $P = 0.0190$) on the farm. On the contrary, if groundnut seed had been purchased from the store, IPCV incidence was low (Pearson $r = 0.524$, $P = 0.0001$).

Discussion

The surveys conducted in the state of Rajasthan confirmed that peanut clump disease (IPCV-D serotype) is widely spread in the major groundnut growing areas. For an investigation

of this type, there was a satisfactory participation of the farmers in filling the questionnaires, which indicated their concern about this pernicious disease. The surveys confirmed previous reports that peanut clump disease occurs mainly in light soil, sandy and sandy-loam soil (Reddy *et al.*, 1999). Heavier soil texture, such as loam, was negatively correlated with the spread of the disease. IPCV is transmitted through seed of groundnut and cereals (Reddy *et al.*, 1999) but little is known about the role of seed inoculum in disease spread and disease establishment in soil harboring non-viruliferous *P. graminis*. This survey among the farmer community in Rajasthan suggested that the use of groundnut, millet and maize produced in infested farms for seed is linked to higher disease incidence on the farm, whereas the use of certified groundnut seed reduced the risk of having high incidence in groundnut crops. Groundnut is not a good host for *P. graminis*. Therefore, groundnut seed carrying the virus is not, a priori, considered as a serious threat that will support establishment of the disease to new areas. However, there is no doubt that the use of virus-infected seed can result in a higher disease incidence in groundnut crops. Maize, pearl millet and wheat are excellent hosts for *P. graminis* isolates from Rajasthan (Legrève *et al.*, 1999) and their seed is suspected to play an important role in disease spread and establishment to new area.

About 250 000 ha are under groundnut cultivation in Rajasthan where groundnut is an important cash crop and a source of protein in the human diet. Peanut clump disease is considered the second-most limiting factor to groundnut production in the state, the major problem being damage caused by white grubs. The surveys indicated that the problem has extended during the last five years, which correspond, to the introduction of groundnut, a host expressing overt symptoms, to new irrigated areas where pearl millet, a symptomless host, was previously the major rainfed crop. It is likely that the cropping systems in force in Rajasthan, which implies rotation of groundnut with cereal hosts for *P. graminis* and IPCV, such as millet, maize and wheat, favor the increase in spread and incidence of peanut clump virus disease. The results of these surveys corroborate epidemiological and ecological studies conducted on IPCV and its vector *P. graminis* (Delfosse *et al.*, 1996, Legrève *et al.*, 1999).

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IDENTIFICATION OF A AND B TYPE BEET NECROTIC YELLOW VEIN VIRUS IN SWEDEN

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Regular surveys for the possible presence of BNYVV have been performed in Sweden since 1986 (Lindsten, 1989). In 1997, rhizomania-like symptoms were seen for the first time on sugar beets in a small area in the South Eastern part of Scania and on one farm on Öland. The presence of the virus was verified by means of ELISA. Soil samples from infected fields were planted with seeds of the highly susceptible sugar beet variety Accord. Four weeks later the roots were harvested and ELISA revealed that they were heavily infected with BNYVV. Single strand conformation polymorphism (SSCP) analyses of BNYVV RNA 1- and 4-derived PCR products were done as described by Koenig et al., 1995. They revealed that the bait plants grown in the soil from Öland contained A type BNYVV, whereas those grown in soil samples from Scania contained B type BNYVV. BNYVV RNA 5 was not detected in the bait plants. The identification of A type BNYVV on Öland and B type BNYVV in Scania indicates that rhizomania has probably been introduced to Sweden from different places abroad. Alternatively, BNYVV may have invaded sugar beet from so far unknown native hosts.

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A detailed description of our work will be given elsewhere (Lennefors, Lindsten and Koenig, manuscript submitted to the European Journal of Plant Pathology).

DIFFERENTIATION OF VIRUS- AND FUNGUS-RELATED RHIZOMANIA RESISTANCE IN SUGARBEET.

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Summary

Nucleic acid based methods have been developed for the quantitative detection of the soil-borne fungus *Polymyxa betae* which is the vector of *beet necrotic yellow vein virus* (BNYVV), the cause of rhizomania disease in sugarbeet. It was shown that the development of *P. betae* in sugarbeet roots could be accurately monitored by dot blot hybridization and competitive PCR, respectively. In growth chamber infection experiments, a combination of nucleic acid based *P. betae* detecion methods with DAS-ELISA for BNYVV detection allowed to follow *P. betae* and BNYVV development in sugarbeet roots. Infection studies revealed that within sugarbeet cultivars and crosses with wild beet tested rhizomania resistance is mainly based on resistance to BNYVV rather than on resistance to its vector *P. betae*.

Introduction

Rhizomania disease of sugarbeet is caused by *beet necrotic yellow vein virus* (BNYVV) which is transmitted from roots of infected sugarbeets to healthy beets by means of zoospores of the soil-borne fungus *Polymyxa betae* (Richards and Tamada ,1992). For assessment of rhizomania resistance in new sugarbeet varieties, breeders routinely determine BNYVV titers in infected roots. However, resistance to BNYVV and its vector *P. betae* can not be distinguished using this conventional screening method. Here we describe the development of nucleic acid based *P. betae* detection methods and their use in combination with established BNYVV detection methods in controlled infection studies under growth chamber conditions for quantitative assessment of virus- and fungus-related rhizomania resistance in sugarbeet varieties and breeding material.

Materials and Methods

Growth of sugarbeet, infection with *P. betae*/BNYVV and sampling:
Emerging seedlings grown in sterile sand after 3 to 4 days of germination were dusted with a root powder obtained from resting spore-containing sugarbeet roots. Seedlings were kept in an illuminated environmental control chamber at 25°C and watered with 0.1 x minimal medium (Steiner, 1984). Batches of 25 to 30 plants were harvested every 4 to 5 days past inoculation until day 26 past inoculation (d.p.i.). One part of the root batches were tested for BNYVV titers by DAS-ELISA (Clark and Adams, 1977). Another part (50 mg fresh weight) was used for DNA extraction with liquid nitrogen and quartz sand using a mortar and pistill according to Dellaporta et al. (1983). DNA was quantified by means of the fluorescent dye, Hoechst 33258 and a TKO100 (Hoefer) fluorimeter.

Cloning of a competitor DNA fragment: The plasmid pPbetaeBS2 which contains a *P. betae*-specific DNA fragment of 1821 bp inside the vector plasmid pBluescript II SK(+) was used for construction and cloning of a competitor DNA fragment applying standard cloning procedures. An internal deletion of 320 bp was introduced and the plasmid obtained was designated p51ΔB (Obermeier, 1998).

Quantification of *P. betae* by competitive PCR: DNA (1 ng) from *P. betae*-infected sugarbeet roots was co-amplified in a titration series with competitor plasmid DNA p51ΔB over a range from 1 pg down to 0.1 fg applying the primers 51LO2 (5'-AATTGAAGATGAGGATGTCG) and 51UP2

(5'-TGCCTCTTTCTTGTGATCAG) (Obermeier, 1998). Amplification products were visualized after agarose gel electrophoresis and ethidium bromide staining on an u.v. transilluminator. An image analysis computer program was used to capture the relative fluorescence intensity of each band and the PCR product ratio (*P. betae* product/internal standard product) was calculated.

Quantification of *P. betae* by dot blot hybridization: DNA (up to 2 µg) from *P. betae*-infected roots was applied after heat denaturation in 500 µl 5 x SSC buffer to a nylon membrane using a vacuum dot blot hybridization apparatus. A series of 25 to 500 pg of plasmid DNA pPbetaeBS2 diluted in 500 µl 5 x SSC buffer was used for calibration. Hybridization and detection was carried out according to the standard protocol described by Boehringer Mannheim in The DIG Systems User's Guide for Filter Hybridization (1995). The probe was digoxigenin-labeled by PCR using primer pair 51LO2/51UP2 and plasmid pPbetaeBS2 and applied in a final concentration of 20 ng/ml.

Results and Discussion

A high-copy-number DNA sequence of the *P. betae* genome has been cloned in *E. coli* (PbetaeBS2) and used as a probe in a dot blot hybridization procedure for quantitative detection of *P. betae* in DNA extracts from infected sugarbeet roots. No signal was obtained with DNA from healthy sugarbeet roots. Extinction values of a calibration series were linear from 25 to 250 pg of *P. betae*-specific DNA fragment. Comparison of data obtained by dot blot hybridization and microscopic analysis of root fragments for *P. betae* resting spore content revealed a good correlation (Fig. 1). Dot blot hybridization can therefore be applied for accurate quantification of *P. betae* colonization in sugarbeet roots. However, relatively large amounts of DNA from root samples (up to 2 µg) were necessary and had to be applied in at least two different amounts to obtain a hybridization signal within the linear range of the calibration series.

A competitive PCR procedure has been developed to improve sensitivity. Competitive PCR is based on the co-amplification of a reference standard (competitor DNA) of known initial copy numbers in the same reaction tube as the sequence of interest. Because both fragments are competing during the PCR for all reaction partners the relative amounts of amplification products obtained reflect the initial copy numbers of both DNA fragments and allow the calculation of the copy number of the sequence of interest (Cross, 1995). A competitor DNA fragment 51ΔB has been obtained by cloning a deletion fragment of the *P. betae*-specific genomic DNA fragment PbetaeBS2. The competitor DNA fragment 51ΔB has been shown to exhibit a similar amplification efficiency compared to PbetaeBS2 and may therefore be used for accurate calculation of copy numbers of PbetaeBS2 in a competitive PCR procedure.

Calculation of the copy number of DNA fragment PbetaeBS2 in total DNA extracted from *P. betae*-infected sugarbeet roots was done by co-amplification of 1 ng of total DNA with a dilution series of competitor plasmid DNA p51ΔB. PCR products amplified from the genomic DNA and the competitor DNA have been separated by agarose gel electrophoresis (products are 888 and 512 bp, respectively) and fluorescence intensities of both fragments have been determined using an image analysis program. The PCR product ratio of competitor to target DNA fragment has been calculated after correcting for the size difference between the competitor and sample fragments. PCR product ratio has been plotted against the number of competitor DNA molecules added per reaction and intersection of calculated regression line with y-axis at x=0 is used for calculation of the copy number of initial genomic PbetaeBS2 DNA fragments as shown in Fig. 2.

A linear relationship was observed between the logarithm of the PCR product ratio and the logarithm of the copy number of the competitor molecules added (R^2 =0.9525 for regression line of sample shown in Fig. 2). Interpolation of the copy number of competitor DNA fragment to the point where its PCR amplification yield equals the yield for the genomic PbetaeBS2 DNA fragment can thus be used for calculation of the copy number of PbetaeBS2 DNA fragment within the DNA samples extracted from *P. betae*-infected roots.

Competitive PCR proved to be at least 2.5×10^4 times more sensitive than dot blot hybridization (detection limit 0.1 fg and 25 pg, respectively) and may therefore also be used for quantification of *P. betae* in small parts of roots from seedlings. However the dot blot hybridization procedure is simpler and more rapid for testing of large numbers of samples.

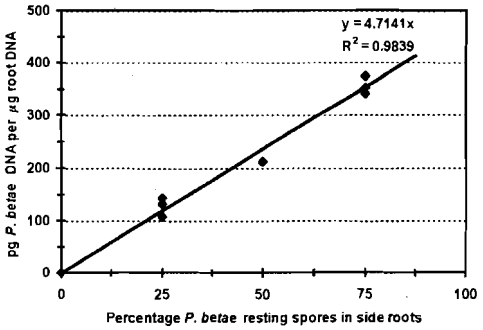

Fig. 1. Relationship between proportion of *P. betae* resting spores in side roots and the amount of *P. betae*-specific DNA fragment PbetaeBS2 per µg root DNA determined by light microscopy and dot blot hybridization, respectively. Microscopic data are based on the examination of *P. betae* resting spore content in at least ten approximately 1 cm long root fragments from side roots.

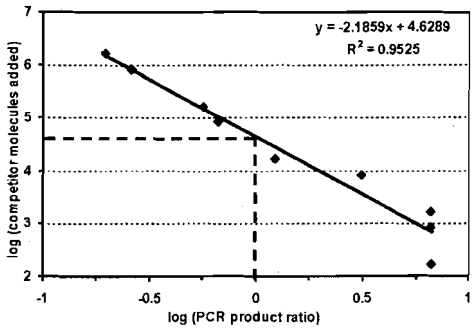

Fig. 2. Calculation of the copy number of DNA fragment PbetaeBS2 in an infected sugar beet root sample by competitive PCR. A logarithmic plot of the number of competitor molecules added to each reaction against the resultant band intensities (PbetaeBS2/competitor DNA ratio) yields a line with point of equivalent fluorescence intensity at 42,550 ($10^{4.6289}$) competitor molecules added.

For quantification of *P. betae*-infection in sugarbeet roots, competitive PCR and dot blot hybridization procedures have been adapted to a rhizomania resistance testing procedure and applied in growth chamber infection experiments. Parallel measurement of BNYVV and *P. betae* titers in roots was possible by using one part of homogenized root material for nucleic acid isolation followed by dot blot hybridization or competitive PCR and the other part for ELISA allowing to compare the development of fungus and virus titers in roots of different sugarbeets.

Results from a rhizomania infection study of sugarbeet (*Beta vulgaris*) cultivars with different rhizomania resistance backgrounds are shown in Fig. 3. Comparison of *P. betae* and BNYVV titers in seedling roots of the rhizomania-susceptible cultivar 'Hilma' (A) with partially rhizomania-resistant cultivar 'Patricia' (B), a line with 'Holly' resistance background (C) and a line with wild beet (*Beta maritima)* background (D) over a time period of 26 days showed weakly to strongly reduced development of virus titers (full lines), but mainly slightly delayed development of fungus titers (dashed lines). The strongest resistance to BNYVV has been found in a line derived from a wild beet population (D). Weaker resistance to BNYVV has been found in the partially field resistant sugarbeet cultivar 'Patricia' (B). Both exhibit a delay in BNYVV development and reduction of BNYVV titers, but the level of reduction of BNYVV titers in the line with *B. maritima* background is much higher compared to cv. 'Patricia' 26 d.p.i. (66% and 19%, respectively). No reduction of virus development was observed in roots of the line with the 'Holly' resistance background under conditions of the test system (C). Although all of the sugarbeet cultivars and lines tested showed some delay in fungus development (B,C,D), most of them reach the same titers as the susceptible control on 26 d.p.i. A reduction of absolute fungus titers on 26 d.p.i. was not observed. Thus fungus-related rhizomania resistance seemed to be weak compared to virus-related resistance.

Results obtained correspondend well to field resistance characteristics of the sugarbeet varieties and lines tested, but the testing procedure may not catch weak resistance influences e.g. in lines with the 'Holly' resistance source. Similar results have been obtained by other authors for sugarbeets with this resistance source (Scholten et al., 1996).

However, different levels of rhizomania resistance were revealed in sugarbeet varieties and lines applying the testing procedure. It was shown that rhizomania resistance in the sugarbeet cultivars tested is mainly based on resistance to BNYVV rather than *P. betae*. Application of this rhizomania resistance testing procedure may help to identify *P. betae* resistance in wild beet which can be useful for breeding of new rhizomania resistant sugarbeet varieties.

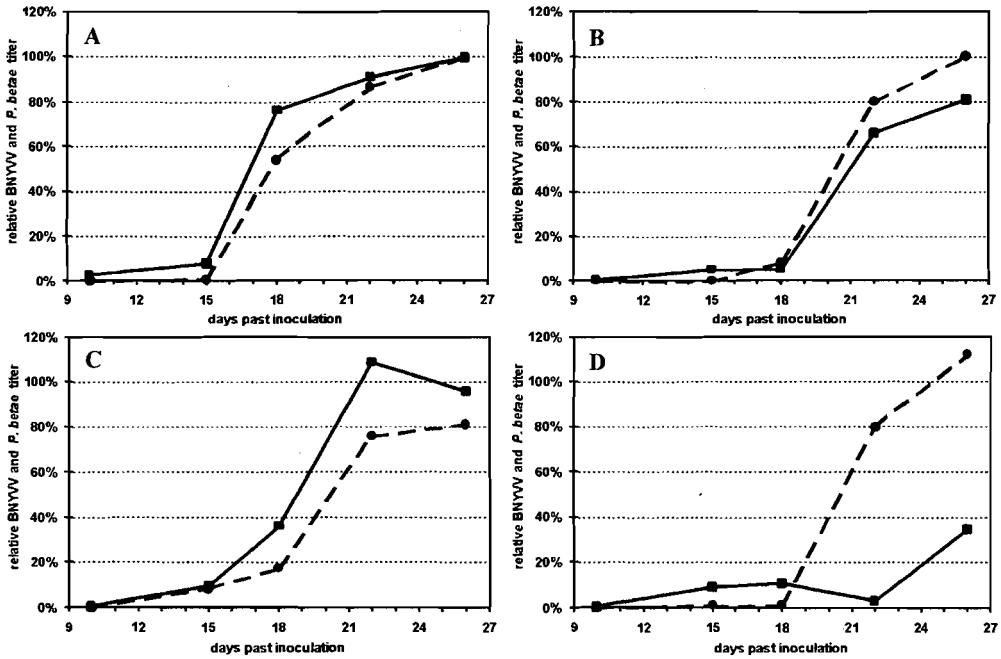

Fig. 3. Development of *P. betae* and BNYVV in roots of sugarbeet cultivars monitored by competitive PCR and DAS-ELISA, respectively. The development of *P. betae* (dashed lines) and BNYVV titers (full lines) are indicated as percentage of titers in roots of the rhizomania-susceptible cultivar 'Hilma' 26 d.p.i. (A) and compared with the development of titers in roots of the partially field resistant cultivar 'Patricia' (B), a partially resistant line originating from the 'Holly' source (C) and a partially resistant line originating from a *B. maritima* source (D).

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THE EFFECTS OF TEMPERATURE ON THE REACTION OF TWO WHEAT CULTIVARS TO DIFFERENT WHEAT YELLOW MOSAIC BYMOVIRUS (WYMV) STRAINS.

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Summary

The use of resistant cultivars is an effective countermeasure to wheat yellow mosaic disease which is caused by wheat yellow mosaic bymovirus (WYMV). In the field, winter wheat cultivar (cv.) Fukuhokomugi showed resistance to WYMV at Morioka (cool temperature) and susceptibility at Ibaraki (warm) while the reverse was true for cv. Nanbukomugi. Temperature may affect resistance of cultivars to WYMV although WYMV strains with different pathogenicities can also be the cause of variation in resistance between cultivars. To more clearly examine the effects of temperature on resistance, cvs. Fukuhokomugi and Nanbukomugi were mechanically inoculated with virus strains from Morioka (WYMV-M) and Ibaraki (WYMV-T), and grown under controlled temperature conditions at 5 °C and 10 °C. Fukuhokomugi was susceptible to WYMV-T both at 5 °C and 10 °C. Nanbukomugi showed resistance to WYMV-T at 10 °C but not at 5 °C. Fukuhokomugi showed immunity to WYMV-M both at 5 °C and 10 °C. Nanbukomugi showed susceptibility to WYMV-M both at 5 °C and 10 °C. When cultivars showed susceptibility, virus incidence was higher at 5 °C than at 10 °C. These result suggest that for some cultivars, resistance to WYMV can be influenced by temperature, and that continuous low temperature results in high virus incidence, promoting symptom expression in susceptible cultivars.

Introduction

Wheat yellow mosaic disease (WYMD) is one of the important diseases in Japanese wheat crops. The disease is induced by wheat yellow mosaic bymovirus (WYMV) mediated by the soil-borne fungal vector *Polymyxa graminis* Led. Use of resistant cultivars is the most effective countermeasure to the disease. In recent years, WYMD has resulted in serious crop yeild reduction in the Tohoku district and Hokkaido which are the northern cold areas of Japan. Because the major cultivars currently grown in these districts are susceptible to the virus, it is necessary to develop new cultivars with high resistance to WYMV as well as cold tolerance and high grain quality. WYMV strains with different pathogenicities have been reported in some locations (Kusume et al., 1997, Saito and Okamoto, 1964). It is therefore necessary to identify gene resources and cultivars that are resistant to the pathogenic viruses at particular locations. However, the resistance of some wheat cultivars in field experiments varied from location to location although the same infested soil had been used as inoculum (Saito and Okamoto, 1964). It was thought that environmental factors such as temperature, precipitation, and day length varied with location, thereby affecting the incidence of WYMD. Thus, the effects of such environmental factors on the resistance of wheat to WYMV strains should be considered when cultivars or gene resouces are tested for resistance to WYMV.

In this study, we conducted experiments focusing on temperature as an environmental factor that may affect the incidence of WYMD. To elucidate the effects of temperature on the resistance of cultivars, cvs. Fukuhokomugi and Nanbukomugi were inoculated with two virus

strains; WYMV-M isolated from Morioka and the WYMV-T isolated from Ibaraki prefecture. Cultivar Fukuhokomugi, which is susceptible to WYMV in Ibaraki prefecture, is immune in Morioka. Cultivar Nanbukomugi, which is susceptible to WYMV in Morioka, is resistant in Ibaraki prefecture (Table 1). Virus propagation and development of symptoms by each virus strain were investigated under controlled temperatures in growth cabinets.

The aim of this study is to determine the effect of temperature on virus propagation, symptom expression and the resistance of wheat cultivars in the fields.

Table 1 Reaction of cvs. Fukuhokomugi and Nanbukomugi to wheat yellow mosaic disease in the field at Morioka and Ibaraki.

Location	Cultivar	
	Fukuhokomugi	Nanbukomugi
Morioka	R	S
Ibaraki*	S	R

R:resistant S:susceptible *Source:Oda and Kashiwazaki, 1989

Materials and Methods

Wheat cultivars and virus strains used. Winter wheat (*Triticum aestivum* Lin.) cvs. Fukuhokomugi and Nanbukomugi were used. Cultivar Fukuhokomugi, which is susceptible to WYMV in Ibaraki prefecture, is immune in Morioka. Cultivar Nanbukomugi, which is susceptible to WYMV in Morioka, was resistant in Ibaraki prefecture (Table 1). Two virus strains, WYMV-M and -T, were used in this study. WYMV-M was isolated at the Tohoku National Agricultural Experiment Station, Morioka in Iwate prefecture, and maintained on cv. Nanbukomugi. WYMV-T, which is Japanese type isolate of WYMV, was isolated in Ibaraki prefecture and maintained on cv. Norin 61. These two strains have been reported to be different in pathogenicity (Kusume et al., 1997).

Preparation of plants and mechanical inoculation. Plastic pots (9 cm diameter) were filled with autoclaved commercial soil and planted with five wheat seeds per pot. Plants were grown to the 3-leaf stage at 20 °C under 12 hour day length (190 μ mol. photon/s/m^2). Inoculum sap for mechanical inoculation was prepared by grinding fresh symptomatic leaves in 0.1 M phosphate buffer (pH 7.0) at a rate of 10 ml/g of leaf. Inoculated plants were lightly sprayed with water immediately after and kept in a dark room for a week at 7 °C to allow recovery from inoculation injury. Inoculated plants were then divided into two sets. One set was placed in a growth cabinet with 12 hr day length (190 μ mol. photon/s/m^2) at 5 °C $\pm$ 1 °C and the other at 10 °C $\pm$ 1 °C. Those temperatures were chosen from observation of symptoms in field experiments at Morioka: 5 °C is the daily mean air temperature when symptoms are most evident, and 10 °C is the daily mean air temperatures when symptoms start disappearing.

Observation of disease progress and monitoring virus incidence. Disease severity was checked weekly by observing plants after the first symptoms developed. Disease index (DI) was rated on a scale of 0 to 4, with 0= without symptoms, 1= slight mosaic on the newest leaf, 2= clear mosaic on upper leaves, 3= mosaic on whole plants and dwarfing, and 4: severe mosaic often with necrosis, deformation of leaves and severe dwarfing. Virus incidence was investigated by enzyme linked immuno-solvent assay (ELISA) at the end of the experiment period. The ELISA procedure was reported previously (Ohto and Naito 1997).

Results

Reaction of the two cultivars to WYMV-M at 5 °C and 10 °C.

When cv. Fukuhokomugi was inoculated with WYMV-M, no symptoms developed and the virus was not detected in any plants at both temperatures (Fig 1A).

When cv. Nanbukomugi was inoculated with WYMV-M, symptoms were developed clearly at both 5 °C and 10 °C. Although symptoms were expressed earlier at 10 °C than 5 °C, they were more severe at 5 °C than 10 °C (Fig. 1A). Symptoms started disappearing about 40 days after inoculation at 10 °C, but were still progressing at 5 °C. Virus incidence was higher at 5 °C than at 10 °C at the end of experiment period (Table 2).

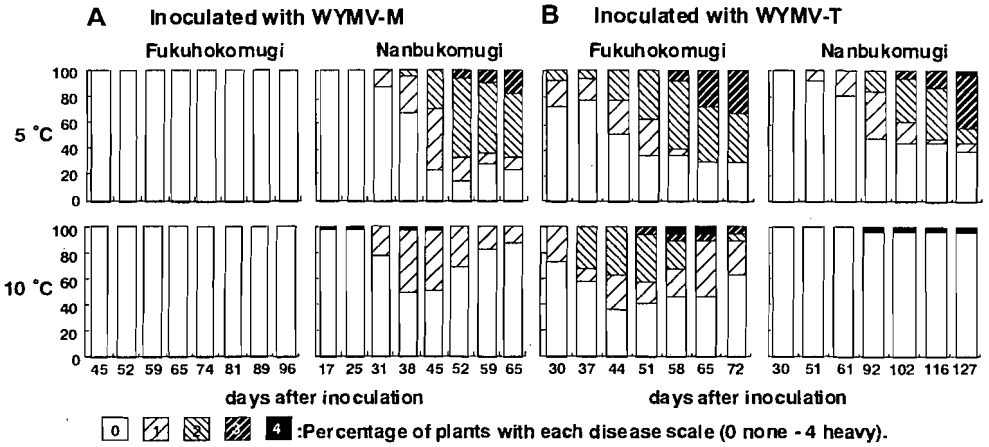

Fig. 1 Symptom development in Fukuhokomugi and Nanbukomugi at 5 °C and at 10 °C after inoculation with WYMV-M and -T.

Reaction of the two cultivars to WYMV-T at 5 °C and 10 °C.

When Fukuhokomugi was inoculated with WYMV-T, symptoms were observed both at 5 °C and 10 °C (Fig. 1B). However, at that time, symptoms were expressed earlier at 10 °C than at 5 °C. Disappearance of symptoms and recovery of diseased plants started earlier at 10 °C than at 5 °C. Virus detection was higher at 5 °C and 10 °C (Table 2).

Table 2 Virus detection (%) in cvs. Fukuhokomugi and Nanbukomugi inoculated with virus strains and incubated at 5 °C and 10 °C.

Virus strain	Cultivar			
	Fukuhokomugi		Nanbukomugi	
	5 °C	10°C	5 °C	10 °C
WYMV-M	ND*	ND	75.0	58.3
WYMV- T	76.0	48.0	64.0	14.0

*: not detected

When cv. Nanbukomugi was inoculated with WYMV-T and grown, symptoms progressed throughout the experiment period at 5 °C. The virus was detected in all symptomatic plants as indicated by high ELISA values (data not shown). In contrast, only slight symptoms in some plants were observed when inoculated plants were grown at 10 °C (Fig. 1B). Compared to the results with WYMV-M inoculated plants, virus incidence was very

low at 10 °C (Table 2). It required about 50 days to express the first symptom at 5 °C for cv. Nanbukomugi, but less than 30 days for cv. Fukuhokomugi. The differences of disease incidence and virus detection between 5 °C and 10 °C were larger in cv. Nanbukomugi than in cv. Fukuhokomugi.

Discussion

It has been reported that some wheat cultivars including cv. Nanbukomugi grown on virus-infested soil from Himeji at both Himeji and Morioka, showed symptoms of WYMD only at Morioka (Saito and Okamoto 1964). In the same experiment, disease severities of cultivars that showed symptoms at both locations were more severe at Morioka than at Himeji. Himeji is in western Japan and was a warmer climate than Morika throughout the year. This result suggests that environmental factors including temperature affect the reaction of wheat cvs. to WYMV as well as genetical factors (eg. cultivar, virus strain).

In this study, we conducted experiments focusing on temperature as an environmental factor that may affect WYMD resistance in some wheat cvs. Our results showed that temperature affected the resistance of wheat cultivars to WYMV. Continuous low-temperatures can promote viral propagation and symptom development on leaves of susceptible cultivars. Compared to western Japan, the wheat growing season in northern Japan has a longer period of time where the temperature is under 10 °C. Thus, some cultivars that show resistance when grown in warm districts (for example cv. Nanbukomugi) may show susceptibility in cold areas. Symptoms in susceptible cvs. by the same virus strains may be more severe in the Tohoku district or Hokkaido (colder) than in western Japan (warmer).

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THE P3 PROTEIN OF BARLEY MILD MOSAIC VIRUS: SYNTHESIS IN *E. COLI* AND PRODUCTION OF DIAGNOSTIC ANTISERA

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Summary

Despite the application of several vector systems all attempts to overexpress the entire P3 gene of an Aschersleben isolate of barley mild mosaic virus (BaMMV) in *E. coli* were unsuccessful. Consequently, a series of subclones representing different parts of the coding sequence was generated and analysed for *in vitro* expression. The region between the amino acid positions 215 and 250 was identified as being extremely toxic for the bacterial host. A fragment corresponding to the N-proximal 60 % of the P3 molecule was purified and used to raise a specific antiserum.

Introduction

Bymoviruses and potyviruses have a common genome organisation although the percentage of their nucleotide sequence identity is very low. Since a reliable and efficient system to infect plants with full-length cDNA clones of bymoviral genomic RNAs is still not available the biological functions attributed to the non-structural proteins have been only partially proven experimentally so far and were in most cases deduced from their position in the initial polyproteins and the presence of characteristic amino acid motifs conserved among potyviruses. The P3 protein represents one of the most divergent parts of the polyproteins encoded by the various bymo- and potyviruses (Meyer and Dessens, 1996; Sakai *et al.*, 1997). To date, its role in virus life cycle is still unknown even for the viruses characterised in most detail. After having detected the P3 of tobacco etch virus (TEV) associated wth nuclear inclusion bodies in infected cells Langenberg and Zhang (1997) supposed an involvement of the protein in early stages of virus replication although in case of potato virus A (PVA) it was not able to bind RNA (Merits *et al.*, 1998). According to Klein *et al.* (1994) small insertions in the tobacco vein mottling virus (TVMV) P3 abolished virus replication in tobacco protoplasts. Recently, Moreno *et al.* (1998) reported that the TVMV P3 gene can confer virus resistance but at the same time has a detrimental effect on transgenic tobacco plants. The objective of the present work was to overexpress the BaMMV P3 gene in *E. coli* in order to raise a specific antiserum that can serve as a diagnostic tool to further investigate the role of the protein in virus life cycle.

Materials and Methods

Starting from a purified RNA preparation of the isolate BaMMV-ASL1 the viral cDNA was synthesised by help of the Marathon kit (Clontech). The two specific primers, 5'-GGTACCATGGAGGAGTTCATTCCTGAAGTGTTCA-3' and 5'-TCTAGATTGCAATATAGGCCCTTTTCTGTTTTTAAG-3' each supplemented with an additional restriction site (underlined) for Kpn I and Xba I, respectively served to amplify the P3 coding region of RNA1 spanning from nucleotide (nt) position 149 to 1024 by PCR utilizing the Advantage KlenTaq Polymerase Mix (Clontech). The amplicons were cloned in pGEM-T vector (Promega), subsequently excised by a Not I/Xba I restriction and recloned in the expression vector pThioHis C (Invitrogen). A modified form of *E. coli* derived thioredoxin serves as a fusion partner in this system. The same strategy was followed for cloning various deleted and truncated forms of the P3 coding sequence, using appropriate PCR primers. Gene expression was induced in 5 ml of cell culture in logarithmic growth stage by addition of IPTG to a final concentration of 2 mM. Cells were harvested 4 hours after induction by brief centrifugation and resuspended immediately in Laemmli (1970) sample buffer (100 µl for 250 µl of cell suspension). Crude cell lysates were analysed by SDS-PAGE in 15 % gels followed by Western blot analysis. The same principle was followed with the PinPoint™ Xa expression system (Promega) to produce the N4D fragment of P3. For preparation of antisera the band corresponding to the fusion protein (P3-N4) was excised from Coomassie brilliant blue stained gels, washed in distilled water, resuspended in 0,9% NaCl and injected into rabbits. Three immunization doses were given intramuscular and subcutan in 10 day intervals. In contrast to this approach the recombinant P3-N4D was purified by affinity chromatography (SoftLink™) according to manufacturer's instructions and a series of 5 intravenous injections

was given to a rabbit. An indirect plate trapped antigen (PTA) ELISA was performed to detect P3 in plants: Material was grinded in a mortar, sap diluted with PBS (leaves) or 0,1 M potassium citrate buffer pH 7,0 (roots) was incubated in the microtiter plate for 1 h at 37 °C, anti P3-IgG (2 µg/ml) was incubated overnight at 4 °C followed by the conjugate (goat-anti-rabbit antibody coupled with alkaline phosphatase) for 1,5 h at 37 °C and the substrate p-nitrophenyl phosphate. For Western blot analysis crude bacterial preparations and total plant protein extracts were resolved by SDS-PAGE and transferred onto PVDF-membrane that was blocked in PBS containing 0,05 % Tween 20 (PBST) and 5 % skimmed milk for 2 h at room temperature. The membranes were incubated for 1,5 h in a 1:1000 dilution of the primary antibody, followed by 3 washing steps (each for 10 min) in PBST and a subsequent incubation with a goat-anti-rabbit secondary antibody conjugated to alkaline phosphatase (AP, Dianova). Finally, the membranes were exposed to a BCIP-NBT substrate solution. The biotinylated products obtained with the PinPoint™ system were visualised by a streptavidin-AP conjugate.

Results and Discussion

Our attempts to overexpress the entire coding region of the putative P3 protein did not reveal products of the expected size regardless of the vector system used. Typically, growth of the induced *E. coli* cells was repressed and on Western blots weak bands as shown in figure 1 (lane P3) became visible only after loading the total extract of a 2,5 ml bacterial culture onto the PA gel. In order to improve this situation and to localise the toxic region of the translation product a series of truncated and deleted subclones was produced (Fig. 2) and overexpressed. As figure 1 demonstrates they behaved differently with respect to the size and the yield of the synthesised P3 fragment. Concluding from these data the region spanning between the aa residues 215 and 250 appears to have a detrimental effect on *E. coli*. It represents a part of an extended hydrophobic domain in the C-terminal quarter of the molecule which appears to be a common feature for the P3 proteins of potyviridae. (Fig. 3). The putative transmembrane nature of this nonstructural protein has been mentioned already (Cerezo and Shaw, 1991; Moreno *et al.*, 1998). In our experiments even the N1 fragment of BaMMV P3 was mainly associated with the unsoluble fraction of the lysed *E. coli* cells.

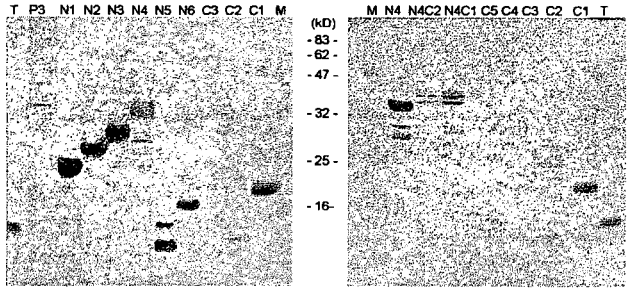

Fig. 1 Western blot analysis after expression of a series of derivative clones of BaMMV P3 gene in (see Fig. 2) in *E. coli* using the antiserum raised against the N4 fragment fused to thioredoxin.

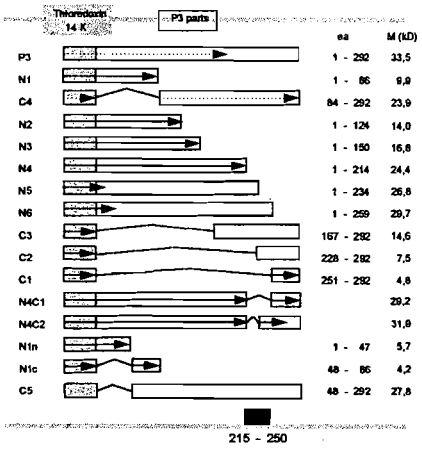

Fig. 2 Scheme of the BaMMV P3 gene fragments cloned in pThioHis vector and expressed in *E. coli*. Shaded boxes represent the fusion partner thioredoxin. Amino acid (aa) positions and the calculated molecular weights (M) give the localisation and size of the cloned sequence. Arrows indicate for both the size of the major translation product and the efficiency (bolt line - high, hatched line - low) of *in vitro* expression determined by Western blotting (see Fig. 1).

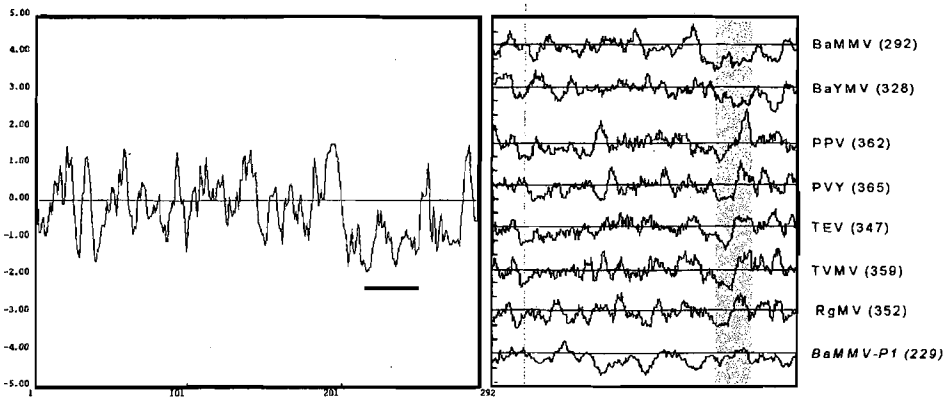

Fig. 3 Hydrophobic profils according to Hopp and Woods for P3 proteins of (a) BaMMV and (b) different bymo-, poty- and rymoviruses. X-axis and numbers in brackets - amino acid residues; Y-axis - hydropathy. Hydrophobic domains are underneath the zero line, hydrophilic domains are above. Bar represents the region between aa residues 215 and 250 in BaMMV P3 that is toxic for *E. coli*. Grey bar marks the corresponding region in the P3 proteins of related viruses.

Due to big losses in the course of purification and separation of the P3 from thioredoxin the N4 fragment was injected into a rabbit as the fusion protein. The resulting antiserum 1 recognised the recombinant polypeptides from *E. coli* (Fig. 1) but did not show any reaction with sap or protein extracts from BaMMV infected plants. To prove the presence of P3 specific antibodies within this antiserum the N-terminal 163 aa comprising P3 fragment was synthesised *in vitro* using the PinPoint^{TM} Xa vector system (Fig. 4). Antiserum 1 did not decorate this polypeptide in Western blots thus indicating that it is specific for thioredoxin only. On the other hand antiserum 2 raised against the N4D fragment clearly detected the recombinant P3 derivatives from the pThioHis expression system, as it is shown in figure 5 for the fragments N1 and N3. In addition it reacts with a number of *E. coli* proteins. Antiserum 2 was applied in ELISA experiments to detect the P3 protein in barley plants. According to the extinction values presented in the table weak positive reactions were observed with infected leaf and root tissue while healthy plant material revealed values above 0,1 only after prolonged substrate incubation.

Taking into account that antiserum 2 was produced against the BaMMV P3 fragment N4D which is fused to a 13 kD subunit of a bacterial transcarboxylase originating from the PinPoint^{TM}Xa vector the positive results in ELISA experiments cannot be assigned to P3 specific antibodies unless the latter have been separated from the putative mixture or an antiserum specific to the fusion component is available. The two approaches are currently being followed to confirm the results.

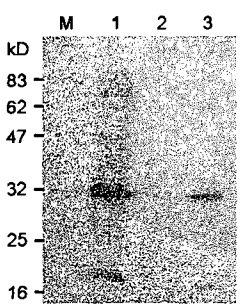

Fig. 4 *In vitro* expression of N4D fragment of BaMMV P3 gene cloned in PinPoint Xa vector. Western blot analysis using a streptavdin/alkaline phosphatase complex for detection of (1) crude bacterial extract, (2) flow through fraction after affinity chromatography on SoftLink^{TM} resin, (3) fraction eluted from the column, (M) molecular weight marker.

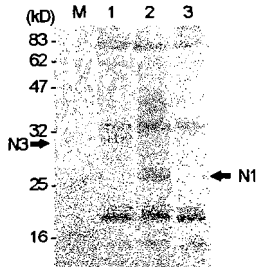

Fig. 5 Western blot analysis of crude bacterial extracts after *in vitro* expression of BaMMV P3 gene clones (1) pThioHisN3 and (2) pThioHisN1. (3) *E. coli* strain JM 109 (control), M-molecular weight marker. Detection with antiserum2 raised against the P3 fragment N4D (see text).

Table Detection of BaMMV P3 protein in infected barley plants by PTA-ELISA. DAS-ELISA for detection of coat protein (CP) serves as a control, h-healthy, i-infected, E_{405}.

Material	Dilution	DAS ELISA (CP)			PTA ELISA (P3)			
		1h	3h	24h*	2h	3h	6,5h	24h*
leaves/i	1/5	2,05	2,08	>3,00	0,39	0,45	0,92	1,32
	1/10	1,66	>3,00	>3,00	0,13	0,15	0,32	0,51
roots/i	1/5	n.t.	n.t.	n.t.	0,13	0,15	0,32	0,49
	1/10	n.t.	n.t.	n.t.	0,12	0,14	0,30	0,47
leaves/h	1/5	0,00	0,01	0,01	0,08	0,09	0,19	0,29
	1/10	0,01	0,01	0,01	0,01	0,01	0,03	0,06
roots/h	1/5	n.t.	n.t.	n.t.	0,04	0,05	0,10	0,16
	1/10	n.t.	n.t.	n.t.	0,05	0,06	0,13	0,22

* After incubation for 3h (DAS) and 6,5 h (PTA), respectively at room temperature the microtiter plates were kept at 4 °C.

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INVOLVEMENT OF BEET NECROTIC YELLOW VEIN VIRUS RNA 3 IN RESTRICTED VIRUS MULTIPLICATION IN RESISTANT LINES OF *BETA VULGARIS* SSP. *MARITIMA*

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Summary

The resistance to beet necrotic yellow vein virus (BNYVV) in sugar beet cultivars such as Rizor is known to be caused by a restriction of virus translocation in the roots. To evaluate the resistance, leaves of the different sugar beet cultivars and several lines of the wild beet *Beta vulgaris* ssp. *maritima* were manually inoculated with sap from BNYVV-infected leaves. The results indicated that BNYVV induced either of two lesion-types in inoculated leaves: necrotic, small or no visible lesions (R reaction) and bright yellow lesions (S reaction). The R reaction in resistant lines was especially dependent on the BNYVV strains. The P25-defective isolates caused only faint chlorotic lesions in inoculated leaves of both resistant and susceptible lines, indicating that the S and R reactions are determined by the presence of the P25. Thus, we suggest that P25 encoded by BNYVV RNA 3 is involved in a restricted virus spread in inoculated leaves of resistant beet plants.

Introduction

Rhizomania disease caused by beet necrotic yellow vein virus (BNYVV) is recently distributed in many sugar beet growing countries and is economically very important (Asher, 1992). Therefore, genetic resistance is the most promising approach for the control of the disease. Several resistant cultivars such as Rizor have been developed and are now grown in rhizomania-infested regions (Asher, 1992). The resistance of such cultivars has been reported to be caused by a restriction of virus multiplication and/or translocation in the roots (Giunchedi *et al.*, 1987; Scholten *et al.*, 1994; Tamada *et al.*, 1999). The genome of BNYVV usually consists of four RNA components. RNAs 1 and 2 encode proteins involved in replication, assembly and cell-to-cell movement, and RNAs 3 and 4 are needed for disease development and spread in nature (Tamada, 1999).

In previous work (Tamada *et al.*,1999), we have shown that the 25 kDa protein (P25) encoded by RNA 3 is directly responsible for the development of rhizomania symptoms on susceptible sugar beet cultivars, and also that it may inhibit virus translocation from rootlets to taproots in the resistant cultivar. In this paper, we describe the results of mechanical inoculation to susceptible and resistant plants, and present evidence that the P25 is involved in a resistant response in inoculated leaves.

Materials and Methods

Plant materials: Nine accessions of *Beta vulgaris* ssp. *maritima* were obtained from Federal Centre for Breeding Research on Cultivated Plants (BAZ), Gene Bank, Braunschweig, Germany. The sugar beet cultivars Monomidori and Rizor were used as susceptible and resistant controls, respectively.

Virus isolates: The wild-type virus isolates O11 (from Obihiro, Hokkaido) and Gw (from Germany), which contain four RNA components, were used. The laboratory isolates O11-4 and Gw-4, both of which lack RNA 3, were obtained from individual original isolates O11 and Gw by single lesion transfers in *Tetragonia expansa* leaves, as described by Tamada *et al.* (1989).

P25-deletion mutant S-3c4, which lost 121 amino acids to the C-terminal part of the P25, was used (Tamada *et al.*, 1999). The other 11 wild-type virus isolates were also used.

Mechanical inoculation: The virus isolates were propagated in inoculated *T. expansa* leaves in a glasshouse or a growth cabinet. Leaves for use as virus inoculum were extracted with 0.1 M phosphate buffer, pH 7.4, containing 0.5 % 2-mercaptoethanol. The extracts were inoculated to well-expanded leaves (about 2 months old) of sugar beets or *B. vulgaris* ssp. *maritima*. Inoculated plants were kept in a growth cabinet at 24 ^{0}C.

Results

Reactions of BNYVV in sugar beet leaves by mechanical inoculation

In previous work (Tamada *et al.*, 1999), wild-type and RNA 3-mutant viruses were inoculated by *Polymyxa betae* to sugar beet seedlings of susceptible cultivar Monomizori and partially resistant cultivar Rizor. The mutant strains lost 94 or 121 amino acids to the C-terminal part of the P25 (219 amino acids) encoded by RNA 3. No differences were found in virus content in rootlets between mutant and wild-type viruses or between susceptible and resistant cultivars after culture for 4 weeks in the growth cabinet. However, when virus-inoculated seedlings were grown in the field for 5 months, the wild-type virus caused typical rhizomania root symptoms in susceptible cultivar, but no symptoms developed in most plants of the resistant cultivar and BNYVV concentrations in the roots were 10 to 20 times lower in these plants than in susceptible plants. In contrast, the mutant viruses caused no symptoms in susceptible or resistant cultivars, and the virus content of roots was not significantly different between both cultivars. These results suggest that wild-type RNA 3 may fail to facilitate virus translocation from rootlets to taproots in the resistant cultivar (Tamada *et al.*, 1999).

To elucidate this resistant mechanism, BNYVV was inoculated mechanically to leaves of two cultivars Monomizori and Rizor. Wild-type virus O11 was used as an inoculum. In susceptible sugar beets, this virus isolate produced chlorotic and yellowish lesions in inoculated leaves at one week after inoculation, and in a few days they became bright yellow lesions. Then, the yellow lesions enlarged and tended to coalesce, spreading along the veins. In resistant cultivar Rizor, however, the virus produced only small yellow or necrotic lesions in inoculated leaves. Such lesions usually did not become larger. The degree of lesion sizes was different with individual plants in the population of the culivar Rizor; about 90% of plants showed various small or necrotic lesions, but the remaining plants showed bright yellow lesions. This indicates that the virus tends to be restricted in inoculated leaves of resistant sugar beet plants. However, when the Gw isolate from Germany was inoculated to this cultivar, it induced the yellow-type lesions in inoculated leaves of most plants.

Reactions of BNYVV in *B. vulgaris* ssp. *maritima* by mechanical inoculation

Nine accessions of *B. vulgaris* ssp. *maritima* were screened by mechanical inoculation with the O11 isolate. Thirty to 60 plants of each accession were tested. The results showed that the O11 isolate produced various types of lesions such as yellow lesions (Fig. 1. D), necrotic lesions (Fig. 1. B), small yellow lesions (Fig. 1. C) or no visible lesions (Fig. 1. A), depending on the accessions of *B. vulgaris* ssp. *maritima*. Some of such lesion types were similar to those in the Rizor plants. Thus, bright large yellow lesions are referred to as a susceptible (S) reaction, whereas no visible, small or necrotic lesions are referred to as a resistant (R) reaction. For the R reaction, the virus is usually restricted in infected sites in inoculated leaves, whereas, for the S reaction, the yellow lesions are spreading gradually in inoculated leaves, followed in some plants by systemic infection, depending on susceptibility of the accessions. Among the 9 *B. vulgaris* ssp. *maritima* accessions, all plants in the following accessions, BGRC49834, 49844 and 54783, showed the S reaction, and the following accessions, BGRC49845, 51424, 51432, 54776, 54778 and 54779, contained plants showing the S and R reaction. For example, 13 to 60 % of plants showed the R reaction, differing with

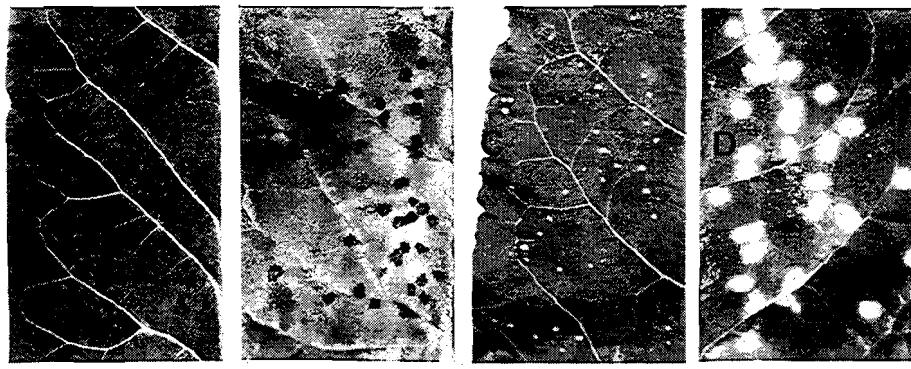

Fig.1. Local lesions in inoculated leaves of susceptible and resistant lines of *Beta vulgaris* ssp. *maritima* inoculated with the O11 isolate of BNYVV. (**A**) no visible lesions, (**B**) necrotic lesions, (**C**) small yellow lesions, and (**D**) bright yellow lesions.

Table 1. Reaction of Japanese and German BNYVV isolates by mechanical inoculation in *B. vulgaris* spp. *maritima* MR1 and MR2

Location	BNYVV isolate	*B. vulgaris* spp. *maritima*		
		MR2	MR1	MR0
Japan	O11, K53, S12, N64, M87	R	R	S
	S113, K80, T101, S34	R	S	S
	T41, H45, T	S	S	S
Germany	Gw	S	S	S

R, resistant reaction ; S, susceptible reaction.

the accessions. This suggests that these *B. vulgaris* ssp. *maritima* accessions were heterogeneous. To obtain plants that were more homogeneous, several sets of seeds were obtained from each plant of two accessions BGRC 54778 and 51424, showing different types of the R reaction. Out of them, two relatively homogeneous lines, which were designated MR1 and MR2, were selected. Several homogeneous lines showing the S reaction were also obtained, and one line was designated MR0. These lines were used for further study.

Reactions of BNYVV isolates in *B. vulgaris* ssp. *maritima* MR1 and MR2

In the first experiments, 12 Japanese and one German isolates were examined by mechanical inoculation for their responses to MR1, MR2 and MR0 (susceptible control) plants. The results (Table 1) showed that BNYVV isolates were divided into three groups based of the reactions: group 1 contains 5 isolates which show the R reaction to both MR1 and MR2 plants, group 2, 4 isolates, the S and R reactions to MR1 and MR2 plants, respectively, and group 3, 4 isolates (including one German isolate), the S reaction in both MR1 and MR2 plants.

In further experiments, to confirm an involvement of RNA 3 in their reactions, laboratory isolates O11-4, Gw-4 and S-3c4 were inoculated to MR1, MR2 or MR0 plants. As a result, these mutant viruses produced only faint chlorotic lesions in inoculated leaves of these plants, and then the lesions became larger. No distinct differences in lesion types were found between

MR1, MR2 and MR0. We therefore concluded that both R and S reactions are due to the presence of P25 encoded by RNA 3.

Discussion

It has been reported that the resistance to rhizomania in sugar beet cultivars is caused by a restriction of virus multiplication and translocation in the roots, but not by resistance to infection by the vector *P. betae* (Asher, 1993; Giunchedi *et al.*, 1987; Scholten *et al.*, 1994). Our previous paper (Tamada *et al.*, 1999) indicated that the RNA 3-coding protein is involved in restriction of virus translocation from rootlets to taproots in the resistant sugar beets and also is essential for symptom development in roots of susceptible plants. In this study, we have tried to do mechanical inoculation to susceptible and resistant sugar beet plants, and the results showed that such a virus restriction in the resistant plants was also observed in inoculated leaves: the resistant (R) reaction consists of necrotic, small, or no visible lesions, whereas the susceptible (S) reaction is bright large yellow lesions. To more clarify the response to virus infection in resistant hosts, the wild beet *B. vulgaris* ssp. *maritima*, from which the resistant genes are derived (Geyl *et al.*, 1995; Whitney, 1989), were screened. As a result, two different lines were able to be selected. Based on the virus reaction to the two lines, BNYVV isolates tested were divided into three groups: group 1 isolates show the R reaction to both lines, group 2, the R reaction to one line and the S reaction to the other line, and group 3, the S reaction to both lines. Thus, there were strain-specific interactions between the two hosts of *B. vulgaris* ssp. *maritima* and BNYVV strains. In addition, experiments using deletion mutants showed that this specificity is determined by the P25 encoded by BNYVV RNA 3.

Acknowledgments

We thank Dr. L. Frese (Federal Centre for Breeding Research on Cultivated Plants (BAZ), Gene Bank, Braunschweig) for providing seed samples of *B. vulgaris* ssp. *maritima* .

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REACTIONS OF DURUM WHEAT (TRITICUM DURUM DESF.) CULTIVARS (CVS) TO SOILBORNE WHEAT MOSAIC FUROVIRUS (SBWMV) IN NORTHERN ITALY DURING 1996-97

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Summary

The reactions to soilborne wheat mosaic furovirus (SBWMV) of thirty-three cvs. of durum wheat (*Triticum durum Desf.*) were evaluated on the basis of symptom expression, DAS-ELISA readings and agronomic performance. The three resistance parameters were closely correlated, yet none of them furnished by itself all the information needed by farmers, policy makers and / or breeders. Cultivars with the highest disease scores and ELISA values suffered grain losses of about 60% and appreciable reductions in test weight, kernel weight and plant height. All cvs. contained at least some SBWMV particles.

Introduction

In Italy, soilborne wheat mosaic furovirus (SBWMV) is widespread, especially in the northern and central regions of the country (Canova and Quaglia 1960; Rubies-Autonell and Vallega 1985, 1987; Vallega and Rubies-Autonell 1985, 1989), where it may cause grain yield reductions of about 50-70% (Toderi 1969; Vallega and Rubies-Autonell 1985; Vallega *et al.* 1997, 1999); often it is found in mixed infection with wheat spindle streak mosaic virus (Rubies-Autonell and Vallega 1987). SBWMV can be effectively controlled by growing genetically resistant cvs. Resistance to this virus may be evaluated on the basis of various parameters, including virus titer, visible symptoms' expression, and agronomic performance.

Material and Methods

The trial comprised thirty-three cultivars of durum wheat sown October 31 (1996) in a field with natural inoculum sources of SBWMV situated near Minerbio (Bologna), in northern Italy. The cultivars were grown in plots of 10-m^2 distributed in the field according to a randomized-block design with three replicates. All cvs., except Bronte, Ceedur, Ciccio, Iride, Lloyd, Louxor, Rusticano, San Carlo and Svevo had been tested also in the previous season (Vallega *et al.* 1999). Symptom severity was evaluated using a 0-4 scale, where 0.0-1.0 = resistant, slight or no symptoms; 1.1-2.0 = mildly resistant, mild mottling and stunting; 2.1-3.0 = mildly susceptible, mottling and stunting; and 3.1-4.0 = susceptible, severe mottling and stunting, with virus-killed plants. Symptom scores, assigned on March 26, April 4, April 16 and April 24, were averaged for presentation and computations. Agronomic performance was evaluated in terms of grain yield, thousand-kernel weight, test weight, heading date and plant height at maturity. Ten plants were collected from each plot on April 4 and May 7 (1997) to perform DAS-ELISA according to the procedure described previously (Vallega *et al.* 1999). Extracts were prepared with the four younger leaves of each plant (April 4) or with the distal half portion of the youngest leaf (May 7). Mean ELISA absorbance for extracts from virus-free leaves of cv. Valgerardo was 0.051. A modified ISEM procedure was used to examine leaf extracts of cvs. with mild symptoms and low ELISA values (Vallega et al 1999).

Results

None of the durum wheats analysed remained asymptomatic and, according to the ISEM tests performed, all contained at least a few SBWMV particles. As in the previous season (Vallega et. al 1999), cultivars Ares and Neodur exhibited low ELISA values (0.049 and 0.042, respectively), very mild symptoms (0.1 and 0.4), and an excellent agronomic performance (fourth and fifth in terms of grain yield). Cultivar Louxor, assayed for the first time, reacted to SBWMV in about the same way as Ares and Neodur. Cultivar Zenit had very low ELISA values, mild symptoms and relatively high yields, but this contrasts with the results of the previous season, and remains unexplained. Among cvs. assayed for the second year, those displaying the greatest susceptibility in terms of symptom severity, ELISA values and grain yield were, again, Grazia, Cirillo, Balsamo, Platani, Giemme and Simeto.

Correlations between either ELISA values or symptom severity scores and each of the agronomic characters considered were mostly significant (Table 1); interestingly, all of the eleven cvs. with symptom scores below 1.0 were among the 13 highest grain yielding wheats (Fig. 1). The correlation coefficient between ELISA values and symptom severity scores (Fig. 2) was very high (0.826**); however, among the eleven cvs. with mild symptoms (disease score < 1.0), mean ELISA values ranged from 0.042 to 0.480, and among the twelve highest yielding cvs. mean ELISA vales ranged from 0.042 to 1,142. As in the previous season, cvs.

Table 1. Simple correlation coefficients between disease ratings, ELISA values and various plant characters, for 33 cvs. of durum wheat grown in a field with natural inoculum sources of SBWMV, near Bologna, Italy, in 1996-97.

	Mean	Range	Disease severity	ELISA values
Grain yield (t/ha)	3.64	1.27 - 5.77	- 0.812 **	- 0.751 **
Test weight (kg/hl)	81.3	72.3 - 85.9	- 0.408 *	- 0.532 **
Heading date (days)	39	37 - 43	0.418 *	0.246
Plant height (cm)	74	64 - 81	- 0.556 **	- 0.468 **
1000-kernel weight (g)	45.7	31.4 - 57.0	- 0.368 *	- 0.412 *
Disease severity	1.5	0.1 - 3.6	-	0.826 **
ELISA values	0.789	0.011 - 1.976	0.826 **	-

* = significant at P = 0.05 ; ** = significant at P = 0.01.

Table 2. Estimated mean effects of SBWMV on symptomatically resistant and symptomatically susceptible cultivars of durum wheat grown in a field with natural inoculum sources of SBWMV near Bologna, Italy, in 1996-97.

Disease severity	Grain yield loss		Test weight reduction		Heading date delay	Plant height reduction		1000-kernel weight reduction	
	Actual (t/ha)	%	Actual (kg/hl)	%	Actual (days)	Actual (cm)	%	Actual (g)	%
0.0 - 1.0	0.32	6 %	0.6	1 %	-1	1	1 %	1.6	3 %
1.1 - 2.0	2.05	39 %	2.0	2 %	-1	6	7 %	3.4	7 %
2.1 - 3.0	2.84	53 %	4.2	5 %	-2	6	8 %	6.4	13 %
3.1 - 4.0	3.01	57 %	2.4	3 %	-5	9	12 %	10.6	22 %

Figure 1. Relationship between grain yield and disease severity (scale = 0 - 4) observed for 33 cvs. of durum wheat grown in a field with natural inoculum sources of SBWMV, near Bologna, Italy, in 1996-97.

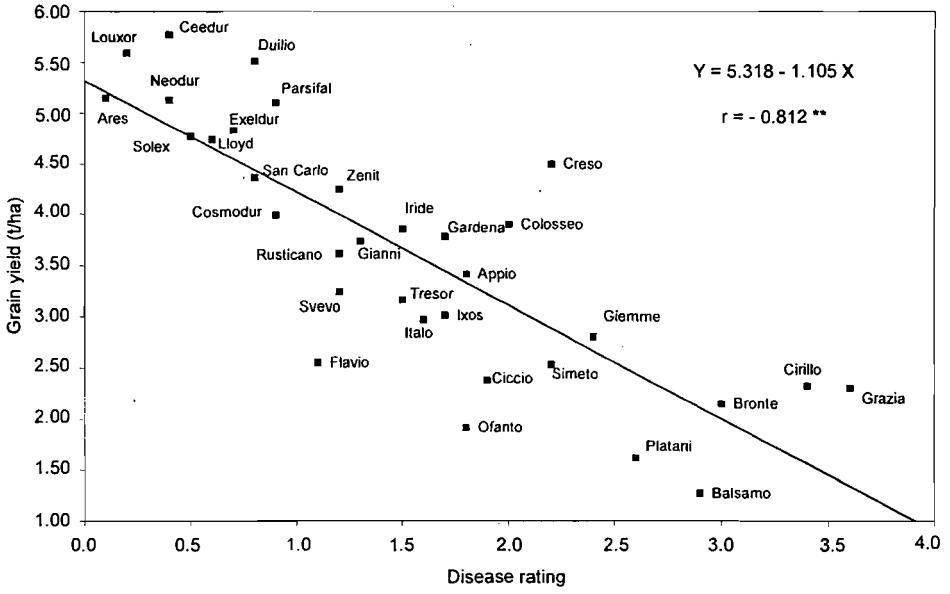
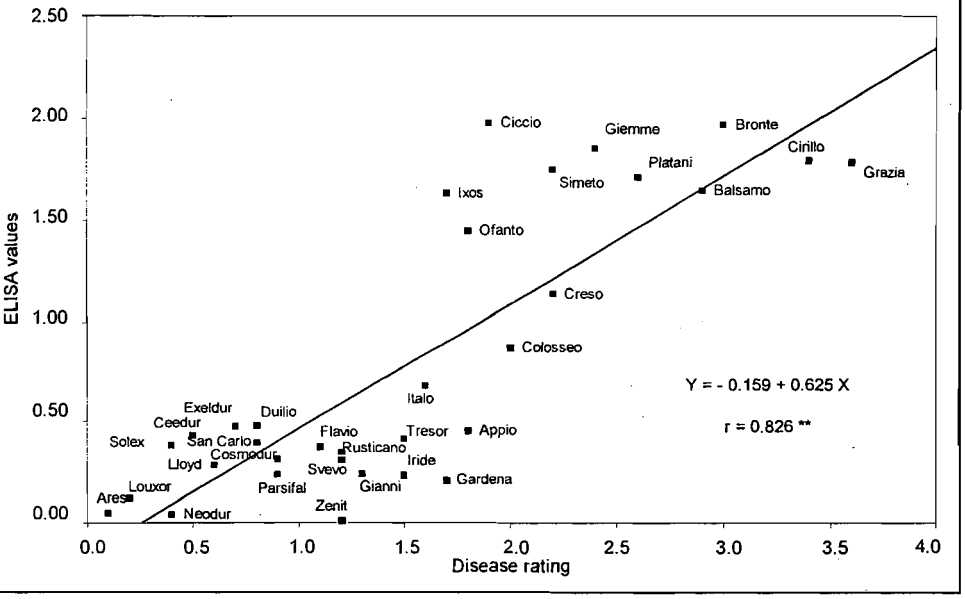


Figure 2. Relationship between ELISA values and disease severity ratings (scale = 0 - 4) observed for 33 cvs. of durum wheat grown in a field with natural inoculum sources of SBWMV, near Bologna, Italy, in 1996-97.



Creso and Parsifal yielded rather well despite moderately severe symptoms and / or high ELISA values. The grain yield rankings of some of the cvs. with intermediate ELISA values (i.e. Duilio, Exeldur, Flavio, Gardena and Solex) contrasted with that recorded in the previous season.

Linear regression equations between disease severity and each of the agronomic characters considered showed that cultivars with mean disease scores above 2.1 suffered grain yield losses of about 55% as well as test weight, 1000-kernel weight and plant height reductions of about 4%, 17%, and 10%, respectively; heading date was also retarded (Table 2). Perhaps more importantly, estimates confirmed (Vallega *et al.* 1997, 1999) that SBWMV may seriously damage even cultivars with relatively mild symptoms; the latter cvs., in fact, suffered grain yield and 1000-kernel weight reductions of about 39% and 7%, respectively.

Discussion

This and previous studies (Vallega *et al.* 1997, 1999) indicate that, although the three resistance parameters investigated are closely related, there exist also noteworthy discrepancies between them. This is of importance because each parameter has a different practical significance. Farmers are interested almost only in yield data, but in the long run would profit even more from cvs. characterized also by low virus titer so as to reduce SBWMV-inoculum in the soil; policy makers may also be interested in cvs. with low virus titer to reduce inoculum sources and virus spread, but can not ignore farmers' yield expectations; finally, breeders should be aware of the fact that they may obtain significant progress by selecting on the basis of symptoms alone, but that this policy does not necessarily guarantee the obtainance of cvs. with low virus titer.

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DIFFERENCES AMONG SUGAR BEET CULTIVARS TO SINGLE AND MIXED INFECTIONS WITH BEET NECROTIC YELLOW VEIN VIRUS AND BEET SOIL-BORNE MOSAIC VIRUS.

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Summary

Eight sugar beet cultivars that range in reaction to rhizomania from uniformly susceptible to resistant were compared for levels of *Beet necrotic yellow vein benyvirus*, as measured by TAS-ELISA in field studies in Salinas, California. Differences in absorbance ($A_{405\ nm}$) values measured among the cultivars closely correlated with the dosage and frequency of the *Rz* allele that conditions resistance to BNYVV. Absorbance values were significantly positively correlated with rhizomania disease index scores and negatively correlated with individual root weight. Seven cultivars were compared in greenhouse pot cultures for reaction to *Beet soil-borne mosaic benyvirus*. All cultivars were highly susceptible to BSBMV, with absorbance readings ranging from 8 to 12 times the healthy root mean. When sequential infections of BNYVV and BSBMV were compared to single infections in a resistant sugar beet cultivar, fresh beet weights were lower than for each virus alone. This was true regardless of whether the seedlings were initially infested with either BNYVV or BSBMV. Resistance to BNYVV did not confer resistance to BSBMV, nor did BSBMV infection moderate the effects of BNYVV.

Introduction: Resistance to rhizomania in most commercial sugar beet cultivars is conditioned by the dominant allele *Rz* (Lewellen, et al., 1987). A number of cultivars with varying degrees of resistance to rhizomania based on different genetic backgrounds have been developed for the diverse production conditions throughout the United States. The dosage of the *Rz* allele (number of alleles in a particular individual) and the frequency (ratio of *Rz* to *rz* alleles in the cultivars) are important in the overall performance of sugar beet cultivars under rhizomania conditions. Previous reports in England (Asher and Kerr, 1996; Asher et al., 1997) and the Netherlands (Tuitert, et al., 1994) showed that sugar beet cultivars with different levels of resistance correlate with the levels of BNYVV detected in roots.

An objective of this study was to determine relative levels of BNYVV in representative commercial and experimental sugar beet cultivars and to relate the BNYVV levels to allelic dosage of these cultivars. Cultivars selected ranged in their reactions to rhizomania from uniformly susceptible to resistant. Selection of rhizomania resistant parental lines of hybrid cultivars is based on their field performance, which includes symptom evaluation and analyses for sugar content and root yield (Lewellen, 1995). In Europe, selections are commonly made by virus concentration as determined by ELISA tests from sugar beet seedlings grown under controlled conditions in greenhouses and growth rooms (Pelsy and Merdinoglu, 1996). At Salinas, representative cultivars were also evaluated for reaction to BSBMV in greenhouse trials. Sequential infections of BNYVV and BSBMV were evaluated in greenhouse studies for their effect on root weight, symptoms, and virus levels.

Materials and Methods: Eight sugar beet cultivars were chosen to represent the geographically diverse growing areas of California and southern Minnesota (Table 1).

Table 1. Sugar beet hybrids evaluated in rhizomania experiments at Salinas.

Identification	Source	Description	Genotype
USH11	USDA-ARS	diploid susceptible	*rzrz*
KWS6770	Betaseed	triploid susceptible	*rzrzrz*
Beta4776R	Betaseed	diploid resistant	*Rzrz*
SS-781R	Spreckels	diploid segregating	*Rzrz:rzrz*
Rival	Holly	diploid resistant	*Rzrz*
HM7072	Novartis	diploid resistant	*Rzrz*
Beta4038R	Betaseed	triploid resistant	*Rzrzrz*
6921H50	USDA-ARS	diploid segregating	*B. maritima* hybrid

A TAS-ELISA was developed in collaboration with Agdia, Inc. that was specific for BNYVV, had no cross-reactions with BSBMV isolates, and had the ability to measure a wide range of absorbance values for BNYVV. Samples were prepared and tested as previously described (Wisler et al., 1999).

Field trials were conducted at the USDA-ARS, U.S. Agricultural Research Station on rhizomania-infested land. The primary test in this study was planted May 1, 1997 in a split-plot design, where harvest dates were the main plot, with eight cultivars (subplots) randomized into three harvest dates (July 14, August 18, October 20), each with eight replications. In each of the three harvests, the 9 randomly selected beets from each plot (72 plants per cultivar; 576 plants per harvest date) were dug by hand, topped just above the lowest leaf scar, and washed free of soil. In the first harvest, because of the small size of the beets, only the TAS-ELISA was done. In the second and third harvests, ELISA tests were performed, tap roots were individually weighed, and each beet root was scored on a scale of 0 to 9 for rhizomania and a disease index (DI) calculated (Wisler et al., 1999). Greenhouse studies were made as standard rhizomania tests in 6-inch pots. Plants were harvested after 6 weeks for ELISA and weight determinations. A standard DAS-ELISA was used to measure BSBMV levels in roots and the TAS-ELISA was used for BNYVV.

Results and Discussion: Differences in absorbance values for BNYVV among the eight cultivars were closely correlated to the dosage and frequency of the *Rz* allele that conditions resistance to BNYVV (Table 2). The diploid *Rzrz* hybrid Beta4776R had a significantly lower value than the similar triploid *Rzrzrz* hybrid Beta4038R. For all cultivars, differences were observed among harvest dates, with progressively lower absorbance values measured as the season progressed, particularly from July 14 to August 18. A highly significant cultivar by date of harvest interaction occurred. This interaction can largely be explained by rate and magnitude of decrease in absorbance values for the susceptible cultivars compared to the resistant ones. Absorbance readings for the July 14 harvest clearly discriminated differences in varietal reactions more than did the subsequent harvests (Table 2).

Table 2. Mean TAS-ELISA readings (A_{405nm}) of BNYVV for varieties, dates of harvest, and varieties X dates.

Variety	Genotype	July 14	August 18	October 22	Mean
USH11	*rzrz*	0.947[a]a[b]	0.365c	0.226efg	0.513b
KWS6770	*rzrzrz*	1.024a	0.414c	0.341cd	0.593a
Beta4776R	*Rzrz*	0.257def	0.150ghi	0.117hi	0.175de
SS-781R	*Rzrz:rzrz*	0.343cd	0.164fghi	0.140ghi	0.216de
Rival	*Rzrz*	0.316cde	0.138ghi	0.128ghi	0.195de
HM7072	*Rzrz*	0.218efg	0.111i	0.138ghi	0.156e
Beta4038R	*Rzrzrz*	0.562b	0.220efg	0.212fgh	0.332c
6921H50	unknown	0.356cd	0.192fghi	0.155ghi	0.234d
Mean		0.503a	0.219b	0.182b	0.302
Healthy sugar beet root		0.105	0.096	0.102	0.101
BNYVV sugar beet root		0.513	0.372	0.482	0.456
Healthy *B. macrocarpa*		0.106	0.098	0.103	0.103
BNYVV *B. macrocarpa*		1.654	1.031	2.345	1.677

[a]Values represent an average of two wells from eight replications of nine beets each.
[b]Within sets, means with a letter in common are not significantly different (p=0.05).

Close correlations were observed between the variables used to evaluate reactions to rhizomania, including absorbance (A_{405nm}), absorbance/healthy, root score and root weight

(Table 3). There was nearly a perfect correlation between absorbance readings of test samples and absorbance of test samples divided by those of healthy roots grown in pasteurized soil (absorbance/healthy), indicating extremely low background reactions and very little plate-to-plate variability and experimental error. The highly significant positive correlations between absorbance/healthy values and root scores showed that visual disease index scores of these roots were highly correlated with virus concentration. Correlations between absorbance/healthy and root weight were negative (Table 3). Root weights and disease scores also were highly inversely correlated.

Table 3. Coefficients of correlation among treatment means from two harvests[a]

	Absorbance (A_{405nm})	Absorbance Healthy[b]	Root Score	Root Weight (g)
Absorbance	----------	0.99**	0.87**	-0.89**
Abs/Healthy	0.99**	----------	0.87**	-0.89**
Root Score	0.95**	0.95**	----------	-0.89**
Root Weight	-0.76*	-0.76*	-0.87**	----------

[a] The correlations for harvest date two (August 18) are above the diagonal and those for date three (October 22) are below.
[b] Absorbance at (A_{405nm}) for test samples divided by that for healthy root samples.
* significant at the 0.05 level of probability; ** at the 0.01 level of probability.

Selected cultivars from the field study, plus 'Rizor' and *Beta vulgaris* subsp. *macrocarpa* were also used in greenhouse studies to evaluate their reaction to BSBMV and BNYVV. Soil was obtained from fields that had been previously tested and were infested with BSBMV only. All seven varieties showed high BSBMV readings in DAS-ELISA from BSBMV soils (Fig. 1). Thus, it appears that resistance to BNYVV does not confer resistance to BSBMV.

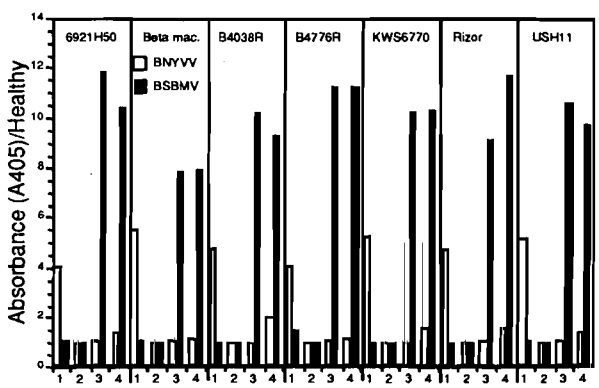

Fig. 1. ELISA tests for seven sugar beet cultivars planted into four soil treatments: (1) BNYVV infested soil, (2) healthy soil, (3) BSBMV-isolate 1 soil, (4) BSBMV-isolate 2 soil.

Cultivar Beta4776R was grown in greenhouse studies to determine the effect of sequential infections of BNYVV and BSBMV in sugar beet. Seed was first planted into (i) healthy soil, (ii) BNYVV-infested soil, or (iii) BSBMV-infested soil. After 6 weeks seedlings were tested for each virus (data not shown), then transplanted into one of the three soil

treatments. After an additional 6 weeks, plants were tested again, evaluated for symptoms and whole plants were weighed (Table 4). Only those beets that were infected with BNYVV showed blackened roots. Otherwise, beets infected with either BSBMV and/or BNYVV were reduced in weight. Other than general yellowing, no leaf symptoms were observed for either virus. The effect of mixed infections ranged from about 25% to 75% fresh weight reduction (Table 4). Neither virus appeared to moderate the infection of the other.

Table 4. Results from Cross-inoculation Experiments; Salinas, California, 1999.

Variety	seedlings	transplants	fresh weight (average of 10 beets)	BNYVV TAS-ELISA (OD/Healthy)	BSBMV DAS-ELISA (OD/Healthy)
B4776R	healthy	healthy	101.3g	1.00[a]	0.976
B4776R	healthy	BSBMV	38.97	1.14	3.788
B4776R	BSBMV	healthy	76.1	1.03	0.964
B4776R	healthy	BNYVV	38.35	2.65	0.996
B4776R	BNYVV	healthy	63.6	4.18	1.028
B4776R	BNYVV	BSBMV	29.14	2.87	4.088
B4776R	BSBMV	BNYVV	25.84	3.03	1.108

[a] Absorbance values are from the second six week transplant period.

In several states in the United States both BNYVV and BSBMV have been found. Resistance to BSBMV was not identified, and the effects of these two distinct viruses appears to be greater than either one alone.

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IMPROVEMENT OF CHINESE WHEAT (*TRITICUM AESTIVUM* L.) CULTIVARS BY TRANSGENESIS FOR RESISTANCE TO SOILBORNE WHEAT MOSAIC VIRUS (SBWMV)

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Summary

Soilborne wheat mosaic virus (SBWMV), one of the most important fungus-transmitted viruses of wheat, can cause high losses of quality and yield and has recently become increasingly important in the winter wheat growing regions in China. Development of virus resistant wheat cultivars could make a great contribution to increasing wheat production. In this study, a spring wheat cultivar and several Chinese wheat varieties were transformed by microprojectile bombardment of immature embryos with plasmids designed to engineer resistance. Plants were regenerated under phosphinothricin (PPT) selection. PCR and Southern blot analyses confirmed the integration of viral genes into the genome of transgenic plants. Transgene stability and virus resistance tests are in progress.

Introduction

SBWMV is one of several important viruses that seriously damage the world wheat production in most winter wheat growing regions. The total yield loss of some susceptible cultivars has been reported during the 1920s (McKinney, 1937). In China, the diseases caused by SBWMV have become increasingly important in winter wheat growing regions and yield losses of wheat varied from 10% to 30% in infested areas and even approached 70% in some instances (Hou *et al.*, 1985, Chen, 1993). SBWMV is transmitted by a soilborne fungus, *Polymyxa graminis*. The resting spores can remain in soil for many years. Once a field is infested with viruliferous fungus it is difficult to eradicate the virus. Therefore the only simple and economical method to control SBWMV is by growing resistant wheat cultivars in the infested fields.

Wheat is an important target for the application of genetic manipulation techniques. The successful recovery of fertile transgenic plants from bombarded regenerable embryogenic callus was first reported by Vasil *et al.* in 1992. Weeks and co-workers (1993) reported a high frequency regeneration of transgenic wheat after particle bombardment of cultured immature embryos of the highly-embryogenic wheat cultivar "Bobwhite", and the transformation frequency was increased to 0.1-0.2%. Since then, many groups have reported the regeneration of fertile transgenic plants from particle bombardment of immature embryos of different wheat cultivars in the past several years (Weeks *et al.*, 1993; Altpeter *et al.*, 1996; Barro *et al.*, 1997). Recently, the regeneration of transgenic wheat with improved properties has been reported by Altpeter *et al.* (1996) and Barro *et al.* (1997). This demonstrated that the functional properties of wheat can be improved by genetic manipulation. To date there has been no reports of successful transferring SBWMV viral sequences conferring resistant to the virus into wheat cultivars.

Here we reported the production of transgenic wheat cultivars containing SBWMV coat protein and cell to cell movement protein coding sequences by microprojectile bombardment of immature embryos.

Materials and Methods

Spring wheat (*Triticum aestivum* L.) cultivar Bobwhite and Chinese wheat cultivars (Yangmai 93-111, Yangmai 94-141, Yanmai 2980) were grown in the glasshouse at 18 °C day and 14 °C night under 12 hours photoperiod. Wheat spikes were harvested and then sterilised with 70% ethanol for 2 minutes followed by two washes with sterile distilled water. Immature embryos (1.5-2.0 mm in length) were isolated under a stereo dissecting microscope. MS medium (Murashige & Skoog, 1962) supplemented with 2.0 mg/l 2.4-D, 500 mg/l glutamate, 100 mg/l casein hydrolysate, 30 g/l maltose, designated as SMS, was used for induction of somatic embryogenesis from immature embryo culture.

After one week culture on the induction medium, immature embryos were plasmolysed on the medium (SMS plus 20% maltose) in the dark for 4 hours. Gold particles (1.0 μm in diameter) were coated with plasmid following the protocol from Iglesias (1994). The pre-cultured immature embryos were bombarded using the Dupont helium-driven Biolistic particle delivery system (PDS 100) under optimal bombardment conditions. For stable transformation, after one week recovery culture on SMS medium, bombarded embryos were transferred onto selection medium (SMS medium with 5 mg/l PPT) and cultured for 6 weeks. The calli were transferred onto selection regeneration medium containing 2 mg/l 6BA, 0.5 mg/l NAA and 5 mg/l PPT for plant regeneration. The regenerants when about 2 cm in length were transferred onto selection rooting medium (1/2 MS supplemented with 0.1 mg/l KT, 0.1 mg/l NAA, 10 g/l maltose and 3 mg/l PPT)

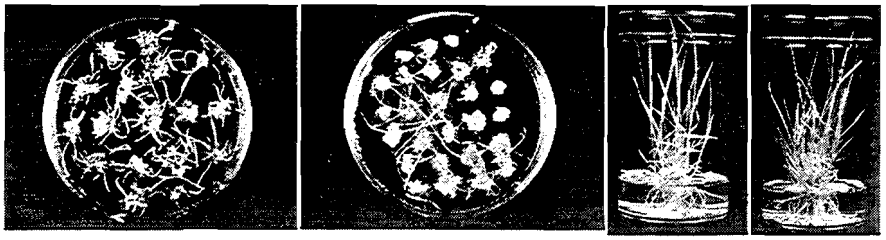
PCR reactions were carried out using several oligonucleotide primers under different programs. PCR amplification products were analyzed by agarose gel electrophoresis. Genomic DNA was isolated from wheat plants using the modified cetyltriethylammoniumbromide (CTAB) extraction method described by Murray and Thompson (1980). Genomic DNA was digested with appropriate restriction enzymes. Agarose gel electrophoresis, Southern blotting and hybridization with radioactive labeled probes were performed following standard procedures.

Results

Recovery of transgenic plants

Immature embryos from Bobwhite and three Chinese cultivars were cotransformed with plasmid pAB1 bearing a chimeric *bar* gene driven by rice *Act1* promoter and a construct containing SBWMV coat protein or movement protein coding sequences under control of maize *Ubiquitin-1* promoter and its intron. After six weeks subculture on the selection medium, masses of somatic embryos appeared on the surface of callus derived from Bobwhite embryos, however only a few of nodular structures which further developed into mature somatic embryos were found from the callus derived from the three Chinese wheat cultivars. After six weeks culture on selection regeneration medium around 60% of somatic embryos from Bobwhite were regenerated to plantlets, whereas only 10% from Chinese varieties were regenerated (Fig. 1 and 2). Following 4 weeks culture on selection rooting medium, transformants grew vigorously with strong roots (Fig. 3 and 4).

Fig.1 Fig. 2 Fig. 3 Fig. 4



PCR and Southern analyses of transgenic plants

Twenty-five transgenic plants were regenerated from 17 independent transformation experiments. The transformation frequency varied from 0.6 to 5%. Full-length sequences of viral CP and MP genes were amplified from most of *bar*-positive transgenic plants and cotransformation frequency was 75%. Figure 5 shows the representative PCR screening results.

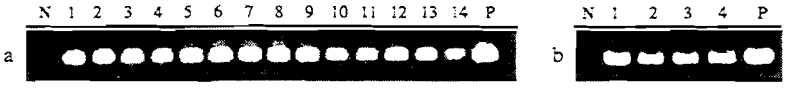


Fig. 5: PCR screening of T_0 plants. a) with CP primers, N) negative control, 1-14) fourteen independent transgenic plants, P) positive control pUbiCP;. b) with MP primers, N) negative control 1-4) 4 independent transgenic plants, P) positive control pUbiMP.

Southern blot analysis of transgenic plants was performed using specific probes and the specific bands corresponding to the intact viral coat protein and movement protein coding genes were detected from transgenic plants (Fig. 6).

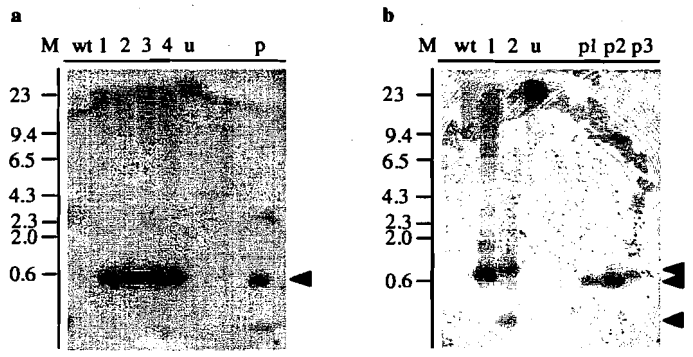


Fig. 6: Southern blot analysis of selected transgenic lines. a) *Bam*HI digested, probed for *cp* gene; b) *Bam*HI and *Bst*I digested, probed for *mp* gene. Arrow indicates the position of *cp* gene (a) and *mp* gene (b).

Discussion

In seventeen independent experiments 12 of 25 transformants were regenerated from Bobwhite. Transformation frequency varied from one experiment to the other and the average stable transformation frequency was 0.8%. These are in agreement with some reports of the production of transgenic wheat by particle bombardment of immature

embryos (Becker *et al.*, 1994; Nehra *et al.*, 1994). However, a lower number of transformants were obtained from most experiments with Chinese wheat cultivars. No transgenic plants were produced from all three Chinese wheat cultivars in two experiments, although the preliminary experiments showed that all these three Chinese cultivars produced comparatively more somatic embryos than other commercial varieties. This is probably due to the suboptimal environmental conditions. The loss of fertility of transgenic wheat plants was found from most of the transgenic plants containing the CP gene. It is well known that infertility problems are mainly contributed from the long-term culture. However, it is unclear if the expression of newly introduced viral gene sequences can significantly interfere with the fertility of transgenic plants. This study has demonstrated that genetic transformation of Chinese winter wheat has been accomplished by particle bombardment of immature embryos. Determination of viral gene expression in transgenic plants and virus resistance testing are in progress.

Acknowledgements

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